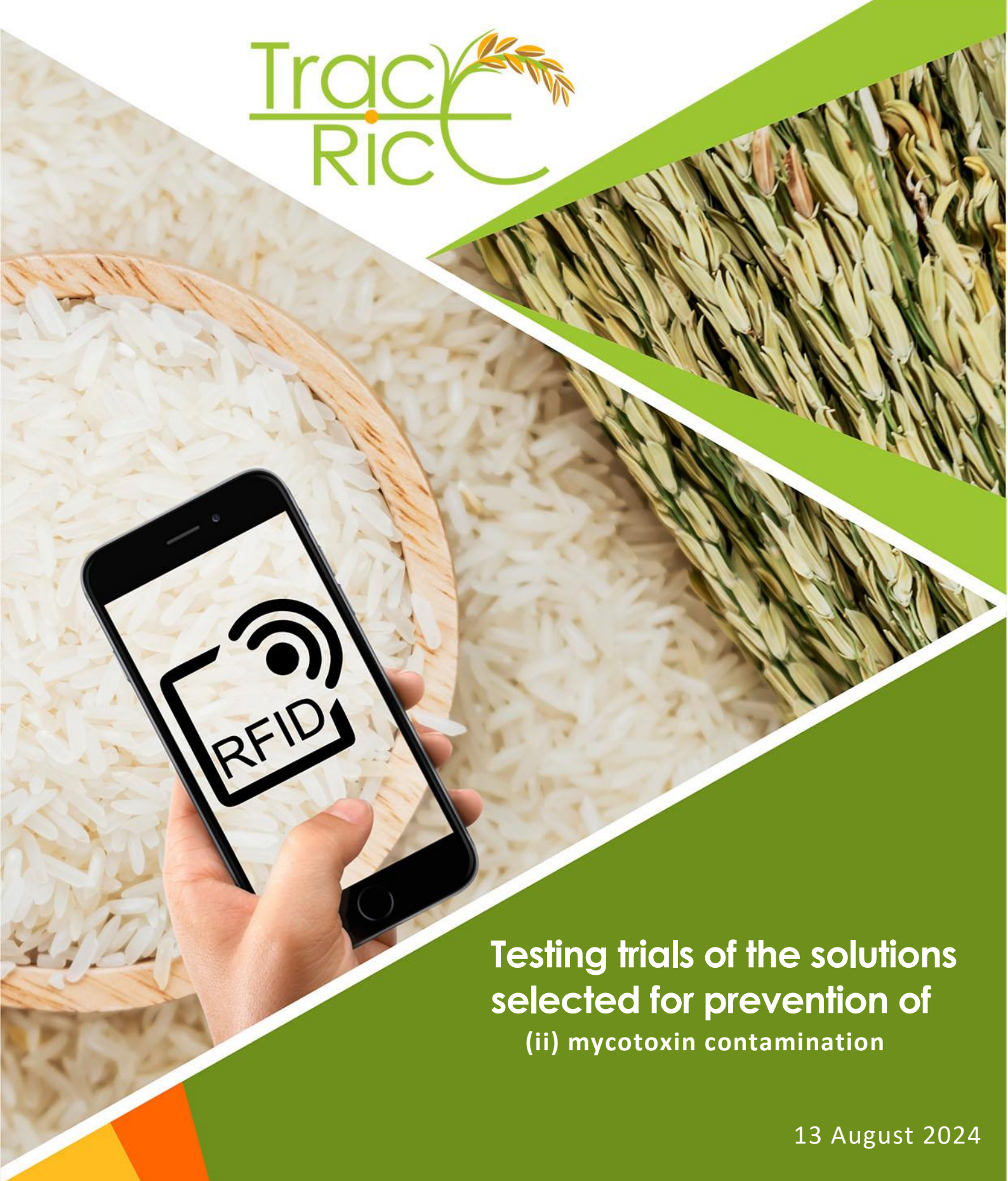

Testing trials of the solutions selected for prevention of

(ii) mycotoxin contamination

13 August 2024

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Report with results of the testing trials of the solutions selected for prevention of (i) insect infestation, (ii) mycotoxin contamination,

HISTORY OF CHANGES			
Date	Beneficiary	Version	Change
26/06/2024	INIAV	V1	Draft version sent to the coordinator
05/08/2024	INIAV	V2	Text revision and addition of an executive summary
13/08/2024	INIAV	V3	Final Version approved by project coordinator

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EXECUTIVE SUMMARY

The TRACE-RICE project aimed at preventing mycotoxins focused on mapping, validating methodologies, and analyzing mycotoxins in various rice varieties. This report summarizes the validation processes and findings concerning both established and emerging mycotoxins.

Key activities included

1. **Mapping Mycotoxins**: Mycotoxins previously documented in deliverable 2.1 and published source (https://doi.org/10.3390/toxins14090647) were mapped.
2. **Immunoassay Validation**: An immunoassay method for detecting multiple mycotoxins, including aflatoxins, fumonisins, ochratoxins, deoxynivalenol, T2 and HT2 toxins and zearalenone was validated and published https://doi.org/10.1016/j.focha.2023.100586.
3. **Emerging Mycotoxins Validation**: Methods for emerging mycotoxins such as citrinin, mycophenolic acid, sterigmatocystin, and ergot alkaloids were validated.
4. **Quantitative Analysis**: Utilizing UHPLC-ToF-MS, all validated mycotoxins were analyzed and quantified in the bran of 22 rice varieties selected for silmutaneous characterization in WP1.

Key Findings

- **Mycotoxin Detection**: Among the 22 varieties analyzed, aflatoxin B1 was detected in the bran of 2 varieties, and zearalenone was detected in 6 varieties. Notably, one variety exceeded the maximum legislated limit for aflatoxin B1, and another for zearalenone.
- **Sample Diversity**: The rice samples were sourced from various seed supply companies and countries (from Portugal, Spain, Italy, Egypt) each utilizing different cultivation techniques.
- **Potential Causes**:
    - The presence of aflatoxin B1 may be attributed to inadequate transport and storage conditions.
    - Further studies are needed to determine if the zearalenone levels are linked to Fusarium diseases occurring in the field.

Conclusion

The results underscore the importance of implementing robust integrated pest management practices from cultivation through distribution to mitigate mycotoxin contamination in rice. Ensuring good storage conditions is also critical to prevent aflatoxin contamination. Continued research is essential to understand the factors contributing to mycotoxin presence and to develop strategies for effective management.

Recommendations

- **Integrated Pest Management**: Adopt best pest management practices to minimize field contamination.
- **Improved Storage**: Enhance storage and transport conditions to reduce aflatoxin risk.
- **Ongoing Monitoring**: Conduct regular monitoring and analysis to detect and address mycotoxin issues promptly.
- **Further Research**: Investigate the underlying causes of mycotoxin contamination, particularly for zearalenone, to develop targeted interventions.

1. INTRODUCTION

1.1 Statement of Problem

Ensuring food safety for consumers is a critical global issue. Recently, the incidence of various types of food contamination, hazardous to both human and animal health, has increased. Globalization and the emergence of a global exchange-based economy have significantly amplified the food market's expansion and impact. Concurrently, this expansion has led to increased exposure to chemical and natural pollutants due to the broader distribution of food products. One of the most pressing new concerns in food safety is the rise in plant diseases associated with toxic fungal species and their secondary metabolites. Among these metabolites, low molecular weight mycotoxins are particularly hazardous to various physiological processes in both humans and animals (Moretti et al., 2017).

The term "mycotoxin" originates from the Greek words "mykes" (fungus or mold) and "toxicum" (poison). Mycotoxin formation is driven by specific environmental conditions, with contamination levels varying based on the region, agricultural practices, and the susceptibility of commodities to fungal invasion during storage and processing (Adeyeye, 2016; Iqbal, 2021). The most prevalent mycotoxigenic fungi belong to the genera *Aspergillus*, *Penicillium*, and *Fusarium*, although *Trichoderma*, *Trichothecium*, and *Alternaria* are also significant as food contaminants or plant pathogens (Adeyeye, 2016).

Despite the numerous species of toxigenic molds, only a few mycotoxins—particularly those affecting groundnuts and cereals such as rice, wheat, barley, and maize—are considered critical for human health. Mycotoxins have been found to exhibit immunotoxic, hepatotoxic, nephrotoxic, mutagenic, teratogenic, and carcinogenic effects, even at low concentrations (Iqbal, 2021; Moretti et al., 2017; Wu et al., 2023).

The mycotoxins of global significance in cereals, as discussed in this report, include aflatoxins (AFs), fumonisins (FBs), ochratoxins (OTs), trichothecenes (TCs), and zearalenone (ZEA) (Iqbal, 2021; Wu et al., 2023). Additionally, emerging mycotoxins were also examined.

Mycotoxins can have both immediate and long-term detrimental effects on human health, depending on the amount consumed and the frequency of exposure. The harmful effects of mycotoxin metabolites can be synergistic or potentiated when multiple mycotoxins are consumed, exacerbating their hazardous impact (Silva et al., 2023).

1.1 Aflatoxins

Aflatoxins are a global concern for food safety due to their wide distribution in foods and feeds and their high toxicity (considered the most toxic), whose impacts are negative for health, the economy, and social life. About 4.5 billion people in the world are subjected to aflatoxins' contamination. Developing countries, such as Gambia, Uganda, Kenya, and Tanzania, located in tropical and sub-tropical regions, are the most affected (Benkerroum, 2020; Popescu et al., 2022; Shabeer et al., 2022). The discovery of AFs made 60 years in 2020, which was identified in England and became known as "turkey X disease". On a poultry farm near London, 100,000 turkeys died of so-called turkey "X" sickness after being given contaminated by a Brazilian groundnut meal (Pickova et al., 2021). All types of aflatoxins are derived from fungal species belonging to the genus *Aspergillus*, including *Aspergillus flavus* or *Aspergillus parasiticus*. Over 20 varieties of aflatoxins are currently recognized, with the most well-known being Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1), Aflatoxin G2 (AFG2), Aflatoxin M1 (AFM1), Aflatoxin M2 (AFM2), aflatoxicol, and aflatoxin Q1. AFM1 and AFM2, for example, are metabolites of AFB1 and AFB2 discovered in the milk of lactating mammals fed with aflatoxins-contaminated feed. Aflatoxins are frequently detected in food and feed items, mainly in foodstuffs, oilseeds, cereals, dried fruits, spices, and dairy products (Benkerroum, 2020; Dhanshetty et al., 2021; Popescu et al., 2022; C. Yang et al., 2020). Because of the public health issues raised by these toxicants as well as their link to genotoxic effects, significant research has been conducted since their discovery to clarify the mechanisms of their carcinogenicity and other toxicities. The carcinogenicity of aflatoxins has long been associated with the liver, where they are first metabolized to release reactive intermediate metabolites. AFB1 exhibits severe carcinogenicity related to hepatocellular carcinomas, and for this reason, the International Agency for Research on Cancer (IARC) has classified this toxin as a group I carcinogen. However, subsequent epidemiological and animal research revealed their carcinogenicity to organs other than the liver, such as the kidney, pancreas, bladder, bone, viscera, central nervous system, among others. Aside from carcinogenicity, they have been shown to be hepatotoxic, genotoxic, mutagenic, teratogenic, immunosuppressive, nephrotoxic, and cytotoxic (Benkerroum, 2020; Dhanshetty et al., 2021; Pickova et al., 2021).

1.2 Fumonisins

Fumonisins are naturally occurring mycotoxins that pose a significant threat to food and animal health and are mainly produced by several species of Fusarium, including *F. verticillioides*, *F. proliferatum*, *F. fujikuroi*, and *F. oxysporum* (T. Li et al., 2022; Qu et al., 2022; Wangia-Dixon & Nishimwe, 2020). They were discovered in 1988 by researchers at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) in Tygerberg, South Africa, and identified and characterized in the late 1980s and early 1990s (Wangia-Dixon & Nishimwe, 2020). Fumonisins can be divided into four categories: A, B, C, and P, including 28 structural analogues. Notably, B-series fumonisins are the most common, with fumonisin B1 (FB1) being the principal and most toxic one (70 to 95% of total fumonisins in feeds and food products), followed by fumonisin B2 (FB2) and fumonisin B3 (FB3). IARC classifies fumonisins into group 2B, which is a possible human carcinogen owing to their harmful effects (T. Li et al., 2022; Qu et al., 2022). Fumonisins easily contaminate maize, maize-based products, rice, and other grains (wheat, barley, rye, and oat). Maize and maize-based products are most infected with fumonisins. Fumonisins can cause damage to the kidneys and livers of several animals that feed on these grains, even causing tumor problems. Additionally, fumonisin toxicity is associated with human esophageal cancer and neural tube defect disease. Fusariosis, caused by Fusarium species infection, is the second-most frequent mold disease in humans (Kamle et al., 2019; T. Li et al., 2022; Yli-Mattila & Sundheim, 2022).

They can cause huge economic losses, the fumonisins are among the most significant mycotoxins in terms of prevalence and possible influence on human health (T. Li et al., 2022).

1.3 Ochratoxins

Ochratoxins are common mycotoxins in various food and feed products discovered in 1965 in South Africa, such as cereals and cereal-based products, wine, tea, coffee, milk and milk products, herbs, poultry, pork, eggs, and cocoa (Fadlalla et al., 2020; Kumar et al., 2020; X. Li et al., 2022). OTs are produced by *Aspergillus* and *Penicillium* species, mostly *A. ochraceus*, *A. carbonarius*, *A. niger* and *P. verrucosum* (Kumar et al., 2020). There are three classes of ochratoxins: Ochratoxin A (OTA), Ochratoxin B (OTB), and Ochratoxin C (OTC). OTA is considered the most abundant as well as the most toxic of the three (Kumar et al., 2020; X. Li et al., 2022). OTB is a non-chlorinated form of OTA and OTC is an ethyl ester form of OTA (Fadlalla et al., 2020; X. Li et al., 2022; Ortiz-Villeda et al., 2021). OTA has been related to various health issues due to its various toxicological effects, such as teratogenicity and carcinogenicity, genotoxicity, mutagenicity, testicular toxicity, embryotoxicity, hepatotoxicity, immunotoxicity, developmental toxicity, neurotoxicity, blood-brain barrier damage, and nephrotoxicity (Kumar et al., 2020). Therefore, OTA has been classified as a class 2B carcinogen (possible human carcinogen) by the IARC since 1993. Consequently, severe control of the OTA contamination in food is very important (Kumar et al., 2020; X. Li et al., 2022).

1.4 Trichothecenes

Trichothecenes are produced by a variety of *Fusarium* fungi like *Fusarium graminearum*, *Fusarium nivale*, and *Fusarium culmorum*. They can be divided into four types: A, B, C, and D. T2 toxin, and HT2 toxin, belong to type A, deoxynivalenol (DON) belongs to type B. T2 toxin, HT2 toxin and DON belong to Group 3 by the IARC (Ostry et al., 2017). These mycotoxins generally are found in barley, wheat, rye, maize, and oats (Ren et al., 2020; Ülger et al., 2020).

1.4.1 Deoxynivalenol

DON was first discovered in moldy wheat and maize and chemically characterized in Japan in 1970 by Yoshizawa. It is one of the top five mycotoxins affecting the safe use of staple crops worldwide, including maize, barley, and wheat (Sumarah, 2022; Yao & Long, 2020). This mycotoxin is produced by *Fusarium graminearum*, *Fusarium asiaticum*, and *Fusarium culmorum* (Mishra et al., 2020; Sumarah, 2022). Due to its stability, DON can stay hazardous in infected wheat for up to four years (Yao & Long, 2020). The other name for DON, *vomitoxin*, is very appropriated because animals tend to reject and vomit after consuming contaminated feed. The most common source of DON is through dietary ingestion, and symptoms in Humans are an upset stomach, vomiting, dizziness, headache, abdominal pain, and diarrhea. The concerns with DON are widespread and is expected to worsen as a result of climate changes (Mishra et al., 2020; Yao & Long, 2020).

1.4.2 HT2 Toxin and T2 Toxin

The T2/HT2 toxin has the highest toxicity of all TCs. T2 is produced by different Fusarium species, like *F. sporotrichioides*, *F. poae*, and *F. acuminatum* (Janik et al., 2021). They are present mostly in cereal grains, for example, wheat, maize, oat, barley, and rice (Steinkellner et al., 2019). To date, the toxicity of T2 on humans and animals has no target organ but can induce a wide range of toxic effects due to its strong toxicity, which primarily impairs heart muscle, nerves, and the immune system. T2 has different toxic effects depending on dosage, age, and ways of exposure (oral, dermal, and aerosol). In general, feed refusal, vomiting, hemorrhages, stomach necrosis, and dermatitis have demonstrated immediate toxicological consequences. It can also cause cardiotoxicity, hepatotoxicity, digestive

toxicity, neurotoxicity, and other multisystemic toxicities that have received widespread attention (Janik et al., 2021; Sun et al., 2022; X. Yang et al., 2020). Additionally, T2 is thought to be a major factor in the development of the gastrointestinal condition known as alimentary toxic aleukia (ATA) illness, which has historically affected Humans, namely soldiers (World War II) in specific parts of the world after consuming contaminated food (Janik et al., 2021).

1.5 Zearalenone

Zearalenone, has attracted particular attention because it shows strong estrogenic activity (Rogowska et al., 2019). This mycotoxin is produced by *Fusarium* and *Gibberella* species, including *F. culmorum*, *F. graminearum*, *F. cerealis*, *F. equiseti*, *F. crookwellense*, *F. semitectum*, *F. sporotrichioides*, *F. oxysporum*, *F. acuminatum*, and *F. verticillioides*, and is found mainly in warm countries (Caglayan et al., 2022; Rai et al., 2020; Ropejko & Twarużek, 2021). High ZEA levels have been linked to symptoms of nausea, vomiting, and diarrhea associated with cereal toxicosis (Caglayan et al., 2022). Wheat, barley, maize, sorghum, rye, rice, maize silage, sesame seed, hay, flour, malt, soybeans, beer, and maize oil have all been found to contain zearalenone. It can also be found in grains for human consumption, baked goods, pasta, morning cereals, and bread. When cows consume ZEA contaminated meals, it can be observed in their milk, making its way into the human food chain (Rogowska et al., 2019). For example, one study revealed the presence of ZEA in 60% of rice grain samples grown in 2017 in Brazil (Rogowska et al., 2019).

1.6. Emerging Mycotoxins

"Emerging mycotoxins" are novel toxins produced by fungi that have drawn the attention of scientists in recent years (Gruber-Dorninger et al., 2017). These new toxins have been defined as “mycotoxins, which are neither routinely determined, nor legislatively regulated; however, the evidence of their incidence is rapidly increasing”. The group's list of poisonous substances is subject to change, since certain molecules may turn out not to be harmful while other mycotoxins may develop toxic characteristics and be classified as "emerging" toxins. Some examples of emerging mycotoxins include enniatins, citrinin, nivalenol, beauvericin, moniliform, fusaproliferin, fusaric acid, culmorin, butenolide, sterigmatocystin, emodin, mycophenolic acid, alternariol and alternariol monomethyl ether, tenuazonic acid (Gruber-Dorninger et al., 2017). The TRACE-RICE 2.2 task was focused specifically on citrinin, mycophenolic acid and sterigmatocystin.

1.6.1 Citrinin

Citrinin (CIT) mycotoxin is a polyketide produced by fungi belonging to the genus: *Aspergillus*, *Penicillium* and *Monascus* (Kamle et al., 2022). Citrinin is generally formed after harvest under storage conditions, and it occurs mainly in stored grains, but can also occur with other products of plant origin, for example, beans, fruits, fruit and vegetable juices, herbs, and spices and also in spoiled dairy products (JH, 2015; Kamle et al., 2022; Rossi et al., 2020). Citrinin is also discovered to be an unwanted contaminant in Red Mold Rice (RMR), which is utilized in Asian cuisine as a colorant and food preservation. CIT was observed to be accumulated more at 20° C in rice. CIT has been shown to be nephrotoxic and hepatotoxic to humans with the kidneys being the primary target organ. CIT is commonly found along with ochratoxin, and an additive or synergic effect has been shown to increase the toxicity, causing kidney disease in humans. Although CIT has been shown antibacterial, anticancer, and neuroprotective properties (JH, 2015; Kamle et al., 2022; Rossi et al., 2020). However, the European Commission has set a maximum limit of 100 µg/kg in rice fermented with red yeast *M.purpureus* (European Commission, 2023).

1.6.2 Mycophenolic Acid

Mycophenolic acid (MPA), a phthalide, is a mycotoxin produced by *Penicillium bialowiezense*, *P. brevicompactum*, *P. carneum*, and *P. roqueforti* (Otero et al., 2020). MPA was isolated in 1893 by an Italian physician, Bartolomeo Gosio, from *Penicillium brevicompactum* (Gruber-Dorninger et al., 2017; Otero et al., 2020). MPA holds the distinction of being the first ever purified antibiotic. In humans, mycophenolic acid is an immunosuppressant which is frequently used for prevention of acute transplant rejection. Mycophenolic acid was identified from samples from food waste, grass silage and blue molded cheeses (Dietrich & Märtlbauer, 2015; Gruber-Dorninger et al., 2017; Otero et al., 2020).

1.6.3 Sterigmatocystin

Sterigmatocystin (STE) was isolated by the first time in 1954 from *Aspergillus versicolor* cultures (Díaz Nieto et al., 2018). Although can be produced by several fungal species belonging to the genera *Aspergillus*, *Bipolaris*, *Botryotrichum*, *Humicola* and *Penicillium* (Zingales et al., 2020). Aflatoxins, recognized as the most potent carcinogenic mycotoxins, share a metabolic pathway with sterigmatocystin (STE). Specifically, AFG1 and AFB1 use STE as a biogenic precursor. In aflatoxigenic species, STE rarely accumulates because it is rapidly converted into O-methylsterigmatocystin, the direct precursor of AFB1 and AFG1. *A. versicolor* and *A. nidulans*, for example, do not appear to be able to convert STE into O-methylsterigmatocystin, most likely because they do not have the genes encoding the particular methyltransferase needed for this conversion (Zingales et al., 2020). Due to fungal infestation at the post-harvest stage, it can happen to grains and products derived from grains. There have occasionally been reports of STE in beer, nuts, spices, and green coffee beans. Cheese is contaminated, especially on the top, during ripening and storage due to fungal deterioration (Biancardi & Dall'Asta, 2015). Since the majority of STE is transformed into aflatoxins, substrates colonized by *A. flavus* and *A. parasiticus* have low levels of STE but substrates colonized by these fungi can have high levels of STE. It is well acknowledged that STE may be a teratogen, mutagen, and carcinogen. STE is considered a strong toxin and carcinogen in animals, but its significance as a health risk to humans is unclear. The IARC categorized STE as a 2B carcinogen. Though the European union have no legislation for STE and therefore no official control/monitoring programmes (Biancardi & Dall'Asta, 2015).

1.7. Ergot Alkaloids

Ergot alkaloids (EAs) are toxic secondary metabolites that are generated by Claviceps fungi, primarily by Claviceps purpurea, a parasitic fungus that feeds on the seed heads of live plants during blooming (Poapolathep et al., 2021). Their production depends on many factors, such as temperature, humidity, insect damage in crops, nutrients, and fungal concentration (Poapolathep et al., 2021; Silva et al., 2023). The main ergot alkaloids produced by Claviceps species are ergometrine, ergotamine, ergosine, ergocristine, ergokryptine, and ergocornine, and the group of agroclavines (Poapolathep et al., 2021). The European Commission has established restrictions on food-related ergot alkaloids across Europe. Numerous organizations, including the Food and Agriculture Organization (FAO), the World Health Organization (WHO), and the European Food Safety Authority (EFSA), consider ergot alkaloids as mycotoxins due to their significance (Silva et al., 2023).

1.8. Analytical Methods to Determine Mycotoxins

Mycotoxin co-contamination in food and feed is widely reported; therefore, interest in the protection of Human and animal health has grown (Adunphatcharaphon et al., 2022; Plotan et al., 2016). For this reason, there is an increasing interest in the development of strategies to prevent food contamination by mycotoxins in order to reduce exposure. In this line, new extraction methodologies, clean-up procedures, and detection methods for diverse food and agricultural commodities have been reported

in the last few years (Adunphatcharaphon et al., 2022; Oswald et al., 2017; Rahman et al., 2019). The majority of analytical methods consist of the following steps: sampling, homogenization, extraction, clean-up (which may involve sample concentration), separation, and detection. These steps are typically carried out either using a chromatographic technique in conjunction with various detectors or by an immunochemical method (Pereira et al., 2014). The developing methods of analysis must be sensitive, simple, easy to use, affordable, and accurate for the effective management and control of mycotoxins (Adunphatcharaphon et al., 2022; Sibanda et al., 2022).

1.8.1 Sampling

Sampling is one of the key steps in the accurate evaluation of mycotoxin levels (Adunphatcharaphon et al., 2022; Zhou et al., 2020). It is critical that the sample used for analysis be representative of the bulk matrix, which is typically challenging in the case of mycotoxins due to the considerable variability of their distribution in contaminated raw and processed foods. All sampling plans require that the entire primary sample be mixed and blended so that the analytical test part contains the same concentration of toxin as the original sample (Pereira et al., 2014). Therefore, to accurately assess the degree of contaminated mycotoxins, choosing an adequate process for sample preparation is essential. In order to recover mycotoxins from a test sample, sample preparation typically involves an extraction procedure utilizing the right solvents, and a clean-up or purification phase to remove any food matrix interferences and concentrate analytes with low mycotoxin abundance (Adunphatcharaphon et al., 2022). The EU has adopted a standard sampling protocol (Commission Regulation No. 2023/915) for the official regulation of mycotoxin levels in foodstuffs to decrease the variability of analytical results (Pereira, Fernandes e Cunha, 2014 Commission of the European Communities, 2023).

1.8.2 Extraction and Clean-up Methodologies

Extraction and clean-up steps are crucial in analytical methods to obtain the cleanest possible extract. The choice depends on many factors, such as matrix type, analyte physicochemical properties, and the ultimate separation and detection method used. There are many types of extraction; the most common is solid-liquid extraction (SLE), and by coincidence, it is the oldest technique using solvents. SLE is widely used for the extraction of mycotoxins. Additionally, to the conventional procedures, more recent techniques, including pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), supercritical fluid extraction (SFE), and microwave-assisted extraction (MAE), were used for the determination of mycotoxins in cereal crops. These techniques have an advantage when compared with conventional SLE because they require smaller volumes of solvent and usually provide better extraction efficiencies. Regardless of their benefits, SFE, MAE, and PLE have not been widely used mycotoxin approaches. This is likely because of challenges with optimization and routine use, as well as the requirement to purchase specialized equipment (Adunphatcharaphon et al., 2022; Pereira et al., 2014).

The clean-up step is crucial because it enables the removal of contaminants that can obstruct the identification of mycotoxins, increasing accuracy and precision. Some examples of clean-up methods are solid-phase extraction (SPE), immunoaffinity columns (IAC), and molecularly imprinted polymers (MIPs). And still exists combined extractive/clean-up extraction, such as quick, easy, cheap, effective, rugged, and safe, better known as QuEChERS (Pereira et al., 2014). The QuEChERS technique employs a small volume of solvents and a small amount of material, allowing for the separation of a wide variety of analytes and the analysis of several samples in a short amount of time (high throughput). There are two steps involved in QuEChERS extraction: First, an extraction step based on the salting-out effect is carried out using acetonitrile (ACN) and an extraction salt mixture of magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) in a 4:1 ratio. Secondly, interferers are cleaned up using adsorbents, often utilizing dispersive solid phase extraction (d-SPE). Sorbents such as primary secondary amine (PSA) or

octadecyl modified silica (C18) are considered the traditional sorbents for the cleanup stage in high lipid matrix (Mateus et al., 2021).

1.8.3 Detection Methods

The analytical methods can be classified into conventional methods and rapid methods for mycotoxin detection (Adunphatcharaphon et al., 2022). Conventional methods, such as high-performance liquid chromatography (HPLC) and ultra-high liquid chromatography coupled with tandem mass spectrometry (UHLPC-MS/MS), are currently the main techniques used for the quantitative detection of mycotoxins (Adunphatcharaphon et al., 2022; Sibanda et al., 2022). Rapid methods have minimal preparation, and most are based on an immunoassay (Adunphatcharaphon et al., 2022).

1.8.3.1 Conventional Methods

Chromatographic methods are the most commonly employed for analyzing mycotoxins in food samples. In a simple way, these methods rely on the physical interaction of a mobile phase and a stationary phase. Mycotoxins are analyzed using thin-layer chromatography (TLC), gas chromatography (GC), and liquid chromatography (LC). TLC is more commonly employed for mycotoxin identification. But a review of current chromatographic techniques for mycotoxins analysis in cereals reveals that liquid chromatography mass spectrometry (LC-MS) techniques have grown in popularity, while gas chromatography mass spectrometry (GC-MS) techniques have been less extensively employed (Mateus et al., 2021b; Santos et al., 2022).

LC is the most commonly used technology for confirming the identity and quantifying mycotoxins and is capable of separating thermolabile, non-volatile, and polar substances due to its high precision, sensitivity, and low detection limit. The stationary phases in an LC analytical column can be classified as normal or reverse phases. In the normal phase, mycotoxins are eluted using a nonpolar or moderately-polar mobile phase(s) via a polar solid phase (consisting of a free or covalently bound particle of phenyl, aluminum, or silica, resulting in a polar stationary phase). Although current methods for aflatoxin analysis primarily rely on reverse-phase HPLC, LC methods for aflatoxin determination encompass both normal and reverse-phase separations. The reverse phase is made up of hydrocarbonated non-polar solid phases, like, C8, C18, or short chains of phenyl, cyanopropyl, and n-alkyl bonded to the silica surface, through which mycotoxins are eluted using binary polar mixtures of water as well as organic solvents (Mateus et al., 2021; Santos et al., 2022). Modern GC combines improved separation on capillary columns with a number of generic or particular detectors, the most common of which is the MS detector, which enables simultaneous identification and quantification of chemicals. GC-MS can be achieved by electron impact (EI) or chemical ionization in positive (PCI) or negative (NCI) mode. Despite the high costs and the need for experienced personnel, LC coupled with MS has been the gold standard in mycotoxin analysis over the last two decades. The ability to simultaneously identify and quantify practically all mycotoxins at low levels without derivatization, as is required in GC methods, is a significant advantage of LC-MS approaches. UHPLC (Ultra High-Performance Liquid Chromatography) systems surpass standard LC's regarding separation capacity. UHPLC is a growing chromatographic separation technology with packing materials with smaller particle sizes (less than 2 μm), which improves analysis speed, resolution, and sensitivity. Another option to overcome the reduced separation capacity of GC capillary columns is to employ multiple MS detectors (LC-MS/MS) or, more recently, high-resolution mass spectrometers such as Time-of-Flight detectors (ToF) or Orbitrap analyzers (high resolution and high accuracy) (Pereira et al., 2014; Santos et al., 2022).

1.8.3.2 Rapid Methods

Immunoassay methods have proven to have numerous advantages in the detection of mycotoxins based on antibody-antigen reactions by developing simple, efficient, and sensitive procedures (Mateus et al., 2021). Enzyme-linked immunosorbent assays (ELISA), flow injection immunoassays (FIIA), lateral flow immunoassays (LFIA), flow immunoassays, and chemiluminescence immunoassays (CLIA) are

some of these techniques. These can be categorized into labeled and label-free sensors, as well as competitive (direct or indirect) and non-competitive assays (Adunphatcharaphon et al., 2022). Due to their relatively small size, mycotoxins are typically detected using competitive rather than non-competitive immunoassays (Maragos, 2009). A classic method, ELISA, is the most commonly used immunoassay, which uses amplification by the enzymatic reaction for detection (Adunphatcharaphon et al., 2022Adunphatcharaphon et al., 2022; Sato, 2020). This method has the advantages of being precise, quick, and simple to use, but it also has some drawbacks, such as the potential for cross-reactivity and dependence on a particular matrix (because matrix effect or interference may lead to under- or overestimation of mycotoxins) and contamination level. Additionally, each kit is designed for a single application and detects only one mycotoxin, making it potentially expensive if multiple tests are needed to identify different mycotoxins (Adunphatcharaphon et al., 2022; Mateus et al., 2021b; Pereira et al., 2014; Santos et al., 2022).

CLIA is an alternative technique for the determination of mycotoxins with the major advantage of requiring simple optical equipment without the need for an external light source. It has already been used to detect mycotoxins in samples of maize (Freitas et al., 2019). Because of the irregularity of the brightness of the reaction and low photon intensity, a catalyst such as an enzyme, transition metal ions, or noble metal nanoparticles is often required to enhance the CLIA signal. A horseradish peroxidase (HRP) enzyme was extensively used as a catalyst in the luminol-hydrogen peroxide (H_2O_2) CLIA system for simultaneous detection of multiple mycotoxins (Adunphatcharaphon et al., 2022; Santos et al., 2022). The Biochip Array Technology (BAT) used by Evidence Investigator is built on a biochip, which serves as both the solid phase and the vessel for miniaturized chemiluminescence immunoassays (Plotan et al., 2016b; Sibanda et al., 2022) In this form of competitive chemiluminescence, the analyte and conjugate compete for binding sites, resulting in an inverse relationship between the analyte concentration and the light produced by the chemical reaction (Jia et al., 2021). This method has already been validated in maize (Freitas et al., 2019). The immunoassay now allows for the determination of the combined T2 and HT2, instead of just T2. In other words, the BAT can detect nine mycotoxins (AFB1, AFG1, OTA, ZEA, DON, FB1+FB2, and T2+HT2), although in two cases it detects and semi-quantifies the sum of two mycotoxins (FB1+FB2 and T2+HT2).

2. Objective

In this context, the objective of this work, in accordance with deliverable 2.4, was twofold: to validate a biochip array technology for screening nine mycotoxins in rice, and to optimize and validate a QuEChERS followed by UHPLC-ToF-MS method for the determination of 25 mycotoxins in rice. These validations aim to provide a more efficient approach for detecting various mycotoxins in rice samples in the future.

3. Validation of a Biochip Array Technology for Multi-Mycotoxins Screening in Rice

3.1 Material and Methods

3.1.1. Chemicals and Reagents

The standards of mycotoxins were purchased from Sigma-Aldrich (Madrid, Spain). A mixed working solution of mycotoxins has the following concentration: 1.5 µg/kg for OTA; 50 µg/kg for ZEA; 1 µg/kg for AFB1 and AFG1; 125 µg/kg for FB1 and FB2; 25 µg/kg for T2; 25 µg/kg for HT2 and 375 µg/kg for DON for the validation of the assay. This solution was prepared from individual stock solutions

prepared in acetonitrile. The MilliQ-plus system from Millipore (Molsheim, France) was used to purify water. Furthermore, methanol and acetonitrile were acquired from Merck (Darmstadt, Germany).

3.1.2. Food Samples

Samples of rice were acquired in local supermarkets in Vila do Conde (Portugal) in July 2022 and evaluated regarding their mycotoxins content through UHPLC-ToF-MS (Freitas et al., 2019). Blank (non-contaminated) samples were used for the validation of the Biochip Chemiluminescent Immunoassay in the present study.

3.1.3. Methodology

3.1.3.1. Extraction

For the extraction, the homogenized samples were weighted first (5 g ± 0.05 g), and extracted with 25 mL of acetonitrile:metanol:water (50:40:10 v/v/v). In the next step the samples were vortexed for 60 seconds, rolled for 10 minutes, and centrifugated for 2 minutes at 1600 rpm. Following that, they were diluted with the working-strength wash buffer included in the kit. In an Eppendorf tube, 50 µL of sample was added to 150 µL of working strength, with a dilution factor of 75. The scheme of the extraction is shown in Figure 1. The diluted sample was applied to the biochip according to the instructions of the manufacturer for the assay Myco 7 (Biochip Array – Randox Food, 2023). Per biochip, Randox can identify a total of 44 antibodies. For this array (Myco 7), Randox has seven antibodies spotted (Freitas et al., 2019).

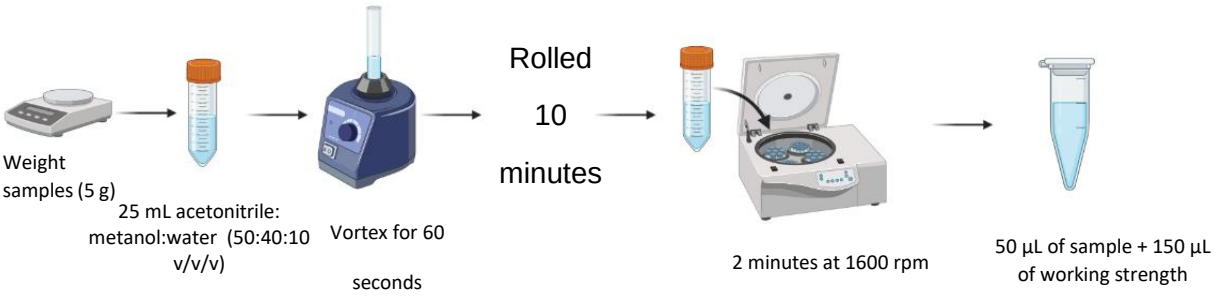

Figure 1. Extraction procedure previous to multi-mycotoxins analysis by chemiluminescence assay.

3.1.3.2. Chemiluminescent Immunoassay Analysis

Evidence Investigator Myco 7 Array (Ev4065) is used for the simultaneous semi-quantitative detection of mycotoxins from a single sample, so the technique is a competitive chemiluminescent immunoassay for the determination of mycotoxins rice samples. Increasing levels of mycotoxins in a sample lead to decreased binding of the conjugate labeled with HRP and therefore a decrease in the chemiluminescence signal emitted. The kit contains six carriers composed of nine biochips each, for a total of 54 biochips, nine calibrators of the mixture of mycotoxins in a range of concentrations, an assay diluent, a control, a multianalyte conjugate, conjugate diluents, washing buffer, a signal reagent, barcodes, and a calibration disc (Figure 2).

Figure 2. Components of the Myco 7 Array. *Nine biochips, control, 9 calibrators, assay diluent, multianalyte conjugate, conjugate diluents, washing buffer and signal reagent.*

For the immunoassay, there are several steps that must be followed. First, 150 µL of assay diluent was pipetted to each carrier, followed by 50 µL of the correspondent sample/control/ calibrator to the appropriate biochip wells, and then the reagents were gently mixed. Furthermore, carriers were incubated at 25°C for 30 minutes at 370 rpm in a thermoshaker (Figure 3). Subsequently, 100 µL of working-strength conjugate was added to each biochip cell, and once again, the carriers were incubated at 25°C for 60 minutes at 370 rpm.

Figure 3. Photography of the termoshaker used to incubate the carriers.

The acquisition of data by digital imaging technology is processed individually. After incubation, the biochip is quickly washed twice and submitted to four two-minute soaks. Each carrier is removed from the handling tray, one by one. For each cycle, all edges of the handling tray were tapped for approximately 10-15 seconds, then the biochips were soaked in dilution buffer for 2 minutes. Lastly, to remove any residues, decanted liquid from the first rack was imaged and tapped on lint-free paper. After tapping, 250 µL of mixed signal reagent were added to each well. Then, they were incubating for

2 minutes and protect from the light. After this, each carrier was placed into Evidence Investigator™ (Figure 4). The whole process is summarized in Figure 5.

Figure 4. Evidence Investigator equipment at Vairão, Vila do Conde (INIAV, I.P.) facilities, (Randox).

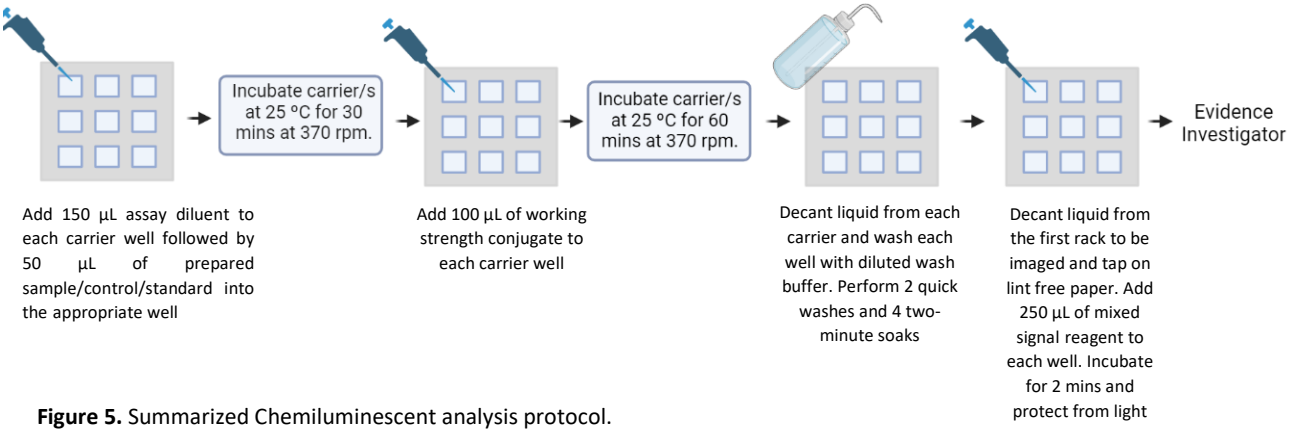

Figure 5. Summarized Chemiluminescent analysis protocol.

3.2 Validation Parameters

The assessment of the method's applicability and robustness, limit of detection, and selectivity/specificity were all necessary for the validation of the screening methodology. All calculations were based on the relative light units (RLU).

Twenty blank samples from different origins were used for validation in rice, and five samples from different origins were used for validation in oat, barley, rye, and wheat. All blank samples were spiked to a concentration of interest (section 2.1.).

RLU is the unit of measurement for the chemiluminescent signal of discrete test regions (DTR) on the biochip, and this light intensity number varies depending on the level of mycotoxins detected. The following equations were used to determine the cut-off (Fm) and the threshold value (T):

$Fm = M - 1.64 \times SD$

M is the mean and SD standard deviation of the signal in the RLU of the spiked samples.

$T = B + 1.64 \times SD_B$

B is the mean, and SD_B is the standard deviation of the signal in RLU of the blank samples.

The cross-reactivity details have been updated regarding Freitas *et al*. (Table 1) (Freitas et al., 2019).

Table 1. Cross-reactivity of the biochip chemiluminescent immunoassay for the simultaneous determination of seven mycotoxins.

Mycotoxin	Cross- reactivity with	% Cross-reactivity
Fumonisins	FB1	100
	FB2	91
	FB3	100
OTA	OTA	100
	OTB	<1
AFG1	AFG1	100
	AFG2	71
	AFB1	8
	AFB2	5
DON	DON	100
	3- Acetyldeoxynivalenol	723
	15- Acetyldeoxynivalenol	3
T2HT2	T2 toxin	100
	HT2 toxin	100
AFB1	AFB1	100
	AFB2	18
	AFG1	15
	AFG2	3
ZEA	ZEA	100
	α- Zearalenol	114
	β- Zearalenol	69
	Zearalanone	65
	α- Zearalanol	51
	β- Zearalanol	52

3.3 Results and Discussion

The T and Fm of the biochip chemiluminescent immunoassay for the different mycotoxins in rice are compiled in Table 2. In the fortified samples, the result obtained should be lower than the cut-off, while blank samples should present results above the cut-off value. The cut-off value is therefore used for compliance purposes. In the expression of results, a result can be considered: compliant, when the signal obtained exceeds the cut-off of the method; or suspected of non-compliance, when the signal is less than or equal to the cut-off established in the validation; in this case, the result should be confirmed by another method (Freitas et al., 2019).

Figure 6 shows the results of each of the 20 blank samples and of the 20 fortified rice samples. In the case of rice, 5% of false negatives and 5% of false positives were found for fumonisins. Moreover, 5% of false negatives were found for the same matrix for ZEA, AFB1, T2+HT2, and DON and 5% of false positives were found for OTA.

In terms of T values, OTA has the highest value for cereals and rice, while ZEA has the lowest value for cereals and rice. In respect of cut-off value, OTA has the highest value for cereals and rice, while ZEA has the lowest value for cereals and rice.

For the screening tests, the criterium is to have a maximum of 5% false negatives or 5% false positives for different mycotoxins in different cereals (Limit of Detection (LOD) should be ≤5%), therefore we were able to successfully validate the method for all the proposed matrices according regulation (EC) No 1881/2006 (Commission of the European Communities, 2021.).

It is important to mention that the preparation of the chemiluminescent method has several critical steps, such as avoiding the formation of bubbles, pipetting solution into the wells of the biochips, not overfilling the wells during washing in order to reduce the potential for well-to-well contamination, carrying out an appropriate number of washes, not leaving carriers to soak for longer than 30 minutes, and at last, protecting carriers awaiting imaging from light (Freitas et al., 2019).

Table 2. Threshold value (T) and Cut-off value (Fm) of the biochip chemiluminescent immunoassay for the different mycotoxins in rice.

	FB1 + FB2		**AFG1**		**ZEA**		**OTA**		**AFB1**		**T2HT2**		**DON**	
	Blank	Spiked	Blank	Spiked	Blank	Spiked	Blank	Spiked	Blank	Spiked	Blank	Spiked	Blank	Spiked
Spiking level (µg/kg)	-	250	-	1	-	50	-	1.5	-	1	-	50	-	375
Mean (RLU)	6815	3742	5283	2182	3179	187	10251	3578	5598	1032	5972	451	8950	1187
SD (RLU)	1220	736	444	446	321	79	1961	974	488	298	883	153	1511	312
T (threshold value) (RLU)	4815		4556		**2653**		**7035**		4798		4524		6472	
Fm (cut-off value) (RLU)	4949		2914		**317**		**5174**		1521		701		1699	

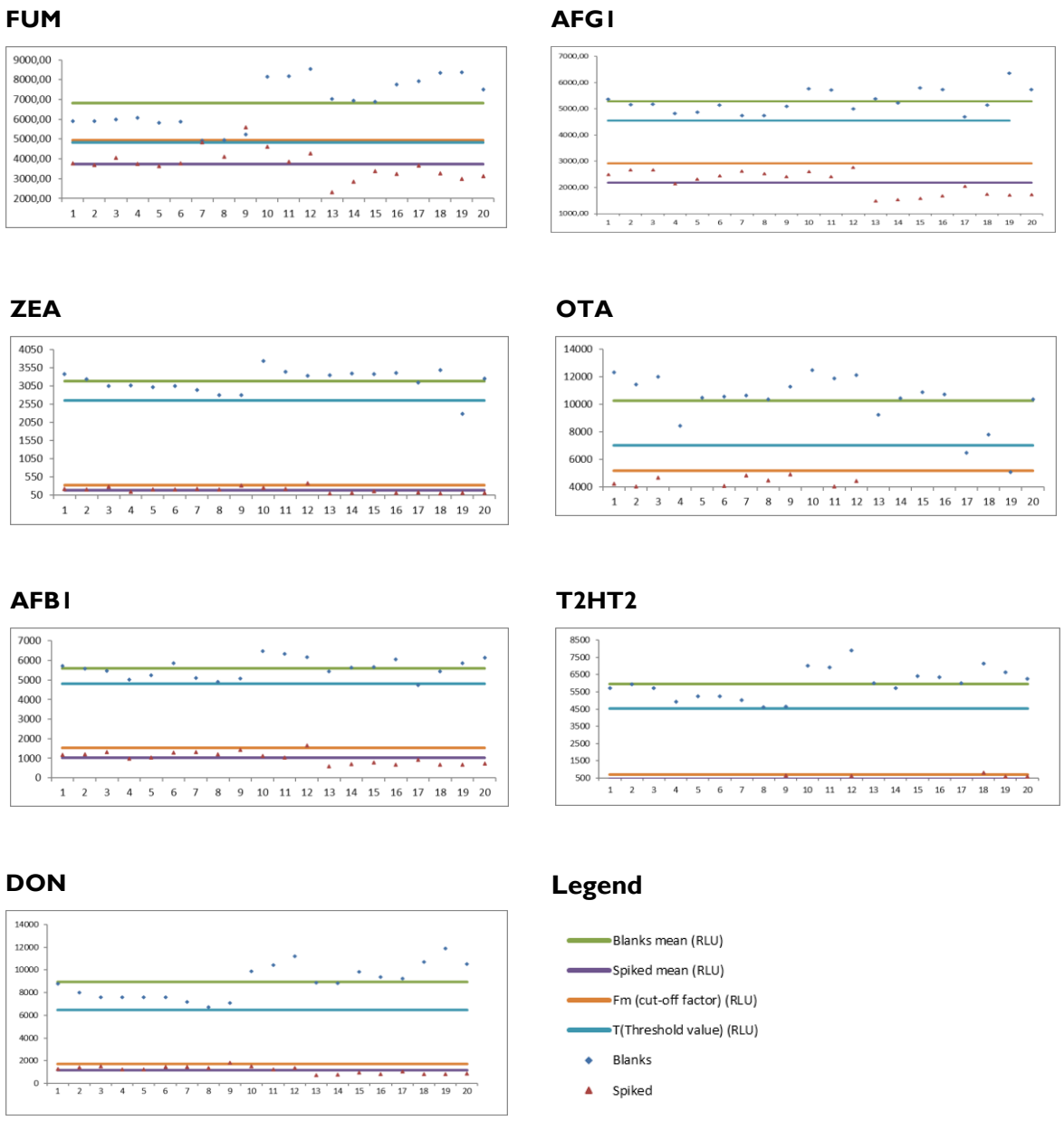

Figure 6. Threshold value (T) and cut-off value (Fm) of each of the mycotoxins analysed by the biochip chemiluminescent immunoassay expressed in RLU, for the 20 blank rice samples and for the 20 spiked rice samples at the level of interest.

4. UHPLC-ToF-MS Method for Determination of Multi-Mycotoxins in Rice

4.1. Experimental

4.1.1. Chemicals and Reagents

Methanol, acetonitrile (both HPLC gradient grade) and formic acid were purchased from Merck (Darmstadt, Germany). Water was purified by Milli-Q plus system from Millipore (Molsheim, France). Mycotoxins standards and internal standard (zearalanone, ZAN) were purchased from Sigma–Aldrich (Madrid, Spain) and were dissolved in acetonitrile (AFB2, AFG1, ZEA, T2, HT2, ZAN and STE), methanol (AFB1, AFG2, OTA, CIT and MPA) or acetonitrile:water (50:50, v/v) (FB1 and FB2). Ergot alkaloids standards were purchased from Dr. EHRENSTORFER and were dissolved in acetonitrile (α-ergocryptine, α-ergocryptinine, ergocornine, ergocorninine, ergocristine, ergocristinine, ergometrine, ergometrinine, ergosine, ergosinine, ergotamine and ergotaminine). Stock solutions were prepared with a concentration of 1 mg/mL, except T2 and HT2, which presented a concentration of 2.5 mg/mL, except, ergocornine, ergometrine, ergosine and ergotamine, which presented a concentration of 0,1 mg/mL; except ergocorninine, ergosinine, ergometrinine, ergocristinine, which presented a concentration of 0,025 mg/mL and except α-ergocryptine and α-ergocryptinine, which presented a concentration of 0,2 mg/mL. These stock solutions were subsequently used to prepare different working solutions for calibrations. Calibration work solution was prepared in acetonitrile with a concentration of 4 ng/mL for AFB1; 8 ng/mL for AFB2, AFG1 and AFG2; 6 ng/mL for OTA; 200 ng/mL for ZEA, T2, HT2, CIT, STE and MPA; 1000 ng/mL for FB1 and FB2 and 20 ng/mL for ergot alkaloids. All standard solutions were stored in amber vials in the dark at- 20° C, for at least 2 years, and before use, they were kept at room temperature for 15 min.

For QuEchERS, magnesium sulfate was purchased from PanReac (Barcelona, Spain) and sodium chloride was purchased from Fluka (Seelze, Germany). For clean-up procedure primary secondary amine-bonded silica (PSA) were acquired from Agilent Technologies (Santa Clara, CA, USA) and anhydrous magnesium sulfate were purchased from PanReac (Barcelona, Spain).

4.1.2. Samples

The samples analyzed consisted of the 22 rice varieties selected in WP1. These samples were provided by various seed companies from Portugal, Spain, Italy, France, and Egypt. The rice paddy grains were milled, and the husk and bran were removed. Multi-mycotoxins were quantified in the bran due to the high probability of incidence in this fraction.

4.1.3. QuEchERS sample preparation

Extraction

About 2 g of rice flour and bran (2.0 ± 0.1 g) was weighted in 50 mL polypropylene tubes. Internal standard (zearalanone) was added (250 µl from a 10 µg/mL). Afterward, samples are hydrated with 4 mL of ultrapure water and 16 mL of acetonitrile with 0.1% of formic acid is added. Then, the sample and the extractant was mixed for 1 min in vortex. Next, mixture of extraction salts for liquid–liquid partitioning step (1 g of magnesium sulfate, and 0.25 g of sodium chloride) were added and mixed for 1 min in vortex, following by centrifugation at 12,669 × g for 5 min at 5 ◦C.

Clean-up

Four mL of supernatant from extract after centrifugation were transferred into a 15 mL Falcon tube with 50 mg PSA and 150 mg anhydrous magnesium sulfate and vortex for 1 min. Then, it was centrifuged at 12,669 × g for 5 min at 5 ◦C. After, 4 mL of the extract was transferred to a 15 mL Falcon tube and evaporated to dryness under a gentle stream of nitrogen at 40 ◦C. Finally, residues were redissolved with 500 µL of acetonitrile 40% (v/v), vortexed for 30 s follow by 15 min in an ultrasonics bath and filtered through a PVDF mini-uniprep™ for injection into the UHPLC-ToF-MS system.

UHPLC-ToF-MS analysis

Detection and quantification were performed with a Nexera X2 Shimadzu UHPLC coupled with a 5600+ ToF-MS detector (SCIEX, Foster City, CA) equipped with a Turbo Ion Spray electrospray ionization source working in positive mode (ESI+). In terms of chromatography conditions, a column HSS T3 (2.1 x 50 mm, 1.8 µm) was used and kept at 30 °C, the autosampler was maintained at 10 °C to refrigerate the samples and a volume of 20 µL of sample extract was injected in the column. The mobile phase consisted of 0.1% formic acid [A] and acetonitrile [B] with a flow rate of 0.4 mL/min and with the following gradient program: 0-13 min from 10% to 60% [B]; 13-15 min from 60% to 90% [B]; and kept until 16 min; back to 10% [B] from 16 to 18 min until the end of the run (total of 18 min). In terms of mass spectrometry, the acquisition was performed in full scan from 100 to 750 Da using the Analyst TF (SCIEX, Foster City, CA) software and with the following settings: ion source voltage of 5500 V; source temperature 575 °C; curtain gas (CUR) 30 psi; Gas 1 and Gas 2 of 55 psi; delustering potential (DP) 100 V. Every 10 injections the ToF-MS detector was calibrated in the mass range of the method, to guarantee the accurate mass resolution.

4.1.3.1 Spiking Experiment

The matrix-matched calibration was prepared by spiking blank sample of rice (5 g) with 8 different levels, using 0.0625 mL to 2 mL of calibration of the work solution (sub-Section 4.1.1) to obtain a concentration range between 0.125 to 4.0 µg/mL of AFB1; 0.250 to 8.0 µg/mL of AFB2, AFG1 and AFG2; 0.19 to 6.0 µg/mL of OTA; 6.25 to 200.0 µg/mL of ZEA, T2, HT2, CIT, STE and MPA; 31.25 µg/mL to 1000 µg/mL of FB1 and FB2; 0.63 µg/mL to 20.0 µg/mL to ergot alkaloids. Subsequently, extraction was performed as described in sub-Section 4.1.3.

4.1.4. Identification of mycotoxins

The identification and data processing were made through the PeakView™ and MultiQuant™ (SCIEX, Foster City, CA) softwares.

In terms of identification criteria three parameters were used: maximum relative retention time deviation (ΔRRT) of 2.5%; difference in the isotope pattern with a tolerance of 10% and exact mass deviation (Δm) with a tolerance of 5 ppm. The isotope match is presented automatically by the PeakView™ software although for the other criteria the following equations were used:

Equation (1): Relative Retention Time (RRT)

$$RRT = \frac{RT_{analyte}}{RT_{internal\ standard}}$$

Where $RT_{analyte}$ is the retention time of the analyte; and the $RT_{internal\ standard}$ is the retention time of the internal standard (zearalanone).

Equation (2): Deviation of RRT (ΔRRT)

$$\Delta\text{RRT} = \left(\frac{RRT_{spiked\ samples} - RRT_{standard}}{RRT_{standard}}\right)$$

Equation (3): Deviation of exact mass (Δm)

$$\Delta\text{m} = \left(\frac{Exact\ mass - Detected\ mass}{Exact\ mass}\right) \times 10^6$$

4.1.5. Validation of Analytical Method

Linearity was evaluated by preparing calibration curve with nine concentrations. (Table 3). Determination coefficients (r^2) of calibration curves were always higher than 0.98, indicating suitability to quantify mycotoxins in the selected calibration range.

Table 3. Linearity and sensitivity of UHPLC-ToF-MS method for the simultaneous determination of mycotoxins in rice.

Mycotoxins	**LOQ (µg/kg)**	**Linear Range (µg/kg)**	**Calibration Curve Parameters**		
			r^2	Slope	Interception
AFB1	0.5	0.5-4.0	0.9910	8439.8	2589.8
AFB2	1.00	1.0-8.0	0.9900	9657.3	9146.9
AFG1	1.00	1.0-8.0	0.9911	23050.2	17614.8
AFG2	1.00	1.0-8.0	0.9904	3645.6	2813.9
OTA	0.75	0.75- 6.0	0.9911	6143.1	4756.1
ZEA	25.0	25.0-200	0.9931	1190.7	25266.4
T2	12.5	12.5-200	0.9909	439.72	10625.8
HT2	12.5	12.5-200	0.9901	284.30	8214.8
FB1	125	125-1000	0.9865	18460.4	-1645111.8
FB2	62.5	62.5-1000	0.9971	18930.4	-564893.1
CIT	50.0	50-200	0.9936	12382.8	-296960.8
STE	12.5	12.5-200	0.9901	11025.2	123065.8
MPA	12.5	12.5-200	0.9931	996.95	3698.5
Ergocornine	1.25	1.25-20	0.9904	11832.1	11071.7
Ergocorninine	1.25	1.25-20	0.9896	37750.6	15495.2
Ergocristine	1.25	1.25-20	0.9898	27392.3	12270.3
Ergocristinine	1.25	1.25-20	0.9933	12879.9	8992.5
Ergometrine	n.a.	n.a.	n.a.	n.a.	n.a.
Ergometrinine	n.a.	n.a.	n.a.	n.a.	n.a.
Ergosine/Ergosinine	5.00	5.0-40	0.9922	22220.2	53605.3
Ergotamine/Ergotaminine	5.00	5.0-40	0.9884	7371.32	34198.2
α-Ergocryptine	1.25	1.25-20	0.9907	14877.85	15748.9
α-Ergocryptinine	1.25	1.25-20	0.9912	12147.09	13691.6

LOQ—limit of quantification; **AFB1**—aflatoxin B1; **AFB2**—aflatoxin B2; **AFG1**—aflatoxin G1; **AFG2**—aflatoxin G2; **FB1/FB2**—fumonisins B1 and B2; **OTA**—ochratoxin A; **T2/HT2**—trichothecenes; **ZEA—**zearalenone; **CIT-** citrinin; **STE-** Sterigmatocystin; **MPA-** Mycophenolic acid. **n.a.**- not applicable

4.2. Results and Discussion

4.2.1. Occurrence of mycotoxins in Rice

The maximum limits (ML) of mycotoxins in rice and/or cereals are regulated by Commission Regulation (EU) No. 2023/915, which establishes maximum levels for certain contaminants in foodstuffs in the European Union (EU) as follows: 5.0 µg/Kg for AFB1; 10.0 µg/kg for the sum of AFB1, AFB2, AFG1 and AFG2; 5.0 µg/kg for OTA; 1250 µg/kg for DON; 100 µg/kg for ZEA; 4000 µg/kg for the sum of FB1 and FB2 and 50 µg/kg for the sum of ergocornine/ergocorninine; ergocristine/ergocristinine; ergocryptine/ergocryptinine (α- and β-form); ergometrine/ergometrinine; ergosine/ergosinine; ergotamine/ergotaminine (Ergot Alkaloids) (European Commission, 2023). Although there are other mycotoxins which have not been classified or legislated up to now, such as, the emerging mycotoxins.

As shown in Table 4, out of the 22 rice bran samples analyzed, FBs, TCs, AFG1, AFG2, and emerging mycotoxins were not detected. The most frequently found mycotoxin was ZEA, present in six samples, followed by AFB1 in two samples, and AFB2 and OTA in one sample each. Two samples exceeded the maximum allowable limits for AFB1 and ZEA: the Giza 177 bran sample contained 41.91 µg/kg of AFB1 and a total of 43.87 µg/kg for the sum of AFB1, AFB2, AFG1, and AFG2. The Ronaldo sample exceeded the ZEA limit with a value of 145.78 µg/kg.

Table 4. Mycotoxins contamination in rice samples.

Samples	Mycotoxins (µg/kg)													
	AFB1	**AFB2**	**AFG1**	**AFG2**	**OTA**	**ZEA**	**T2**	**HT2**	**FB1**	**FB2**	**CIT**	**STE**	**MPA**	**ERGOTS**
Ronaldo	n.d	n.d.	n.d.	n.d.	n.d.	**145. 8 ± 7.898**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caravela	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Giza 177	**41.91 ± 0.536**	1.959 ± 0.208	n.d.	n.d.	2.135 ± 0.002	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Elettra	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.
Basmati	2.530 ± 0.263	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Maçarico	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Giza 181	<LOQ	n.d.	n.d.	n.d.	<LOQ	<LOQ	n.d.	n.d.	<LOQ	n.d.	<LOQ	n.d.	n.d.	n.d.
Albatroz	n.d.	n.d.	n.d.	n.d.	n.d.	81.15 ± 0.594	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tipo III	<LOQ	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.
CL-28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.
Manobi	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ariete	n.d.	n.d.	n.d.	n.d.	n.d.	38.84 ± 2.435	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sendra	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Carnaroli	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.
Bomba	<LOQ	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Arborio	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.
Lusitano	n.d.	n.d.	n.d.	n.d.	n.d.	49.08 ± 0.392	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Teto	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ulisses	n.d.	n.d.	n.d.	n.d.	n.d.	39.06 ± 2.494	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Arelate	n.d.	n.d.	n.d.	n.d.	n.d.	33.142 ± 0.618	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Puntal	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gageron	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.

LOQ- Limit of Quantification; **n.d.-** not detect

5. Conclusion

Foods contaminated with mycotoxins are associated with adverse human health effects. Therefore, it is crucial to develop simple and cost-effective analytical methodologies to enhance food safety and prevent potential harm from contaminants, particularly in cereals like rice.

This study successfully validated two analytical methodologies. The first method, based on Biochip Array Technology, was validated for the multi-mycotoxin analysis of nine mycotoxins (AFB1, AFG1, OTA, ZEA, DON, FB1+FB2, and T2+HT2). This technology can detect and semi-quantify the sum of two mycotoxins (FB1+FB2 and T2+HT2), making it an excellent screening tool for multi-mycotoxins in cereals. It offers significant advantages, including high throughput and cost-effective, rapid screening of multiple mycotoxins from cereal-based samples. Only positive samples require confirmatory testing by liquid chromatography with a mass spectrometry detector.

The second method, based on QuEChERS followed by ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-MS), was used for the simultaneous detection of twenty-five mycotoxins. This includes ten regulated mycotoxins (AFB1, AFB2, AFG1, AFG2, OTA, ZEA, T2, HT2, FB1, and FB2), three emerging mycotoxins (CIT, STE, MPA), and twelve ergot alkaloids (α-ergocryptine, α-ergocryptinine, ergocornine, ergocorninine, ergocristine, ergocristinine, ergometrine, ergometrinine, ergosine, ergosinine, ergotamine, and ergotaminine) in rice samples. This method is an excellent tool for monitoring mycotoxin levels in rice and is suitable for screening and routine analysis following European Regulations. It was confirmed to be an accurate, precise, and sensitive methodology, capable of detecting mycotoxins at low concentrations. Our research demonstrated the applicability of QuEChERS for detecting biological contaminants and the excellent sensitivity achieved using UHPLC-MS.

Of the 22 rice bran samples analyzed with the QuEChERS method followed by UHPLC-MS, only two samples exceeded the maximum limits established by the EU. One sample had AFB1 at 41.91 µg/kg and a combined total of AFB1, AFB2, AFG1, and AFG2 at 43.87 µg/kg. Another rice bran sample exceeded the maximum permitted value for ZEA, with a concentration of 145.78 µg/kg. These results were obtained in the bran, which is the fraction of the grain most subject to contamination, and lower values are expected in milled rice (white grain). The presence of aflatoxin B1 may be attributed to inadequate transport and storage conditions, and further studies are needed to determine if the zearalenone levels are linked to Fusarium diseases occurring in the field.

The results underscore the importance of implementing robust integrated pest management practices from cultivation through distribution to mitigate mycotoxin contamination in rice. Ensuring good storage conditions is also critical to prevent aflatoxin contamination. Continued research is essential to understand the factors contributing to mycotoxin presence and to develop effective management strategies.

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