FACULTY OF PHARMACEUTICAL SCIENCES

OCCURRENCE AND CO-OCCURRENCE OF 19 MYCOTOXINS IN SOY AND ALMOND-BASED BEVERAGES USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

The global consumption of plant-based beverages, including soy-based and almond-based beverages, is experiencing a notable increase. However, with the rising consumption comes the necessity to ensure their quality, particularly regarding the presence of mycotoxins. These toxic substances are produced by fungi that can grow on crops such as soybeans and almonds. Testing for mycotoxins in plant-based beverages is crucial because they can be harmful to both human and animal health. Therefore, this thesis investigates the presence of 19 mycotoxins in 31 soy-based beverage samples and 33 almond-based beverage samples purchased in Cranfield, United Kingdom.

The tested mycotoxins include aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), ochratoxin A (OTA), fumonisin B1 (FB1), fumonisin B2 (FB2), zearalenone (ZEN), deoxynivalenol (DON), HT-2 toxin (HT-2), T-2 toxin (T-2), alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), enniatin A (ENNA), enniatin A1 (ENNA1), enniatin B (ENNB), enniatin B1 (ENNB1), and beauvericin (BEA).

The occurrence of mycotoxins ranged from 0% (for AFG2) to 100% (for BEA). The highest concentration in this study was 2.2 µg/L, observed for both HT-2 and BEA in almond beverages. Additionally, the co-occurrence was examined, showing combinations ranging from two to twelve mycotoxins per sample.

Given the potential adverse health effects associated with mycotoxins, it is important that mycotoxins are regulated. Currently, the legislation does not include maximum limits for mycotoxins in plant-based beverages. Therefore, this thesis contributes to the sparse literature on mycotoxin concentrations in plant-based beverages, enhancing the understanding of food safety within plant-based beverages. Nonetheless, further research is necessary to gather additional occurrence and exposure data of mycotoxins in plant-based beverages to establish appropriate safety guidelines and limits if needed.

SAMENVATTING

De populariteit van plantaardige dranken zoals sojamelk en amandelmelk neemt wereldwijd alsmaar toe. Echter, met de stijgende consumptie komt ook de noodzaak om de kwaliteit ervan te waarborgen, onder andere wat betreft de aanwezigheid van mycotoxinen. Deze giftige stoffen worden geproduceerd door schimmels die kunnen groeien op gewassen zoals sojabonen en amandelen. Het testen op mycotoxinen in plantaardige dranken is cruciaal, omdat ze schadelijk kunnen zijn voor de gezondheid van mens en dier. Daarom wordt in deze thesis de aanwezigheid van 19 mycotoxinen getest in 31 sojamelkstalen en 33 amandelmelkstalen aangekocht in Cranfield in het Verenigd Koninkrijk.

De geteste mycotoxinen zijn aflatoxine B1 (AFB1), aflatoxine B2 (AFB2), aflatoxine G1 (AFG1), aflatoxine G2 (AFG2), ochratoxine A (OTA), fumonisine B1 (FB1), fumonisine B2 (FB2), zearalenone (ZEN), deoxynivalenol (DON), HT-2 toxine (HT-2), T-2 toxine (T-2), alternariol (AOH), alternariol monomethyl ether (AME), tentoxine (TEN), enniatine A (ENNA), enniatine A1 (ENNA1), enniatine B (ENNB), enniatine B1 (ENNB1) en beauvericin (BEA).
Het voorkomen van mycotoxinen lag tussen 0% (AFG2) en 100% (BEA). De hoogste concentratie die geobserveerd is in deze studie was 2.2 µg/L voor zowel HT-2 als BEA in amandeldrank. Ook het aantal mycotoxinen per staal werd onderzocht en er werden combinaties van twee tot twaalf mycotoxinen per staal gevonden.

Gezien de mogelijke nadelige gezondheidseffecten verbonden aan mycotoxinen, is het van belang dat mycotoxinen meer gereguleerd worden. De huidige wetgeving omvat momenteel geen maximum limieten voor mycotoxinen in plantaardige dranken. Daarom draagt deze thesis bij aan de schaarse literatuur over het voorkomen van mycotoxinen in plantaardige dranken, opdat de voedselveiligheid binnen deze dranken beter begrepen wordt. Desalniettemin is verder onderzoek nodig om meer gegevens te verzamelen over het voorkomen en de blootstelling aan mycotoxinen in plantaardige dranken, zodat indien nodig geschikte veiligheidsrichtlijnen en limieten kunnen worden opgesteld.

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LIST OF USED ABBREVIATIONS

ADI	Acceptable daily intake
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AME	Alternariol monomethyl ether
AOH	Alternariol
BEA	Beauvericin
CID	Collision-induced dissociation
CONTAM Panel	Panel of Contaminants in the Food Chain
DC	Direct current
DON	Deoxynivalenol
EFSA	European Food Safety Authority
ENNA	Enniatin A
ENNA1	Enniatin A1
ENNB	Enniatin B
ENNB1	Enniatin B1
ENNs	Enniatins
ESI	Electrospray ionization
ESI-	Negative electrospray ionization
ESI+	Positive electrospray ionization
EU	European Union
FAO	Food and Agriculture Organization
FB1	Fumonisin B1
FB2	Fumonisin B2
HPLC	High-Pressure Liquid Chromatography
HT-2	HT-2 toxin
IARC	International Agency of Research on Cancer
JECFA	Joint FAO/WHO Expert Committee on Food Additives

LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass-to-charge
MeCN	Acetonitrile
MeOH	Methanol
$MgSO_4$	Magnesium sulfate
MRM	Multiple reaction monitoring
MS/MS	Tandem Mass Spectrometry
MS^2	Tandem Mass Spectrometry
N.d	Not detected
NOAEL	No observed adverse effect level
OTA	Ochratoxin A
Q	Quantifier
q	Qualifier
Q1	First quadrupole
Q2	Second quadrupole
Q3	Third quadrupole
QqQ	Triple quadrupole mass spectrometer
R^2	Coefficient of determination
RF	Radiofrequency
RPLC	Reversed Phase Liquid Chromatography
S/N	signal-to-noise
SALLE	Salting-out-assisted liquid-liquid extraction
SIM	Single ion monitoring
T-2	T-2 toxin
TDI	Tolerable daily intake
TEN	Tentoxin
UHPLC	Ultra-High-Pressure Liquid Chromatography
WHO	World Health Organization
ZEN	Zearalenone

1. INTRODUCTION

1.1 GROWTH OF THE PLANT-BASED MARKET

Over the past decade, there has been an increase in the consumption of plant-based beverages on a global scale. The global plant-based beverages market was estimated to be worth US$19.7 billion in 2023 (1,2). While the largest market for plant-based beverages is found in Asia and Australia, Europe and Russia are experiencing the most rapid growth in this sector (3).

Plant-based beverages, however, cannot be regarded as novel because these beverages hold a significant historical and cultural legacy in both Eastern and Western societies (4). For instance, beverages like Sikhye from South Korea, composed of malt extract, cooked rice, and sugar, and the widely known soy drink from China have long been part of culinary traditions. Notably, the popularity of soy beverages increased by 50% upon their introduction as a viable alternative for individuals with lactose intolerance (1,5). Customers choose cow milk substitutes because of lactose intolerance, cow milk allergies, concerns about calories, the incidence of hypercholesterolemia, and a growing preference for vegan diets (6). Lactase non-persistence, affecting over 50% of populations in South America, Africa, and Asia, and nearing 100% in certain Asian regions, further underscores the significance of plant-based alternatives (7). Plant-based milk has also experienced an increase in consumption due to its absence of cholesterol and lactose. This quality makes it suitable for individuals with heart conditions and lactose intolerance (6,8). Moreover, the affordability of plant-based milk alternatives has made them increasingly attractive, particularly in developing countries with lower incomes and regions facing challenges with the availability of cow’s milk (6).

1.2 MYCOTOXINS

Plant-based beverages represent a fast-growing market, but these plant-based products may also harbor mycotoxins. Mycotoxins are natural secondary metabolites produced by certain fungal species that can contaminate food sources (9). The advent of modern mycotoxicology can be traced back to the identification of aflatoxins in 1960 (10,11). Since then, numerous other mycotoxins have been identified as noteworthy contaminants in food,

often resulting in intoxications. Exposure to mycotoxins can occur through direct consumption of contaminated food items or indirectly through the ingestion of animal products derived from animals fed with mycotoxin-contaminated feed, with dairy products such as milk being particularly susceptible (12). Over time, more than 500 distinct mycotoxins have been documented, and classified based on their biological origin, chemical structure, or time of generation, ranging from preharvest stages in plant cultivation to postharvest phases encompassing storage, transportation, and processing (10,13,14). These fungi find habitat in a diverse array of crops and foods, including nuts, cereals, legumes, dried fruits, spices, apples, and coffee beans. Importantly, fungal colonization is not confined to surface growth but can penetrate deeply into food matrices. The fungi often thrive in warm and humid conditions, and in consequence, climate change is potentially linked to alterations in mycotoxin presence and fungal growth dynamics (15).

Fungi are traditionally classified into two categories: field fungi and storage fungi. Field fungi, such as species of *Alternaria*, *Fusarium*, *Cladosporium*, and *Botrytis*, infect corps during the vegetative phase and their prevalence diminishes during storage. Conversely, storage fungi, which include genera such as *Aspergillus*, *Penicillium*, *Rhizopus*, and *Mucor*, predominantly infect crops post-harvest and during storage (16). The primary concern regarding fungal contamination in soybeans is with field fungi during the cultivation period. The leading producers of soybeans globally include the United States, Brazil, Argentina, China, and India. The climate conditions prevalent in soybean-growing regions, characterized by moderate mean temperatures and relative humidity levels between 50% and 80% create an optimal environment for fungal proliferation. The most prevalent fungal species associated with soybeans belong to the field fungi, particularly those belonging to the genera *Alternaria*, *Fusarium* producing fumonisins, tentoxin, alternariol, trichothecenes etc. These fungi are predominantly observed in mature seeds prior to storage (16,17). Almonds, with their high lipid content, are highly prone to contamination by storage fungi, notably *Aspergillus* and *Penicillium* species, which thrive in the lipid-rich environment, prevalent during storage. These fungi have the capacity to produce various mycotoxins, including aflatoxins and ochratoxin A, posing substantial health risks (18).

1.2.1 Aflatoxins

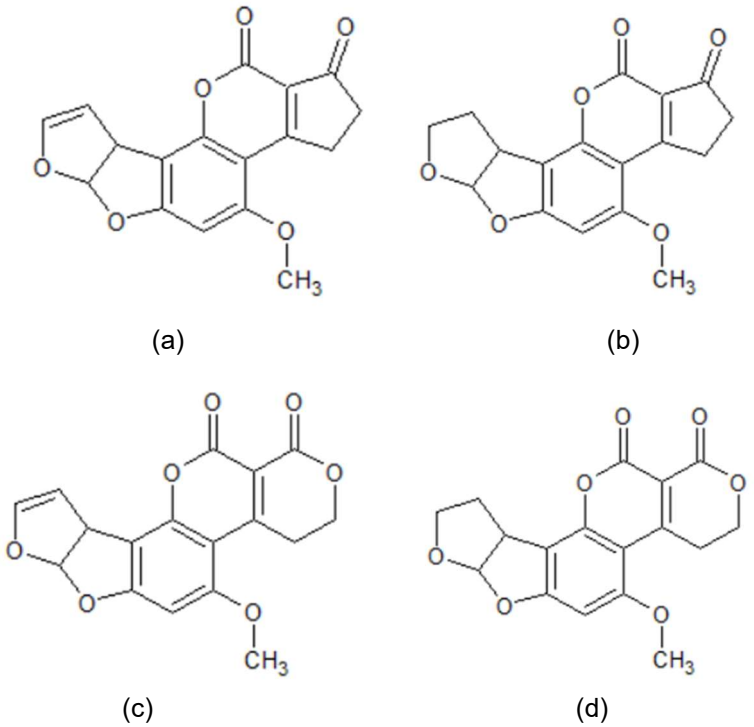


Figure 1.1: Chemical structure of (a) aflatoxin B1, (b) aflatoxin B2, (c) aflatoxin G1, (d) aflatoxin G2 (19)

Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) (**Figure 1.1**) are difuranocoumarin derivatives primarily produced by *Aspergillus flavus* and *A. parasiticus*. While *A. parasiticus* strains can produce AFB1, AFB2, AFG1, and AFG2, *A. flavus* strains are limited to AFB1 and AFB2 production (20). Following the outbreak of Turkey X disease, which resulted in the deaths of 100000 young turkeys in Great Britain in 1960, aflatoxins were identified as the causative agents. This discovery prompted extensive research on these compounds, making them the most scrutinized mycotoxins to date (21–23). Aflatoxin-producing fungi colonize a variety of food commodities, including cereals (maize, rice, barley, oats, and sorghum), peanuts, ground nuts, pistachio nuts, almonds, walnuts, and cottonseeds (20,24). Aflatoxins predominantly affect the liver, exhibiting teratogenic, immunosuppressive, hepatotoxic, and carcinogenic properties. Both acute toxicity and long-term carcinogenicity have been documented in human and animal populations exposed to aflatoxins (20). Notably, the International Agency of Research on Cancer (IARC) has categorized aflatoxins as Group 1 carcinogens, indicating a high risk of hepatocellular carcinoma development among individuals exposed to aflatoxins (23).

1.2.2 Ochratoxin A

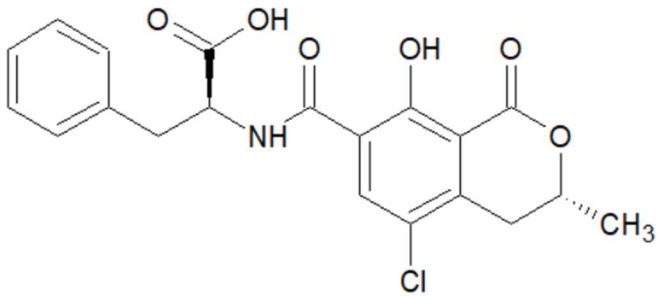
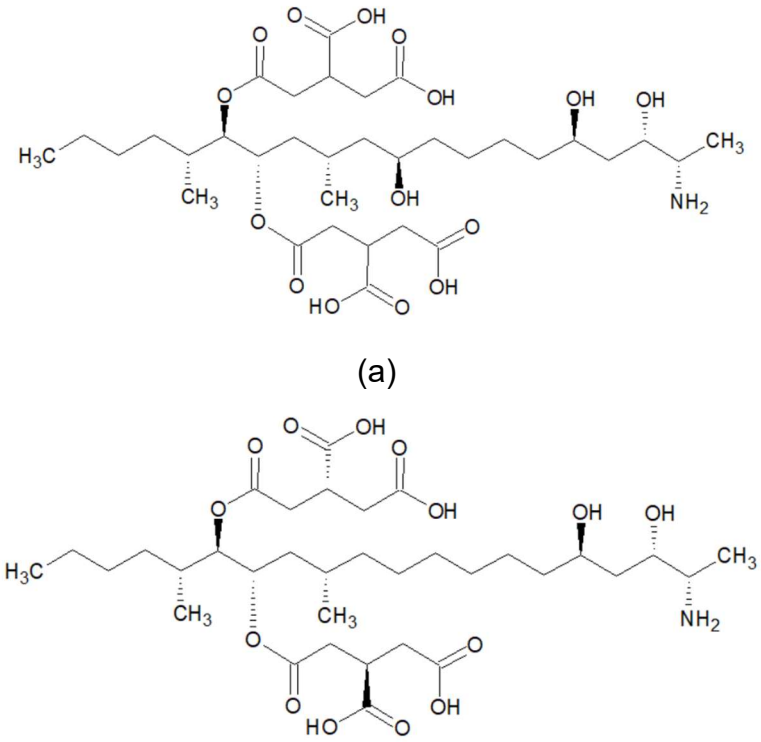


Figure 1.2: Chemical structure of Ochratoxin A (19)

Ochratoxin A (OTA) (**Figure 1.2**) is the predominant toxin among ochratoxins, primarily synthesized by *Aspergillus ochraceus*, *A. carbonarius*, and *Penicillium verrucosum*. *P. verrucosum* is known to produce OTA in cool-temperature conditions, while *A. ochraceus* thrives in hot tropical regions (23,25). Ochratoxins have been detected in various agricultural commodities, including corn, barley, flour, wheat, coffee, oats, rice, rye, peas, beans, and mixed feeds. They are particularly prevalent in wine, grape juice, and dried vine fruits (26). OTA exhibits remarkable stability in acidic environments and resilience to high thermal processing, rendering its presence persistent in cereal products and beers, and difficult to remove through routine cooking processes (20,27). The IARC has categorized OTA as a Group 2B substance, signifying its potential carcinogenicity in humans (28). Besides its carcinogenic properties, OTA demonstrates significant nephrotoxic and hepatotoxic effects (23). Furthermore, it has been documented to induce immunotoxicity, neurotoxicity, genotoxicity, teratogenicity, and embryotoxicity in both animal and human studies (29).

1.2.3 Fumonisins



(a)

(b)

Figure 1.3: Chemical structure of (a) fumonisin B1, and (b) fumonisin B2 (19)

Fumonisins (**Figure 1.3**), comprising a class of mycotoxins that exhibit hydrophilic properties and possess structurally distinctiveness from the majority of other mycotoxins, which are typically soluble in organic solvents (23). It is hypothesized that fumonisins are synthesized by condensation of the amino acid alanine into an acetate-derived precursor (20). Fumonisins are primarily produced by *Fusarium verticillioides*, *F. proliferatum* and are prevalent in maize kernels, wheat, barley, sorghum, soybean, asparagus spears, figs, black tea, and medicinal plants (20,30). Currently, over 28 fumonisins have been identified and categorized into four groups: A, B, C, and P (23,31). Among them, fumonisin B1 (FB1), constituting 70-80% of total fumonisins, represents the most frequently encountered variant and is also recognized as the most toxic of them. The IARC classified FB1 as Group 2B, possibly carcinogenic to humans (28). The liver and kidney are the primary targets of fumonisin toxicity, eliciting severe adverse effects in experimental animal models (23,32).

1.2.4 Zearalenone

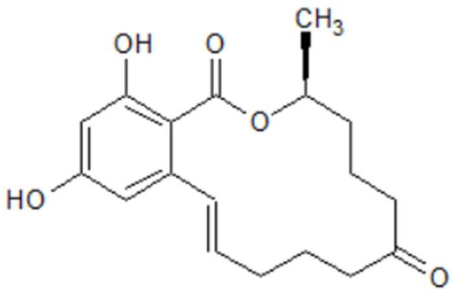


Figure 1.4: Chemical structure of zearalenone (19)

Zearalenone (ZEN) (**Figure 1.4**) is a macrocyclic β-resorcyclic acid lactone synthesized by *Fusarium graminearum* and *F. semitectum* and commonly detected in corn, barley, wheat, sorghum, and rye. The production of ZEN is enhanced by environmental factors such as high humidity and low temperatures. While ZEN exhibits stability when subjected to typical cooking temperatures, it undergoes partial degradation under heat conditions (23,33). Due to its structural resemblance to natural estrogens, ZEN is considered an estrogenic mycotoxin capable of inducing estrogenic effects in both humans and animals (20). This potent estrogenic activity raises concerns for public health. Currently, ZEN is categorized in Group 3 by IARC, indicating that there is insufficient evidence for carcinogenicity to humans (28). ZEN competitively binds to estrogen receptors across various animal species, resulting in modifications and lesions within the female reproductive system (27). Furthermore, research has indicated that ZEN possesses hepatotoxic, hematotoxic, immunotoxic, and genotoxic potential (34–36).

1.2.5 Trichothecenes

Trichothecenes are a group of chemically related compounds characterized by a shared tetracyclic sesquiterpenoid 12-13-epoxytrichothec-9-ene ring system. The classification of the roughly 170 trichothecenes is based on distinctions in the hydroxyl and acetoxy side groups, resulting in four types (A-D). Deoxynivalenol is the most prevalent and extensively researched mycotoxin within the trichothecene class (37).

1.2.5.1 Deoxynivalenol

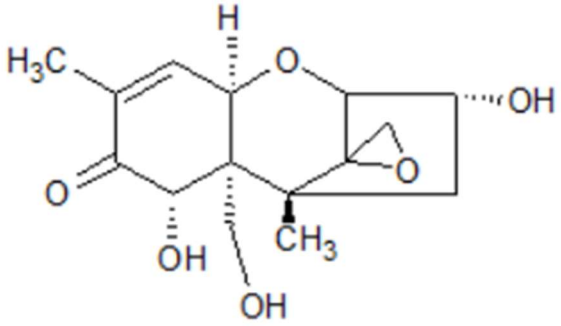
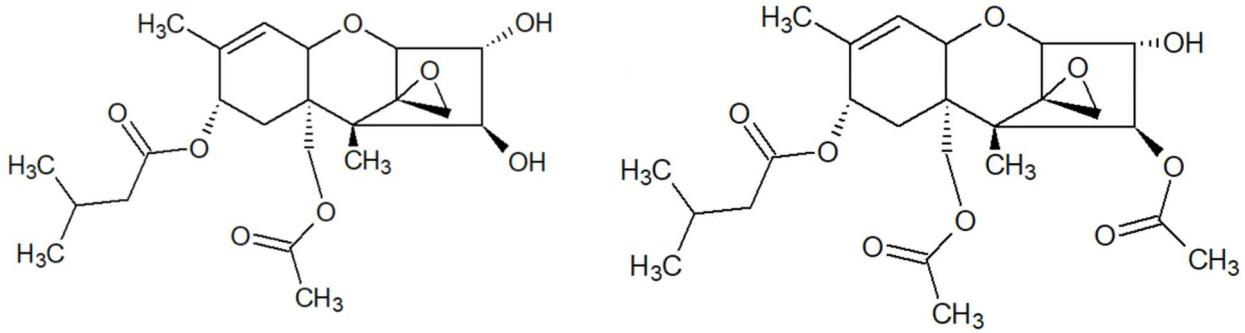


Figure 1.5: Chemical structure of deoxynivalenol (19)

Deoxynivalenol (DON) (**Figure 1.5**) is a type B trichothecene and is primarily biosynthesized by *Fusarium graminearum*, *F. culmorum,* and *F. cerealis* (37). These fungi are responsible for *Fusarium* Head Blight, a highly detrimental disease affecting cereal grain crops on a global scale (23). Cereal grains including wheat, oats, rye, maize, barley, rice, and soybeans are the primary substrates of trichothecene contamination (38). DON is considered one of the less toxic *Fusarium* mycotoxins and is classified in Group 3 (not classifiable as to its carcinogenicity to humans) by the IARC (20,28). Nonetheless, human exposure to grains contaminated with DON has been linked to vomiting, nausea, diarrhea, abdominal pain, headache, dizziness, and fever (23,39).

1.2.5.2 HT-2 and T-2 toxin



(a) (b)

Figure 1.6: Chemical structure of (a) HT-2 toxin and (b) T-2 toxin (19)

HT-2 toxin (HT-2) and T-2 toxin (T-2) (**Figure 1.6**) belong to the type A trichothecenes characterized by the presence of a hydroxyl group at C-8, an ester function at C-8, or a lack of oxygen substitution at C-8 (40). The primary T-2 and HT-2-producing species are *Fusarium langsethiae*, *F. sporotrichioides*, *F. acuminatum,* and *F. poae*. T-2 is rapidly metabolized *in vivo* to yield the metabolite HT-2 (37). European Food Safety Authority (EFSA) has concluded that HT-2 and T-2 possess the capacity to inhibit the synthesis of proteins, DNA, and RNA, consequently initiating processes such as necrosis, apoptosis, and lipid peroxidation. Additionally, EFSA has confirmed that the principal adverse effects of T-2 toxicity encompass immunological and hematological perturbations (41,42). While T-2 and HT-2 are known to induce anorexia and emesis across various species, a comprehensive elucidation of their mechanism of action remains pending (42). The IARC determined that due to limited evidence of carcinogenicity in animals, T-2 is not classified as a human carcinogen (Group 3) (43).

1.2.6 *Alternaria* toxins

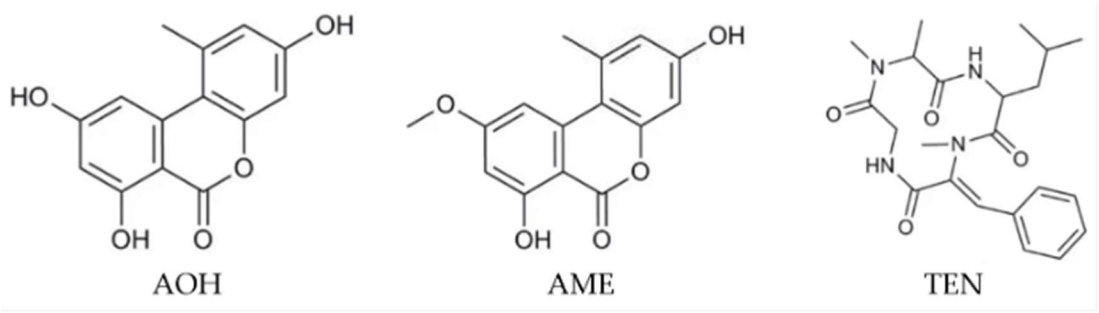


Figure 1.7: Chemical structure of alternariol (AOH), alternariol monomethyl ether (AME), and tentoxin (TEN) (44)

Alternaria mycotoxins constitute a subset of over 70 phytotoxins synthesized by members of the *Alternaria* genus. These toxins are categorized into three distinct structural groups: dibenzopyrone derivates encompassing alternariol (AOH) and alternariol monomethyl ether (AME); perylene derivatives; and tetramic acid derivatives including tentoxin (TEN) (**Figure 1.7**). *Alternaria* mycotoxins are commonly encountered in cereals, fruits, vegetables, juices, and wine. Due to the remarkable adaptability of *Alternaria* species to diverse environmental conditions and their ability to synthesize mycotoxins even under

low-temperature regimes, their potential to infiltrate every level of the food chain is significant. AOH and AME are noted for their mutagenic and clastogenic properties across various *in vitro* systems (37). Furthermore, the presence of TEN influences the growth and development of plants, acting as an inhibitor for chloroplast production and leading to chlorosis (44).

1.2.7 Emerging *Fusarium* mycotoxins

The group of emerging *Fusarium* mycotoxins comprises various toxic secondary metabolites such as enniatins (ENNs) and beauvericin (BEA) (**Figure 1.8, Table 1.1**). These mycotoxins are designated as ‘’emerging’’ due to the incomplete understanding of their toxicological properties. The scarcity of available information on these metabolites stems from their delayed identification and late comprehension of their function as mycotoxins. ENNs and BEA belong to a group of structurally related cyclic hexadepsipeptides, composed of alternating 3 d-2 hydroxycarboxylic acid and N-methylamino acid residues (37). They are commonly found contaminating cereal crops, especially, maize, wheat, oats, and barley (45).

Currently, there are 29 naturally occurring enniatin analogues, with ENNA, ENNA1, ENNB, and ENNB1 being the most commonly detected in food and animal feed samples (46). ENNs possess diverse biological activities including antifungal, phytotoxic, anti-yeast, antibacterial, and insecticidal agents. Their cytotoxic effects have been investigated across various cell types, demonstrating their impact on mammalian and cancer cells at relatively low micromolar concentrations, underscoring the necessity for further investigation (46–49). BEA exhibits both insecticidal and phytotoxic properties, contributing to the development of insect and plant diseases (46,50). Additionally, BEA has been observed to facilitate apoptosis, interfere with smooth muscle contraction, and impede cholesterol accumulation in liver cells, potentially leading to steatosis (46). However, BEA also has beneficial attributes including antifungal, antiviral, and antibiotic properties (46,51,52).

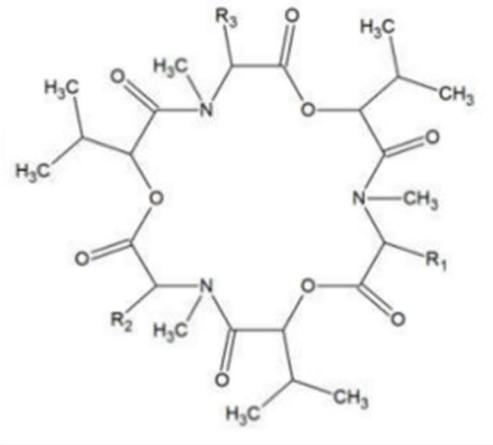


Figure 1.8: enniatins and beauvericin structure (46)

Table 1.1: Side chains of enniatin A (ENNA), enniatin A1 (ENNA1), enniatin B (ENNB), enniatin B1 (ENNB1), and beauvericin (BEA) (46)

Compound	R_1	R_2	R_3
ENNA	Sec-butyl	Sec-butyl	Sec-butyl
ENNA1	Sec-butyl	Sec-butyl	Iso-propyl
ENNB	Iso-propyl	Iso-propyl	Iso-propyl
ENNB1	Iso-propyl	Iso-propyl	Sec-butyl
BEA	Phenylmethyl	Phenylmethyl	Phenylmethyl

1.3 REGULATORY FRAMEWORK OF MYCOTOXINS

1.3.1 Regulatory Authorities

The European Food Safety Authority (EFSA) was established in 2002 and its headquarters are located in Parma, Italy. EFSA is responsible for carrying out risk assessments on food and feed safety for the European Union (EU) (53). The activities of EFSA encompass the acquisition of scientific information and knowledge and offering impartial and up-to-date scientific recommendations on food safety concerns. EFSA's work also involves disseminating its scientific findings to the general public and collaborating with European Union (EU) member states, global organizations, and other relevant stakeholders (53). One of the collaborations of EFSA is with the Joint FAO/WHO Expert Committee on Food Additives (JECFA). While EFSA focuses on providing scientific advice and risk

assessments within the EU, JECFA operates globally, evaluating the safety of food additives, contaminants, and residues for international use. EFSA and JECFA share scientific data, research findings, and methodologies relevant to food safety assessments (54).

JECFA is an international scientific expert committee jointly managed by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). Since 1956 JECFA has assessed the health risks associated with food additives, contaminants, residues of veterinary drugs in food, and natural toxins including mycotoxins. JECFA conducts risk and exposure assessments and offers guidance to FAO, WHO, member countries of both organizations, and the Codex Alimentarius Commission (55). Based on JECFA assessments, the Codex Alimentarius Commission develops international guidelines and codes of practice to reduce exposure to mycotoxins (12).

1.3.2 Risk Assessment

Risk assessment is a crucial process aimed at evaluating the potentially harmful effects on human health resulting from exposure to contaminated food. This assessment includes 4 steps: identifying hazards, characterizing hazards, assessing exposure, and characterizing risks (10). The main objective of hazard identification is to assess if exposure is probable to result in a negative health outcome in humans (56). The primary goal of hazard characterization is to predict potential risks to humans at low exposure levels by utilizing data from animal studies. The determination of a ‘safe dose’ represents the endpoint of the hazard characterization process (10). The internationally recognized reference value to indicate the safe level of consumption of intentionally used chemicals is the acceptable daily intake (ADI). ADI is defined as an estimate of the quantity of a substance present in food and/or drinking water, expressed in relation to body weight, that can be consumed daily over an individual’s lifetime without posing any significant health risks. The ADI is typically derived from the lowest no observed adverse effect level (NOAEL) determined from animal research and is adjusted by a safety factor, often 10, to account for differences between species and individuals (57). In the case of contaminants and naturally occurring chemicals, the reference intake value is denoted as the tolerable daily intake (TDI) (58).

1.3.3 Recommendations and Legislation

Properly dried and stored foods are typically less susceptible to mold growth, making effective drying and maintenance of dry conditions crucial strategies for mitigating mold formation and mycotoxin production. To minimize the danger of mycotoxins, the WHO recommends vigilance for signs of mold on whole grains, nuts, and dried figs, as these foods are frequently contaminated with aflatoxins, with AFB1 being the most potent carcinogen among mycotoxins. Nonetheless, each mycotoxin may exhibit distinct acute and long-term toxicological properties, including genotoxicity, carcinogenic toxicity, immunotoxicity, mutagenicity, nephrotoxicity, and teratogenicity attributes (14,59,60).

Furthermore, the WHO advises the prevention of grain damage before, during, and after harvest, as damaged grains are more susceptible to mold growth and subsequent mycotoxin contamination. Consumers are encouraged to purchase nuts and grains as fresh as possible and store them under appropriate conditions, avoiding prolonged exposure to warmth and ensuring protection from insects. WHO encourages everyone to have a varied diet, not only to reduce mycotoxin exposure but also to enhance overall nutrition (12).

In the realm of legislation, the Codex Alimentarius Commission established international standards and codes of practice aimed at curtailing exposure to mycotoxins in specific foodstuffs. Under EU regulations, aflatoxins, OTA, patulin, DON, ZEN, and fumonisins are subject to legislative measures in certain food items, each with prescribed maximum levels. For nuts, including almonds, when utilized as an ingredient in foodstuffs, the prescribed threshold for AFB1 is defined at 8.0 µg/kg, while the cumulative limit for AFB1, AFB2, AFG1, and AFG2 is set at 10.0 µg/kg. Regulatory frameworks do not govern the presence of other mycotoxins in nuts, almonds, or almond-based beverages, nor do they specify maximum levels for any mycotoxin in soy and its derivatives (61). The other mycotoxins are not subject to specific regulatory standards, however, certain among them have recommended indicative levels. The recommended indicative levels for both AOH and AME vary from 2.0 µg/kg in cereal-based foods intended for infants and young children to 30 µg/kg in sunflower and sesame seeds (62). Indicative levels also exist for the sum of

HT-2 and T-2, serving as thresholds beyond which investigations should be conducted, particularly upon repetitive detection. These indicative levels are established for T-2 and HT-2, specifically in cereals and cereal products, ranging from 15 µg/kg to 2000 µg/kg (63). In summary, legislation encompasses legally enforceable regulations established by the EU institutions. In contrast, recommendations and indicative levels provide non-binding guidance and advice intended to strengthen food safety practices and policies (64).

Thus regulatory oversight is currently limited to a few mycotoxins, applicable to only a restricted range of food items. There remains a pressing necessity for additional regulatory measures encompassing other mycotoxins as well as extending regulatory coverage to a broader spectrum of food products. For soy and almond beverages specifically, the European Commission currently lacks a clear stance on the maximum permissible levels of mycotoxins (65,66).

1.4 MYCOTOXIN ANALYSIS

1.4.1 Sample Collection

Sample collection is a pivotal phase within all laboratory methodologies, serving as the cornerstone for attaining accurate and reliable results in research studies and scientific experiments. The efficacy of subsequent analyses is intricately tied to the quality and integrity of the gathered samples (67). The paramount consideration in sample collection lies in ensuring the sample's representativeness.

To consider a sample representative, several criteria must be met. Firstly, it should be acquired utilizing suitable equipment and must be obtained using a sampling pattern devised to encompass all regions of the lot. Additionally, the sample size should be commensurate with the dimensions of the lot and the nature of the commodity. Furthermore, to preserve its representativeness, proper handling is imperative. This entails storing samples in a cool and dry environment. Shipping samples in plastic bags should be avoided, as these may foster growth if the sample's moisture level exceeds 14%. Lastly, the sample must be appropriately identified and labeled on the container (68,69).

The substantial sampling error observed in mycotoxin testing is due to two principal factors: the low concentration of mycotoxin within a given commodity and the uneven distribution throughout the lot. For instance, within a corn batch, the vast majority of kernels typically lack mycotoxins, with less than 0.1% exhibiting contamination. However, individual kernels have been documented to contain aflatoxin levels as high as 400000000 parts per billion. The uneven growth of molds within a field leads to the deposition of toxins in select kernels, so-called ‘hot spots’ while leaving others unaffected. Accurate analysis entails determining the average contamination level across the entire lot. Failure to adhere to proper sampling protocols increases the likelihood that results either underestimate or overestimate the actual mycotoxin concentration (68).

1.4.2 Sample Treatment

When sample clean-up is not required, salting-out-assisted liquid-liquid extraction (SALLE) emerges as a simple and efficient approach for sample treatment (66). Adding an appropriate amount of salt to a mixture of aqueous sample and water-miscible organic solvent helps to decrease the mutual miscibility and induce phase separation (70). This facilitates the selective transfer of polar analytes from the aqueous phase to the polar organic phase. The use of nonpolar, water-immiscible organic solvents presents a limitation due to their low dielectric constants, making them less efficient in extracting highly polar or charged solutes. This limitation is particularly notable when dealing with water-soluble compounds that necessitate extractions at extremely low or high pH values. More polar solvents, which offer solubility for these compounds, are often water-miscible and unsuitable for conventional liquid-liquid extraction (71).

1.4.3 Sample Analysis

The combination of liquid chromatography with ultraviolet-visible and fluorescence detectors has been employed for the analysis of different mycotoxins, offering suitable detectability and performance. However, this approach has certain limitations, such as the absence of structural information, the need to detect a large number of analytes in a single run, and the coelution of structurally related mycotoxins (72). Consequently, there has been a shift towards a more advanced detection technique. Mass spectrometry has emerged as

one of the most powerful tools for multi-mycotoxin analysis, due to its selectivity, sensitivity, high throughput, and ability to differentiate co-eluting compounds based on their molecular masses (72,73). Currently, Liquid Chromatography-Tandem Mass spectrometry (LC-MS/MS) techniques are the preferred methods for the quantitative multi-trace determination of mycotoxins (72).

1.4.3.1 Ultra-High-Pressure Liquid Chromatography (UHPLC)

Liquid chromatography involves the separation of components by distributing them between 2 phases: a fixed stationary phase and a mobile phase. The rate at which solutes move through the column is determined by their interactions with the stationary phase. These interactions dictate the time analytes spend inside the column and influence peak broadening, ultimately affecting the elution time of compounds from the column. The choice of liquid chromatography type is influenced by the nature, chemical structure, and molecular weight of the analytes (74). For the determination of mycotoxins Ultra-High-Pressure Liquid Chromatography (UHPLC) is often used. UHPLC uses sub-2 μm particles and high linear solvent velocities to significantly enhance resolution, sensitivity, and analysis speed. To accommodate the reduction in particle size below 2 μm, specialized instrumentation capable of operating within the 6000-15000 psi pressure range is necessary. The UHPLC system typically generates peak widths in the range of 1-2 seconds for a 10-minute separation (75). UHPLC reduces the consumption of organic solvents, making it more environmentally friendly than high-pressure liquid chromatography (HPLC). Additionally, it provides enhanced chromatographic resolution and higher sensitivity, resulting in more accurate and reliable results (76).

Reversed-phase liquid chromatography (RPLC) frequently serves as a preferred method for the analysis of mycotoxins. RPLC is distinguished by the partitioning of compounds between a polar mobile phase and a less polar stationary phase (77). C18 is a traditional reversed-phase material used for the chromatographic separation of a wide array of compounds, ranging from compounds with small molecular weights to proteins (73). The mobile phase in RPLC consists of an aqueous solution that significantly influences the

retention mechanism. The presence of water is essential for the distinctive characteristics of RPLC separation, as it is a solvent with high cohesive energy, hydrogen-bond acidity, and dipolarity. These properties contribute to the retention behavior in RPLC, where larger compounds tend to have higher retention times, and polar compounds capable of hydrogen bonding and dipole interactions with water exhibit lower retention times. To elute compounds effectively in RPLC, water is typically mixed with an organic solvent (77).

The ability to separate neutral and ionic compounds simultaneously is made possible by the rapid equilibrium of the stationary phase with changes in mobile phase composition, allowing the utilization of gradient elution (77). In gradient elution, the mobile phase undergoes continuous changes during the separation process. These changes can manifest in different forms, including alterations in the proportion of solvents comprising the mobile phase, variations in pH levels, adjustments in ionic strength, or the introduction of specific additives. Gradient elution serves four primary purposes: (I) reducing the overall duration of separations, (II) modifying the retention times in cases where the separation between specific compounds is inadequate, (III) cleansing the chromatographic column, (IV) narrowing the chromatographic peaks (78).

1.4.3.2 Tandem mass spectrometry

Mass spectrometry involves the ionization of samples, followed by their separation based on the mass-to-charge (*m/z*) ratio by altering their velocities and trajectories in an electric or magnetic field (79,80). Analyzing the *m/z* ratio provides valuable insights into the elemental composition of the compounds present in the samples. In mass spectrometry, the chemical reactions occur in the gas phase, where sample molecules generate both ionic and neutral species (81). The ions derived from the sample molecules are called precursors, while fragments are the ions produced as a result of fragmentation. The predominant method to achieve the correlation between precursors and fragments involves isolating a singular *m/z* ratio before fragmentation, commonly referred to as tandem mass spectrometry, also known as MS^2 or MS/MS (80). Mass spectrometers are composed of three primary elements: the ion source, responsible for generating gaseous ions from the

substance being investigated, the mass analyzer, which differentiates the ions based on their distinctive mass components and *m/z* ratio, and the detector system, which detects the ions and records the relative abundance of each resolved ionic species (82).

Electrospray ionization (ESI) is often employed as the ionization technique, due to its superior sensitivity compared to other methods for detecting a wide range of mycotoxins in multi-class/ multi-trace analyses (72). Both positive electrospray ionization (ESI+) and negative electrospray ionization (ESI-) exist and LC-MS/MS instruments can switch fast between ESI+ and ESI- (83). In ESI+ positively charged ions are produced, while in ESI- negatively charged ions are generated. When liquid chromatography is coupled with ESI/MS, the ESI+ is typically favored due to the higher likelihood of compounds ionizing in this mode. Nonetheless, ESI- offers the significant benefit of lower background noise (84). ESI involves the nebulization of a solution at atmospheric pressure, followed by its exposure to a powerful electric field that induces ionization (81). This electric field is generated by applying a potential difference of 3-6 kV between the capillary where the liquid flows through and the counter-electrode, resulting in an electric field of approximately 10^6 V m^{-1}. Consequently, charge accumulates at the liquid surface located at the end of the capillary, leading to the formation of highly charged droplets. Subsequently, these droplets pass through either a heated curtain of inert gas or a heated capillary to eliminate any remaining solvent molecules. To confine the spray within a limited space, a gas is introduced coaxially at a low flow rate. As the solvent within the droplets evaporates, they shrink in size, resulting in an increase in their charge per unit volume. Deformation of the droplet is initiated by the intense electric field, causing the droplet to shrink due to solvent evaporation, and giving rise to additional offspring. Once the electric field reaches a critical level on the droplet's surface, ions begin to desorb from the surface (82). These ions can go to the mass analyzer and finally to the ion detector.

For analyzing the mycotoxin concentrations multiple reaction monitoring mass spectrometry (MRM-MS) is applied. MRM refers to a targeted MS/MS scan mode that is generally coupled with a triple quadrupole (QqQ) (85). Prior to initiating MRM analysis, it is crucial to establish transitions for the target analyte by selecting precursor ions and their

corresponding product ions based on specific criteria (86). QqQ is composed of two quadrupole mass analyzers connected in series. Between these analyzers, a radio frequency-only quadrupole is positioned to serve as a collision cell, enabling collision-induced dissociation (CID). QqQ exhibits the capability to selectively choose a precursor ion in the first quadrupole (Q1), induce fragmentation within the collision cell (Q2), and subsequently record the resulting fragments using the last quadrupole (Q3) (**Figure 1.9**) (87).

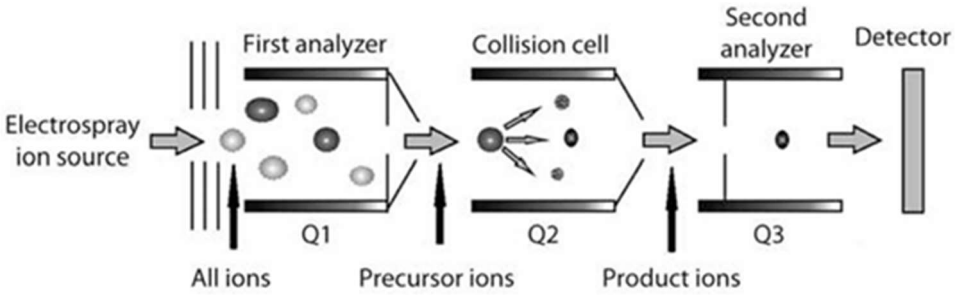


Figure 1.9: Schema of triple quadrupole (QqQ) mass spectrometry analysis in multiple reaction monitoring (MRM) mode. Q1 = first quadrupole, Q2 = second quadrupole, Q3 = third quadrupole (88)

A quadrupole mass analyzer is composed of four cylindrical or elliptical rods in a parallel configuration. The rods are interconnected with radiofrequency (RF) and direct current (DC) generators, with adjacent rods having opposite radiofrequency phases. When specific RF and DC potentials are applied, only ions within a narrow *m/z* range will follow a stable path and successfully reach the detector. Conversely, ions outside this specific *m/z* range will collide with the rods, leading to their neutralization (89,90).

Another scan mode is single ion monitoring (SIM), another targeted mass spectrometry technique where the instrument is set to monitor the intensity of pre-determined *m/z* values. By fixing the voltages at a specific value, it is feasible to isolate a single ion with a particular *m/z*. While MRM monitors specific transitions between precursor ions and their corresponding product ions, SIM focuses solely on monitoring the precursor ions without inducing fragmentation. Consequently, SIM is a simpler technique but potentially less specific than MRM, which provides additional selectivity through monitoring product ions (90,91).

2. OBJECTIVES

In recent years, plant-based beverages have surged in popularity due to environmental and ethical considerations, as well as lactose intolerance, cow milk allergies, and hypercholesterolemia. However, these plant-based products may also serve as potential hosts for mycotoxins. Mycotoxins are secondary metabolites produced by fungi that can exert adverse effects on both human and animal health.

For this reason, this study aimed to investigate the occurrence of 19 mycotoxins in 33 almond-based beverage samples and 31 soy-based beverage samples from Cranfield, United Kingdom. The targeted mycotoxins were aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, ochratoxin A, fumonisin B1, fumonisin B2, zearalenone, deoxynivalenol, HT-2 toxin, T-2 toxin, alternariol, alternariol monomethyl ether, tentoxin, enniatin A, enniatin A1, enniatin B, enniatin B1, and beauvericin.

An analytical method based on salting-out assisted liquid-liquid extraction followed by Ultra-High-Pressure Liquid Chromatography-Tandem Mass Spectrometry is employed to quantify the mycotoxin levels present in these samples. In addition to assessing the individual occurrence of mycotoxins, this study also investigated their co-occurrence. This is the first study that examines the occurrence and co-occurrence of 19 mycotoxins in plant-based beverages purchased in the United Kingdom.

In light of the potential adverse health consequences linked to mycotoxins, it is important that mycotoxins are more regulated. Legislation currently encompasses only a select number of mycotoxins, applicable solely to a narrow spectrum of food products. Currently, mycotoxins in plant-based beverages are not regulated. More specifically on soy and almond beverages, there is no legislation in place for soy, while regulations for almonds are limited to aflatoxins only. Hence, this shows that further research on the occurrence of mycotoxins in plant-based beverages is still necessary to ascertain whether regulatory measures are needed.

3. MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

A total of 64 plant-based beverages were bought from five supermarkets in Cranfield, United Kingdom during January and February 2024. 31 samples of soy-based beverages and 33 samples of almond-based beverages were collected and analyzed in duplicate. The detailed information on the plant-based beverage samples is listed in **Attachment 1** and **Attachment 2**.

3.2 MYCOTOXINS

Analytical standards of AFB1, AFB2, AFG1, AFG2 were supplied by Fermentek.Ltsd (Jerusalem, Israel). FB1, FB2, AOH, AME, TEN, ZEN, OTA, HT-2, T-2 , ENNB, ENNB1, ENNA, ENNA1, BEA, and DON were purchased from Romer Labs (Tulln, Austria). AFB1, AFB2, AFG1, AFG2, and OTA were reconstituted from powder at 1000 µg/mL in acetonitrile (MeCN). ENNs and BEA were similarly prepared from powder at 1000 µg/mL in methanol (MeOH) and 2000 µg/mL in MeCN, respectively. For these solutions, an intermediate stock solution at 100 µg/mL was prepared in MeCN. For HT-2, T-2, AOH, AME, TEN, ZEN, and DON stock solutions of 100.0 µg/mL in MeCN were used, while for FB1 and FB2, a mixture at a concentration of 50.3 µg/mL in MeOH:water 50:50 (v/v) was employed. Finally, a 1 µg/mL stock solution in MeCN containing all mycotoxins was prepared.

3.3 SAMPLE TREATMENT

The experiment is conducted at the Food and Drug Department of the University of Parma, Italy. The reagents used for mycotoxin extraction are listed in **Table 3.1**. Mycotoxin extraction is performed by adding 5 mL of plant-based beverage and 3 mL of acidified MeCN with formic acid at 1.5% (v/v) to a 15 mL Falcon tube. Next, the mixture is vortexed for 1 minute, and then, 2 g of anhydrous magnesium sulfate ($MgSO_4$) is added and vortexed again for 1 minute. The mixture obtained is centrifuged at 10000 rpm for 5 minutes at 4°C (5810 R centrifuge, Eppendorf, Hamburg, Germany). After phase separation, 1 mL of the supernatant is recovered and transferred to a glass vial. The samples are evaporated

to dryness under a gentle nitrogen stream. Before injection into the HPLC system, the obtained residue was reconstituted with 200 µL of a mixture of MeOH:water 75:25 (v/v) and vortexed for 10 seconds. The sample preparation led to a concentration factor of 8.3.

Table 3.1: Reagents for mycotoxin extraction

Reagent	Supplier	Product code	Lot number
Acetonitrile (HPLC-grade)	VWR Chemicals	83639.320	23F034003
Formic acid 99% (LCMS-grade)	CARLO ERBA Reagents	405824	P0I099190M
Anhydrous magnesium sulfate	PanReac AppliChem	212486.1211	0002325137
Methanol (HPLC-grade)	VWR Chemicals	20864.320	23H74004
Water (HPLC-grade)	VWR Chemicals	23595.328	22J124005

3.4 METHOD CHARACTERIZATION

The method characterization and validation were performed earlier, but the analytical characteristics of the SALLE-HPLC-MS/MS technique were re-evaluated with regard to its linearity, limits of detection (LOD), and limits of quantification (LOQ). In order to evaluate linearity, calibration curves with corresponding coefficients of determination (R^2) are generated. For each mycotoxin, a calibration curve is created using 10 curve points 0.1 µg/L, 0.5 µg/L, 1 µg/L, 2.5 µg/L, 5 µg/L, 10 µg/L, 25 µg/L, 50 µg/L, 75 µg/L, and 100 µg/L. These are prepared by spiking a sample blank extract solution for the soy-based beverages and a sample blank extract solution for the almond-based beverages. The standard curves were injected three times: at the beginning, after 20 samples, and at the end of the sequence. Standards are then plotted with the concentrations of the mycotoxin as the horizontal axis and the peak area as the vertical axis. The LODs and LOQs are determined as the minimal analyte concentrations corresponding to signal-to-noise ratios of 3 and 10, respectively. These LODs and LOQs are then divided by the concentration factor of 8.3.

3.5 SAMPLE ANALYSIS

3.5.1 UHPLC-MS/MS conditions

The analysis was performed using a UHPLC Dionex Ultimate 3000 coupled to a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific Inc., San Jose,

CA, USA) and equipped with an ESI source. The chromatographic separation was carried out on a XSelect® HSS T3 column (2.1 i.d. x 150 mm; 2.5 µm) from Waters (Wexford, Ireland) using as mobile phases ultrapure water with 0.2 % acetic acid and 5 mM ammonium acetate (Phase A) and methanol with 0.2 % acetic acid (Phase B). The reagents used for mobile phases A and B are provided in **Table 3.2** and **Table 3.3**, respectively. The chosen gradient was programmed as follows: 0 min, 5 % B; 8 min, 90 % B; 11 min, 90 % B; 12 min, 5 % B; 18 min, 5 % B. A flow rate of 0.4 mL/min was used, with the column temperature set at 40 °C and an injection volume of 3 µL.

Table 3.2: Reagents mobile phase A

Reagent	Supplier	Product code	Lot number
Water (HPLC-grade)	VWR Chemicals	23595.328	22J124005
Acetic acid (HPLC-grade)	VWR Chemicals	87023.260	FA643448
Ammonium acetate (LCMS-grade)	Sigma-Aldrich	73594-25G-F	10257139

Table 3.3: Reagents mobile phase B

Reagent	Supplier	Product code	Lot number
Methanol (HPLC-grade)	VWR Chemicals	20864.320	23H74004
Acetic acid (HPLC-grade)	VWR Chemicals	87023.260	FA643448

The mass spectrometry analysis was conducted using both positive (ESI +) and negative (ESI -) ionization modes, depending on the specific mycotoxin being studied. The spray voltage was set at 3,500 V, with the capillary and vaporizer temperatures maintained at 270 °C and 200 °C, respectively. The flow rates of sheath gas (N_2) and auxiliary gas (N_2) were set at 50 and 5 units, respectively. MRM acquisition mode was utilized for the analysis. The optimized MS/MS parameters for the monitored mycotoxins can be found in **Table 3.4**. For each mycotoxin, two *m*/*z* transitions were monitored. The identification of the analyte was based on the evaluation of the retention time, as well as the quantifier and qualifier transitions (92). The Thermo Xcalibur Qual Browser was used for analyzing the MS data. The obtained chromatograms of the 10 µg/L standard in soy beverage can be found in **Attachment 3**.

Table 3.4: MS/MS parameters

Mycotoxin	Retention time (min)	Precursor ion (*m/z*)	Molecular ion	Product ions (*m/z*)	Collision energy (eV)
AFB1	7.57	313.1	$[M+H]^+$	285.1 (Q) 241.2 (q)	25 42
AFB2	7.37	315.2	$[M+H]^+$	259.0 (Q) 287.0 (q)	25 30
AFG1	7.16	329.0	$[M+H]^+$	243.0 (Q) 311.0 (q)	25 20
AFG2	6.94	331.3	$[M+H]^+$	285.0 (Q) 313.3 (q)	30 25
AOH	8.32	257.0	$[M-H]^-$	213.0 (Q) 215.0 (q)	25 29
AME	9.32	415.0	$[M+H]^+$	256.0 (Q) 228.0 (q)	42 20
TEN	8.51	271.0	$[M-H]^-$	312.1 (Q) 302.0 (q)	25 32
FB1	8.61	722.4	$[M+H]^+$	334.0 (Q) 352.0 (q)	43 42
FB2	9.43	706.4	$[M+H]^+$	318.1 (Q) 354.0 (q)	42 42
HT-2	8.33	442.0	$[M+NH_4]^+$	168.9 (Q) 105.0 (q)	12 26
T-2	8.81	484.4	$[M+NH_4]^+$	185.0 (Q) 215.0 (q)	22 21
OTA	8.87	404.5	$[M+H]^+$	238.7 (Q) 101.7 (q)	21 68
ZEN	9.12	317.2	$[M-H]^-$	131.0 (Q) 175.0 (q)	32 32
ENNB	10.94	640.6	$[M+H]^+$	196.2 (Q) 186.2 (q)	29 37
ENNB1	11.14	654.2	$[M+H]^+$	196.0 (Q) 210.0 (q)	25 25
ENNA	11.60	682.2	$[M+H]^+$	210.0 (Q) 99.99 (q)	25 47
ENNA1	11.36	668.2	$[M+H]^+$	210.0 (Q) 100.0 (q)	10 26
BEA	11.10	801.0	$[M+NH_4]^+$	244.0 (Q) 134.0 (q)	36 54
DON	4.48	355.2	$[M+Ac]^-$	295.0 (Q) 265.0 (q)	13 17

AFB1: aflatoxin B1, AFB2: aflatoxin B2, AFG1: aflatoxin G1, AFG2: aflatoxin G2, AOH: alternariol, AME: alternariol monomethyl ether, TEN: tentoxin, FB1: fumonisin B1, FB2: fumonisin B2, HT-2: HT-2 toxin, T-2: T-2 toxin, OTA: ochratoxin A, ZEN: zearalenone, ENNB: enniatin B, ENNB1: enniatin B1, ENNA: enniatin A, ENNA1: enniatin A1, BEA: beauvericin, DON: deoxynivalenol, min: minutes, *m/z*: mass-to-charge ratio, eV: elektronvolt, Q: quantifier, q: qualifier

4. RESULTS

4.1 METHOD CHARACTERIZATION

4.1.1 Soy-based beverage

The data presented in **Table 4.1** display the limits of detection (LOD) and limits of quantification (LOQ) for soy-based beverages determined by signal-to-noise ratios (S/N) of 3 and 10, respectively. The lowest LOQ is 0.0067 µg/L for BEA, while the highest LOQ is 1.5 µg/L for DON. Additionally, **Table 4.1** shows the linearity equations of the calibration curves with an overall coefficient of determination (R^2) > 0.98.

Table 4.1: Linearity and sensitivity of the LC/MS-MS method for soy-based beverages

MYCOTOXIN	LOD (µg/L)	LOQ (µg/L)	Linearity equation	R^2
AFB1	0.052	0.17	$y = 2988.6\,x - 933.2$	0.99
AFB2	0.060	0.20	$y = 4062.9\,x - 3378.9$	0.99
AFG1	0.090	0.30	$y = 4711.3\,x - 6215.4$	0.99
AFG2	0.018	0.060	$y = 848.04\,x + 13825$	0.98
AOH	0.024	0.080	$y = 183.6\,x + 0.553$	0.99
AME	0.012	0.040	$y = 1998.5\,x + 43.021$	0.99
TEN	0.0090	0.030	$y = 5465.7\,x - 173.25$	0.99
FB1	0.018	0.060	$y = 672.66\,x - 1519.8$	0.99
FB2	0.010	0.034	$y = 1073.3\,x - 2336$	0.99
HT-2	0.018	0.060	$y = 314.97\,x + 1344.1$	0.99
T-2	0.018	0.060	$y = 375.78\,x - 8.0201$	0.99
OTA	0.014	0.048	$y = 882.5\,x - 710.04$	0.99
ZEN	0.014	0.048	$y = 291.17\,x + 16.693$	0.99
ENNB	0.0036	0.012	$y = 39356\,x + 24847$	0.99
ENNB1	0.0036	0.012	$y = 30737\,x + 5991.8$	0.99
ENNA	0.0030	0.010	$y = 24908\,x + 30245$	0.98
ENNA1	0.0060	0.020	$y = 23343\,x + 15819$	0.99
BEA	0.0020	0.0067	$y = 31002\,x - 19777$	0.99
DON	0.45	1.5	$y = 38.289\,x - 34.883$	0.99

AFB1: aflatoxin B1, AFB2: aflatoxin B2, AFG1: aflatoxin G1, AFG2: aflatoxin G2, AOH: alternariol, AME: alternariol monomethyl ether, TEN: tentoxin, FB1: fumonisin B1, FB2: fumonisin B2, HT-2: HT-2 toxin, T-2: T-2 toxin, OTA: ochratoxin A, ZEN: zearalenone, ENNB: enniatin B, ENNB1: enniatin B1, ENNA: enniatin A, ENNA1: enniatin A1, BEA: beauvericin, DON: deoxynivalenol, LOD: limit of detection, LOQ: limit of quantification, R^2: coefficient of determination

4.1.2 Almond-based beverage

Table 4.2 illustrates the LOD and LOQ, which were established using S/N of 3 and 10, respectively. The lowest LOQ recorded is 0.017 µg/L for ENNB1, whereas the highest LOQ observed is 0.24 µg/L for HT-2. Moreover, **Table 4.2** exhibits the linear equations of the calibration curves for each mycotoxin with again an R^2 greater than 0.98.

Table 4.2: Linearity and sensitivity of the LC/MS-MS method for almond-based beverages

MYCOTOXIN	LOD (µg/L)	LOQ (µg/L)	Linearity equation	R^2
AFB1	0.010	0.034	$y = 8377.7\,x - 76.619$	0.99
AFB2	0.012	0.040	$y = 5846.5\,x + 4020.2$	0.99
AFG1	0.018	0.060	$y = 9251.1\,x + 7701.6$	0.99
AFG2	0.060	0.20	$y = 1967.5\,x + 1064.8$	0.99
AOH	0.012	0.040	$y = 390.36\,x - 96.163$	0.99
AME	0.018	0.060	$y = 4226.8\,x + 203.49$	0.99
TEN	0.0072	0.024	$y = 8543.6\,x + 5414.7$	0.99
FB1	0.012	0.040	$y = 614.82\,x - 1841.9$	0.98
FB2	0.090	0.30	$y = 636.85\,x - 1259.4$	0.99
HT-2	0.072	0.24	$y = 542.17\,x + 207.19$	0.98
T-2	0.018	0.060	$y = 426.44\,x + 184.64$	0.99
OTA	0.018	0.060	$y = 840.2\,x + 518.94$	0.98
ZEN	0.018	0.060	$y = 517.26\,x + 28.971$	0.99
ENNB	0.0060	0.020	$y = 13058\,x - 1232$	0.99
ENNB1	0.0052	0.017	$y = 7009.2\,x + 3047.6$	0.98
ENNA	0.0060	0.020	$y = 3489.4\,x - 547.04$	0.98
ENNA1	0.0090	0.030	$y = 3896.8\,x + 1136.5$	0.99
BEA	0.010	0.034	$y = 2092.6\,x - 716.08$	0.99
DON	0.018	0.060	$y = 67.714\,x + 66.048$	0.99

AFB1: aflatoxin B1, AFB2: aflatoxin B2, AFG1: aflatoxin G1, AFG2: aflatoxin G2, AOH: alternariol, AME: alternariol monomethyl ether, TEN: tentoxin, FB1: fumonisin B1, FB2: fumonisin B2, HT-2: HT-2 toxin, T-2: T-2 toxin, OTA: ochratoxin A, ZEN: zearalenone, ENNB: enniatin B, ENNB1: enniatin B1, ENNA: enniatin A, ENNA1: enniatin A1, BEA: beauvericin, DON: deoxynivalenol, LOD: limit of detection, LOQ: limit of quantification, R^2: coefficient of determination

4.2 MYCOTOXIN OCCURRENCE

The occurrence of 19 mycotoxins was studied in 31 soy drink samples and 33 almond drink samples in duplicate. Of the 19 mycotoxins, AFG2, ENNB, ENNA, and ENNA1 were never detected above LOD in soy-based beverages, while AFB2, AFG1, and AFG2 were not detected in the almond beverage samples. The most frequently detected mycotoxin is BEA, which is found in all the samples. **Figure 4.1** depicts the occurrence of mycotoxins in soy- and almond-based beverages presented as a heat map.

	Soy-based beverage	Almond-based beverage	Soy-based beverage + almond-based beverage
AFB1	35,48%	9,09%	21,88%
AFB2	61,29%	0,00%	29,69%
AFG1	29,03%	0,00%	14,06%
AFG2	0,00%	0,00%	0,00%
AOH	54,84%	75,76%	65,63%
AME	16,13%	30,30%	23,44%
TEN	61,29%	12,12%	35,94%
FB1	67,74%	60,61%	64,06%
FB2	54,84%	63,64%	59,38%
HT-2	9,68%	42,42%	26,56%
T-2	48,39%	24,24%	35,94%
OTA	90,32%	9,09%	48,44%
ZEN	22,58%	12,12%	17,19%
ENNB	0,00%	96,97%	50,00%
ENNB1	9,68%	6,06%	7,81%
ENNA	0,00%	93,94%	48,44%
ENNA1	0,00%	12,12%	6,25%
BEA	100,00%	100,00%	100,00%
DON	3,23%	21,21%	12,50%

Figure 4.1: Heat map with a graded color scale that shows the occurrence (%) of the mycotoxins in the 31 soy drink samples, in the 33 almond drink samples, and in the total 64 samples. ■ = Lowest value, ■ = 50th percentile, ■ = Highest value.
AFB1: aflatoxin B1, AFB2: aflatoxin B2, AFG1: aflatoxin G1, AFG2: aflatoxin G2, AOH: alternariol, AME: alternariol monomethyl ether, TEN: tentoxin, FB1: fumonisin B1, FB2: fumonisin B2, HT-2: HT-2 toxin, T-2: T-2 toxin, OTA: ochratoxin A, ZEN: zearalenone, ENNB: enniatin B, ENNB1: enniatin B1, ENNA: enniatin A, ENNA1: enniatin A1, BEA: beauvericin, DON: deoxynivalenol

4.3 MEAN, MEDIAN, MINIMUM, AND MAXIMUM

4.3.1 Soy-based beverage

Table 4.3 shows the mean, median, minimum, and maximum concentrations of mycotoxins observed in the soy-based beverage samples. The maximum observed concentration in the soy samples was 0.87 µg/L for ZEN. The mean, median, minimum, and maximum values have been calculated considering only positive samples.

Table 4.3: Mean, median, minimum, and maximum contamination values for the studied mycotoxins in soy-based beverages considering the positive samples

Mycotoxin	Mean (µg/L)	Median (µg/L)	Min (µg/L)	Max (µg/L)
AFB1	0.079	0.069	0.053	0.14
AFB2	0.11	0.11	0.10	0.15
AFG1	0.18	0.17	0.16	0.23
AFG2	N.d.	N.d.	N.d.	N.d.
AOH	0.057	0.050	0.032	0.099
AME	0.019	0.019	0.014	0.027
TEN	0.047	0.036	0.0093	0.15
FB1	0.29	0.28	0.27	0.37
FB2	0.27	0.27	0.26	0.31
HT-2	0.19	0.13	0.078	0.37
T-2	0.081	0.071	0.040	0.21
OTA	0.19	0.11	0.098	0.70
ZEN	0.34	0.36	0.033	0.87
ENNB	N.d.	N.d.	N.d.	N.d.
ENNB1	0.026	0.031	0.0074	0.039
ENNA	N.d.	N.d.	N.d.	N.d.
ENNA1	N.d.	N.d.	N.d.	N.d.
BEA	0.12	0.086	0.083	0.32
DON	0.47	0.47	0.47	0.47

AFB1: aflatoxin B1, AFB2: aflatoxin B2, AFG1: aflatoxin G1, AFG2: aflatoxin G2, AOH: alternariol, AME: alternariol monomethyl ether, TEN: tentoxin, FB1: fumonisin B1, FB2: fumonisin B2, HT-2: HT-2 toxin, T-2: T-2 toxin, OTA: ochratoxin A, ZEN: zearalenone, ENNB: enniatin B, ENNB1: enniatin B1, ENNA: enniatin A, ENNA1: enniatin A1, BEA: beauvericin, DON: deoxynivalenol, N.d: Not detected

4.3.2 Almond-based beverage

Table 4.4 displays the mean, median, as well as the minimum and maximum concentrations identified in the almond drink samples. The calculations for the mean, median, minimum, and maximum values were derived solely from the positive samples. The highest concentration observed in the almond samples amounted to 2.2 µg/L for HT-2 and BEA.

Table 4.4: Mean, median, minimum, and maximum contamination values for the studied mycotoxins in almond-based beverages considering the positive samples

Mycotoxin	Mean (µg/L)	Median (µg/L)	Min (µg/L)	Max (µg/L)
AFB1	0.076	0.029	0.014	0.19
AFB2	N.d.	N.d.	N.d.	N.d.
AFG1	N.d.	N.d.	N.d.	N.d.
AFG2	N.d.	N.d.	N.d.	N.d.
AOH	0.075	0.049	0.033	0.56
AME	0.086	0.057	0.018	0.41
TEN	0.13	0.11	0.023	0.28
FB1	0.37	0.37	0.18	0.49
FB2	0.25	0.25	0.24	0.29
HT-2	0.37	0.099	0.074	2.2
T-2	0.19	0.11	0.033	0.60
OTA	0.30	0.30	0.18	0.42
ZEN	0.096	0.11	0.025	0.13
ENNB	0.060	0.029	0.015	0.68
ENNB1	1.0	1.0	0.30	1.7
ENNA	0.042	0.024	0.020	0.52
ENNA1	0.66	0.39	0.18	1.7
BEA	0.25	0.090	0.052	2.2
DON	0.32	0.19	0.045	0.79

AFB1: aflatoxin B1, AFB2: aflatoxin B2, AFG1: aflatoxin G1, AFG2: aflatoxin G2, AOH: alternariol, AME: alternariol monomethyl ether, TEN: tentoxin, FB1: fumonisin B1, FB2: fumonisin B2, HT-2: HT-2 toxin, T-2: T-2 toxin, OTA: ochratoxin A, ZEN: zearalenone, ENNB: enniatin B, ENNB1: enniatin B1, ENNA: enniatin A, ENNA1: enniatin A1, BEA: beauvericin, DON: deoxynivalenol, N.d: Not detected

4.4 MYCOTOXIN CO-OCCURRENCE

4.4.1 Soy-based beverage

In this study, a total of 30 combinations of mycotoxins were identified considering the 31 soy drink samples. Only one mycotoxin mixture (AFB1 + AFB2 + AFG1 + AOH + AME + TEN + FB1 + FB2 + T-2 + OTA + ZEN + BEA) occurred more than once. All the identified combinations of mycotoxins in soy-based beverages are listed in **Table 4.5**. The mycotoxin mixtures in soy-based beverages ranged from two mycotoxins to 12 mycotoxins. How often a certain number of mycotoxins occurs in soy-based beverages is shown in **Figure 4.2.** It shows that most samples contain five mycotoxins.

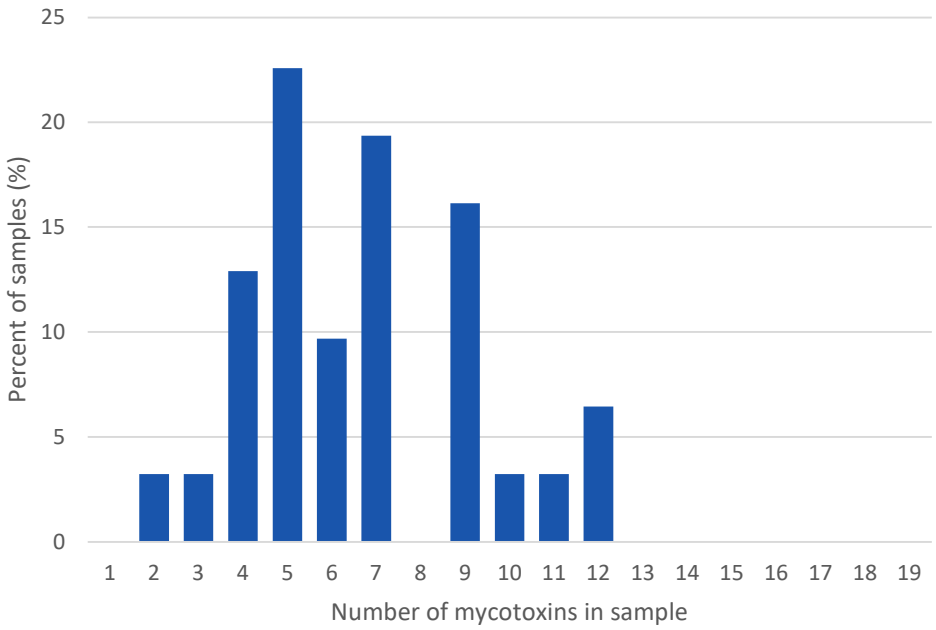


Figure 4.2: A clustered column chart depicting the percentage distribution of samples containing a specific number of mycotoxins. The percentages are calculated based on a total of 31 soy-based beverage samples.

Table 4.5: Mycotoxin mixtures found in soy-based beverages

Mycotoxin mixtures	Occurrence
AFB1 + AFB2 + AFG1 + AME + TEN + FB1 + FB2 + T-2 + OTA + ZEN + BEA	1
AFB1 + AFB2 + AFG1 + AOH + TEN + FB1 + FB2 + OTA + BEA	1
AFB1 + AFB2 + AFG1 + AOH + TEN + FB1 + FB2 + T-2 + OTA + BEA	1
AFB1 + AFB2 + AME + TEN + FB1 + FB2 + OTA + ZEN + BEA	1
AFB1 + AFB2 + AOH + TEN + FB1 + T-2 + OTA + BEA + DON	1
AFB1 + AFB2 + TEN + FB2 + T-2 + OTA + BEA	1
AFB1 + AFB2 + TEN + OTA + BEA	1
AFB1 + AOH + TEN + FB1 + FB2 + T-2 + OTA + ZEN + BEA	1
AFB1 + TEN + FB1 + OTA + BEA	1
AFB2 + AFG1 + AOH + TEN + FB1 + FB2 + T-2 + OTA + BEA	1
AFB2 + AOH + FB1 + BEA	1
AFB2 + AOH + FB1 + FB2 + OTA + BEA	1
AFB2 + AOH + FB1 + FB2 + T-2 + OTA + BEA	1
AFB2 + AOH + OTA + BEA	1
AFB2 + AOH + TEN + FB2 + T-2 + OTA + BEA	1
AFB2 + FB1 + FB2 + OTA + BEA	1
AFB2 + FB1 + HT-2 + OTA + BEA	1
AFB2 + FB1 + T-2 + OTA + BEA	1
AFB2 + OTA + BEA	1
AFG1 + AOH + FB1 + HT-2 + OTA + ENNB1 + BEA	1
AFG1 + TEN + OTA + BEA	1
AFG1 + TEN + T-2 + OTA + BEA	1
AOH + AME + TEN + T-2 + ZEN + ENNB1 + BEA	1
AOH + FB1 + FB2 + T-2 + OTA + BEA	1
AOH + TEN + FB1 + FB2 + T-2 + OTA + BEA	1
AOH + TEN + FB2 + OTA + BEA	1
FB1 + BEA	1
HT-2 + OTA + ENNB1 + BEA	1
TEN + FB1 + FB2 + OTA + ZEN + BEA	1
AFB1 + AFB2 + AFG1 + AOH + AME + TEN + FB1 + FB2 + T-2 + OTA + ZEN + BEA	2

AFB1: aflatoxin B1, AFB2: aflatoxin B2, AFG1: aflatoxin G1, AFG2: aflatoxin G2, AOH: alternariol, AME: alternariol monomethyl ether, TEN: tentoxin, FB1: fumonisin B1, FB2: fumonisin B2, HT-2: HT-2 toxin, T-2: T-2 toxin, OTA: ochratoxin A, ZEN: zearalenone, ENNB: enniatin B, ENNB1: enniatin B1, ENNA: enniatin A, ENNA1: enniatin A1, BEA: beauvericin, DON: deoxynivalenol

4.4.2 Almond-based beverage

Considering 33 almond-based beverage samples 27 unique combinations of mycotoxins were found. Four mycotoxin combinations occurred twice, one mycotoxin mixture occurred three times, and the other combinations only occurred once. The mycotoxin mixtures found in almond beverages are provided in **Table 4.6**. The frequency of occurrence for each number of mycotoxins in almond beverages is visualized in **Figure 4.3**. This figure shows that the combinations range from three (AOH + FB2 + BEA) to 12 (AFB1 + AME + TEN + FB1 + HT-2 + T-2 + OTA + ZEN + ENNB + ENNA + ENNA1 + BEA) mycotoxins. Additionally, it demonstrates that one-third of the samples have a combination of seven mycotoxins. In **Attachment 4,** the mycotoxin combinations in the total 64 samples of soy and almond beverages are presented. None of the mycotoxin combinations detected in soy drinks were present in almond beverages, resulting in a cumulative count of 57 unique mycotoxin mixtures across 64 samples.

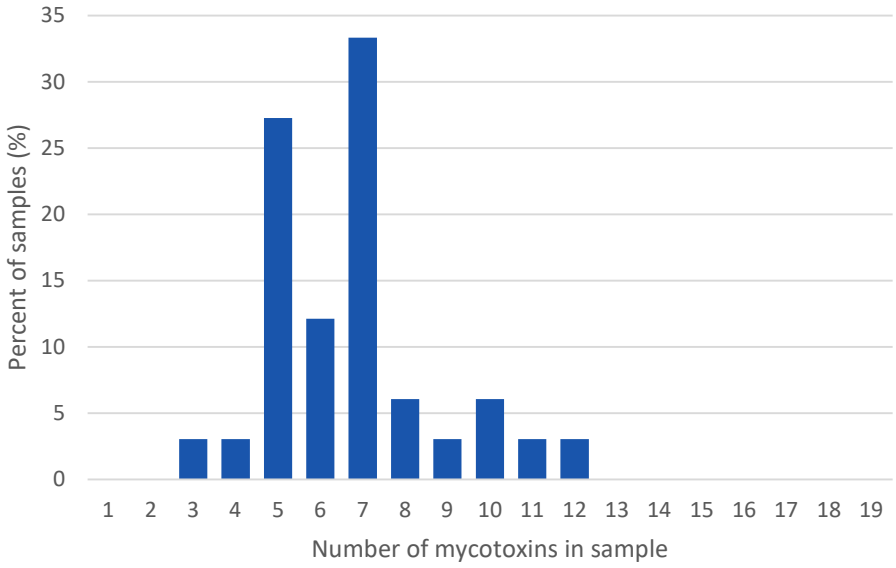


Figure 4.3: Clustered column chart that illustrates the percentage distribution of samples containing precisely ‘’x’’ number of mycotoxins. The percentage is computed on a total of 33 almond-based beverage samples.

Table 4.6: Mycotoxin mixtures found in almond-based beverages

Mycotoxin mixtures	Occurrence
AFB1 + AME + TEN + FB1 + HT-2 + T-2 + OTA + ZEN + ENNB + ENNA + ENNA1 + BEA	1
AFB1 + AME + TEN + HT-2 + T-2 + ENNB + ENNA + BEA	1
AFB1 + AOH + ENNB + ENNA + BEA	1
AOH + AME + ENNB + ENNA + BEA	1
AOH + AME + FB1 + FB2 + HT-2 + T-2 + ZEN + ENNB + ENNA + BEA + DON	1
AOH + AME + FB2 + HT-2 + ENNB + ENNA + BEA	1
AOH + AME + FB2 + HT-2 + ZEN + ENNB + ENNB1 + ENNA + ENNA1 + BEA	1
AOH + AME + T-2 + ENNB + ENNA + BEA + DON	1
AOH + AME + TEN + FB1 + FB2 + ENNB + ENNA + BEA	1
AOH + FB1 + FB2 + ENNB + ENNA + BEA	1
AOH + FB1 + FB2 + T-2 + ENNB + ENNA + BEA	1
AOH + FB1 + HT-2 + T-2 + ENNB + ENNB1 + ENNA + ENNA1 + BEA + DON	1
AOH + FB2 + BEA	1
AOH + HT-2 + ENNB + ENNA + BEA	1
AOH + TEN + FB1 + HT-2 + ENNB + ENNA + BEA	1
AOH +FB2 + HT-2 + ENNB + ENNA + BEA	1
ENNB + ENNA + BEA + DON	1
FB1 + FB2 + ENNB + ENNA + BEA	1
FB1 + FB2 + HT-2 + ENNB + ENNA + BEA	1
FB1 + FB2 + HT-2 + ENNB + ENNA + BEA + DON	1
FB1 + FB2 + T-2 + OTA + ZEN + ENNB + ENNA + ENNA1 + BEA	1
FB2 + HT-2 + T-2 + OTA + ENNB + BEA	1
AOH + AME + FB1 + FB2 + ENNB + ENNA + BEA	2
AOH + FB1 + FB2 + ENNB + ENNA + BEA + DON	2
AOH + FB1 + FB2 + HT-2+ ENNB + ENNA + BEA	2
AOH + FB2 + ENNB + ENNA + BEA	2
AOH + FB1 + ENNB + ENNA + BEA	3

AFB1: aflatoxin B1, AFB2: aflatoxin B2, AFG1: aflatoxin G1, AFG2: aflatoxin G2, AOH: alternariol, AME: alternariol monomethyl ether, TEN: tentoxin, FB1: fumonisin B1, FB2: fumonisin B2, HT-2: HT-2 toxin, T-2: T-2 toxin, OTA: ochratoxin A, ZEN: zearalenone, ENNB: enniatin B, ENNB1: enniatin B1, ENNA: enniatin A, ENNA1: enniatin A1, BEA: beauvericin, DON: deoxynivalenol

5. DISCUSSION

5.1 METHOD CHARACTERIZATION

The overall coefficient of determination for both the soy and almond beverage samples was higher than 0.98. All compounds demonstrated good linearity across the concentration range examined. Also, acceptable LOQ values were achieved, ranging from 0.0067 µg/L (for BEA) to 1.5 µg/L (for DON) in soy beverages, and from 0.017 µg/L (for ENNB1) to 0.30 µg/L (for FB2) in almond drinks.

The obtained LOQs are similar, or even lower compared to previously reported values in the literature. For instance, previous reports indicated LOQs of 0.3 µg/L – 0.8 µg/L for enniatins in soy drinks, whereas the LOQs achieved in this study for enniatins in soy drinks ranged between 0.010 µg/L and 0.020 µg/L (93). Additionally, another study reported higher LOQ values than in this thesis, with LOQs spanning from 0.5 µg/L for aflatoxins to 7.3 µg/L for HT-2 in almond beverages. For soy drinks, the LOQ varied from 0.4 µg/L for aflatoxins to 6.5 µg/L for HT-2 (94). Only the LOQ for DON in the soy drink is notably high in this thesis compared to the LOQ determined for DON in soy beverages in other studies, which stands at 0.3 µg/L – 0.48 µg/L (14,95).

5.2 MYCOTOXIN OCCURRENCE

5.2.1 Soy-based beverage

In each analyzed sample of soy beverage, mycotoxin contamination was observed. BEA was uniformly detected across all samples, whereas ENNB, ENNA, ENNA1, and AFG2 were not detected. AFB1 was found in 11 of the 31 samples with a concentration range between 0.053 µg/L and 0.14 µg/L. 19 out of 31 samples were positive for AFB2 and 9 out of 31 were positive for AFG1. The AFB2 content varied between 0.10 µg/L and 0.15 µg/L, while the concentration of AFG1 ranged from 0.16 µg/L to 0.23 µg/L. Aflatoxins are recognized as the most mutagenic and carcinogenic toxins, with AFB1 acknowledged as the most potent (96). The IARC has classified aflatoxins as a group 1 carcinogen and has determined the toxicity order of aflatoxins as follows: AFB1 > AFG1 > AFB2 > AFG2 (97).

Legislation regarding mycotoxins in soy is absent. However, comparing concentrations with the maximum levels of aflatoxins in other foods can provide insight into the significance of the detected concentrations. In Commission regulation No 1881/2006, the maximum levels for AFB1 range from 0.10 μg/kg for processed cereal-based foods and baby foods for infants and young children, to 12.0 μg/kg for almonds, pistachios and apricot kernels subjected to physical treatment (61). The maximum concentration of AFB1 in soy beverages is higher than the maximum level of AFB1 in cereal-based foods for babies and children. More specifically two samples exceeded this limit with concentrations of 0.13 μg/L and 0.14 μg/L. Given that this threshold has been surpassed, further investigation may be necessary to estimate the potential hazard posed by aflatoxins in soy-based products.

Following a request from the European Commission, the Panel of Contaminants in the Food Chain (CONTAM panel) has issued a scientific assessment regarding the potential human health hazards associated with aflatoxins found in food. The risk evaluation incorporated over 200000 analytical results on the occurrence of aflatoxins. The data derived from this risk assessment showed that AFB1 was found in soy drinks with contents ranging from 0.00 μg/L to 0.21 μg/L. The concentrations of AFB1 discovered in this study fall within this range. AFB2 had concentrations up to 0.20 μg/L in soy drinks. AFG1 in soy drinks had concentrations between 0.00 μg/L and 0.30 μg/L, while AFG2 between 0.0 μg/L and 0.20 μg/L (96). Also, the concentrations of AFB2 and AFG1 observed in this research fall within the ranges found in the risk assessment of the EFSA (96). In an Iranian study, AFB1 was detected in soy beverages within a range of 1.6-3.9 ng/L, while another study conducted in Spain reported a mean concentration of AFB1 at 0.05 μg/L (95,98). Another recent investigation examining 10 soy drink samples did not detect aflatoxins at all (65). In comparison to these studies, our findings exhibit higher levels of AFB1 presence.

AOH was detected in 55% of the soy beverage samples with a maximum content of 0.099 μg/L. 5/31 samples were positive for AME with a mean value of 0.019 μg/L, while 19/31 samples were positive for TEN with concentrations between 0.0093 μg/L and 0.15 μg/L. Regarding TEN, only one other study on its occurrence in soy drinks has been published. In that study, TEN was not detected. However, it is noteworthy that their LOQ was set at

0.31 µg/L, a relatively high threshold, which may account for the absence of contaminated samples (65). *Alternaria* mycotoxins are currently not regulated and do not have official maximum limits, but there are indicative levels for AOH and AME published by EFSA. The indicative levels for both AOH and AME range from 2.0 µg/kg in cereal-based foods for infants and young children, to 30 µg/kg in sesame and sunflower seeds (62). The concentrations in this thesis are significantly below these indicative thresholds.

FB1 and FB2 were detected in 21 and 17 samples with concentrations ranging from 0.27-0.37 µg/L and 0.26-0.31 µg/L, respectively. Despite the concentrations of FB1 and FB2 were always above the LOQ, these concentrations are negligible compared to the maximum limits of fumonisins in other foods which go from 200 µg/kg to 4000 µg/kg (61). In previous studies, fumonisins have not been detected in soy beverages. It is pertinent to consider that these studies encompassed sample sizes of 9, 3, and 10 soy beverage samples, respectively (14,65,95). This factor may elucidate the absence of detection.

Regarding the trichothecenes, HT-2 was found in 3 soy drink samples with a maximal concentration of 0.37 µg/L. T-2 toxin was detected in 48% of the samples with concentrations ranging from 0.040 to 0.21 µg/L. EFSA also did a risk assessment for HT-2 and T-2, but there is no data specific to soy beverages. However, data regarding these toxins in soybeans are available, indicating concentrations ranging from 0.00 µg/kg to 1.97 µg/kg for HT-2 and from 0.00 µg/kg to 0.71 µg/kg for T-2 (99). The concentration levels observed in this research align with these reported ranges. In other studies on soy-based beverages HT-2 and T-2 were not detected; however, these studies had small sample sizes of 9, 3, and 10 (14,65,95). DON was detected in one soy drink sample, exhibiting a concentration of 0.47 µg/L. Similar observations are noted in other studies where DON detection in soy beverages was also infrequent. Two studies in Spain reported no detection of DON in any sample, while another study in Latvia identified DON in one of the ten samples, registering a concentration of 2.0 µg/L (14,65,100).

OTA was observed in 90% of the samples with contents ranging from 0.098 µg/L to 0.70 µg/L. The maximum levels set up for OTA in other foods vary from 0.5 µg/kg for cereal-based food for infants and children, to 10 µg/kg for dried vine fruits (61). The maximum concentration identified in this thesis surpasses the established maximum level for OTA in cereal-based food intended for infants and children. It is difficult to determine to what extent values in soy-based beverages are comparable to values in cereal-based foods for children. Nonetheless, the exceeding of this limit and the high occurrence signify a concerning trend, indicating the necessity for additional investigations into regulatory thresholds for plant-based beverages. The maximum limit for OTA in unprocessed cereals is 5.0 µg/kg (61). Compared to this value the concentrations found in this study do not exceed this limit.

23% of the samples were positive for ZEN with a maximal concentration of 0.87 µg/L, this is the highest concentration found for any mycotoxin in soy drink in this study. ZEN was studied in soy beverages in two other studies, of which one reported a maximum concentration of 0.27 µg/L, and the other study published a non-specified value below LOQ (14,65). Compared to ZEN in soybeans and soybean meals, values of 10 to 807 µg/kg are reported (101). These higher concentrations observed in soybeans are understandable, as the mycotoxin concentration inherent to soybeans becomes diluted within soy beverages. In the soy beverage samples in this thesis, soy content ranges from 5.7% to 15%, thus contributing to this dilution effect.

Among the enniatins, only ENNB1 was detected in 3 of the 31 samples with a mean concentration of 0.026 µg/L. In an occurrence study carried out in Latvia, ENNA and ENNA1 were also not detected, while ENNB and ENNB1 were detected in a range of 0.15 µg/L to 0.32 µg/L (65). High concentrations of ENNB and ENNB1 within the range of 11 µg/L to 22 µg/L were reported in a study in Spain (93). ENNA and ENNA1 had a concentration ranging from <LOD to 2.2 µg/L. In another study, ENNB, ENNB1, and ENNA were once detected with a concentration of 0.5 µg/L, 0.29 µg/L, and 0.19 µg/L, respectively. ENNA1 was twice detected with a concentration of 0.12 µg/L and 0.23 µg/L (95). Significant variations in concentrations were observed in these studies. The fact that the used raw

materials originate from different regions with different climates may contribute to the diverse concentrations. Lastly, BEA had the highest frequency of detection among mycotoxins in soy drinks, being present in every sample with concentrations ranging from 0.086 µg/L to 0.32 µg/L. A study by Rodríguez-Cañás et al. reported an incidence rate of BEA in soy beverages of 66% with a mean concentration of 0.39 µg/L, and another study by Pavlenko et al. documented a mean concentration of 0.12 µg/L (65,95). These concentration levels align closely with our findings.

5.2.2 Almond-based beverage

In almond beverage samples, the detection frequency of aflatoxins was notably low. AFB2, AFG1, and AFG2 were not detected, while AFB1 was identified in three of the 33 samples, ranging in concentrations from 0.014 µg/L to 0.19 µg/L. The European Commission has established maximum levels for aflatoxins in nuts used as ingredients in food products, setting the maximal concentration for AFB1 at 5.0 µg/kg and 10.0 µg/kg for the sum of AFB1, AFB2, AFG1, and AFG2. The concentrations found in the three samples did not exceed these maximum levels. The risk assessment data provided by the CONTAM Panel regarding aflatoxins in food indicated the presence of AFB1 in almond-based beverages, with concentrations ranging from 0.00 µg/L to 0.52 µg/L. The concentrations found in this study fall within this reported range. The range for AFB1 in almond drinks is higher compared to the concentration range found in the risk assessment for AFB1 in soy drinks, which is 0.00 µg/L to 0.21 µg/L (96). However, in this thesis, the AFB1 contamination is higher in soy drinks than in almond drinks. A plausible rationale for this discrepancy is that there exist regulations governing aflatoxin levels in almonds and not in soybeans. This may prompt the use of almonds of higher quality and the adoption of a more stringent processing methodology for almonds to mitigate fungal and mycotoxin contamination. Additionally, the undisclosed origin of the almonds and soybeans utilized in the production of plant-based beverages may further contribute to this differentiation.

In a study performed in Spain, AFB1 was detected in almond drinks with a mean concentration of 0.03 µg/L, and AFB2 was not detected (95). In another study carried out in Valencia, AFB1, AFG1, and AFG2 were not detected, while AFB2 was detected with a

maximum concentration of 0.7 µg/L (94). In a last comparative study, AFG1 and AFG2 were not detected in nut-based beverages, whereas AFB1 and AFB2 were found within a range of 0.048 µg/L to 0.067 µg/L and 0.010 µg/L to 0.024 µg/L, respectively (65). Thus AFG1 and AFG2 were consistently undetected across all four studies, whereas AFB1 and AFB2 were occasionally identified.

Regarding the *Alternaria* toxins, AOH was observed in 76% of the samples with a maximum concentration of 0.56 µg/L. In 10 samples AME was found with contents ranging from 0.018 µg/L to 0.41 µg/L. These concentrations are lower than the indicative levels set by EFSA, which range from 2.0 µg/kg to 30 µg/kg for AOH and AME in other food products (62). TEN was detected in 12% of the samples with a maximum concentration of 0.28 µg/L. In a comparative study, AOH and AME were not detected, and TEN exhibited a high mean positive concentration of 82.81 µg/L (94). Another investigation reported the detection of AME in all almond-based beverage samples, with a mean concentration of 0.3 µg/L. AOH and TEN were not assessed in this study (95). Lastly, another study found no detection of AME, AOH, or TEN in nut-based beverages (65). Significant disparities exist in the findings concerning the occurrence and concentration of these toxins, underscoring the need for further research.

Concentrations of FB1 and FB2 were found in 61% and 64% of the samples, respectively. The detected concentrations for FB1 ranged from 0.18 µg/L to 0.49 µg/L, while FB2 concentrations ranged from 0.24 µg/L to 0.29 µg/L. These concentrations are significantly lower in comparison to the maximum allowable levels of fumonisins found in other food products, spanning from 200 µg/kg to 4000 µg/kg (61). In a study conducted in Spain, FB1 and FB2 were detected in 42% of the samples, with concentrations ranging from 0.72 µg/L to 0.84 µg/L and 0.75 µg/L to 1.3 µg/L, respectively (95). So the detected concentrations in this thesis appear relatively low compared to those reported in the Spanish study. The risk posed by FB1 to agricultural crops is more frequently reported in regions located within temperature tropical regions (102). Hence, this could potentially account for the observed concentration disparities between the United Kingdom and Spain.

Concerning the trichothecenes, HT-2 was detected in 14 out of the 33 samples, with contents ranging from 0.074 µg/L to 2.2 µg/L. T-2 was observed in 24% of the samples with a maximum concentration of 0.60 µg/L. Slightly higher concentrations were reported in a study conducted by Juan et al., where maximum concentrations of 3.6 µg/L for HT-2 and 2.2 µg/L for T-2 were observed (94). No data concerning the presence of HT-2 and T-2 in almond drinks or nuts were incorporated in the risk assessment of EFSA. Indicative levels for the presence of HT-2 and T-2 are available solely for cereals and cereal products, with ranges spanning from 15 µg/kg to 2000 µg/kg for the sum of T-2 and HT-2. Although 2.2 µg/L is the highest concentration observed in this study, it can be deemed relatively low in comparison to the indicative maximum concentrations specified for cereals. 7 of the 33 samples were positive for DON with contents ranging from 0.045 µg/L to 0.79 µg/L. No data regarding DON presence in almond drinks is available, however, it has been observed in almonds. In a study conducted in Portugal, DON was detected in 36% of the almond samples, with a mean concentration of 2.85 µg/L (103).

OTA was detected in 3 samples with a maximum concentration of 0.42 µg/L, which is lower than the maximum levels set up for OTA, which vary from 0.5 µg/L to 8 µg/L. In previous studies involving almond beverages, OTA was either not detected or detected with a maximum concentration of 0.186 µg/L (94,95). Compared to these findings, the maximum concentration found in this thesis appears high. The risk assessment of EFSA did not include almond beverages, however, 513 almond samples were incorporated, yielding a mean upper bound concentration of 1.21 µg/kg (104). The maximum concentration of OTA observed in almond-based beverages within this study is beneath this upper bound concentration. ZEN was found in four samples with a mean concentration of 0.11 µg/L. In another study, ZEN was detected in almond beverages with a maximum concentration of 3.17 µg/L (94). In comparison to this study, our concentration was lower.

In contrast to the soy-based beverage samples, enniatins have exhibited frequent detection in the almond beverage samples. ENNB and ENNA were observed in 97% and 94% of the samples with contents ranging from 0.015 µg/L to 0.68 µg/L and 0.020 µg/L to

0.52 µg/L, respectively. ENNB1 and ENNA1 were detected less frequently, appearing in two samples for ENNB1 and four samples for ENNA1. While their occurrence was less frequent, the concentrations detected for ENNB1 and ENNA1 were relatively high compared to those for ENNB and ENNA. The maximum concentrations observed for both ENNB1 and ENNA1 were 1.7 µg/L. In a study conducted in Spain, ENNB and ENNB1 were detected in all the samples, while ENNA and ENNA1 were observed in 85% of the samples (95). In another study performed in Latvia, enniatins were not detected in their 23 nut beverage samples (65). Hence, substantial variability in findings and concentrations has been documented in the literature, potentially partly attributable to climate disparities and different crop management practices to reserve quality (105,106). Just as in the soy beverage samples, BEA was found in all almond drink samples with concentrations varying between 0.052 µg/L and 2.2 µg/L. A study conducted in Spain also observed a 100% occurrence of BEA in almond drinks, with contents ranging between 0.05 µg/L and 2.0 µg/L (95). These concentration levels consistently align with our findings.

5.3 MYCOTOXIN CO-OCCURRENCE

5.3.1 Soy-based beverage

30 different mixtures of mycotoxins were found in 31 samples ranging from two mycotoxins (FB1 + BEA) to 12 mycotoxins (AFB1 + AFB2 + AFG1 + AOH + AME + TEN + FB1 + FB2 + T-2 + OTA + ZEN + BEA). The mixture of these 12 mycotoxins occurred twice. All other combinations of mycotoxins found in the 31 soy-based beverage samples only occurred once. As shown in **Figure 4.2**, most samples had a combination of five mycotoxins.

Interesting to see is that in seven of the nine cases that AFB1 was detected, it co-occurred with FB1. Multiple animal and *in vitro* investigations have been conducted to examine the effects of co-exposure to aflatoxins and fumonisins. These studies have revealed either additive or synergistic effects on the development of precancerous lesions or liver cancer. For instance, a recent experiment involving mice showed that oral administration of pure AFB1 and FB1 resulted in an increase in relative spleen weight and elevated activity of enzymes linked to oxidative stress (107,108). Another study conducted on broiler chicks revealed that simultaneous exposure to AFB1 and FB1 resulted in additive effects on body

weight, liver structure, and immunological response (109). Additionally, a rat feeding trial indicated that exposure to pure AFB1 or FB1 alone or in sequence had effects on body weight that were less than additive, while the effects on certain liver enzymes were synergistic. This supports the hypothesis that fumonisins might act as a promoter for aflatoxin-induced liver cancer (108,110).

In the literature, there are also synergistic outcomes reported concerning the co-occurrence of FB1 and OTA. In the 31 samples, OTA and FB1 are detected together 19 times. In a recent study on rat liver cells OTA and FB1 exhibited a synergistic cytotoxic influence on rat liver cells by promoting apoptosis (111). Also, observations in C6 glioma cells, Caco-2 cells, and Vero cells revealed a synergistic relationship between OTA and FB1. While FB1 is minimally cytotoxic or non-cytotoxic, OTA displays moderate cytotoxicity. When these toxins were combined, the observed cytotoxicity exceeded the additive effects of the toxins when tested separately (112,113). However, these are *in vitro* studies and more research is needed to further investigate these effects *in vivo*.

5.3.2 Almond-based beverage

A total of 27 different mycotoxin mixtures were identified in the 33 samples analyzed, varying in composition from three mycotoxins (AOH + FB2 + BEA) to 12 mycotoxins (AFB1 + AME + TEN + FB1 + HT-2 + T-2 + OTA + ZEN + ENNB + ENNA + ENNA1 + BEA). Four mycotoxin combinations occurred twice (AOH + AME + FB1 + FB2 + ENNB + ENNA + BEA, AOH + FB1 + FB2 + ENNB + ENNA + BEA + DON, AOH + FB1 + FB2 + HT-2+ ENNB + ENNA + BEA, and AOH + FB2 + ENNB + ENNA + BEA). The mycotoxin mixture that was detected the most frequently was AOH + FB1 + ENNB + ENNA + BEA, which occurred three times. All other mycotoxin mixtures occurred only once. As shown in **Figure 4.3**, most mycotoxin mixtures consisted of seven different mycotoxins.

In these almond beverage samples, it is noticeable that AME often co-occurred with AOH, specifically in seven mixtures. A study conducted in 2014 examined the combined effects of AOH and AME on human intestinal cell line HCT116 cells. Exposure to low doses of alternariols resulted in moderate cytotoxicity, leading to decreased cell viability through

activation of the mitochondrial apoptotic pathway. When combined, AME and AOH demonstrated a notable increase in their toxic effects. Overall, the study indicated that the combination of AOH and AME exhibits an additive effect (114). Additivism describes a situation where the combined effects of mycotoxins are equivalent to the sum of the effects observed when each mycotoxin is administered individually.

The literature delineates the potential for both synergistic and additive effects arising from specific combinations of mycotoxins. Consequently, the concurrent occurrence of mycotoxins necessitates consideration when delineating maximum limits.

5.4 STUDY LIMITATIONS AND STRENGTHS

The strength of this study lies in the application of a sophisticated analytical methodology. The use of UHPLC-MS/MS offers high sensitivity and specificity for the detection and quantification of mycotoxins, enhancing the reliability of the results. However, this study is limited by the relatively small sample size, encompassing 31 soy-based beverage samples and 33 almond-based beverage samples. Another constraint is the exclusive sourcing of samples from Cranfield, thereby rendering the results non-generalizable to the whole United Kingdom. Lastly, only nineteen mycotoxins have been included in this study, while many more mycotoxins exist.

6. CONCLUSIONS

First, the presence of 19 mycotoxins in 31 soy-based beverage and 33 almond-based beverage samples from Cranfield, United Kingdom was determined using UHPL-MS/MS. Each sample exhibited mycotoxin contamination. The occurrence of mycotoxins ranged from 0% (for AFG2) to 100% (for BEA). The highest concentration in almond beverages was 2.2 µg/L, observed for both HT-2 and BEA, whereas in soy beverages, the maximum observed concentration was 0.87 µg/L for ZEN. Currently, no established maximum limits exist for mycotoxins in plant-based beverages, although regulatory standards are in place for certain mycotoxins in other food categories. The maximum concentrations for AFB1 and OTA found in soy-based beverages exceeded the maximum levels of AFB1 and OTA in cereal-based foods for babies and children. It is difficult to determine to what extent soy beverage values are comparable to cereal-based foods for children. Nevertheless, surpassing these limits suggests a concerning trend, highlighting the need to further explore regulatory thresholds for mycotoxins in plant-based beverages.

Secondly, the co-occurrence of mycotoxins was examined in these samples. All samples exhibited contamination by at least two mycotoxins, with a maximum of 12 mycotoxins detected. The literature describes potential synergistic and additive effects resulting from specific combinations of mycotoxins. Hence, the simultaneous presence of mycotoxins should be taken into account when establishing maximal limits or conducting risk assessments.

In conclusion, the swift growth of the plant-based dairy alternatives market, largely driven by health considerations and awareness of sustainability, raises the need for further research to evaluate the quality and safety of these beverages. In addition, the impact of climate change may increase the risk associated with mycotoxins. Hence, this study opted to examine mycotoxin levels in a Northern European country, the United Kingdom, where data on this matter are currently lacking. This research enriches the limited literature concerning mycotoxin levels in plant-based beverages, thereby advancing the understanding of food safety within this specific product category. Policymakers should

consider regulating mycotoxins in plant-based drinks, particularly in response to emerging dietary trends. However, additional investigation is warranted to provide more incidence data and assess the risk to consumers, facilitating the formulation of regulatory measures in plant-based beverages.

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8. ATTACHMENTS

Attachment 1: Soy-based beverages sample list

Code	Size	Ingredients	Nutritional information (100g)
S1	1L	Soya base (98.9%) (water, hulled soya beans (8.7%)), acidity regulators (potassium phosphates), calcium (calcium carbonate), flavourings, sea salt, stabiliser (gellan gum), vitamins B2, B12, D2.	Energie: 33 kcal; Fat: 1.8g, of which saturates: 0.3g; Carbohydrate: 0g, of which sugars: 0g; Fibre: 0.6g; Protein: 3.3g; Salt: 0.09g
S2	1L	Water, Soya Bean (9%), Sugar, Tricalcium Phosphate, Sea Salt, Stabiliser (Gellan Gum), Potassium Iodide, Vitamin B12, Riboflavin, Vitamin D2.	Energie: 42 kcal; Fat: 1.6g, of which saturates: 0.3g; Carbohydrate: 3.1g, of which sugars: 2.7g; Fibre: 0.4g; Protein: 3.5g; Salt: 0.20g
S3	1L	Soya base (97%) (water, hulled soya beans (8%)), sugar, acidity regulators (potassium phosphates), calcium (calcium carbonate), flavouring, sea salt, stabiliser (gellan gum), potassium iodide, Vitamins B2, B12, D2.	Energie: 39 kcal; Fat: 1.8g, of which saturates: 0.3g; Carbohydrate: 2.5g, of which sugars: 2.5g; Fibre: 0.5g; Protein: 3.0g; Salt: 0.08g
S4	1L	Soya base (89%) (water, hulled soya beans (7.4%)), sugar, cocoa (2%), chicory root fibre, maltodextrin, calcium (Tri-calcium phosphate), sea salt, natural flavourings, stabiliser (carrageenan), vitamins B2, D2.	Energie: 68 kcal; Fat: 2.1g, of which saturates: 0.6g; Carbohydrate: 8.0g, of which sugars: 6.4g; Fibre: 2.2g; Protein: 3.3g; Salt: 0.14g
S5	750 mL	Soya base (93.5%) (water, hulled soya beans (5.7%)), vegetable oils and fats (sunflower, shea), sugar, chicory root fibre, emulsifier (sucrose esters of fatty acids), sea salt, stabilisers (Locust bean gum, Gellan gum), Flavourings.	Energie: 61 kcal; Fat: 4.4g, of which saturates: 0.6g; Carbohydrate: 2.0g, of which sugars: 1.9g; Fibre: 1.6g; Protein: 2.1g; Salt: 0.15g
S6	1L	Soya base (96.9%) (water, hulled soya beans (13.1%)), sugar, acidity regulators (potassium phosphates), calcium (calcium carbonate), flavouring, sea salt, stabiliser (gellan gum), vitamins B2, B12, D2.	Energie: 57 kcal; Fat: 2.8g, of which saturates: 0.5g; Carbohydrate: 2.5g, of which sugars: 2.5g; Fibre: 0.9g; Protein: 5.0g; Salt: 0.10g
S7	1L	Water, peeled soybeans (9%), calcium carbonate, maltodextrin, stabiliser (gellan gum), natural flavouring, vitamins (riboflavin (B2), cyanocobalamin (B12), ergocalciferol (D2)).	Energie: 33 kcal; Fat: 1.7g, of which saturates: 0.2g; Carbohydrate: 1.4g, of which sugars: 0.5g; Fibre: 0.2g; Protein: 3.0g; Salt: 0.02g
S8	1L	Water, peeled soybeans (9%), apple extract, calcium carbonate, maltodextrin, stabiliser (gellan gum), sea salt, natural flavouring, vitamins (riboflavin (B2), cyanocobalamin (B12), ergocalciferol (D2)).	Energie: 42 kcal; Fat: 1.8g, of which saturates: 0.2g; Carbohydrate: 3.4g, of which sugars: 2.3g; Fibre: 0.3g; Protein: 3.0g; Salt: 0.12g
S9	1L	Soya base (92.8%) (water, hulled soya beans (7.8%)), sugar, calcium (calcium carbonate), flavourings, black carrot concentrate, sea salt, acidity regulator (potassium phosphates), stabiliser (gellan gum), vitamins B2, B12, D2.	Energie: 57 kcal; Fat: 1.8g, of which saturates: 0.3g; Carbohydrate: 6.8g, of which sugars: 6.8g; Fibre: 0.5g; Protein: 3.0g; Salt: 0.14g
S10	1L	Soya base (water, hulled soya beans (12%)), sugar, fat-reduced cocoa (1.6%), calcium (calcium carbonate), flavourings, sea salt, acidity regulator (potassium phosphates), stabiliser (gellan gum), vitamins (B2, D2).	Energie: 69 kcal; Fat: 2.8g, of which saturates: 0.6g; Carbohydrate: 5.3g, of which sugars: 5.0g; Fibre: 1.3g; Protein: 5.0g; Salt: 0.16g

S11	1L	Soya base (90.5%) (water, hulled soya beans (6.6%)), maltodextron, raw cane sugar, fructose, sunflower oil, calcium (calcium carbonate), acidity regulator (potassium phosphates), flavourings, emulsifier (lecithins (sunflower)), stabiliser (gellan gum), iron pyrophosphate, potassium iodide, vitamins b2, B12, C, D2.	Energie: 65 kcal; Fat: 2.1g, of which saturates: 0.3g; Carbohydrate: 8.6g, of which sugars: 2.5g; Fibre: 0.4g; Protein: 2.5g; Salt: 0.04g
S12	1L	Water, organic soya beans (12%), organic rice syrup, sea salt.	Energie: 42 kcal; Fat: 1.4g, of which saturates: 0.2g; Carbohydrate: 4.0g, of which sugars: 2.0g; Fibre: 0.7g; Protein: 3.0g; Salt: 0.1g
S13	1L	Water, soya beans (9%), concentrated apple extract (3%), tricalcium phosphate, sea salt, stabiliser (gellan gum), potassium iodide, vitamin B12, riboflavin, vitamin D2.	Energie: 44 kcal; Fat: 1.9g, of which saturates: 0.3g; Carbohydrate: 3.0g, of which sugars: 2.5g; Fibre: 0.5g; Protein: 3.4g; Salt: 0.14g
S14	1L	Soya base (92.9%) (water, hulled soya beans (12%)), sugar, fat-reduced cocoa (1.6%), calcium (calcium carbonate), flavouring, sea salt, acidity regulator (potassium phosphates), stabiliser (gellan gum), vitamins B2, D2.	Energie: 69 kcal; Fat: 2.8g, of which saturates: 0.6g; Carbohydrate: 5.3g, of which sugars: 5.0g; Fibre: 1.3g; Protein: 5.0g; Salt: 0.16g
S15	1L	Spring water, organic soybeans 8%.	Energie: 32 kcal; Fat: 1.6g, of which saturates: 0.3g; Carbohydrate: 0.8g, of which sugars: 0g; Fibre: 0.6g; Protein: 3.2g; Salt: 0g
S16	1L	Water, soya bean (8%), tricalcium phosphate, flavouring, stabiliser (gellan gum), potassium iodide, vitamin B12, riboflavin, and vitamin D2.	Energie: 28 kcal; Fat: 1.3g, of which saturates: 0.2g; Carbohydrate: 0.7g, of which sugars: 0.3g; Fibre: 0.5g; Protein: 3.0g; Salt: 0.05g
S17	1L	Soya base (98.8%) (water, hulled soya beans (8.7%)), acidity regulators (potassium phosphates), calcium (calcium carbonate), flavourings, sea salt, stabiliser (gellan gum), vitamins B2, B12, D2.	Energie: 33 kcal; Fat: 1.8g, of which saturates: 0.3g; Carbohydrate: 0g, of which sugars: 0; Fibre: 0.6g; Protein: 3.3g; Salt: 0.09g
S18	1L	Water, soya bean (9%), tricalcium phosphate, flavouring stabiliser (gellan gum), potassium iodide, vitamin B12, riboflavin, vitamin D2.	Energie: 38 kcal; Fat: 2.1g, of which saturates: 0.3g; Carbohydrate: 1.1g, of which sugars: 0.5; Fibre: 0.5g; Protein: 3.4g; Salt: 0.09g
S19	1L	Water, pea protein isolate (2.4%), sunflower oil, rapeseed oil, fermented pea, calcium carbonate, natural flavourings, stabilisers (guar gum, gellan gum), sea salt, acidity regulator (potassium carbonate), iodine, vitamins (B12, D).	Energie: 25 kcal; Fat: 1.9g, of which saturates: 0.3g; Carbohydrate: 0.2g, of which sugars: 0.5; Fibre: 0.1g; Protein: 2.0g; Salt: 0.13g
S20	1L	Soya base (97%) (water, hulled soya beans (8.7%)), sugar, acidity regulators (potassium phosphates), calcium (calcium carbonate), sea salt, stabiliser (gellan gum), natural flavouring, potassium iodide, vitamins B2, B12, D2.	Energie: 42 kcal; Fat: 1.9g, of which saturates: 0.3g; Carbohydrate: 2.6g, of which sugars: 2.5; Fibre: 0.6g; Protein: 3.3g; Salt: 0.09g
S21	250mL	Soya base (water, hulled soya beans (8.6%)), sugar, natural flavourings, calcium (calcium carbonate), sea salt, flavouring, acidity regulator (potassium phosphates), stabiliser (gellan gum), vitamins (B2, B12, D2).	Energie: 52 kcal; Fat: 1.9g, of which saturates: 0.3g; Carbohydrate: 5.7g, of which sugars: 5.7; Fibre: 0.6g; Protein: 3.3g; Salt: 0.15g

S22	250m L	Soya base (water, hulled soya beans (7%)), sugar, fat-reduced cocoa (1.5%), calcium (calcium carbonate), stabilisers (carrageenan, guar gum, xanthan gum), acidity regulator (potassium phosphates), flavouring, sea salt, natural flavouring, vitamins (B2, D2).	Energie: 61 kcal; Fat: 1.8g, of which saturates: 0.4g; Carbohydrate: 7.9g, of which sugars: 7.6; Fibre: 0.9g; Protein: 3.1g; Salt: 0.14g
S23	1L	Water, soybeans (15%), tapioca syrup, sea salt, hatomugi (Job's tears), acidity regulator (calcium carbonate).	Energie: 58 kcal; Fat: 2.2g, of which saturates: 0.3g; Carbohydrate: 5.4g, of which sugars: 2.7; Protein: 4.1g; Salt: 0.12g
S24	1L	Water, hulled soya beans (7%), tri-calcium phosphate, maltodextrin, flavouring, stabiliser: gellan gum; vitamin B12, vitamin D2, riboflavin (B2).	Energie: 33 kcal; Fat: 1.9g, of which saturates: 0.3g; Carbohydrate: <0.5g, of which sugars: <0.5; Fibre: 0.6g; Protein: 3.4g; Salt: 0.09g
S25	1L	Water, protein from peas (1.9%), rapeseed oil, acodoty regulator (dipotassium phosphate), calcium carbonate, calcium phosphates, natural flavouring, gluten-free oat oil, salt, vitamins (riboflavin, vitamin D, vitamin B12).	Energie: 24 kcal; Fat: 1.8g, of which saturates: 0.2g; Carbohydrate: 0.3g, of which sugars: 0; Protein: 1.6g; Salt: 0.1g
S26	1L	Water, hulled soya bean (9%), tricalcium phosphate, flavouring, stabiliser (gellan gum), vitamin B12, vitamin B2, and vitamin D.	Energie: 35 kcal; Fat: 1.9g, of which saturates: 0.3g; Carbohydrate: 0.8g, of which sugars: <0.5; Fibre: 0.6g; Protein: 3.4g; Salt: 0.03g
S27	1L	Water, soya bean (9%), tricalcium phosphate, sea salt, stabiliser: gellan gum; flavouring, vitamins B12, B2, and D2.	Energie: 33 kcal; Fat: 1.6g, of which saturates: 0.2g; Carbohydrate: 1.2g, of which sugars: <0.5; Fibre: <0.5g; Protein: 3.4g; Salt: 0.14g
S28	1L	Water, hulled soya bean (7%), apple extract (3%), tri-calcium phosphate, maltodextrin, sea salt, stabiliser: gellan gum; vitamin B12, vitamin D2, riboflavin (B2).	Energie: 42 kcal; Fat: 1.9g, of which saturates: 0.3g; Carbohydrate: 2.6g, of which sugars: 2.5; Fibre: 0.6g; Protein: 3.4g; Salt: 0.13g
S29	1L	Water, hulled soya beans (9%), apple concentrate (3%), tricalcium phosphate, sea salt, stabiliser (gellan gum), vitamin B12, vitamin B2, and vitamin D.	Energie: 41 kcal; Fat: 1.9g, of which saturates: 0.3g; Carbohydrate: 2.6g, of which sugars: 2.4; Fibre: 0.0g; Protein: 3.4g; Salt: 0.15g
S30	1L	Water, soya bean (9%), sugar, tricalcium phosphate, sea salt, stabiliser: gellan gum, potassium iodide, vitamins B12, B2, D2.	Energie: 42 kcal; Fat: 1.6g, of which saturates: 0.3g; Carbohydrate: 3.1g, of which sugars: 2.7; Fibre: <0.5g; Protein: 3.5g; Salt: 0.20g
S31	1L	Water, rapeseed oil, protein from peas (2.5%), agave syrup, acidity regulator (dipotassium phosphate), calcium carbonate, calcium phosphates, gluten-free oat oil, salt, vitamins (riboflavin, vitamin D, vitamin B12).	Energie: 40 kcal; Fat: 2.7g, of which saturates: 0.2g; Carbohydrate: 2.0g, of which sugars: 1.8; Protein: 2.1g; Salt: 0.1g

Attachment 2: Almond-based beverages sample list

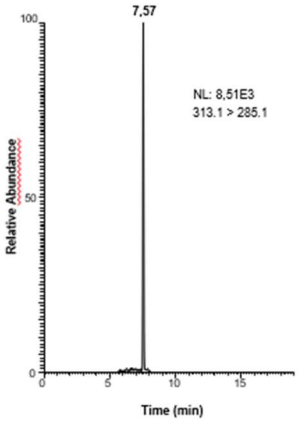
Code	Size	Ingredients	Nutritional information (100g)
A1	1L	Spring water, rice, Italian almonds (1%), sunflower oil, calcium carbonate, and sea salt.	Energie: 56 kcal; Fat: 1.5g, of which saturates: 0.3g; Carbohydrate: 10.0g, of which sugars: 4.7g; Fibre: <0.5g; Protein: <0.5g; Salt: 0.08g
A2	1L	Water, Almond (2%), tricalcium phosphate, sea salt, stabilisers (Locust Bean Gum, Gellan Gum), emulsifier (Lecithins), potassium iodide, vitamin E, vitamin B12, riboflavin, vitamin D2.	Energie: 15 kcal; Fat: 1.0g, of which saturates: 0.1g; Carbohydrate: 0.7g, of which sugars: 0.3g; Fibre: 0.5g; Protein: 0.6g; Salt: 0.3g
A3	1L	Water, sugar, hazelnuts (2.8%), calcium (tri-calcium phosphate), sea salt, stabilisers (locust bean gum, gellan gum), emulsifier (lecithins), vitamins B2, B12, E, D2.	Energie: 29 kcal; Fat: 1.6g, of which saturates: 0.2g; Carbohydrate: 3.1g, of which sugars: 3.1g; Fibre: 0.3g; Protein: 0.4g; Salt: 0.13g
A4	750 mL	Water, Almond (2.4%), Sugar, Fructose, acidity regulators (potassium phosphates), calcium (calcium carbonate), natural flavourings, stabilisers (gellan gum, guar gum), sea salt, vitamins B12, E, D2.	Energie: 25 kcal; Fat: 1.2g, of which saturates: 0.1g; Carbohydrate: 2.5g, of which sugars: 2.5g; Fibre: 0.2g; Protein: 0.5g; Salt: 0.08g
A5	1L	Water, Almond (2.3%), sugar, calcium (calcium carbonate), sea salt, stabilisers (guar gum, gellan gum), natural flavouring, emulsifier (lecithins), potassium iodide, vitamins B12, D2, E-	Energie: 24 kcal; Fat: 1.1g, of which saturates: 0.1g; Carbohydrate: 2.6g, of which sugars: 2.3g; Fibre: 0.3g; Protein: 0.5g; Salt: 0.15g
A6	1L	Water, Almond (2.3%), Calcium (calcium carbonate), sea salt, stabilisers (guar gum, gellan gum), emulsifier (lecithins), natural flavouring, potassium iodide, vitamins B12, D2, E.	Energie: 15 kcal; Fat: 1.1g, of which saturates: 0.1g; Carbohydrate: 0g, of which sugars: 0g; Fibre: 0.3g; Protein: 0.4g; Salt: 0.14g
A7	1L	Water, Sugar, Almond (2%), Tricalcium phosphate, sea salt, stabilisers (locust bean gum, gellan gum), emulsifier (Lecithins), Potassium Iodide, Vitamin E, Vitamin B12, Riboflavin, Vitamin D2.	Energie: 25 kcal; Fat: 1.0g, of which saturates: 0.1g; Carbohydrate: 3.0g, of which sugars: 2.8g; Fibre: 0.5g; Protein: 0.7g; Salt: 0.20g
A8	750 mL	Coffee (83%) (water, coffee extract), almond drink (water, almonds (2%)), cane sugar, acidity regulator (potassium citrate), calcium carbonate, sea salt, emulsifier (sunflower lecithin), stabilisers (guar gum, gellan gum).	Energie: 33 kcal; Fat: 1.2g, of which saturates: 0.1g; Carbohydrate: 4.9g, of which sugars: 4.0g; Fibre: 0.4g; Protein: 0.7g; Salt: 0.18g
A9	750 mL	Almond drink (water, almonds (2%)), coffee (10%) (water, coffee extract, cane sugar, pumpkin puree (0.75%), calcium carbonate, flavourings, cinnamon, emulsifier (sunflower lecithin), sea salt, ginger, stabilisers (guar gum, gellan gum), acidity regulator (potassium citrate), nutmeg.	Energie: 34 kcal; Fat: 1.2g, of which saturates: 0.1g; Carbohydrate: 5.0g, of which sugars: 4.3g; Fibre: 0.4g; Protein: 0.5g; Salt: 0.2g

A10	750 mL	Almond drink (water, almonds (2%)), coffee (30%) (water, coffee extract), cane sugar, cocoa powder (1%), flavourings, calcium carbonate, sea salt, emulsifier (sunflower lecithin), acidity regulator (potassium citrate), stabilisers (guar gum, gellan gum).	Energie: 35 kcal; Fat: 1.3g, of which saturates: 0.2g; Carbohydrate: 5.7g, of which sugars: 4.4g; Fibre: 0.7g; Protein: 0.8g; Salt: 0.18g
A11	1L	Water, cane sugar, 2.3% almonds, sea salt, stabiliser: gellan gum, emulsifier: sunflower lecithins.	Energie: 27 kcal; Fat: 1.5g, of which saturates: 0.1g; Carbohydrate: 2.8g, of which sugars: 2.7g; Fibre: 0.2g; Protein: 0.5g; Salt: 0.11g
A12	1L	Spring water, organic cashews 5%, sea salt	Energie: 32 kcal; Fat: 2.5g, of which saturates: 0.4g; Carbohydrate: 1.5g, of which sugars: 0g; Fibre: 0.3g; Protein: 0.9g; Salt: 0.1g
A13	1L	Spring water, almonds (2%), calcium carbonate, sea salt, emulsifier (sunflower lecithin), stabiliser (gellan gum), natural flavouring, vitamins (D2, E, B12).	Energie: 13 kcal; Fat: 1.1g, of which saturates: <0.1g; Carbohydrate: 0.2g, of which sugars: 0g; Protein: <0.5g; Salt: 0.15g
A14	1L	Water, 2.3% Almonds, sea salt, stabiliser: gellan gum; emulsifier: sunflower lecithins.	Energie: 16 kcal; Fat: 1.5g, of which saturates: 0.1g; Carbohydrate: 0g, of which sugars: 0g; Fibre: 0.2g; Protein: 0.5g; Salt: 0.15g
A15	1L	Water, gluten-free oats (13%), almonds (2%), sunflower oil, sea salt, gellan gum	Energie: 63 kcal; Fat: 2.4g, of which saturates: 0.3g; Carbohydrate: 8.8g, of which sugars: 4.5g; Fibre: 0.7g; Protein: 1.1g; Salt: 0.10g
A16	1L	Water, almond (2%), tricalcium phosphate, sea salt, stabilisers (locust bean gum, gellan gum), emulsifier (lecithins), potassium iodide, vitamin E, vitamin B12, riboflavin, vitamin D2.	Energie: 15 kcal; Fat: 1.0g, of which saturates: 0.1g; Carbohydrate: 0.5g, of which sugars: 0.5g; Fibre: 0.5g; Protein: 0.7g; Salt: 0.17g
A17	1L	Water, Almond (2-5%), sugar, fructose, acidity regulators (potassium phosphates), calcium (calcium carbonate), natural flavourings, stabilisers (gellan gum, guar gum), and sea salt.	Energie: 25 kcal; Fat: 1.2g, of which saturates: 0.1g; Carbohydrate: 2.5g, of which sugars: 2.5g; Fibre: 0.2g; Protein: 0.5g; Salt: 0.08g
A18	1L	Spring water, organic almonds 5%, sea salt.	Energie: 29 kcal; Fat: 2.5g, of which saturates: 0.2g; Carbohydrate: 0.4g, of which sugars: 0g; Fibre: 0.5g; Protein: 1g; Salt: 0.1g
A19	1L	Water, sugar, cashew nut 3.2%, cocoa powder 1.7%, cocoa butter 0.7%, pea protein 0.3%, fructose, salt, flavour, calcium carbonate, acidity regulator: dipotassium phosphate, sodium citrate; stabiliser: cellulose, cellulose gum, gellan gum.	Energie: 59 kcal; Fat: 1.8g, of which saturates: 0.8g; Carbohydrate: 8.4g, of which sugars: 7.1g; Protein: 1.8g; Salt: 0.20g
A20	1L	Spring water, organic rice, organic Italian almonds (1%), organic cold-pressed sunflower oil, and sea salt.	Energie: 56 kcal; Fat: 1.5g, of which saturates: 0.3g; Carbohydrate: 10g, of which sugars: 4.7g; Fibre: <0.5g; Protein: <0.5g; Salt: 0.08g

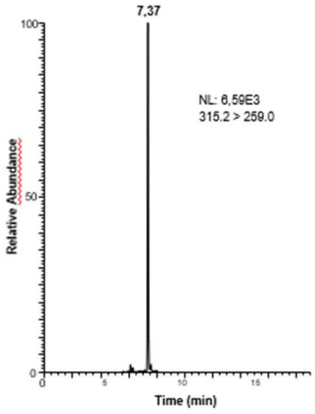
A21	1L	Almond (2.3%), sugar, calcium (calcium carbonate), sea salt, stabilisers (guar gum, gellan gum), natural flavouring, emulsifier (lecithins), vitamins B12, D2, E, potassium iodide.	Energie: 24 kcal; Fat: 1.1g, of which saturates: 0.1g; Carbohydrate: 2.7g, of which sugars: 2.4g; Fibre: 0.3g; Protein: 0.5g; Salt: 0.15g
A22	1L	Spring water, organic rice, organic tiger nuts (6%), sea salt	Energie: 49 kcal; Fat: 1.2g, of which saturates: 0.3g; Carbohydrate: 9g, of which sugars: 3g; Fibre: 0g; Protein: 0.5g; Salt: 0.05g
A23	1L	Same as A13	Same as A13
A24	1L	Same as A18	Same as A18
A25	1L	Water, almond (2%.3%), tri-calcium phosphate, sea salt, stabilisers: locust bean gum, gellan gum; emulsifier: lecithins; vitamin E, vitamin B12, vitamin B2, vitamin D2.	Energie: 18kcal; Fat: 1.3g, of which saturates: 0.1g; Carbohydrate: 1.1g, of which sugars: <0.5g; Fibre: <0.5g; Protein: <0.5g; Salt: 0.22g
A26	1L	Spring water, organic almonds (2.5%), organic carob seed flour.	Energie: 18kcal; Fat: 1.5g, of which saturates: 0.1g; Carbohydrate: <0.5g, of which sugars: 0.0g; Fibre: 0.6g; Protein: 0.6g; Salt: 0.0g
A27	1L	Water, almonds (2%), tricalcium phosphate, sea salt, stabilisers (locust bean gum, gellan gum), emulsifier (rapeseed lecithin), vitamin E, vitamin B12, riboflavin (B2), vitamin D2.	Energie: 15kcal; Fat: 0.8g, of which saturates: 0.1g; Carbohydrate: 0.8g, of which sugars: 0.5g; Fibre: 0.5g; Protein: 0.8g; Salt: 0.18g
A28	1L	Water, rice (10%), almond (2.5%), sea salt	Energie: 43kcal; Fat: 1.5g, of which saturates: 0.1g; Carbohydrate: 7.3g, of which sugars: 4.6g; Fibre: <0.5g; Protein: <0.5g; Salt: 0.08g
A29	1L	Water, organic almonds (6%).	Energie: 38kcal; Fat: 3.2g, of which saturates: 0.8; Carbohydrate: 0.8, of which sugars: 0g; Fibre: 0.8g; Protein: 1.5g; Salt: 0g
A30	1L	Water, rice (11%), hazelnut (2%), sea salt.	Energie: 53kcal; Fat: 1.4g, of which saturates: 0.2; Carbohydrate: 9.2, of which sugars: 6.7g; Fibre: 0.4g; Protein: 0.6g; Salt: 0.08g
A31	1L	Water, sugar, almond (2.3%), tri-calcium phosphate, sea salt, stabilisers: locust bean gum, gellan gum; emulsifier: lecithins; vitamin W, vitamin B12, vitamin B2, vitamin D2-	Energie: 13kcal; Fat: 1.0g, of which saturates: 0.1; Carbohydrate: <0.5, of which sugars: 0.0g; Fibre: 0.0g; Protein: 0.7g; Salt: 0.17g
A32	1L	Water, almonds (2%), tricalcium phosphate, sea salt, stabiliser (locust bean gum, gellan gum), emulsifier (rapeseed lecithin), vitamin E, vitamin B12, vitamin B2, vitamin D2.	8nergie: 27kcal; Fat: 1.4g, of which saturates: 0.2; Carbohydrate: 3.1, of which sugars: 3.0g; Fibre: <0.5g; Protein: <0.5g; Salt: 0.14g
A33	750 mL	Water, almonds (2.3%), calcium carbonate, emulsifier (sunflower lecithin), sea salt, flavourings, stabilisers (guar fum, gellan gum), acidity regulator (potassium citrate).	Energie: 17kcal; Fat: 1.4g, of which saturates: 0.1; Carbohydrate: 0.4, of which sugars: 0.1g; Fibre: 0.2g; Protein: 0.7g; Salt: 0.15g

Attachment 3: Chromatograms derived from the 10 parts per billion (ppb) standard in soy drink

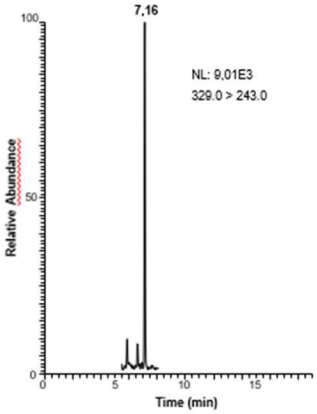
AFB1 (10 ppb)



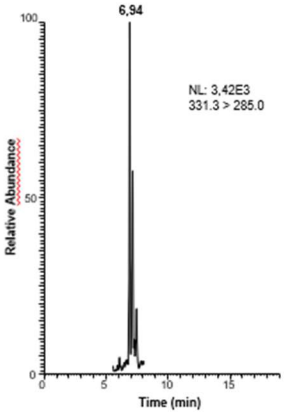
AFB2 (10 ppb)



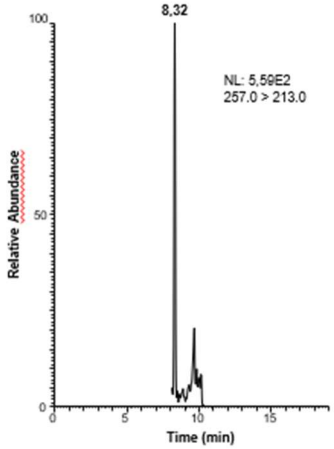
AFG1 (10 ppb)



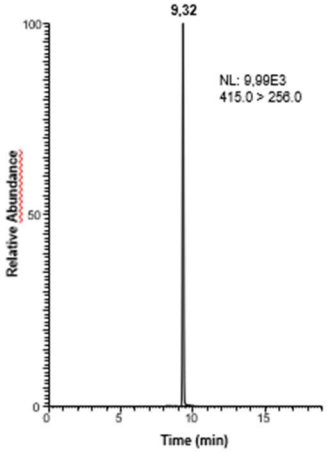
AFG2 (10 ppb)



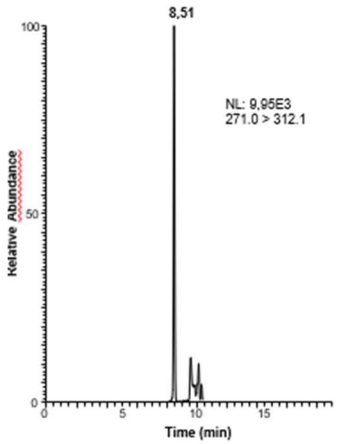
AOH (10 ppb)



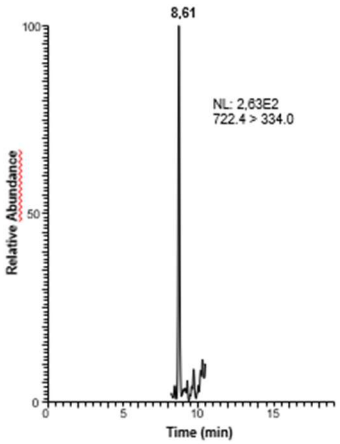
AME (10 ppb)



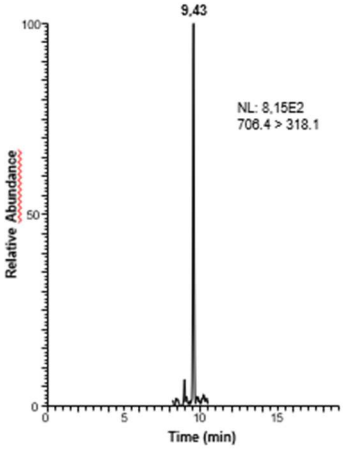
TEN (10 ppb)



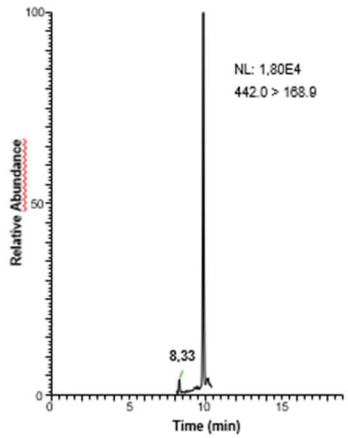
FB1 (10 ppb)



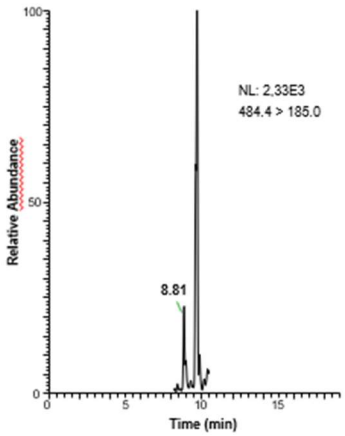
FB2 (10 ppb)



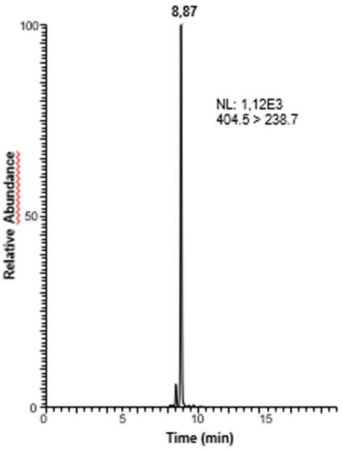
HT-2 (10 ppb)



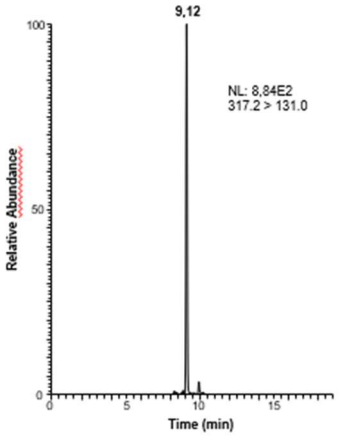
T2 (10 ppb)



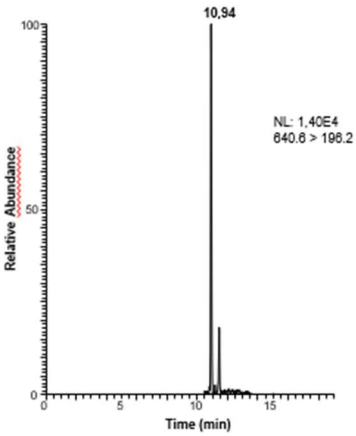
OTA (10 ppb)



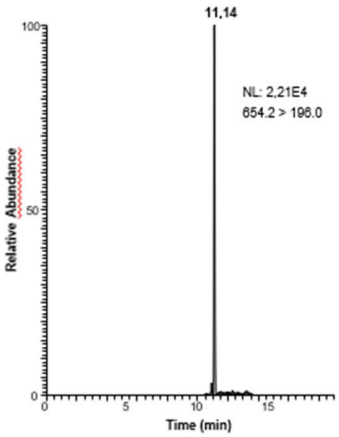
ZEN (10 ppb)



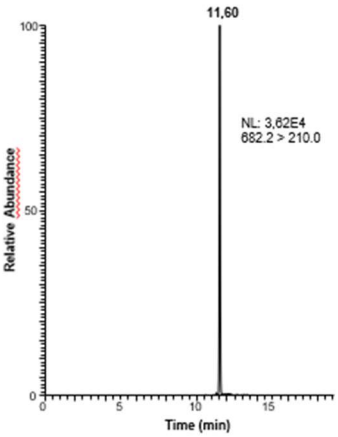
ENNB (10 ppb)



ENNB1 (10 ppb)



ENNA (10 ppb)



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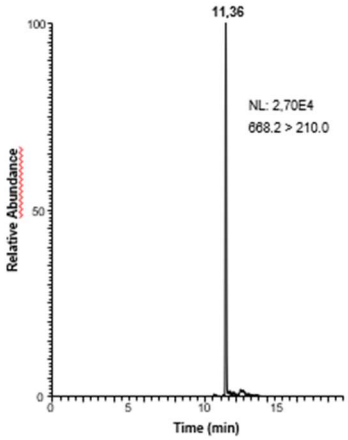
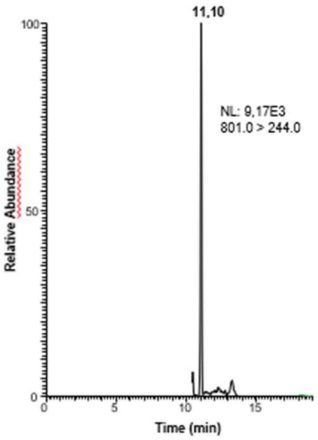
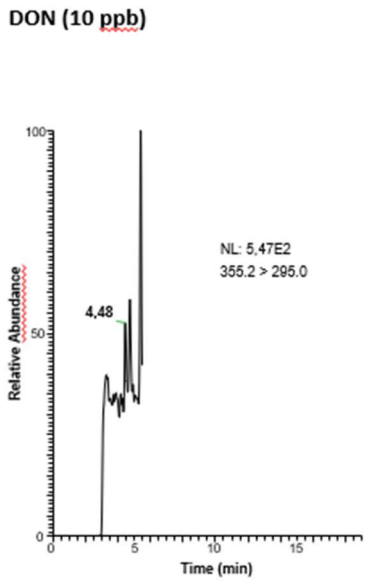


BEA (10 ppb)





Attachment 4: Mycotoxin mixtures found in the total 64 samples

Mycotoxin mixtures	Occurrence
AFB1 + AFB2 + AFG1 + AME + TEN + FB1 + FB2 + T-2 + OTA + ZEN + BEA	1
AFB1 + AFB2 + AFG1 + AOH + AME + TEN + FB1 + FB2 + T-2 + OTA + ZEN + BEA	2
AFB1 + AFB2 + AFG1 + AOH + TEN + FB1 + FB2 + OTA + BEA	1
AFB1 + AFB2 + AFG1 + AOH + TEN + FB1 + FB2 + T-2 + OTA + BEA	1
AFB1 + AFB2 + AME + TEN + FB1 + FB2 + OTA + ZEN + BEA	1
AFB1 + AFB2 + AOH + TEN + FB1 + T-2 + OTA + BEA + DON	1
AFB1 + AFB2 + TEN + FB2 + T-2 + OTA + BEA	1
AFB1 + AFB2 + TEN + OTA + BEA	1
AFB1 + AME + TEN + FB1 + HT-2 + T-2 + OTA + ZEN + ENNB + ENNA + ENNA1 + BEA	1
AFB1 + AME + TEN + HT-2 + T-2 + ENNB + ENNA + BEA	1
AFB1 + AOH + ENNB + ENNA + BEA	1
AFB1 + AOH + TEN + FB1 + FB2 + T-2 + OTA + ZEN + BEA	1
AFB1 + TEN + FB1 + OTA + BEA	1
AFB2 + AFG1 + AOH + TEN + FB1 + FB2 + T-2 + OTA + BEA	1
AFB2 + AOH + FB1 + BEA	1
AFB2 + AOH + FB1 + FB2 + OTA + BEA	1
AFB2 + AOH + FB1 + FB2 + T-2 + OTA + BEA	1
AFB2 + AOH + OTA + BEA	1
AFB2 + AOH + TEN + FB2 + T-2 + OTA + BEA	1
AFB2 + FB1 + FB2 + OTA + BEA	1
AFB2 + FB1 + HT-2 + OTA + BEA	1
AFB2 + FB1 + T-2 + OTA + BEA	1

AFB2 + OTA + BEA	1
AFG1 + AOH + FB1 + HT-2 + OTA + ENNB1 + BEA	1
AFG1 + TEN + OTA + BEA	1
AFG1 + TEN + T-2 + OTA + BEA	1
AOH + AME + ENNB + ENNA + BEA	1
AOH + AME + FB1 + FB2 + ENNB + ENNA + BEA	2
AOH + AME + FB1 + FB2 + HT-2 + T-2 + ZEN + ENNB + ENNA + BEA + DON	1
AOH + AME + FB2 + HT-2 + ENNB + ENNA + BEA	1
AOH + AME + FB2 + HT-2 + ZEN + ENNB + ENNB1 + ENNA + ENNA1 + BEA	1
AOH + AME + T-2 + ENNB + ENNA + BEA + DON	1
AOH + AME + TEN + FB1 + FB2 + ENNB + ENNA + BEA	1
AOH + AME + TEN + T-2 + ZEN + ENNB1 + BEA	1
AOH + FB1 + ENNB + ENNA + BEA	3
AOH + FB1 + FB2 + ENNB + ENNA + BEA	1
AOH + FB1 + FB2 + ENNB + ENNA + BEA + DON	2
AOH + FB1 + FB2 + HT-2+ ENNB + ENNA + BEA	2
AOH + FB1 + FB2 + T-2 + ENNB + ENNA + BEA	1
AOH + FB1 + FB2 + T-2 + OTA + BEA	1
AOH + FB1 + HT-2 + T-2 + ENNB + ENNB1 + ENNA + ENNA1 + BEA + DON	1
AOH + FB2 + BEA	1
AOH + FB2 + ENNB + ENNA + BEA	2
AOH + HT-2 + ENNB + ENNA + BEA	1
AOH + TEN + FB1 + FB2 + T-2 + OTA + BEA	1
AOH + TEN + FB1 + HT-2 + ENNB + ENNA + BEA	1
AOH + TEN + FB2 + OTA + BEA	1
AOH +FB2 + HT-2 + ENNB + ENNA + BEA	1
ENNB + ENNA + BEA + DON	1
FB1 + BEA	1
FB1 + FB2 + ENNB + ENNA + BEA	1
FB1 + FB2 + HT-2 + ENNB + ENNA + BEA	1
FB1 + FB2 + HT-2 + ENNB + ENNA + BEA + DON	1
FB1 + FB2 + T-2 + OTA + ZEN + ENNB + ENNA + ENNA1 + BEA	1
FB2 + HT-2 + T-2 + OTA + ENNB + BEA	1
HT-2 + OTA + ENNB1 + BEA	1
TEN + FB1 + FB2 + OTA + ZEN + BEA	1

Master dissertation submitted to the faculty of Pharmaceutical Sciences, performed in collaboration with the Laboratory of the Food and Drug Department at the University of Parma, Italy.

Promotor: Prof. Dr. Apr. Marthe De Boevre
Second promotor: Prof. Dr. Chiara Dall'Asta

Commissioners: Dr. Tullia Tedeschi and Dr. Kokeb Tesfamariam Hadush

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