UNIVERSIDADE D
COIMBRA

Filipa Simões dos Santos Monteiro Carreiró

Validation of Analytical Methodologies for Determination of Mycotoxins and Pesticides in Cereals

Dissertação no âmbito do Mestrado em Segurança Alimentar orientada pelo Professor Doutor Fernando Jorge dos Ramos e coorientada pela Doutora Ana Sanches Silva apresentada à Faculdade de Farmácia da Universidade de Coimbra.

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RESUMO

Os cereais são as espécies de plantas mais antigas que os Humanos plantaram e constituem uma fonte essencial de energia e nutrientes (apresentam elevado valor nutritivo) da dieta humana. Mais de 60% de toda a produção agrícola é composta por culturas de cereais. Estima-se que 2.799 milhões de toneladas de cereais foram produzidas em 2022, com uma grande quantidade de grãos, principalmente de trigo, milho e arroz. Os cereais devem ser estudos no que respeita aos seus potenciais contaminantes, como as micotoxinas, bem como resíduos de pesticidas, para garantir a segurança alimentar e consequentemente a saúde dos consumidores. Este estudo validou duas metodologias analíticas diferentes, uma tecnologia de *biochip* (BAT) para o *screening* de multi-micotoxinas em cereais e um método LC-MS/MS para a determinação de resíduos de pesticidas em arroz. Além disso, avaliaram-se os Fatores de Processamento e percentagens de redução em amostras contaminadas de arroz agulha submetidos a diferentes métodos de processamento (lavagem com água, lavagem com vinagre, cozimento convencional e cozimento a vapor e suas combinações).

O estudo de micotoxinas foca-se na validação de um BAT para o *screening* de multi-micotoxinas em arroz, estendida adicionalmente a outros cereais, como aveia, cevada, centeio e trigo. O *Evidence Investigator Myco 7* (RANDOX Food Diagnostic), baseado num imunoensaio quimioluminescente competitivo, foi utilizado para a deteção simultânea semiquantitativa de micotoxinas: aflatoxina B1 (AFB1) e aflatoxina G1 (AFG1), ocratoxina A (OTA), zearalenona (ZEA), toxina T2 e HT2 (soma de T2 e HT2), fumonisinas (soma de FB1 e FB2) e deoxinivalenol (DON). Foi utilizada uma extração com acetonitrilo:metanol:água (50:40:10, v/v/v). De acordo com os resultados da validação, as amostras de arroz fortificado apresentaram resultados falsos ≤5%, de acordo com os critérios impostos na legislação da União Europeia. Nas amostras fortificadas dos outros cereais, não houve falsos positivos e apenas 5% de falsos negativos para as micotoxinas FB1+FB2, OTA, AFB1 e T2+HT2. O imunoensaio BAT fornece vantagens importantes para a triagem rápida e eficiente de várias micotoxinas em amostras de cereais e amostras derivadas de cereais. O procedimento de triagem permite que apenas amostras suspeitas de contaminação sejam submetidas a testes de confirmação usando uma técnica mais precisa, como o LC-MS/MS.

No estudo dos pesticidas, foi validado um método para determinar 121 resíduos de pesticidas em amostras de arroz, de acordo com as diretrizes do documento SANTE/11312/2021. O método de extração escolhido para análise de resíduos de pesticidas foi o QuEChERS (Rápido, Fácil, Barato, Eficaz, Robusto e Seguro) e o extrato foi analisado por Cromatografia Líquida acoplada a Espectrometria de Massa (HPLC-MS/MS). As gamas de trabalho das curvas de

calibração encontraram-se entre 5–100, 10–100 ou 50-100 µg/L, dependendo do pesticida. O limite de quantificação foi de 5, 10 ou 50 µg/kg, dependendo também da molécula. A metodologia mostrou-se precisa (recuperação 70 - 119%) e foi aplicada a amostras comerciais de arroz. Quatro amostras de arroz comercial, entre elas uma de arroz agulha, uma de arroz basmati, uma de arroz integral e uma de arroz carolino foram analisadas quanto à sua potencial contaminação no que respeita aos 121 resíduos de pesticidas incluídos no método HPLC-MS/MS validado. Todas as amostras foram negativas para os resíduos de pesticidas analisados. Outro objetivo do estudo foi avaliar os efeitos da lavagem, com vinagre (5%, v/v) e sem vinagre, do cozimento convencional, do cozimento a vapor e da sua combinação nos níveis de resíduos de 121 pesticidas em arroz agulha. Uma porção de 200 g de amostra de arroz não contaminada foi submersa em 400 mL de água mineral contendo os pesticidas numa concentração final de 50 µg/kg e, em seguida, os efeitos de processamento (da lavagem, e/ou do cozimento e a sua combinação), foram investigados. Em relação à lavagem, a que apresentou maior eficácia na redução dos resíduos de pesticidas foi a lavagem com vinagre (5%, v/v), em 26,8-80,3%. A remoção de resíduos de pesticidas devido ao processamento é afetada pelo grau de absorção dos pesticidas pelos grãos dos cereais, pela solubilidade dos resíduos de pesticidas na água e pela degradação induzida pelo calor. As amostras de arroz submetidas aos dois métodos de processamento (lavagem com vinagre e cozimento) foram as que apresentaram maiores fatores de redução. Portanto, recomenda-se a utilização, a nível doméstico e industrial, de ambos os métodos para melhor garantir a segurança alimentar do arroz.

Palavras-chave: cereais; micotoxinas; imunoensaio de quimioluminiscência; resíduos de pesticidas; QuEChERS; HPLC-MS/MS; lavagem; cozimento; arroz; fatores de processamento.

ABSTRACT

Cereal crops are the earliest plant species that Humans have planted, and they constitute a key source of energy and nourishment (high nutritive qualities) in the human diet globally. More than 60% of all agricultural production is comprised of cereal crops. An estimated 2799 million tons of cereals were produced in 2022, with a large amount of coarse grains, mainly of wheat, maize, and rice. Cereals must be screened for contaminants such as mycotoxins as well as pesticide residues both to protect the health of consumers and due to food security.

This study validated two different analytical methodologies, a Biochip Array Technology (BAT) for multi-mycotoxins screening in cereals and a LC-MS/MS method to determine pesticides residues in rice. Moreover, it has evaluated the processing factors and reduction percentages in contaminated long-grain rice samples submitted to different processing methods (washing with mineral water, washing with vinegar, conventional cooking and steam cooking and their combinations).

The study of mycotoxins focuses on the validation of a BAT for multi-mycotoxins screening in rice, additionally extended to other cereals, like, oat, barley, rye, and wheat. The Evidence Investigator Myco 7 (RANDOX Food Diagnostic), based in a competitive chemiluminescent immunoassay, was used for the simultaneous semi-quantitative detection of the mycotoxins immunoassays: aflatoxin B1 (AFB1) and aflatoxin G1 (AFG1), ochratoxin A (OTA), zearalenone (ZEA), toxin T2 and HT2 (sum of T2 and HT2), fumonisins (sum of FB1 and FB2) and deoxynivalenol (DON). A single extraction step with acetonitrile:methanol:water (50:40:10, v/v/v) was used. According to validation results, spiked rice samples showed false results ≤5 %, in agreement with European Union legislation performance criteria. In the spiked samples of other cereals, any false positives were found and only 5% of false negatives were found for FB1+FB2, OTA, AFB1 and T2+HT2. The BAT immunoassay provides important benefits for the rapid and efficient screening of several mycotoxins from feed and food cereal-based samples at various levels. The screening procedure is further made easier by the multi-analytical approach because only samples suspected of contamination need confirmation testing using a more precise technique, like LC-MS/MS.

In the study of pesticides, a method was validated allowing to determine 121 pesticide residues in rice samples, according to the guidance document SANTE/11312/2021. QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) was chosen for pesticides extraction method and the extract was analyzed by high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). The linear ranges of the calibration curves were between 5–100, 10–100 or 50-100 µg/L, depending on the pesticide. The limit of quantification was 5, 10, or 50 µg/kg.

The methodology was shown to be precise and accurate (recovery 70.0-119%) and was applied to commercial samples of rice. Four commercial rice samples, one long grain rice, one basmati, one brown rice and one *Carolino* (short grain) rice were analyzed regarding their content in the 121 pesticide residues included in the HPLC- MS/MS method. All samples were negative for pesticide residues. Another objective of the study was to evaluate the effects of washing, washing with vinegar (5%, v/v), conventional cooking, steam cooking and their combination on residue levels of 121 pesticides in long grain contaminated rice samples. A 200 g portion of non-contaminated rice sample was soaked in 400 mL of water containing the pesticides at final concentration 50 μg/kg and then, the effects of processing (washing and/or cooking and their combination) were assessed. Regarding washing, the method using vinegar (5%, v/v) showed the highest effectiveness in reducing pesticides residues (from 26.8-80.3%). The washing effect was not associated with the water solubility of the pesticides or their chemical structure. The removal of pesticide residues due to processing is affected by the degree of adsorption of pesticides by the cereals' grains, pesticide residues' solubility in water and heat-induced breakdown. The rice samples submitted to both processing methods (wash with vinegar and cooking) presented higher reduction factors. Therefore, it is recommended to use, at domestic and industrial levels, both methods in order to better guarantee food safety of rice.

Keywords: cereals; mycotoxins; chemiluminescence immunoassay; pesticide residues; QuEChERS; HPLC-MS/MS; washing; cooking; rice; processing factors.

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LIST OF SYMBOLS, ACRONYMS AND ABBREVIATIONS

AChE- Acetylcholinesterase

ACN- Acetonitrile

AFB1- Aflatoxin B1

AFB2- Aflatoxin B2

AFG1- Aflatoxin G1

AFG2- Aflatoxin G2

AFM1- Aflatoxin M1

AFM2- Aflatoxin M2

AFs- Aflatoxins

ASE- Accelerated solvent extraction

ATA- Alimentary toxic aleukia

BAT- Biochip array technology

BEA- Beauvericin

CLIA- Chemiluminescent immunoassays

CO_2- Carbon dioxide

DDT- Dichloro-diphenyl-trichloroethane

DNC- 1,3-bis(4-nitrophenyl)urea

DON- Deoxynivalenol

DTR- Discrete test regions

EI- Electron impact

ELISA- Enzyme-linked immunosorbent assays

ENNs- Enniatins

ESI- Electrospray ionization

EU- European Union

FAO- Food and Agriculture Organization

FB1- Fumonisin B1

FB2- Fumonisin B2

FB3- Fumonisin B3

FIIA- Flow injection immunoassays

Fm- Cut-off value

FUMs- Fumonisins

GC- Gas chromatography

GC-MS- Gas chromatography mass spectrometry

GMP- Good Manufacturing Practice

H_20_2- Hydrogen peroxidase

HCH- Hexachlorocyclohexane

HPLC- High-performance liquid chromatography

HPLC-DAD- High-performance liquid chromatography-diode array detection

HRP- Horseradish peroxidase

IAC- Immunoaffinity columns

IARC- International Agency for Research on Cancer

INE- National Institute of Statistics

LC- Liquid chromatography

LC-MS- Liquid Chromatography mass spectrometry

LC-MS/MS- Liquid chromatography with tandem mass spectrometry

LFIA- Lateral flow immunoassays

LLE- Liquid-liquid extraction

LMs-LFIA- Latex microsphere lateral flow immunoassay

LOD- Limit of Detection

LOQ- Limit of Quantification

mAb- monoclonal antibody

MAE- Microwave-assisted extraction

MIPs- Molecularly imprinted polymers

ML- Maximum level

MRL- Maximum residue levels

MRM- Multiple reaction monitoring

MS- Mass spectrometry

MSPD- Matrix solid-phase extraction

NCI- Negative ionization in negative

OCPs- Organochloride pesticides

OPs- Organophosphates

OTA- Ochratoxin A

OTB- Ochratoxin B

OTC- Ochratoxin C

OTs- Ochratoxins

PCI- Chemical ionization in positive

PF- Processing factor

PLE- Pressurized liquid extraction

POPs- Persistent organic pollutants

PROMEC- Programme on Mycotoxins and Experimental Carcinogenesis

PSA- Primary secondary amine bonded silica

QuEChERS- Quick, Easy, Cheap, Effective, Rugged, and Safe

R^2- Determination coefficient

RASFF- Rapid Alert System for Food and Feed

RLU- Relative light units

RSD_R- Intra-laboratory reproducibility

RSD_r- Repeatability

RT- Retention time

SFE- Supercritical fluid extraction

SLE- Solid-liquid extraction

SPE- Solid-phase extraction

SPME- Solid-phase microextraction

T- Threshold value

TCs- Trichothecenes

TLC- Thin-layer chromatography

TOF- Time-of-flight

TPP- Triphenylphosphate

U- Expanded uncertainty

UHPLC-MS/MS- Ultra-high performance liquid chromatography tandem mass spectrometry

UHPLC-TOF-MS- Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry

UPLC- Ultra performance liquid chromatography

WHO- World Health Organization

ZEA- Zearalenone

PART I- INTRODUCTION

1. CONTAMINANTS IN CEREALS

Cereal crops are the earliest plant species that Humans have planted, and they constitute a key source of energy and nourishment (high nutritive qualities) in the human diet globally. More than 60% of all agricultural production is comprised of cereal crops. An estimated 2799 million tons of cereals were produced in 2022, with a large amount of coarse grains, mainly of wheat, maize, and rice (Alkuwari *et al.*, 2022). In the European Union (EU), crop cultivation takes up an average of 13% of the land area. After France and Poland, Germany has the third-largest EU cereal-producing area (6.7 million hectares) (Kresse *et al.*, 2019). Cereals can become contaminated with mold at various stages of development, processing, and storage. These infections can be divided into two groups: pathogenic, which cause plant illnesses and low productivity, and toxic, which cause toxic metabolites to build up and reduce output. Both times, the quantity and quality of the cereals are harmed, causing significant annual financial losses in the global agriculture industry *(Alkuwari et al., 2022; Kresse et al., 2019; Kumar et al.*, 2022; Zhao *et al*., 2022).

One of the most crucial aspects of growing grain production is the application of pesticides, including pre- and post-emergence herbicides, insecticides, and fungicides, at different phases of cultivation. However, the use of these pesticides has an impact on the soil and water. In addition to the use of authorized pesticides, a significant concern is the presence of not allowed pesticides in cereals, which is the reason why Good Manufacturing Practice (GMP) plays a crucial role. The absence of pesticides in agriculture practices would result in crop losses for a number of reasons, including the growth of fungus that would contaminate grains with mycotoxins, especially in humid climates. Cereals must be screened for pollutants such as mycotoxins as well as pesticide residues both to protect the health of consumers and due to food security (Kresse *et al.*, 2019; Shakoori *et al.*, 2018).

1.1. RICE

For a substantial portion of the global human population, rice is the most significant and primary cereal crop. In seventeen countries in Asia and the Pacific, nine in North and South America, and eight in Africa, rice is the main energy source (Shakoori *et al.*, 2018). 20% of the calories consumed globally come from rice, which is essential for the existence of more than 3.5 billion people. Rice is a good source of thiamine, riboflavin, niacin, glutamic acid, and aspartic acid, yet it cannot provide all the components required for proper nutrition on its own. Rice that has not been processed has a considerable amount of nutritional fiber.

Rice is an annual plant that is a member of the Oryza genus, which has roughly 22 different species. *Oryza sativa*, which is grown all over the world, and *Oryza glaberrima*, which is grown in some regions of West Africa, are the only two species of rice that are thought to be significant as food species for Humans and are widely cultivated (Shakoori *et al.*, 2018).

According to the *Ricepedia,* the annual consumption of milled rice per person varies from 6–18 kilograms in southern Europe to 3.5–5.5 kg in northern Europe's non-rice-growing nations. Italy and Spain produce over 80% of the rice consumed in the EU, with Greece and Portugal producing 12% more (*Rice Production in Europe - Ricepedia.org*, 2023). According to the National Institute of Statistics (INE), the Portuguese consumed 15 tons of rice between 2021 and 2022 (*Portal do INE*, 2023).

2. MYCOTOXINS

Fungi, also known as molds, produce various secondary metabolites like mycotoxins (low-molecular weight compounds ~700 Da). They can colonize a huge variety of food and feed commodities and generate mycotoxins during the pre-harvest or post-harvest stages of the food processing chain. In this line, food safety concerns have grown worldwide (Ekwomadu, Akinola e Mwanza, 2021; Habschied *et al.*, 2021; Janik *et al.*, 2021; Mamo *et al.*, 2020). Mycotoxins are almost unavoidable in food and are highly influenced by edaphoclimatic conditions; however, controlling them is challenging, particularly due to pollution, mechanical damage to kernels, high moisture and temperature, and geographical location (Buszewska-Forajta, 2020; Ekwomadu, Akinola e Mwanza, 2021). According to the Food and Agriculture Organization (FAO), mycotoxins impact approximately one-fourth (25%) of global crops, resulting in billions of dollars in annual losses (Eskola *et al.*, 2020; Mamo *et al.*, 2020). Some of the most contaminated crops by mycotoxins are important from an agricultural point of view, including maize, peanuts, rice, wheat, oat, rye, and barley, as well as fruits, coffee, and spices (Eskola *et al*., 2020; Habschied *et al*., 2021). Mycotoxins have diverse chemical structures, and their toxicities are well characterized (Table 1). Dietary intake of mycotoxins is associated with many chronic conditions, including immunosuppression, cancer, gastrointestinal, hematological, and neurological damage, which is associated with significant health risks (Mamo *et al.*, 2020; Ülger *et al.*, 2020). To date, 400 mycotoxins have been identified, the most relevant being aflatoxins (AFs), fumonisins (FUMs), ochratoxins (OTs), trichothecenes (TCs), and zearalenone (ZEA) (Mamo *et al*., 2020; Ülger *et al*., 2020).

Table 1. Mycotoxins and their toxicity (Singh e Mehta, 2020; Wolf e Schweigert, 2018). Chemical structures obtained by (ChemSpider | Search and Share Chemistry, 2023).

Mycotoxins	Aflatoxins	Fumonisins	Ochratoxins	T-2/HT-2 toxin	Deoxynivalenol	Zearalenone
Toxicity	Nephrotoxic Acute aflatoxicosis Carcinogenic Mutagenic Pulmonary carcinogen Teratogenicity Hepatotoxic Liver carcinogen Liver disease and immunosuppressive Formation of DNA adducts Lipid peroxidation Bioactivation by cytochromes P450 Conjugation to GS-transferases	Immunotoxic Necrosis Carcinogenic Hepatotoxic	Teratogenic Hepatotoxic Effect on protein synthesis Carcinogenic Mutagenic Detoxification by peptidases Nephrotoxic Inhibition of ATP production	Immunodepressants Gastrointestinal Nausea Mutagenic induction of apoptosis in haemopoietic progenitor cells Emesis Effect on protein synthesis, abnormal changes to immunoglobulins Abdominal pain Diarrhea Dermal necrosis	Diarrhea Abdominal stress Emesis Increased salivation Neurotoxic Malaise Mutagenic	Carcinogenic Estrogenic activity Genotoxic

2.1. 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, Thorat e Banerjee, 2021; Popescu *et al.*, 2022; Yang, Song e Lim, 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 1 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 (Table 1) (Benkerroum, 2020; Dhanshetty, Thorat e Banerjee, 2021; Pickova *et al.*, 2021).

2.2. 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* (Li *et al.*, 2022; Qu *et al.*, 2022; Wangia-Dixon e 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 e 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 (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; Li *et al.*, 2022; Yli-Mattila e 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 (Li *et al.*, 2022).

2.3. 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; 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; 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; 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).

2.4. 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 HT-2 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).

2.4.1. 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 e 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 e 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 e Long, 2020).

2.4.2. HT-2 Toxin and T-2 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 T-2 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. T-2 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; Yang *et al.*, 2020). Additionally, T-2 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).

2.5. 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, Şahin e Üstündağ, 2022; Rai, Das e Tripathi, 2020; Ropejko e Twarużek, 2021). High ZEA levels have been linked to symptoms of nausea, vomiting, and diarrhea associated with cereal toxicosis (Caglayan, Şahin e Üstündağ, 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).

2.6. EMERGING MYCOTOXINS

Emerging mycotoxins are a class of mycotoxins that have not yet been regularly identified or regulated by law, despite the fact that evidence of their occurrence has been growing quickly over the past several decades. This class of mycotoxins, including enniatins (ENNs) and beauvericin (BEA), is generated by numerous filamentous fungi. Fusarium spp., Alternaria spp., Halosapheia spp., and Verticillum spp. are the primary producers of ENNs, whereas Beauveria spp., Paecilomyces spp., Polyporus spp., and Fusarium spp. are the primary producers of BEA. According to studies, new mycotoxins can co-occur with other kinds of mycotoxins and are widespread around the world. They could therefore pose a risk to both human and animal health. Although several studies have discussed potential hazards connected with their consumption due to their ionophoric qualities, no evidence of mycotoxicosis induced by BEA and ENNs has been discovered. In order to assess their health risk and finally establish regulation levels, more research must be conducted (Mateus *et al.*, 2021; Santos *et al.*, 2022).

2.7. MYCOTOXINS LEGISLATION AT EUROPEAN UNION

Because mycotoxins are difficult to eliminate, some countries have set their own maximum tolerance limits. When comparing mycotoxin regulations around the globe, the EU appears to have implemented the lowest maximum permitted levels for mycotoxins, with strict standards established at levels as low as μg/kg (Sibanda *et al.*, 2022; Zong *et al.*, 2021). Table 2 summarizes the maximum levels for cereals in EU.

Table 2. Adapted from Commission Regulation (EU) No. 2023/915 (European Commission, 2023) and its amendments indicating the maximum permitted levels of mycotoxins in cereals and from Commission Recommendation of 27 March 2013 (European Commission, 2013) reporting the indicative levels for the sum of T-2 and HT-2 (μg/kg) from which investigations should be performed.

	AFB1*	AFB1 + AFB2 + AFG1 + AFG2*	OTA*	DON*	ZEA*	T-2+HT-2 toxins**	FB1+FB2
Cereals for direct consumption (μg/kg)	2.0	4.0	3.0	750	75	100	-
Baby Foods for Infants and Young Children (μg/kg)	0.10	-	0.50	200	20	15	-
Maize and rice to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs (μg/kg)	5.0	10.0	-	-	-	-	-
Unprocessed cereals (μg/kg)	-	-	5.0	-	100	-	-
Wheat gluten not sold directly to the consumer (μg/kg)	-	-	8.0	-	-	-	-
Unprocessed cereals other than durum wheat, oats and maize (μg/kg)	-	-	-	1250	-	-	-
Unprocessed durum wheat and oats (μg/kg)	-	-	-	1750	-	-	-
Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling (μg/kg)	-	-	-	1750	350	-	4000
Pasta (dry) (μg/kg)	-	-	-	750	-	-	-
Bread (including small bakery wares), pastries, biscuits, cereal snacks, and breakfast cereals (μg/kg)	-	-	-	500	-	-	-

	AFB1*	AFB1 + AFB2 + AFG1 + AFG2*	OTA*	DON*	ZEA*	T-2+HT-2 toxins**	FB1+FB2
Unprocessed cereals other than maize (µg/kg)	-	-	-	-	100	-	-
Maize intended for direct human consumption, maize-based snacks, and maize-based breakfast cereals (µg/kg)	-	-	-	-	100	-	-
Processed maize-based foods for infants and young children (µg/kg)	-	-	-	-	20	-	200
Unprocessed cereals: barley (including malting barley) and maize (µg/kg)	-	-	-	-	-	200	-
Unprocessed cereals: oats (with husk) (µg/kg)	-	-	-	-	-	1000	-
Unprocessed cereals: wheat, rye and other cereals (µg/kg)	-	-	-	-	-	100	-
Cereal grains for direct human consumption: oats (µg/kg)	-	-	-	-	-	200	-
Cereal grains for direct human consumption: maize (µg/kg)	-	-	-	-	-	100	-
Cereal products for human consumption: oat bran and flaked oats (µg/kg)	-	-	-	-	-	200	-
Cereal products for human consumption: cereal-based foods for infants and young children (µg/kg)	-	-	-	-	-	15	-

Legend: * maximum permitted levels; ** Indicative levels

2.8. ANALYTICAL METHODS

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, Fernandes e Cunha, 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).

2.8.1. 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, Fernandes e Cunha, 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. 401/2006) 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, 2006).

2.8.2. Extraction and Clean-up Methodologies

Extraction and clean-up steps are very important for the determination of analytical methods, for the extract to be as clean as possible. 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, Fernandes e Cunha, 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 (this method will be fully addressed in Part III) (Pereira, Fernandes e Cunha, 2014).

2.8.3. 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).

2.8.3.1. Conventional Methods

As was mentioned previously, conventional methods can be chromatographic methods, for example. 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.*, 2021; 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, Fernandes e Cunha, 2014; Santos *et al.*, 2022).

2.8.3.2. Rapid Methods

There are several types of rapid methods, such as immunoassays and biosensors, for example. But the work will focus primarily on explaining some different types of immunoassay 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 a few of these techniques. Furthermore, they can be divided into two categories: labeled and label-free sensors, as well as competitive (direct or indirect techniques) and non-competitive assays (Adunphatcharaphon *et al.*, 2022). Because

mycotoxins are relatively small molecules, they have, in general, been detected using competitive, rather 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.*, 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 made for a single application and only detects one mycotoxin. Additionally, it might become expensive if numerous tests are required to identify different mycotoxins (Adunphatcharaphon *et al.*, 2022; Mateus *et al.*, 2021; Pereira, Fernandes e Cunha, 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. 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.*, 2016; Sibanda *et al.*, 2022) The analyte and conjugate compete for the binding sites in this form of competitive chemiluminescence, and the relationship between the analyte concentration and the light produced by the chemical reaction is inverse (Jia *et al.*, 2021). The method has already been validated in maize (Freitas *et al*, 2019). But the immunoassay now allows the determination of the sum of T-2 and HT-2 instead of just T-2; in other words, the BAT can detect nine mycotoxins (AFB1, AFG1, OTA, ZEA, DON, FB1+FB2, and T-2+HT-2), although in two cases it detects and semi-quantifies the sum of two mycotoxins (FB1+FB2 and T-2+HT-2). In Table 3 the main advantages and drawbacks of some rapid methods used to detect mycotoxins are compiled.

Table 3. Advantages and drawbacks of rapid methods for mycotoxins analysis (Posthuma-Trumpie, Korf e Amerongen, 2008; Singh e Mehta, 2020; Wolf e Schweigert, 2018).

	Advantages	Drawbacks	Results (qualitative/semi-quantitative/quantitative)
ELISA	• Simple sample preparation • Inexpensive equipment • High sensitivity • Simultaneous analysis of multiple samples • Suitable for screening • Not having to use radioisotopes or an expensive radiation counter • Low expenditure at time	• Cross-reactivity with related mycotoxins • Matrix interference problems • Possible false-positive/negative results • High costs	Qualitative and Semi-Quantitative
Lateral Flow detection	• One-step assay • No washing step necessary • Fast and low cost • Low sample volume • Simple test procedure • Portable	• Qualitative or semi quantitative results • Imprecise sample volume reduces precision • High cost	Semi-quantitative
Fluorescense polarizaton immunoassay	• Multi-analyte immunoassay is feasible • Wide detection range • Long lived luminescence in comparison with conventional fluorophore • Low time expenditure per sample • Sensitive • No washing and separation steps	• Background interference in sample • Longer incubation times is required for better reproducibility • High costs • Currently only for certain mycotoxins available (in research)	Qualitative or semi-quantitative
Microarray technology	• High-throughput screening miniaturized • Multiplexed • Parallel processing method	• Not common because of their variability and reproducibility • Intensive labor requirement	Semi-quantitative

2.9. DECONTAMINATION STRATEGIES

There are several types of decontamination strategies for mycotoxins, including physical, chemical, and biological. However, no single method has been demonstrated to be effective against the large range of mycotoxins that may exist concurrently in a food product. The techniques should be capable of entirely eliminating, deactivating, or destroying the poison with any lingering spores of fungus. It must also maintain the commodity's technological capabilities and nutritional value. Table 4 summarizes the advantages and disadvantages of each method (Santos *et al.*, 2022).

Table 4. Different decontamination means of mycotoxins in food, their advantages, and disadvantages, adapted from (Santos *et al.*, 2022).

	Physical decontamination	**Chemical decontamination**	**Biological decontamination**
Examples	Sorting Sieve cleaning Density segregation Washing De-hulling Steeping Extrusion cooking Steam heating Infrared heating Microwave heating Radio frequency heating Irradiation Cold plasma Photocatalytic detoxification	Organic acids Hydrochloric acid Ammonium hydroxide Hydrogen peroxide Sodium bisulphite Chlorinating agents Ozone Formaldehyde Natural substances such as herbs, spices, and their extracts	Bacteria Yeasts Mold Algae
Advantages	Effective against some mycotoxins Low change in food properties Does not involve usage of chemicals	Effective against some mycotoxins Affordable	Effective against some mycotoxins Inexpensive Environment friendly Does not involve usage of chemicals
Disadvantages	Impractical Might be limited to large-scale industries with sophisticated equipment Time-consuming Expensive In case of thermal treatment possible changes in color and food quality	Possible health effects Formation of toxic byproducts Enhancing bioavailability of masked mycotoxins Time consuming Environmentally toxic	Time consuming Impractical More effective in controlled laboratory settings

3. PESTICIDES

The group of substances known as pesticides pertains to substances used as insecticides (kill insects and other arthropods), fungicides (kill fungi, including blights, mildews, molds, and rusts), herbicides (kill weeds and other plants that grow where they are not wanted), rodenticides (control mice and other rodents), molluscicides (kill snails and slugs), and nematicides (kill nematodes) (Kresse *et al.*, 2019). The following citation is the definition of pesticides provided by the FAO: "*Pesticide means any substance or mixture of substances or biological ingredients intended for repelling, destroying or controlling any pest or regulating plant growth*" (Food and Agriculture Organization, 2021). Three billion kilograms of pesticides are used worldwide every year, while only 1% of total pesticides are effectively used to control insect pests on target plants. This not only increases the cost of agricultural production but also affects the quality and safety of agricultural products and the ecological environment (Tudi *et al.*, 2021). Recent research demonstrates that around 2 million tons of pesticides are utilized, with herbicides accounting for 47.5%, insecticides for 29.5%, fungicides

for 17.5%, and other pesticides for 5.5% (Bondareva e Fedorova, 2021). Different classification terms, such as chemical classes, functional groups, modes of action, and toxicity, are used to categorize pesticides. Pesticides are categorized into organic and inorganic components according to chemical classifications. Copper sulfate, ferrous sulfate, copper, lime, and sulfur are examples of inorganic pesticides. Organic pesticides have more complex ingredient lists. Organic pesticides can be classified according to their chemical structure, such as chlorohydrocarbon insecticides, organophosphorus insecticides, carbamate insecticides, synthetic pyrethroid insecticides, metabolite and hormone analog herbicides, synthetic urea herbicides, triazine herbicides, benzimidazole nematocides, metaldehyde molluscicides, metal phosphide rodenticides, and D group vitamin-based rodenticides. Table 5 summarizes examples of pesticides by their functional class.

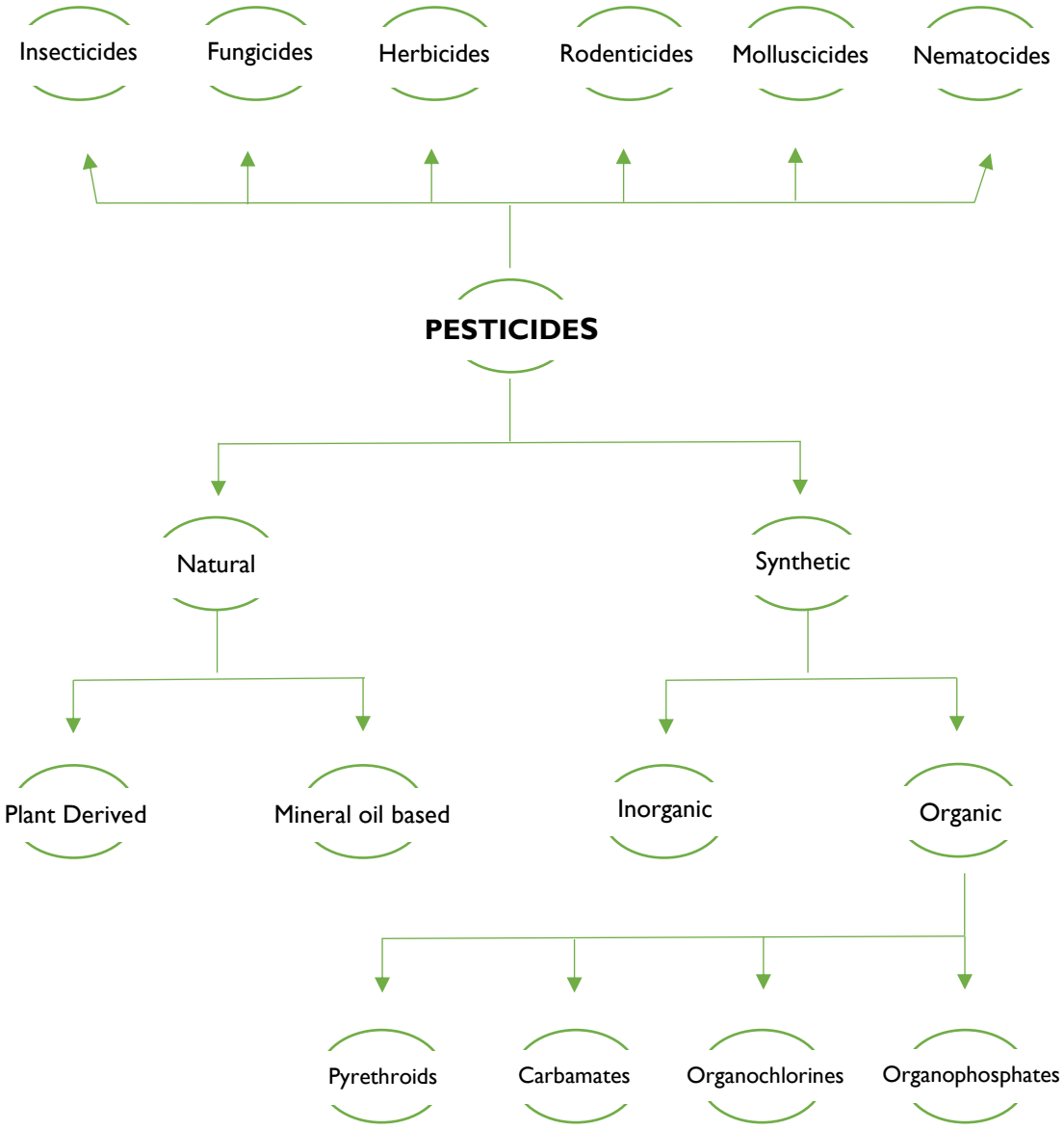

Figure 1. Classification of Pesticides according to target organisms, origin and chemical structure.

3.1. CLASSIFICATION OF PESTICIDES BY THEIR CHEMICAL STRUCTURE

3.1.1. Pyrethroids

Pyrethroids have been deemed safe for both people and animals. After 1945, the first pyrethroids were used as insecticides. Pyrethroids are now widely employed in a variety of fields, including crop protection, the forestry, wood, and textile industries, as well as human and veterinary medicine to treat parasitic crustacean infestations. In the form of soaking mosquito nets, sprays, or gels, they are also employed to protect individuals from insects. Importantly, the World Health Organization (WHO) advises using pyrethroids as a preventative approach against mosquito outbreaks in order to fight malaria and the Zika virus (Hołyńska-Iwan e Szewczyk-Golec, 2020; Werner e Young, 2017).

Historically, pyrethroids were a class of organic substances that were isolated from the *Tanacetum cinerariaefolium* plant's (formerly known as *Chrysanthemum cinerariaefolium*) flowers (Hołyńska-Iwan e Szewczyk-Golec, 2020; Pfeil, 2014; Werner e Young, 2017).

Pyrethroids consist of an acid and an alcohol as their two main structural components. Chrysanthemic acid, a cyclopropane ring attached to a carboxylic acid moiety, and other halogenated and nonhalogenated substituents were added to most pyrethroids to boost their photostability. It depends on the heterocyclic structure, but the alcohol component is either a primary or secondary alcohol. Additionally, certain pyrethroids include cyano groups attached to the α-methylene of the alcohol, increasing the compound's ability to kill insects (Pfeil, 2014; Riar, 2014; Werner e Young, 2017).

Type I pyrethroids are substances without the α-cyano substituent (e.g., allethrin, bifenthrin, etofenprox, permethrin, phenothrin, resmethrin, and tefluthrin), whereas type II pyrethroids are substances with the α-cyano group, such as cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, esfenvalerate, fenpropathrin, and t-fluvalinate (Pfeil, 2014; Werner e Young, 2017).

Natural pyrethroids are unstable substances that quickly decrease when exposed to light, so they have not been frequently employed in agriculture. Thus, it has been developed to synthesize derivatives that are more radiation-resistant and more deadly to insects (Hołyńska-Iwan e Szewczyk-Golec, 2020; Pfeil, 2014).

3.1.2. Carbamates

Despite their intriguing medical uses, including the treatment of glaucoma, Alzheimer's disease, and myasthenia gravis, among others, carbamates are primarily employed as insecticides around the world. Esters of N-methyl carbamic acid, or carbamates, were initially isolated from the Calabar bean, *Physostigma venenosum*, a plant native to West Africa. Due to their quick hydrolysis into typically less harmful metabolites like amine and carbon dioxide (CO_2), which are rapidly expelled from the organism, this class of compounds is recognized to lack residual persistency in the environment and in mammalian species (Mdeni *et al.*, 2022; Moreira *et al.*, 2022).

Carbamate pesticides can be subdivided into three groups: carbamates, thiocarbamates, and dithiocarbamates. Some examples of carbamates are carbendazim, carbaryl, carbofuran, aminocarb, thiodicarb, and mancozeb (Gupta, 2014).

Despite the differences, the compounds from the carbamate family share the common trait of being both acetylcholinesterase (AChE) inhibitors; in other words, as temperature, pH, or both increase, the chemical breakdown of these pesticides accelerates, and their toxicity is due to the disruption of the nervous system of an invertebrate or a vertebrate through the inhabitation of cholinesterase enzymes (Mdeni *et al.*, 2022; Moreira *et al.*, 2022).

3.1.3. Organochlorines

In a variety of applications such as agriculture, industry, medicine, and the home, organochlorine pesticides (OCPs), which are organic compounds with at least one covalently bound chlorine atom, are used to kill insects (such as mosquitoes, termites, head lice, and fire ants) and even to control insect-borne diseases (Jayaraj, Megha e Sreedev, 2016; Tsai, 2014). Organochlorine pesticides can be categorized into three main categories: cyclodienes (like chlordane), dichlorodiphenylethanes (like dichloro-diphenyl-trichloroethane (DDT)), and hexachlorocyclohexanes (like lindane). As a result, they are categorized as persistent organic pollutants (POPs), and, especially at high latitudes and high elevations, they can still be found in the environment and in organisms at detectable concentrations. Although OC pesticides were once used successfully to combat malaria and typhus, they are now outlawed in the majority of industrialized nations (Jayaraj, Megha e Sreedev, 2016; Roark, 2020; Tsai, 2014).

According to statistics on the use of various pesticides, 40% of all pesticides used belong to the chemical class of organochlorines. Organochlorine insecticides like DDT, hexachlorocyclohexane (HCH), aldrin, and dieldrin are among the most extensively used

pesticides in developing countries in Asia because of their low cost and the need to combat diverse pests (Jayaraj, Megha e Sreedev, 2016).

3.1.4. Organophosphates

Nearly 34% of the pesticides made and sold for agricultural use globally are organophosphates (OPs). Include all insecticides that contain phosphorus because these chemicals are created when phosphoric acid and alcohol undergo the esterification reaction (Ajiboye *et al.*, 2022; Mukesh Doble, 2005; Richardson e Makhaeva, 2014). These substances are the primary ingredients in pesticides, insecticides, and herbicides. The environment is polluted by their excessive and unchecked use. They are the most toxic of all pesticides to vertebrates, and they also have toxic effects on plants and animals that have an impact on agricultural productivity and yield. Organophosphorus insecticides are unstable or nonpersistent, and their toxicity is dependent on the quantity of sulfur and the valency of the phosphorus present. OPs can also be categorized by their lethal dosage (LD50) values as low-toxic, moderately toxic, highly toxic, and virulent. Additionally, OPs make up the majority of nerve gas. They contain substances such as malathion, ethyl parathion, and diazinon (Adeyinka, Muco e Pierre, 2023; Ajiboye *et al*., 2022; Mukesh Doble, 2005; Richardson e Makhaeva, 2014).

Table 5. Functional Classes of representative Pesticides.

Pesticide	**Functional Class**	**Pesticide**	**Functional Class**
Acetamiprid	Insecticide, acaricide	Chlorpyrifos-methyl	Insecticide
Azoxystrobin	Fungicide	Clofentezine	Insecticide, acaricide
Bitertanol	Fungicide	Cymoxanil	Fungicide
Bixafen	Fungicide	Cyproconazole	Fungicide
Boscalid	Fungicide	Cyprodinil	Fungicide
Bupirimate	Fungicide	Demeton-S-methylsulfone	Insecticide
Buprofezin	Insecticide	Diazinon	Insecticide, acaricide
Cadusafos	Insecticide	Dichlorvos	Insecticide, acaricide
Carbaryl	Insecticide, plant growth regulator	Difenoconazole	Fungicide
Carbendazim	Fungicide	Diflubenzuron	Insecticide
Carbofuran	Insecticide	Dimethoate	Insecticide, acaricide
Carbofuran-3-hydroxy	Insecticide	Dimethomorph	Fungicide
Carboxin	Fungicide	Diniconazole	Fungicide
Chlorantraniliprole	Insecticide	DMST	Fungicide
Chlorfenvinphos	Insecticide	EPN	Insecticide, acaricide
Epoxiconazole	Fungicide	Fenthion oxon sulfone	Insecticide

Pesticide	Functional Class	Pesticide	Functional Class
Ethiofencarb	Insecticide	Fenthion oxon sulfoxide	Insecticide
Ethoprophos	Insecticide, nematicide	Fenthion sulfone	Insecticide
Etrimfos	Insecticide	Fenthion sulfoxide	Insecticide
Fenamidone	Fungicide	Fluopyram	Fungicide
Fenamiphos sulfone	Nematicide	Fluquinconazole	Fungicide
Fenamiphos sulfoxide	Nematicide	Flutriafol	Fungicide
Fenarimol	Fungicide	Fonofos	Insecticide
Fenitrothion	Insecticide	Fosthiazate	Insecticide, nematicide
Fenoxycarb	Insecticide	Hexythiazox	Acaricide
Fenpropathrin	Insecticide, acaricide	Indoxacarb	Insecticide
Fenpropidin	Fungicide	Iprodione	Fungicide
Fenpropimorph	Fungicide	Iprovalicarb	Fungicide
Fenpyroximate	Acaricide	Isoprocarb	Insecticide
Fenthion oxon	Insecticide	Isoprothiolane	Fungicide
Isoproturon	Herbicide	Metribuzin	Herbicide
Kresoxim-methyl	Fungicide	Mevinphos	Insecticide, acaricide
Linuron	Herbicide	Monocrotophos	Insecticide, acaricide
Lufenuron	Insecticide	Oxadixyl	Fungicide
Malaoxon	Insecticide	Paclobutrazole	Regulator of systemic plant growth
Malathion	Insecticide	Paraoxon-ethyl	Insecticide
Mepanipyrim	Bactericide, fungicide	Paraoxon-methyl	Insecticide
Metaflumizone	Insecticide	Parathion	Insecticide
Metalaxyl	Fungicide	Parathion-methyl	Insecticide
Metalaxyl-M	Fungicide	Penconazole	Fungicide
Metazachlor	Herbicide	Pencycuron	Fungicide
Metconazole	Fungicide	Pendimethalin	Herbicide
Methiocarb	Insecticide	Phentoate	Insecticide, acaricide
Methomyl	Insecticide, acaricide	Phosalone	Insecticide
Metobromuron	Herbicide	Phosmet	Insecticide, acaricide
Phosphamidon	Insecticide, acaricide	Spinosad D	Insecticide
Phoxim	Insecticide	Spiroxamine	Fungicide
Pirimiphos-ethyl	Insecticide, acaricide	Tebuconazole	Fungicide
Pirimiphos-methyl	Insecticide, acaricide	Tebufenpyrad	Acaricide
Prochloraz	Fungicide, herbicide	Terbuthylazine	Herbicide
Profenofos	Insecticide, acaricide	Tetraconazole	Fungicide
Propiconazole	Fungicide	Thiabendazole	Fungicide
Propoxur	Insecticide, acaricide	Thiacloprid	Insecticide, molluscicide

Pesticide	Functional Class	Pesticide	Functional Class
Propyzamide	Herbicide	Thiodicarb	Insecticide
Prothioconazole-desthio	Fungicide	Triadimefon	Fungicide
Pyrazophos	Fungicide	Triadimenol	Insecticide, fungicide
Pyrimethanil	Fungicide	Tricyclazole	Fungicide
Quinoxyfen	Fungicide	Triflumuron	Insecticide
Rotenone	Insecticide, acaricide	Zoxamide	Fungicide
Spinosad A	Insecticide	Fludioxonil	Fungicide

Legend: DMST- N,N-dimethyl-N'-p-tolysulphamide; EPN- O-ethyl O-4-nitrophenyl phosphonothiate.

3.2. PESTICIDES LEGISLATION AT EUROPEAN UNION

The use of pesticides is still a reality, and in fact, it is essential to prevent food loss, despite the fact that efforts to decrease or find alternatives are rapidly developing. However, in addition to causing environmental disturbances (air, soil, and water), pest resistance, pest resurgence, acute and long-term effects on non-target organisms in agroecosystems, and toxicity for both users and food consumers, pesticides also cause pest resurgence (Melo *et al.*, 2020).

To maintain public safety as well as domestic and international trade, it is crucial to control pesticide residues in food, and in the European Union, this is backed by regulation. Regulation (EC) No. 396/2005 and its amendments and Regulation (EU) No. 2018/62 set down the rules for pesticide usage in the EU. In Regulation 396/2005, the European Commission established standardized maximum residue levels (MRL) to prevent different Member States from having different MRLs for the same pesticide in the same product (European Commission, 2005, 2018). In addition, a very useful tool for searching the different MRLs is the website of the European Commission, which has a search bar to search the different MRLs and even for different matrices (European Commission, 2023; Carrasco Cabrera e Medina Pastor, 2022; *Melo et al.*, 2020).

3.3. ANALYTICAL METHODS

Like mycotoxins, pesticides residues also require sampling, extraction, and clean-up steps before the actual analysis of the compounds. The sampling step is as important for mycotoxins as it is for pesticides (section 2.8.1). The commission directive 2002/63/EC of 11 July 2002 provides sampling procedures to enable a representative sample to be obtained from a lot for analysis to determine compliance with MRLs (European Commission, 2002).

3.3.1. Extraction and Clean-Up Methodologies

Most methods for determining pesticide residues involve these two crucial steps: cleaning up the target analytes from the coextractives and removing them from the majority of the matrix. Traditional methods are generally still utilized, although they take a long time, require a lot of work, are expensive, difficult, and produce a lot of waste. The preparation of food and environmental samples for extraction and measurement of pesticide residues has benefited from the development of various innovative analytical techniques in recent years (Sandín-España e Dagnac, 2023).

It is well known that traditional sample preparation methods like LLE and SPE typically use a lot of organic solvents and are laborious and time-consuming. To address the shortcomings of traditional sample preparation. Although traditional methods like LLE and SPE are still used to extract pesticides, there are other approaches that can be used instead, including matrix solid-phase dispersion (MSPD), the QuEChERS method, and solid-phase microextraction (SPME) (Martins *et al.*, 2013; Sandín-España e Dagnac, 2023).

Since its development by *Anastassiades et al.* (2003), the QuEChERS approach has grown to be one of the most widely used methods for preparing samples for pesticide detection, mostly on fruits and vegetables, but has since been modified for use in other food crops, such as dried agricultural commodities (Cho *et al.*, 2016). The QuEChERS method has been extensively used in a variety of matrices for the determination of a wide range of pesticides, proving its benefits in terms of low cost, simplicity, quick extraction times, and minimal organic solvent usage. Additionally, this method aids in overcoming several difficulties brought on by the co-extraction of matrix components, such as co-elution, chemical background noise, or signal amplification or suppression (Sandín-España e Dagnac, 2023). The QuEChERS method has also been applied to mycotoxin analysis. *Mateus et al.* (2021), developed and validated a QuEChERS followed by UHPLC-ToF-MS for determination of multi-mycotoxins in pistachio nuts. In this study different methods of cleaning up high lipid matrices using dispersive solid phase extraction were assessed. Classic sorbents like C18 (octadecylmodified silica) and PSA (primary secondary amine) were utilized for this goal, as well as modern sorbents such as EMR-Lipid (enhanced matrix removal-lipid) and Z-Sep (modified silica gel with zirconium oxide). The best analytical performance for aflatoxins (AFB1, AFB2, AFG1 and AFG2), OTA, ZEA, toxin T2, and HT2 in pistachios was provided by the QuEChERS method, followed by Z-Sep d-SPE clean-up (Mateus *et al.*, 2021).

3.3.2. Detection Methods

For the analysis of pesticides, the most common is to use chromatographic methods, such as LC and GC, already reported in Section 2.8.3.1, which allow identification and quantitation of pesticide residues in complex matrices. Because of their polarity, poor thermal stability, or lack of volatility, some pesticides cannot be determined by GC; hence, LC is an option for the determination of these substances (Pareja *et al.*, 2011).

3.4. DECONTAMINATION STRATEGIES

When pesticides are applied, some of them accumulate in the soil and have an impact on the soil's microbial inhabitants. Some studies claim that only approximately 5% of all pesticides used actually reach their intended targets; the remainder gets into the soil and water supply. Human exposure to pesticides might occur in jobs, agriculture, and homes, as well as when people ingest contaminated water and food and breathe contaminated air. The skin, mouth, eyes, and respiratory systems are all entry points for pesticides into the human body. The molecular structure, dosage, and exposure times all affect how hazardous a pesticide is (Maqbool *et al.*, 2016; Raffa e Chiampo, 2021).

Due to these factors, it is essential to employ efficient remediation strategies to lower the residual pesticide content in the soil. Bioremediation is an environmentally acceptable, economically viable, and reasonably effective technique that can be used as an alternative to more costly and harmful techniques like chemical (hydrolysis, oxidation, and reduction mechanisms) and physical (use of incineration, sorbent materials, or simply burying or washing contaminated surfaces to minimize the risk of exposure) ones (Maqbool *et al.*, 2016; Raffa e Chiampo, 2021; Thakur, Medintz e Walper, 2019).

Utilizing microorganisms' microbial activity allows for elimination during biodegradation. Pesticides are transformed by microorganisms, primarily bacteria or fungi, into less complex substances like CO_2, water, oxides, or mineral salts that can be used as energy, mineral, and carbon sources. The enzymes play a crucial role in these processes because they function as catalysts. In other words, the simplest definition of bioremediation is the utilization of naturally occurring biological processes, such as plants and bacteria, that degrade or consume environmental toxins (Raffa e Chiampo, 2021; Thakur, Medintz e Walper, 2019).

The biodegradation of pesticides can occur in aerobic or anaerobic circumstances depending on the type of microorganisms involved and can be accomplished in a variety of ways. In addition, depending on whether the remediation treatment is carried out *on-site*, *ex situ*, or *in*

situ, the bioremediation approaches can be categorized into three groups (Raffa e Chiampo, 2021).

In the *in situ* method, the treatment is carried out in the contaminated area, and the procedure is often aerobic. Natural attenuation, bioaugmentation, biostimulation, bioventing, and biosparging are the primary *in situ* approaches. The contaminated soil is removed from polluted sites and transported to other locations for treatment in *ex situ* methods. *Ex situ* treatments incorporate bioreactors, composting, land farming, and biopiles. The *on-site* method involves cleaning up polluted soil in the surrounding area while removing it from its original location to avoid any negative effects from its transportation (Raffa e Chiampo, 2021).

PART II- VALIDATION OF A BIOCHIP ARRAY TECHNOLOGY FOR MULTI-MYCOTOXINS SCREENING IN CEREALS

1. MATERIAL AND METHODS

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).

1.2. Food samples

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

1.3. METHODOLOGY

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 2. 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).

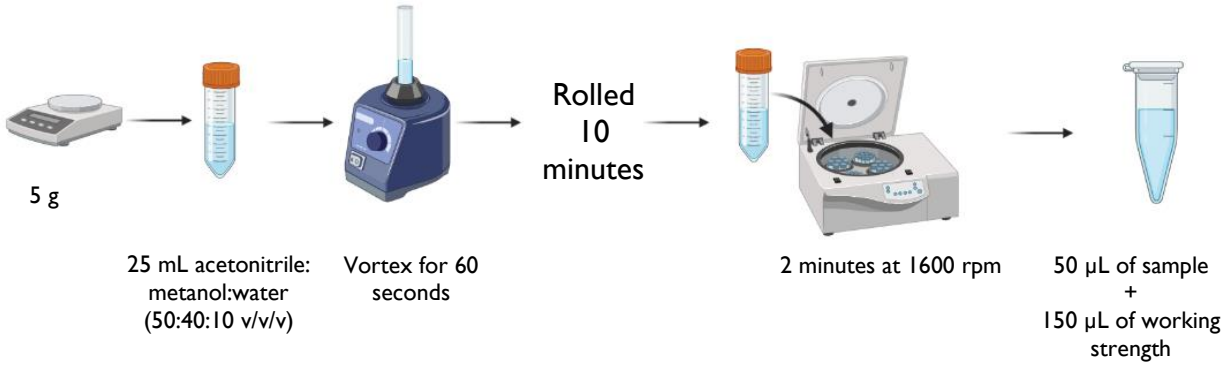

Figure 2. Extraction procedure previous to multi-mycotoxins analysis by chemiluminescence assay.

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 in cereals such as rice, oat, barley, rye, and wheat 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 3).

Figure 3. 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 4). 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 4. 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 incubate for 2 minutes and protect from the light. After this, each carrier was placed into Evidence Investigator™ (Figure 5). The whole process is summarized in Figure 6.

Figure 5. Evidence Investigator equipment at Vairão, Vila do Conde (INIAV, I.P.) facilities, (Randox).

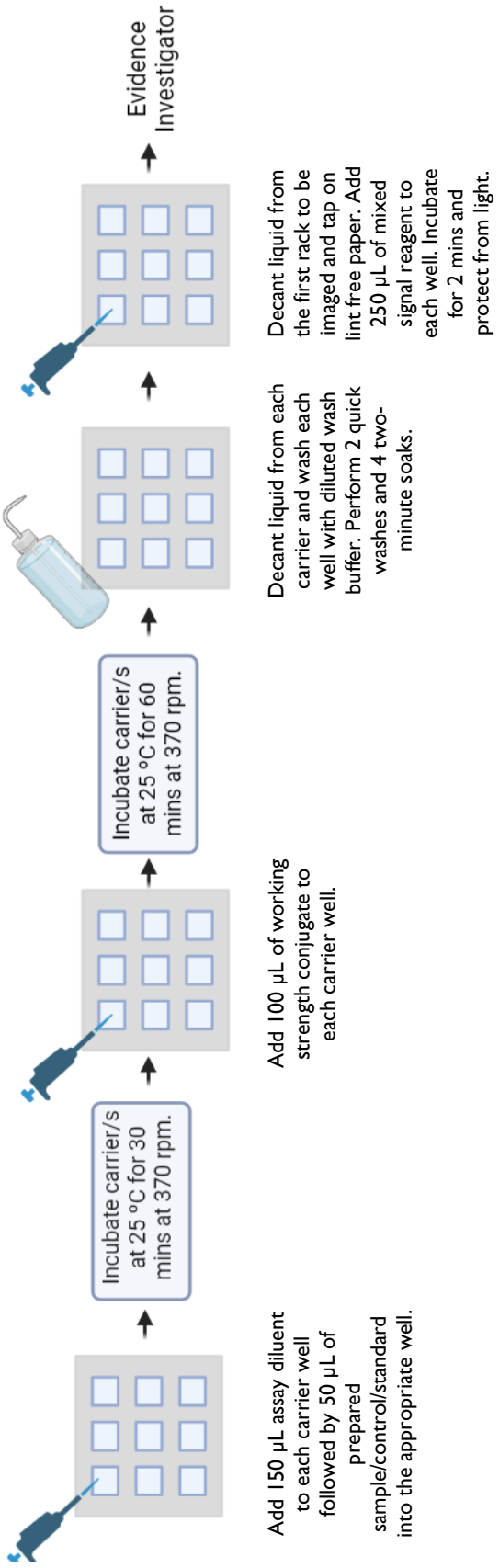

Figure 6. Summarized Chemiluminescent analysis protocol.

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 6) (Freitas *et al.*, 2019).

Table 6. 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

Mycotoxin	Cross- reactivity with	% Cross-reactivity
	Zearalanone	65
	α- Zearalanol	51
	β- Zearalanol	52

3. RESULTS AND DISCUSSION

The T and Fm of the biochip chemiluminescent immunoassay for the different mycotoxins in rice, oats, rye, barley, and wheat are compiled in Tables 7–8. 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 7 shows the results of each of the 20 blank samples and of the 20 fortified rice samples; Figure 8 shows the results of the 5 blank samples and of the 5 fortified samples of oat, rye, barley, and wheat.

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, OTA, AFB1, T2+HT2, and DON. The other validated cereals (oat, rye, barley, and wheat) have not shown any false positives. In addition, 5% of false negatives were found for FB1+FB2, OTA, AFB1, and T2+HT2.

Table 7. 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		479		4524		6472	
Fm (cut-off value) (RLU)	4949		2914		317		5174		1521		701		1699	

Table 8. Threshold value (T) and cut- off value (Fm) of the biochip chemiluminescent immunoassay for the different mycotoxins in cereals (oat, rye, barley, and wheat).

	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)	8174	4561	5882	2811	3546	294	13543	4751	6579	1369	5328	436	10420	1679
SD (RLU)	997	542	571	264	423	53	2032	975	362	160	1279	82	956	276
T (threshold value) (RLU)	6539		4947		2852		10201		5986		3230		8852	
Fm (cut-off value) (RLU)	5450		3244		381		6350		1632		571		2131	

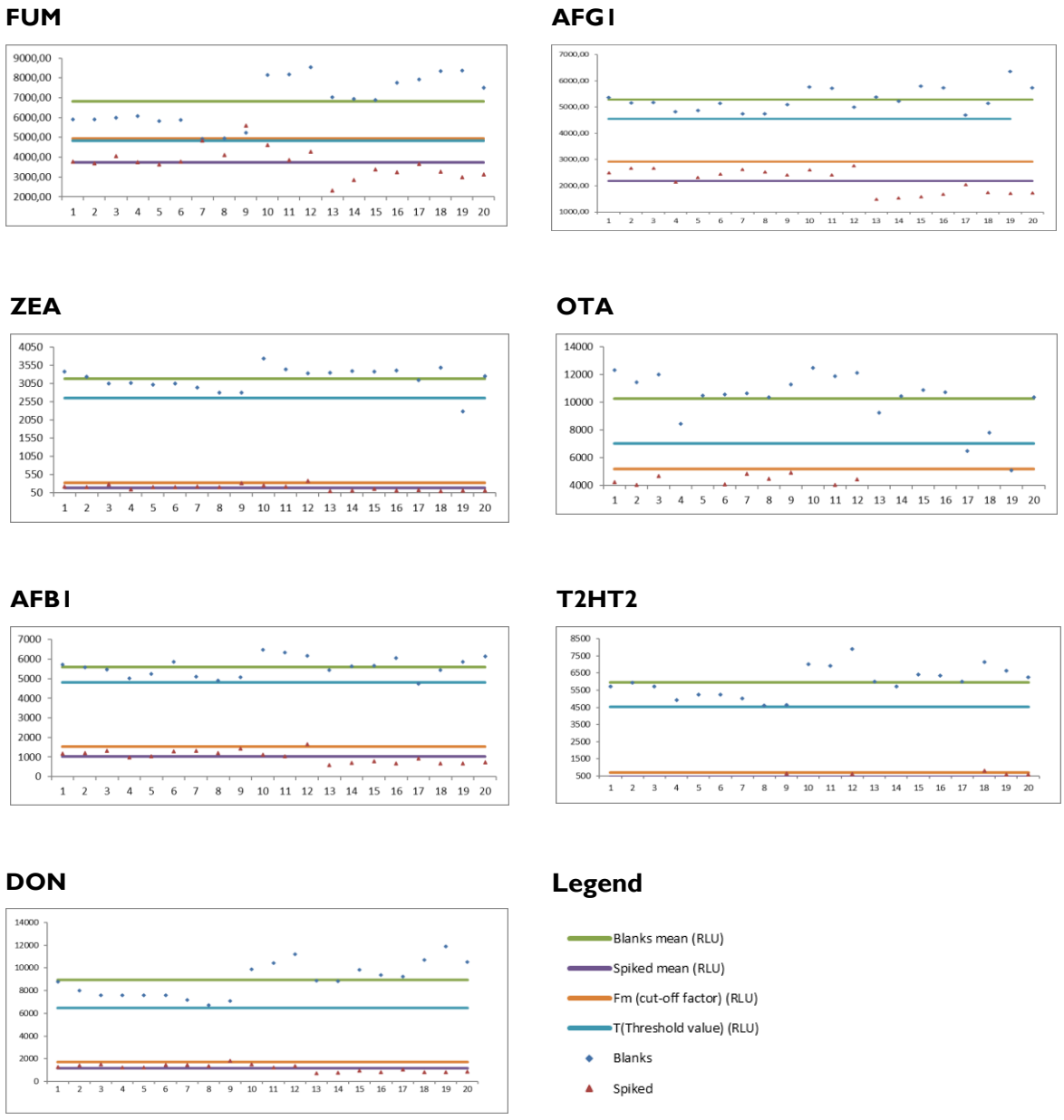

Figure 7. Threshold value (T) and cut-off value (Fm) of each of the mycotoxins analysed by the biochip chemiluminescent immunoassay expressed in RLU, for the 20 blank rice samples and for the 20 spiked rice samples at the level of interest.

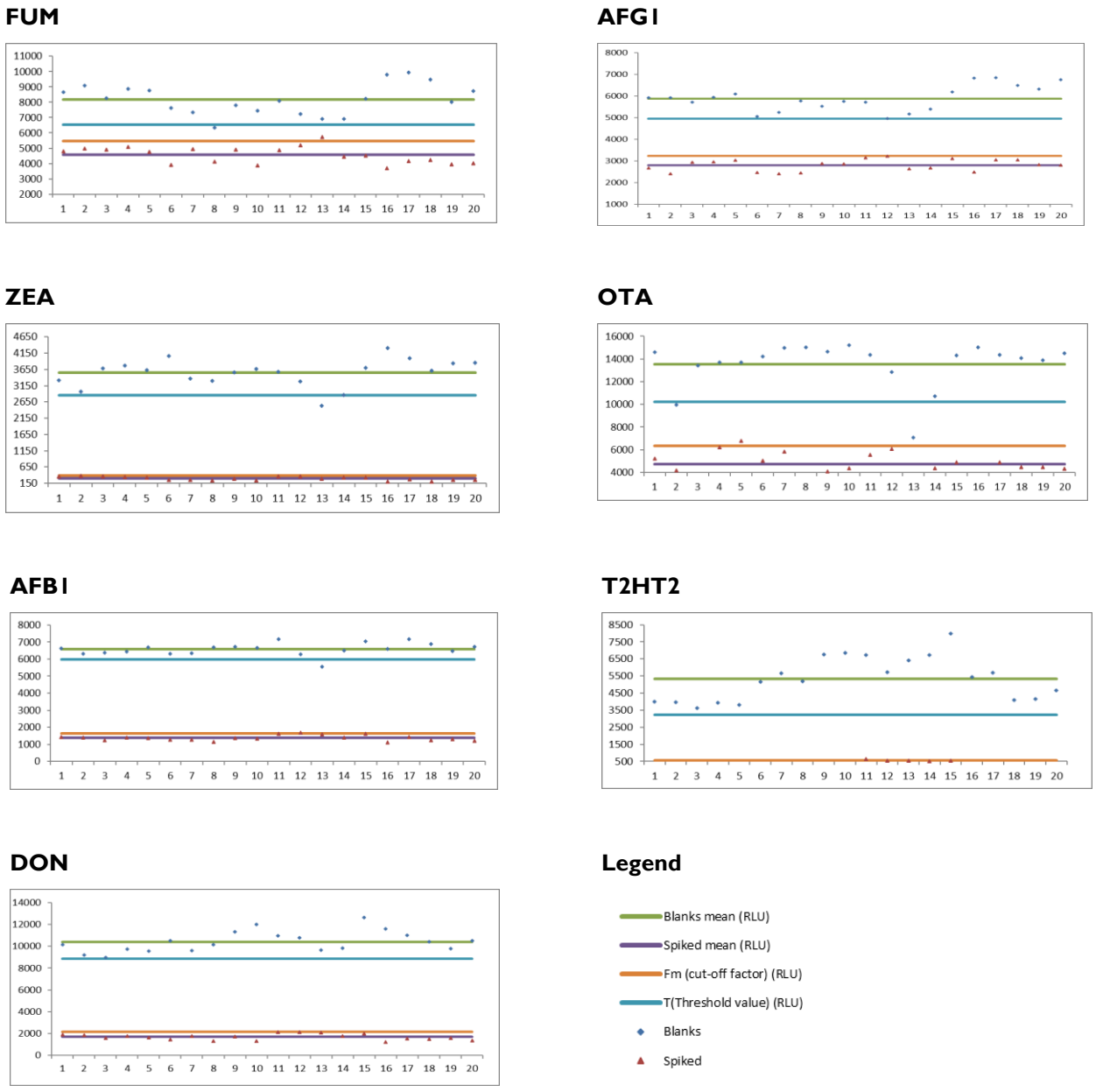

Figure 8. Threshold value (T) and cut-off value (Fm) of each of the mycotoxins analysed by the biochip chemiluminescent immunoassay expressed in RLU, for the 5 blank samples and for the 5 spiked samples of each different cereal at the level of interest.

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 (Commission of the European Communities, 2002).

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).

Few papers have addressed Biochip Chemiluminescent Immunoassay for Multi-Mycotoxins Screening. *Plotan et al.* validated a Biochip Chemiluminescent Immunoassay for Multi-Mycotoxins Screening in feed samples. The following parameters were determined: linearity, specificity/cross-reactivity, precision, stability, screening decision level, CCα, CCβ, repeatability, within-laboratory R, and trueness. A BAT-based competitive multi-analyte chemiluminescence immunosensor was established for the semiquantitative detection of multiple prevalent mycotoxins in feed samples because the biochip array increases screening capacity and allows for the simultaneous determination of multiple prevalent mycotoxins from a single sample (Plotan *et al.*, 2016).

The Myco 7 kit's test technique is simple to carry out, and the findings are easy to decipher. The kit (Cat. No.: EV4065) was reportedly used to simultaneously detect HT-2/T-2, ZEA, AFB1, AFB2, AFG1, AFG2, DON, OTA, FB1, FB2, and FB3, and in their results, all samples (poultry feed, rabbit feed, dairy feed, equine feed, dried distillers' grains with soluble (DDGS), dog food, and swine feed) were below the measuring ranges and reported as negative. For five tests, including T2, OTA, AFB1, ZEA, and DON, all 17 samples reported values within the necessary measurement limits. For fumonisins, 15 of 17 samples and 10 of 17 for the AFG1 test fell within the measurement limits. In this interlaboratory investigation, the repeatability of the Randox multiplex biochip array for the simultaneous detection of seven mycotoxins in feed matrices was established (Sibanda *et al.*, 2022).

Freitas et al. validated a biochip chemiluminescence detection method for multiple mycotoxins in maize *(Zea mays L.)*. AFB1 and AFG1, OTA, ZEA, T2, fumonisins (sum of FB1 and FB2), and DON were among the mycotoxins that were screened. In this research, mycotoxins were detected in samples of maize kindly provided by InovMilho (Portuguese National Competence Center for Maize and Sorghum Cultures). The calibration curves (n=5) for the AFB1, AFG1, FB1, OTA, DON, ZEA, and T2 were obtained at the same time and presented correlation coefficients (r) values that satisfied the acceptance requirement of r >0.95, ranging from 0.9947 to 0.9995. The precision data (CVs) for the immunoassays were below 10.1%, except for the total of FB1 and FB2, which was 21.2%. Despite the screening of AFB1, AFG1, FB1, FB2, OTA, DON, ZEA, and T2, only significant levels of the presence of fumonisins in maize samples were revealed in this study. Furthermore, with the exception of one sample, all of the samples had FB1 + FB2 concentrations less than 300 µg/kg. This sample should be analyzed further using LC-MS or liquid chromatography with tandem mass spectrometry (LC-MS/MS). According to *Freitas et al*., the validated BAT immunoassay is reliable, cost-effective, fast, semi-quantitative, and environmentally friendly, and it covers the regulated mycotoxins (Freitas *et al.*, 2019).

On the other hand, there is more data on other types of immunoassays. ELISA is the most widely used immunoassay. *Omar et al.* reported the validation of ELISA for detection and quantification of mycotoxins in different food samples, where aflatoxins were determined in wheat, AFB1 (LOD=0.05 µg/Kg), AFB2 (LOD=0.04 µg/Kg), AFG1 (LOD=0.06 µg/Kg) and AFG2 (LOD=0.07 µg/Kg), while with the Myco 7 Biochip Array obtained values were substantially higher (0.5 µg/Kg for AFG1 and 0.25 µg/Kg for AFB1 (Omar, Haddad e Parisi, 2020).

Yanshen et al., developed a quick and accurate ELISA method for detecting T-2 toxin in rice, and produced a highly sensitive and specific anti-T-2 toxin monoclonal antibody (mAb). The LOD value was 5.80 µg/kg. Despite the fact that the LOD value in a Myco7 Biochip array is 7 µg/Kg. In order to confirm the efficacy of this developed ELISA technique, commercial rice samples (20) were bought from local grocery stores. Both the UHPLC-MS/MS method and the standard ELISA protocol were followed in the processing and analysis of the samples. From all commercial rice samples, 5 were detected with a trace amount of T-2 toxin, sample 2 (11.2 µg/Kg), sample 5 (15.8 µg/Kg), sample 12 (9.6 µg/Kg), sample 14 (35.2 µg/Kg) and sample 17 (28.1 µg/Kg). In conclusion, the study developed and characterized a selective anti-T-2 toxin mAb. An ELISA technique for the determination of T-2 toxin in rice was developed using this mAb. The findings of the study were validated by UHPLC-MS/MS, and there was a strong correlation between the two procedures. This newly proposed approach might be a useful

tool for the specific, quick, semiquantitative, and quantitative detection of T-2 toxin in rice, which will help to prevent contamination caused by this fungal toxin (Li *et al.*, 2014).

A very interesting study carried out by *Ching-Kuo Yang et al.* looked at the prevalence of mycotoxins in feed and feed ingredients between 2015 and 2017 in Taiwan. The mycotoxins analyzed were aflatoxins, zearalenone, fumonisins, and deoxynivalenol using the ELISA method. A total of 820 feed samples (maize-based swine feed for pregnancy and nursery diets and maizemeal) were provided by various feed and animal producers in Taiwan between 2015 and 2017. Results showed that 91.4% of the feed samples were contaminated with DON. The second most common mycotoxin was ZEA, accounting for 70.2% of positive samples, followed by AFs and FUM with 58.0% and 50.4%, respectively (Yang *et al.*, 2019).

In 2016, *Maragos* reported a paper concerning "Multiplexed Biosensors for Mycotoxins" and presented a summary table of different biosensors. Such as an antigen-immobilized planar microarray with a matrix of cereals and a LOD of AFB1: 0.9 μg/kg; OTA: 1.1 μg/kg, and FB1: 159 μg/kg. DON: 40.5 μg/kg with a time of analysis of 19 minutes; an antigen-immobilized suspension microarray with a matrix of cereals and a LOD of AFB1: 1.19 pg/mL FB1: 0.60 pg/mL; OTA: 0.73 pg/mL with a time of analysis of over 1 hour; an antigen-immobilized lateral flow device with a matrix of peanut, maize, and rice and a LOD of AFB1: 0.25 ng/mL; OTA: 0.5 ng/mL ZEA: 1 ng/mL with a time of analysis of 20 minutes; Aptamer immobilized, planar suspension with a matrix of maize, wheat, and rice and a LOD of OTA: 0.25 pg/mL; FB1: 0.16 pg/mL with a time of analysis of 1.5 h, for example. It can be seen that different biosensors have different LODs and analysis times (Maragos, 2016). A portable smartphone-based self-programming App detection devices were used to rapidly quantify the results in the study of *Chen et al.,* and red, blue, and green latex microspheres lateral flow immunoassays were applied to label AFB1, ZEA, and T2. The first to detect three mycotoxins concurrently was a rainbow "traffic light" latex microsphere lateral flow immunoassay (LMs-LFIA) linked with a portable and user-friendly smartphone-based device. The method's cut-off values for AFB1/T-2/ZEA in cereals were 1/15/40 μg/kg, with detection limits of 0.04/0.40/1.21 μg/kg, respectively. The rainbow LMs-LFIA created and integrated with the smartphone-based device has the potential to provide a promising multi-target analysis tool for portable, sensitive, fast, and on-site screening of mycotoxins or other hazardous substances (Chen *et al.*, 2022).

PART III- VALIDATION OF LC-MS/MS METHOD TO DETERMINE PESTICIDES RESIDUES IN RICE AND EVALUATION OF PROCESSING FACTORS

1. MATERIAL AND METHODS

1.1. Chemicals and Reagents

Methanol, acetonitrile (both HPLC gradient grade), toluene, acetone, n-hexane, ethyl acetate, and formic acid were purchased from Merck (Darmstadt, Germany). Water was purified by the Milli-Q Plus system from Millipore (Molsheim, France) with a resistivity of 18.2 M Ω x cm at 25°C.

Pesticide standards and internal standards (triphenylphosphate-TPP and dinitrocarbanilide or 1,3-bis(4-nitrophenyl)urea-DNC) were acquired from Sigma-Aldrich (Madrid, Spain), and dissolved at a concentration of 5 mg/Kg in toluene, acetonitrile, acetone, methanol, ethyl acetate, and chloroform. After preparing the stock solutions, working solutions were prepared in acetonitrile.

For QuEChERS, magnesium sulfate and sodium chloride were acquired from Fluka (Seelze, Germany). Sodium citrate dibasic sesquihydrate was purchased from Sigma–Aldrich (Madrid, Spain). Tri-Sodium Citrate 2-hydrate was purchased by AppliChem (Darmshtadt, Germany).

For clean-up, primary secondary amine bonded silica (PSA) was purchased from Supelco (Supelclean™, Bellefonte, PA, USA). Anhydrous magnesium sulfate was purchased from AppliChem (Darmshtadt, Germany).

For the contamination of rice, cooking and washing, mineral water was used. For the washing with vinegar, apple vinegar was used, at a concentration of 5% (v/v).

1.2. Samples and Sampling Procedure

Twenty samples of different types of rice were purchased in different supermarkets in Portugal between April and June of 2023 for determination of pesticide residues. Rice belongs to the types: 5 long-grain rice samples, 5 samples of medium-grain rice of the Portuguese variety Carolino, 5 samples of Basmati rice and 5 samples of parboiled rice. Each laboratory sample (1 kg) was homogenized by grinding (Retsch rotor mill SK 300 with a sieve of trapezoid holes of 1.00 mm) and the flours were mixed carefully to assure complete homogenization. Each sample was placed in separate sample collection tubes (50 g approx.) and preserved at −20°C until analysis.

The sample CF17 (wheat kernels) from European Union Reference Laboratory for Pesticides in Cereals (DTU National Food Institute, Denmark) was received and extracted immediately according to the method described in 1.3.1.

1.3. METHODOLOGY

1.3.1. Extraction

A QuEChERS procedure was used to extract pesticides from rice. First, weight10 g of sample for a tube of 50 mL, add 20 mL of cold water, and stand by for 1 hour. Next, add 10 mL of acetonitrile (ACN) and vortex. Next, 6.5 g of a mixture of extraction salts (4 g of magnesium sulfate, 1 g of sodium chloride, 1 g of sodium citrate, and 0.5 g of disodium hydrogen citrate sesquihydrate) was added for the liquid-liquid partitioning step and vortexed for 1 minute, followed by a centrifugation at 4000 rpm during 5 minutes. After centrifugation, 6 mL of the extract were added to a mixture with PSA and anhydrous magnesium sulfate (1.05 g), which corresponds to a clean-up step called dispersive solid-phase extraction. After mixing and centrifugation at 4500 rpm for 2 minutes, 1 mL of the extract was added to 220 µL of ACN on the Eppendorf. Finally, 500 µL of the extract was added to 25 µL of an internal standards solution in a mini-uniprep™. The extract was analyzed by ultra high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) with a triple quadrupole instrument using electrospray ionization (ESI).

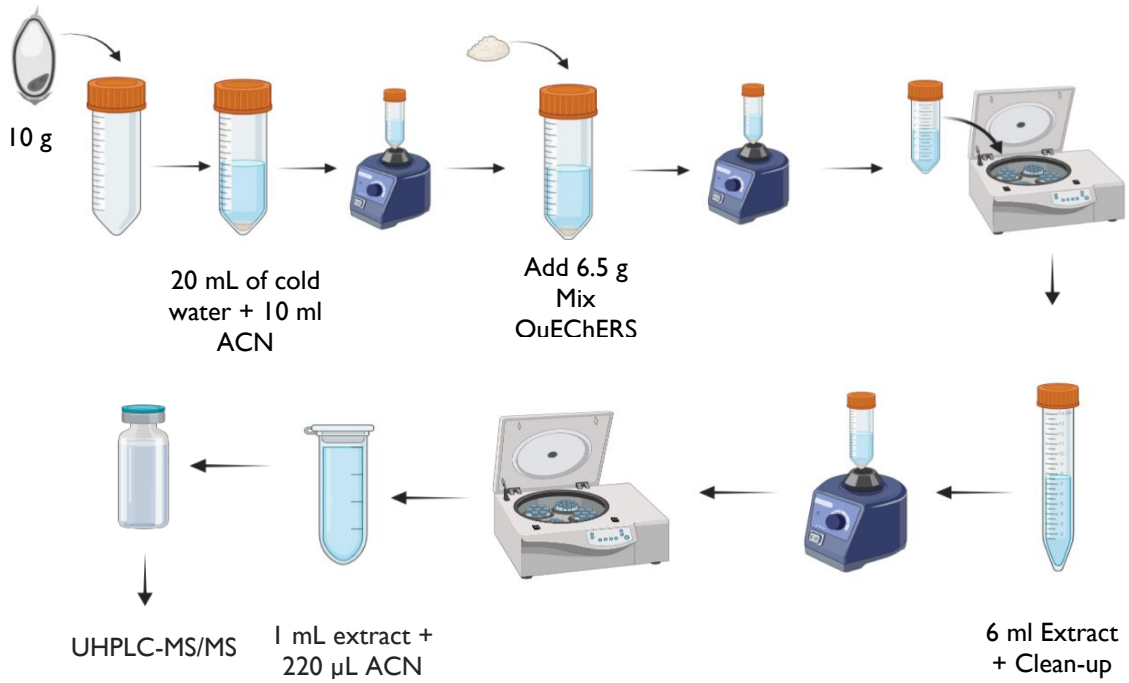

Figure 9. Summarized QuEChERS protocol used for extraction of pesticides residues from rice samples.

1.3.2. Matrix-matched calibration

A matrix matched calibration curve was prepared with 10 levels of concentration, i.e., at 5, 10, 20, 30, 40, 50, 60, 70, 80, and 100 µg/kg. Before this, a solvent calibration curve is prepared with defined volumes of the mix of internal standards at 2.5 ng/µL, a working solution at 250 µg/kg (the pesticides that are in this solution are found in table 10), and ACN (5-200 µg/kg). Then, 500 µL is taken from the levels of the solvent calibration curve (for instance, for the construction of the 5 µg/kg level, 500 µL is taken from the 10 µg/kg level of the solvent curve), and 500 µL of a blank sample and 10 µL of a solution of internal standards at 2.5 ng/µL are added to each level (5 to 100) (Figure 10). Following, each vial is injected into the UHPLC-MS/MS.

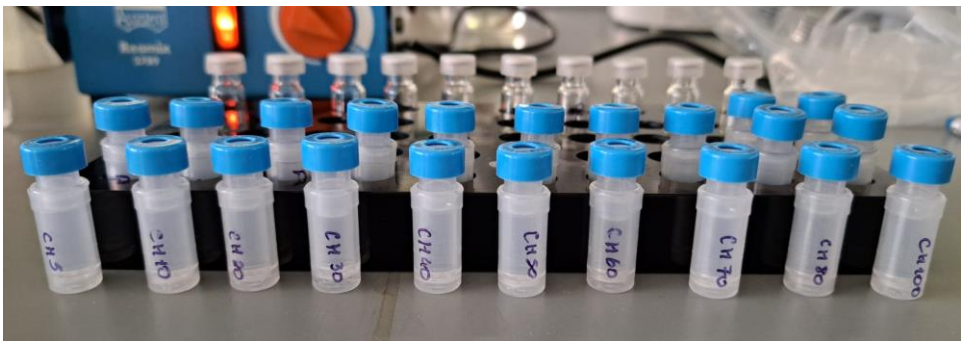

Figure 10. Photography of the vials with the Matrix Matched Calibration points.

1.3.3. Spiking Experiment

To determine the recovery of the target analytes, spiking experiments were performed. Blank samples of rice (10 g) were spiked at three different concentrations (5,10 and 50 µg/kg) using a multi-pesticide standard solution in acetonitrile (v/v). After fortification the solution was left in contact with the matrix at room temperature in the dark for 30 minutes. Subsequently, extraction was performed as described in Section 1.3.1.

1.3.4. Sample preparation for processing

For this step, the paper of *Shakoori et. al.* (2018) was used as guidance and adapted. Before the cooking process, one solution with a concentration of 50 µg/kg was prepared for the contamination of the long rice in a beaker with water. For a concentration of 50 µg/kg, 4 mL of mixed pesticide solution (5000 µg/kg) were dissolved in 400 mL in water. And 200 g of long rice were submerged (Figure 11). Followed by air-drying at room temperature for 24 hours.

To dry the rice, it was spread out on trays and left in the sun until completely dry. For each process (including washing and cooking), 20 g rice were used.

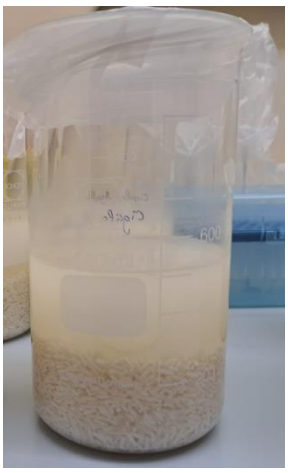

Figure 11. Contamination of long grain rice with a solution of pesticides at a level of 50 µg/kg.

1.3.4.1. Washing

A 20 g portion of the rice samples was washed with mineral water and soaked in 100 mL of this water for 20 minutes. The samples were grounded and then analyzed.

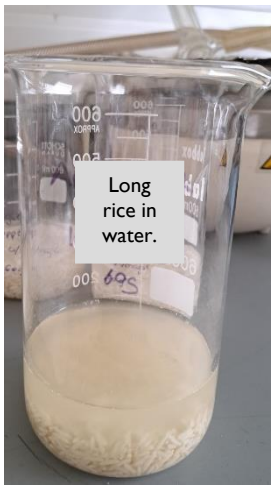

Figure 12. Washing process of the contaminated long grain rice samples.

1.3.4.2. Washing with Vinegar

A 20 g portion of the rice samples was washed with mineral water and soaked in 95 mL of this water and 5 mL of vinegar for 20 minutes. The samples were grounded and then analyzed.

1.3.4.3. Cooking

For each sample, a mixture of 20 g of the rice sample, and 40 mL of water was placed in a beaker. The mixture was boiled on a stove until the water evaporated, approximately 10 minutes (Figure 13). Then, the cooked rice sample was completely crushed and analyzed.

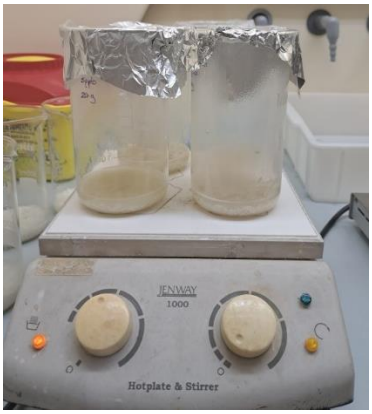

Figure 13. Cooking process of the contaminated long grain rice samples.

1.3.4.4. Steam cooking

For each sample, a mixture of 20 g of the rice sample was placed in a small round stainless-steel sieve with a fine wire mesh (Figure 14). Then, the rice was cooked with steam cooking from a beaker with 500 ml of boiling water (Figure 14). Each sample took approximately 1 hour to cook. Then, the cooked rice sample was completely crushed and analyzed.

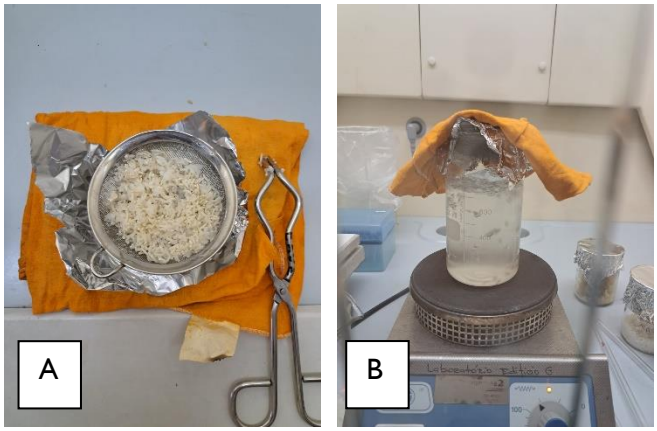

Figure 14. Small round stainless-steel sieve with a fine wire mesh with cooked rice (A) and steam cooking of the contaminated long-grain rice samples (B).

1.5. HPLC-MS/MS Parameters

A UHPLC Nexera X2 (Shimadzu, Kyoto, Japan) and QTRAP 5500+ MS/MS detector (AB SCIEX, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source operating simultaneously in both positive and negative modes (ESI+ and ESI-) were used for both detection and quantification (Figure 15). The autosampler was kept at 10°C to keep the samples cold, and a volume of 10 µL of sample extract was injected in the column at the following chromatographic conditions: an analytical column Synergi 4 µm Fusion-RP 80A 50 2 mm (Phenomenex, Torrance, CA, USA) was used.

Figure 15. UHPLC-MS/MS equipment at INIAV, I.P. facilities (Vairão campus, Vila do Conde).

With a flow rate of 0.25 mL/min, the mobile phase was composed of the gradient shown in Table 9 using formic acid 0.1% in ultrapure water as mobile phase [A] and formic acid 0.1% in methanol as mobile phase [B].

Table 9. Gradient elution program for the determination of pesticide residues in rice by HPLC-MS/MS.

Time	Mobile Phase [A]	Mobile Phase [B]
0	95	5
0.5	95	5
8	10	90
13	10	90
15	95	5
18	95	5

The 18-minute runtime was the total. Mass spectrometry data were acquired in MRM mode from 100 to 750 Da using the Analyst® TF software (SCIEX, Foster City, CA, USA) and the following parameters: ion spray voltage of 4500 V; source temperature 600 C; curtain gas (CUR) at 35 psi; and gas 1 and gas 2 at 40 and 60 psi, respectively.

Tables A1 and A2 give the parameters for the determination of pesticide residues in rice using MS/MS in the ESI+ and ESI- modes, respectively. Following the direct injection of each individual standard solution at a concentration of 1 µg/mL into the detector, data acquisition in the multiple reaction monitoring (MRM) mode was adjusted. Thus, two ion transitions were selected for each compound, a quantifier, and a qualifier MRM.

1.6. Identification of Pesticide Residues in Rice

The identification and data processing of pesticide residues in rice were made through the MultiQuant™ software (SCIEX, Foster City, CA, USA).

According to the SANTE/11312/2021, two parameters were utilized as identification criteria: ion ratio tolerance below 30% and retention time (RT) with a tolerance of 0.1 min in relation to the RT of the analyte in calibration standard (may need to be matrix-matched). In mass spectrometry methodologies, using an internal standard is advised to access any variances throughout the analytical process. In this case, two internal standards were used TPP and DNC for the pesticides residues determined in positive and negative mode, respectively.

Equation 1: Deviation of RRT,

$$\Delta RRT = (RT_{sample} - RT_{mean\ calibration}),$$

where RT_{sample} is the retention time of the analyte in a sample and $RT_{mean\ calibration}$ corresponds to the mean of retention time obtained, for the same analyte, in a set of calibrations (matrix-matched calibration curves were used). The ratio between the areas measured for both ion transitions of each analyte is used to calculate the ion ratio.

Equation 2: Ion ratio (IR, %),

$$IR = \left(\frac{A \text{ ion with lowest intensity}}{A_{ion\ with\ highest\ intensity}}\right) \times 100$$

where, $A_{ion\ with\ lowest\ intensity}$ corresponds to the area of the ion with the lowest intensity and the $A_{ion\ with\ highest\ intensity}$ to the area of the ion with the highest intensity.

Equation 3: Deviation of IR (ΔIR, %),

$$\Delta IR = \frac{IR_{Sample} - IR_{mean\ calibration}}{IR_{mean\ calibration}} \times 100,$$

where $IR_{mean\ calibration}$ refers to the mean ion ration achieved for a batch of calibration of the same analyte and IR_{sample} corresponds to the ion ratio obtained for a target compound present in a sample.

The positive identification is reached if both criteria are fulfilled (ΔRRT < 0.1 min and ΔIR < 30% - Equations 2 and 3).

1.7. Validation of HPLC–MS/MS Method

The method was validated through different parameters, namely the specificity, concentration range, linearity, the limit of quantification (LOQ), precision (repeatability and intra-laboratory reproducibility) and accuracy (using recovery assays). Expanded uncertainty was also evaluated.

For the determination of repeatability (RSD_r) and intra-laboratory reproducibility (RSD_R), blank samples of rice were spiked at 3 different levels (n=5), taking in account the maximum level (ML) of each pesticide residue. In the case of RSD_R determination, experiments were carried out in 3 different days by the same operator. The accuracy of the method was evaluated using recovery assays and certified reference materials.

1.8. Processing factors (PF) evaluation

Pesticide residue reduction during processing was evaluated by calculating the processing factor (PF) according to the equation,

PFs = $\frac{C_a}{C_b}$

Where,

PF < 1 PF > 1

Ca = concentration of pesticide in processed samples (mg/kg)

C_b = concentration of pesticide in raw samples (mg/kg)

PF < 1, it means the reduction of pesticide concentration

PF >1, it means the increase of pesticide residue concentration

2. RESULTS AND DISCUSSION

2.1. Validation of the Method

The method was validated according to the criteria defined by SANTE/11312/2021, which establishes the validation parameters for the official control of the pesticides in foodstuffs in the EU, and 121 pesticides in total were validated (SANTE, 2021).

Linearity was evaluated by matrix-matched calibration curves (mean of six curves) in different ranges for different pesticide residues (see Table 10). The linear range of the calibration curves ranged between 5–100, 10–100 or 50-100 µg/L, depending on the pesticide. The limit of quantification was 5, 10, or 50 µg/kg which is sensitive enough to meet the requirements imposed by EU regulations for the MRL of pesticide residues in rice (European Commission, 2005). The determination coefficients varied between 0.9532 and 0.9983, indicating suitability for pesticides quantification. Table 10 shows the results of linearity, repeatability, precision, recovery, and expanded uncertainty for the different pesticide residues in a blank rice sample spiked at 3 concentration levels. Recoveries for the 121 analyzed pesticides ranged between 70.0 and 119%. The specificity criteria were met for all pesticides at 5 µg/kg, except for Chlorantraniliprole, Chlorfenvinphos and Metazachlor where they were met at 10 µg/kg and for Hexythiazox and Fludioxanil where they were met at 50 µg/kg. For the pesticides with LOQ of 5 µg/kg, the mean of the three spiking levels (5, 10, and 50 µg/kg) was used to calculate

precision, repeatability, and recovery. For the pesticides with LOQ of 10 µg/kg, the mean of levels 10 and 50 µg/kg, was used to calculate precision, repeatability, and recovery and for the pesticides with LOQ of 50 µg/kg, just the data achieved at this level are used present the same validation parameters.

The recoveries of the method were all within the appropriated range of the SANTE/11312/2021 criteria. Repeatability of the method was evaluated by the Relative Standard Deviation RSD_r. RSD_r was between 5.71 and 17.1%. Reproducibility was evaluated by the Relative Standard Deviation RSD_R at 3 different days of analysis, different concentration levels and values were considered acceptable (varied between 6.62 and 19.7%).

The expanded uncertainty ranged between 8% for Fenamiphos sulfoxide and 49% for Profenofos. Then, it was concluded that the pesticide residue results do not have to be adjusted for recovery due to the fact that the mean recovery is within the range of 70%–120% and the criteria of 50% expanded measurement uncertainty is achieved, according to the SANTE/11312/2021.

There is a vast literature on the extraction of pesticides from rice (Arias *et al.*, 2014; Teló *et al.*, 2017); however, there are many different results from each other. Tauseef *et al.* (2021), validated a method that allows to determine 25 pesticides residues in rice, using a slightly different QuEChERS extraction method (acetate buffered QuEChERS method without PSA cleanup), and obtained excellent results, for example, for the coefficient of determination of the matrix curve, they had results between 0.994 and 0.999, also using LC-MS/MS. The selected 25 pesticides residues met the EU-SANTE and FAO/WHO Codex Alimentarius Commission method validation guidelines. The interday repeatability of the optimized method was between 4 and 18% (n=6). The expanded uncertainty calculated for the optimized method ranged from 22 to 48% (Tauseef *et al.*, 2021).

Whereas *Tsochatzis et. al.* (2010) used MSPD extraction for the determination of nine pesticides residues instead of the traditional QuEChERS, also allowing to obtain good results for the recovery (97.1- 104.6%) and linearity (coefficient of determination: 0.9948-0.9999). Samples were further analyzed by a high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) (Tsochatzis *et al.*, 2010).

An excellent study carried out by *Tran-Lam et. al.* (2021), determined 656 pesticide residues using UHPLC-MS/MS and GC-MS/MS in combination with QuEChERS extraction and mixed-mode SPE clean-up. Validating the method followed SANTE/12682/2019. Linear regressions of all analyzes exhibit coefficient of determination greater than 0.999, which is an indicator of

excellent goodness-of-fit for the calibration points. All analyzes displayed recovery between 70 and 120% with RSD_r and RSD_R less than 20%. Furthermore, the maximal LOQs were 10 µg/kg in both MS methods (SANTE, 2019; Tran-Lam *et al.*, 2021).

At INIAV, this same method had already been validated for the validation of 155 pesticide residues, also with very good results, for all parameters, for example, recovery varied between 77.1 and 111.5% and with a linear range between 5-50, 5-60, or from 5-70 µg/kg, depending on the pesticide. The other parameters were in accordance with SANTE/11813/2017. The present method allows to quantify pesticides residues in rice at higher levels of concentration (up to 100 µg/kg) (Melo *et al.*, 2020).

Nowadays, it is increasingly important to develop analytical methodologies for the detection of multi-pesticide residues, at very low limits of quantification, in order to evaluate if food samples meet the established maximum residues levels at EU level.

Table 10. Results of the validation of the HPLC-MS/MS method to determine 121 pesticides in rice: determination coefficient (r^2) in matrix-matched curves, recovery, repeatability (RSD_r) and precision (RSD_R), limit of quantification (LOQ) and expanded uncertainty (U).

Pesticide	Linear Range (µg/L)	r^2 matrix	LOQ (µg/kg)	RSD_R	RSD_r	Recovery (%)	U (%)
Acetamiprid	10-100	0.9917	10	8.34	7.36	113.5	12
Azoxystrobin	10-100	0.9834	10	13.9	7.02	91.82	39
Bitertanol	10-100	0.9848	10	13.1	13.6	102.8	19
Bixafen	10-100	0.9830	10	11.0	6.16	80.40	30
Boscalid	5-100	0.9874	5	11.4	7.50	81.25	29
Bupirimate	10-100	0.9786	10	8.48	6.79	98.89	12
Buprofezin	10-100	0.9774	10	15.5	6.98	90.69	49
Cadusafos	5-100	0.9885	5	12.9	7.01	94.50	36
Carbaryl	10-100	0.9868	10	10.6	6.21	103.5	26
Carbendazim	10-100	0.9747	10	16.3	14.6	70.02	32
Carbofuran	10-100	0.9830	10	8.03	6.38	107.9	13
Carbofuran-3-hydroxy	10-100	0.9830	10	17.0	10.9	103.7	43
Carboxin	10-100	0.9841	10	7.7	6.37	103.2	10
Chlorantraniliprole	10-100	0.9819	10	8.1	6.81	109.1	13
Chlorfenvinphos	10-100	0.981	10	12.3	6.34	82.10	35
Chlorpyrifos-methyl	10-100	0.9958	10	15.2	7.07	92.22	38
Clofentezine	10-100	0.9831	10	9.13	5.90	83.99	20
Coumaphos	50-100	0.9720	50	14.4	8.86	80.90	31
Cymoxanil	10-100	0.9983	10	13.4	12.31	104.1	22
Cyproconazole	10-100	0.9782	10	8.23	6.17	117.1	14
Cyprodinil	10-100	0.9809	10	9.84	5.89	89.96	25
Demeton-S-methylsulfone	10-100	0.9800	10	15.9	11.3	103.8	39
Diazinon	10-100	0.9854	10	8.93	5.23	101.4	23
Dichlorvos	10-100	0.9825	10	11.3	7.41	105.7	31
Difenoconazole	10-100	0.9756	10	13.5	7.48	100.7	39
Diflubenzuron	10-100	0.9814	10	11.6	6.67	85.69	29
Dimethoate	5-100	0.9874	5	12.4	9.72	102.9	32
Dimethomorph	10-100	0.9768	10	12.1	6.96	99.78	26
Diniconazole	10-100	0.9810	10	12.6	7.49	106.8	38
DMST	10-100	0.9842	10	10.2	6.51	101.1	28
EPN	10-100	0.9766	10	8.49	6.99	89.67	9
Epoxiconazole	10-100	0.9759	10	10.4	7.37	110.0	19
Ethiofencarb	10-100	0.9864	10	8.54	6.48	110.4	17
Ethoprophos	5-100	0.9857	5	12.9	6.26	91.34	37

Pesticide	Linear Range (µg/L)	r^2 matrix	LOQ (µg/kg)	RSD_R	RSD_r	Recovery (%)	U (%)
Etrimfos	50-100	0.9767	50	8.08	6.97	90.23	15
Fenamidone	10-100	0.9774	10	10.4	7.06	109.8	25
Fenamiphos sulfone	5-100	0.9880	5	7.02	6.31	94.75	12
Fenamiphos sulfoxide	10-100	0.9809	10	7.12	5.50	100.4	8
Fenarimol	10-100	0.9750	10	10.2	7.66	96.38	23
Fenitrothion	10-100	0.9759	10	14.6	9.55	96.56	32
Fenoxycarb	10-100	0.9777	10	13.6	6.17	109.5	39
Fenpropathrin	10-100	0.9750	10	9.19	3.56	78.34	20
Fenpropidin	5-100	0.9839	5	9.42	7.68	100.9	13
Fenpropimorph	10-100	0.9778	10	8.72	7.56	105.0	8
Fenpyroximate	50-100	0.9639	50	13.6	9.79	91.31	24
Fenthion oxon sulfone	10-100	0.9839	10	10.2	8.01	114.2	19
Fenthion oxon sulfoxide	10-100	0.9845	10	9.18	7.30	108.7	15
Fenthion oxon	10-100	0.9812	10	13.5	8.18	99.60	35
Fenthion sulfone	10-100	0.9863	10	9.14	5.74	104.3	21
Fenthion sulfoxide	10-100	0.9844	10	10.9	6.87	109.1	28
Fluopyram	5-100	0.9863	5	8.80	6.06	99.78	21
Fluquinconazole	10-100	0.9788	10	13.7	7.18	87.06	33
Flutriafol	5-100	0.9864	5	15.7	7.99	106.4	46
Fonofos	50-100	0.9798	50	9.71	14.5	80.60	31
Fosthiazate	10-100	0.9801	10	9.58	6.62	111.2	20
Hexythiazox	10-100	0.9743	10	14.0	9.18	90.38	28
Indoxacarb	10-100	0.9761	10	8.92	6.19	109.8	19
Iprodione	10-100	0.9781	10	11.8	6.72	72.66	29
Iprovalicarb	10-100	0.9765	10	8.66	6.31	107.2	15
Isoprocarb	10-100	0.9791	10	9.67	6.10	103.9	23
Isoprothiolane	50-100	0.9802	50	11.1	7.33	101.9	24
Isoproturon	10-100	0.9817	10	10.6	5.75	98.80	28
Kresoxim-methyl	10-100	0.9772	10	13.8	8.66	118.1	36
Linuron	10-100	0.9845	10	7.65	5.85	100.4	13
Lufenuron	5-100	0.9817	5	15.3	9.83	73.05	46
Malaoxon	10-100	0.9827	10	15.3	7.85	97.47	48
Malathion	10-100	0.9760	10	10.5	6.95	91.70	24
Mepanipyrim	10-100	0.9823	10	10.9	6.45	85.49	28
Metaflumizone	50-100	0.9790	50	10.8	6.90	79.90	20
Metalaxyl	5-100	0.9804	5	6.62	5.39	104.4	8
Metalaxyl-M	10-100	0.9810	10	8.29	6.01	100.6	12

Pesticide	Linear Range (µg/L)	r^2 matrix	LOQ (µg/kg)	RSD_R	RSD_r	Recovery (%)	U (%)
Metazachlor	10-100	0.9818	10	12.3	6.64	109.7	34
Metconazole	10-100	0.9815	10	11.5	7.69	113.9	27
Methiocarb	10-100	0.9828	10	14.4	6.96	90.97	44
Methomyl	10-100	0.9772	10	13.7	11.5	100.3	32
Metribuzin	10-100	0.9897	10	9.79	7.96	105.4	18
Mevinphos	5-100	0.9878	5	13.9	9.43	108.7	34
Monocrotophos	5-100	0.9842	5	12.5	9.83	97.15	32
Oxadixyl	10-100	0.9673	10	9.08	7.00	108.6	17
Paclobutrazole	10-100	0.9821	10	10.3	6.76	115.7	25
Paraoxon-ethyl	10-100	0.9845	10	10.2	6.51	94.53	22
Paraoxon-methyl	10-100	0.9803	10	9.80	7.41	100.7	17
Parathion	10-100	0.9779	10	12.9	7.18	97.65	37
Parathion-methyl	10-100	0.9811	10	14.9	8.19	89.33	40
Penconazole	10-100	0.9819	10	7.31	11.3	110.0	30
Pencycuron	10-100	0.9811	10	16.5	8.55	102.7	47
Pendimethalin	50-100	0.9760	50	13.2	13.70	73.72	28
Phenthoate	10-100	0.9802	10	9.20	7.06	87.69	14
Phosalone	10-100	0.9827	10	10.9	8.24	76.55	27
Phosmet	10-100	0.9788	10	12.5	5.31	97.77	36
Phosphamidon	10-100	0.9837	10	8.00	6.37	100.4	12
Phoxim	5-100	0.9831	5	12.3	8.07	100.6	35
Pirimiphos-ethyl	50-100	0.9532	50	11.5	8.36	83.72	21
Pirimiphos-methyl	5-100	0.9868	5	10.8	6.88	93.54	29
Prochloraz	10-100	0.9781	10	12.7	5.80	91.00	37
Profenofos	10-100	0.9747	10	17.1	7.46	94.80	49
Propiconazole	10-100	0.9799	10	9.01	6.82	96.89	13
Propoxur	10-100	0.9830	10	9.95	7.33	103.9	23
Propyzamide	10-100	0.9811	10	9.94	5.62	96.97	24
Prothioconazole-desthio	10-100	0.9803	10	9.35	8.02	111.3	15
Pyrazophos	5-100	0.9869	5	12.8	9.56	80.78	24
Pyrimethanil	10-100	0.9809	10	7.92	6.84	96.80	10
Quinoxyfen	5-100	0.9872	5	13.4	9.59	92.62	31
Rotenone	10-100	0.9815	10	11.7	7.37	86.62	29
SpinosadA	5-100	0.9866	5	14.6	7.33	97.08	46
SpinosadD	10-100	0.9830	10	8.55	5.24	108.8	20
Spiroxamine	5-100	0.9862	5	12.0	7.50	95.58	25
Tebuconazole	50-100	0.9797	50	8.96	6.43	98.45	16
Tebufenpyrad	10-100	0.9848	10	13.3	9.44	78.46	34

Pesticide	Linear Range (µg/L)	r^2 matrix	LOQ (µg/kg)	RSD_R	RSD_r	Recovery (%)	U (%)
Terbuthylazine	10-100	0.9809	10	10.1	6.64	91.52	24
Tetraconazole	10-100	0.9856	10	12.9	7.96	93.76	32
Thiabendazole	10-100	0.9761	10	19.7	16.0	112.7	49
Thiacloprid	10-100	0.9882	10	9.18	6.53	113.2	20
Thiodicarb	10-100	0.9819	10	11.1	8.79	83.66	16
Triadimefon	10-100	0.9805	10	10.6	7.92	116.4	22
Triadimenol	10-100	0.9768	10	10.3	7.87	119.1	19
Tricyclazole	10-100	0.9800	10	6.62	5.93	96.80	10
Triflumuron	10-100	0.9786	10	9.48	5.24	74.62	19
Zoxamide	5-100	0.9881	5	8.69	5.77	80.53	20
Fludioxonil	10-100	0.9740	10	11.9	6.10	80.95	36

Legend: DMST- N,N-dimethyl-N'-p-tolysulphamide; EPN- O-ethyl O-4-nitrophenyl phosphonothiate; U-

2.2. Effect of cooking and washing process in pesticides residues in rice

The study of pesticide residues in various commodities, including rice, is a major challenge in food safety. Rice is processed by different methods all around the world. So, it is important to evaluate the effect of different processes such as washing and cooking in the levels of pesticides residues.

The aim of the study was to analyze the effect of washing, washing with apple vinegar, cooking, and steam cooking and the combination of washing and cooking methods in the reduction of pesticides in long grain rice samples.

2.2.1. Unprocessed Samples

To calculate the pesticide reduction throughout the washing and cooking processes, it is important to ascertain the pesticide concentration in unprocessed rice samples. Two replicates of each rice sample (unprocessed, washed, and cooked) are shown in Table 11 and 12, respectively.

Of the 121 pesticides validated, only 97 pesticides could be quantified, although some pesticides in the unprocessed samples were lower than the LOQ. Such as Carboxin (carboxanilide), Coumaphos (Organophosphate), Etrimfos (Organophosphate), Fonofos (Organophosphate), Isoprothiolane (Dithiolane), Metaflumizone (Semicarbazone), Phosphamidon (Organophosphate), Pirimiphos-ethly (Organophosphate) and Tebuconazole (Triazole).

The mean concentration of the studied pesticides was in the range of 11.27- 41.73 µg/kg. Many pesticides are degraded because of volatilization, or even temperature. As mentioned before, the rice was dried in the sun until it was completely dry, so at this stage the concentration of the different pesticides could already have decreased.

In Figure 16, the color of the different extracts can be compared, where the extract of the long grain rice washed with apple vinegar followed by conventional cooking is the clearest.

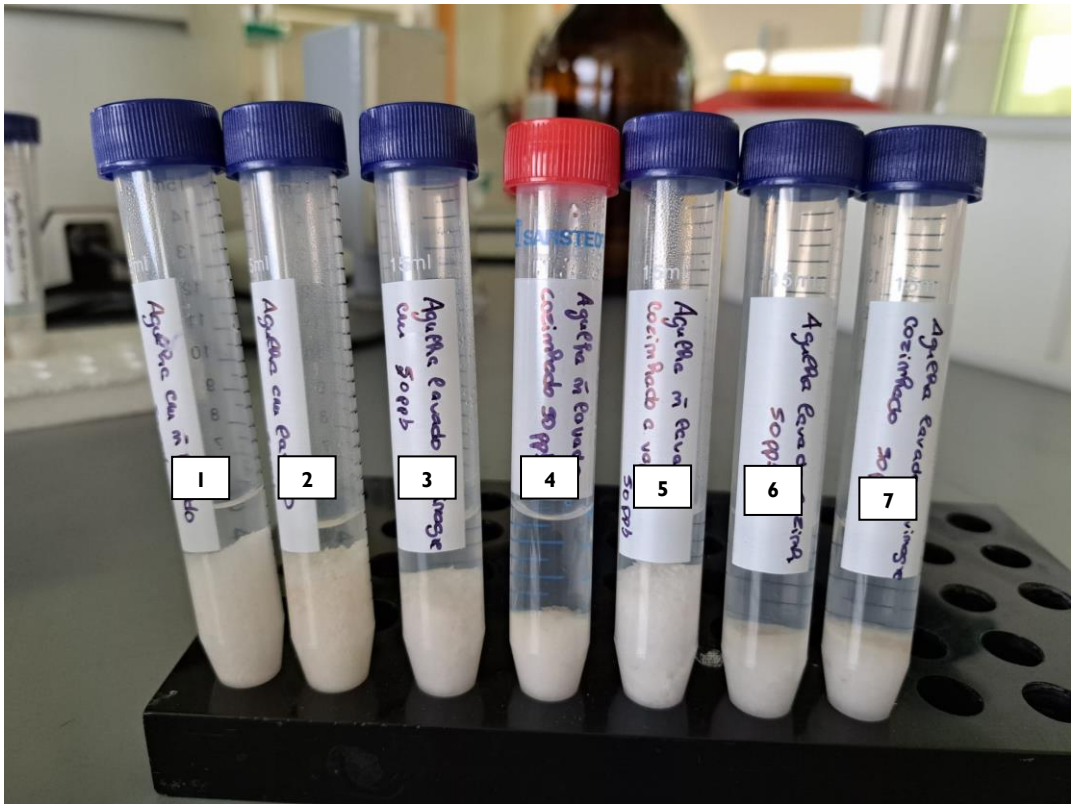

Figure 16. Comparison of the color of extracts from the QuEChERS Method. *(1)-Unprocessed Sample, (2)-Washed, (3)- Washed with Vinegar, (4)- Cooked, (5)- Steam Cooked, (6)- Washed and Cooked, (7)- Washed with Vinegar and Cooked.*

2.2.2. Effects of Washing and Washing with Vinegar

Of the 97 pesticides, it was only possible to calculate the percentage reduction for 86 pesticides. As mentioned above, some pesticides are below the LOQ, so it was not possible to quantify their reduction (section 2.2.1.).

Some pesticides are below the LOQ after washing and washing with vinegar. Therefore, the corresponding LOQ of the pesticide was used to calculate the minimal percentage reduction (Table 11).

The percentage reduction after washing ranged from 0.21 to 73%, with diazinon (organophosphate), indoxacarb (oxadiacin), zoxamide (benzamide), and fludioxanil (phenylpyrrole) not being significantly removed by washing. The highest reduction was 73% for pirimiphos-methyl (organophosphate), followed by 65.9% for fenamidone (imidazole), 65.8% for cadusafos (organophosphate) and 65.4% for propiconazole (azole). The pesticides that are below the LOQ after washing are EPN (organophosphate), fenamiphos sulfoxide (organophosphate), fenthion oxon (organophosphate), and fenthion oxon sulfoxide (organophosphate).

More than 40% of the pesticides presented a reduction between 40-60% and more than 10% of pesticides presented a reduction higher than 60% just after washing with mineral water (Figure 17).

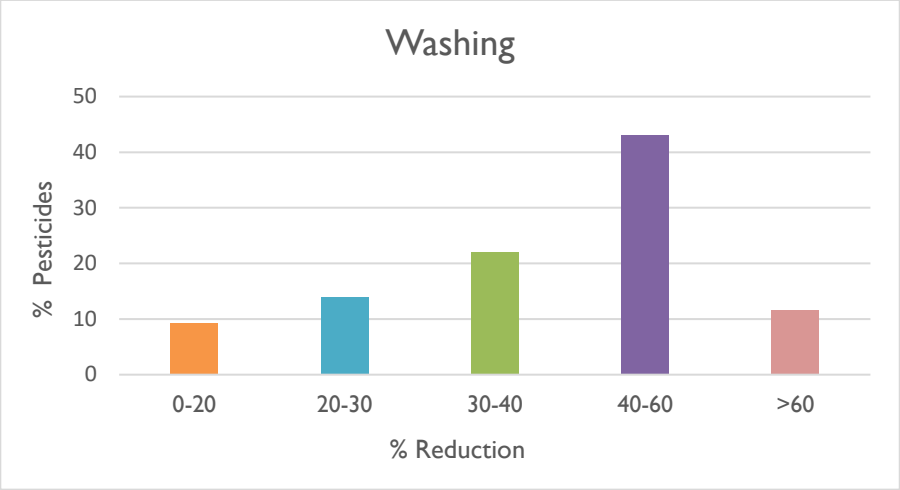

Figure 17. Reduction of pesticides in a contaminated rice samples after washing with mineral water.

The percentage reduction after washing with vinegar (5%, v/v) varies from 26.8 to 80.3%, for pesticides above their LOQ. Fludioxonil is not affected by vinegar washing while most of the pesticides are significantly reduced by vinegar washing. The highest reduction occurs for the pesticide pirimiphos-methyl. As found when washing the samples with mineral water, concentrations of pesticides below the LOQ were also found after washing with vinegar. In addition to those mentioned above, more pesticides were below the LOQ, such as Acetamiprid (neonicotinoid), Carbofuran (carbamate), Fenpropidin (unclassified), Fosthiazate (organophosphate), or Metribuzin (1,2,4-triazinone), among others.

Figure 18 shows that there is a higher percentage of pesticides, with a 60% reduction of pesticides contamination when washing with vinegar (5%, v/v) compared to washing with just mineral water.

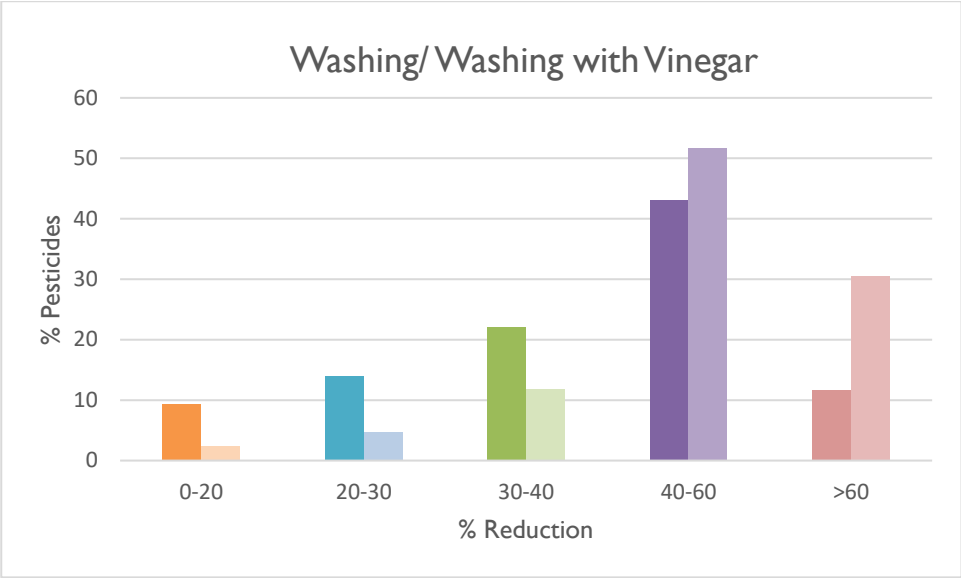

Figure 18. Comparison of the effect of washing with vinegar (5%, v/v) and washing just with water in the reduction of the initial concentration of pesticides in contaminated rice samples.

Regarding washing with vinegar, it is generally observed that there is a greater reduction than just washing with water, and this can be seen in figures 19 and 20. In figure 19, four different pesticides are used as examples, and one of them, Fenoxycarb (Carbamate) has about the same reduction when washing with mineral water and with vinegar (5%, v/v).

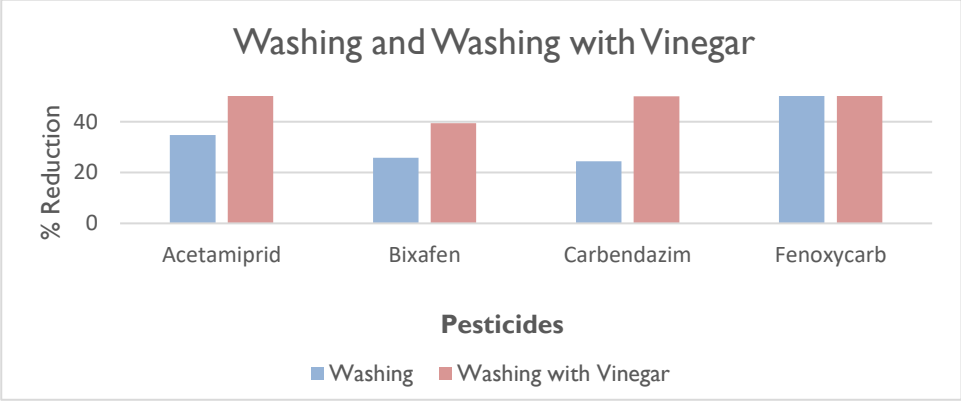

Figure 19. Effect of Washing and Washing with Vinegar in the reduction of four different representative pesticides in rice samples.

Figure 20 shows that approximately 40% of the pesticides are reduced by 5 to 10% more in the process involving washing with vinegar than in washing with just mineral water. So, in general, washing rice with vinegar reduces more than washing with water and should be recommended.

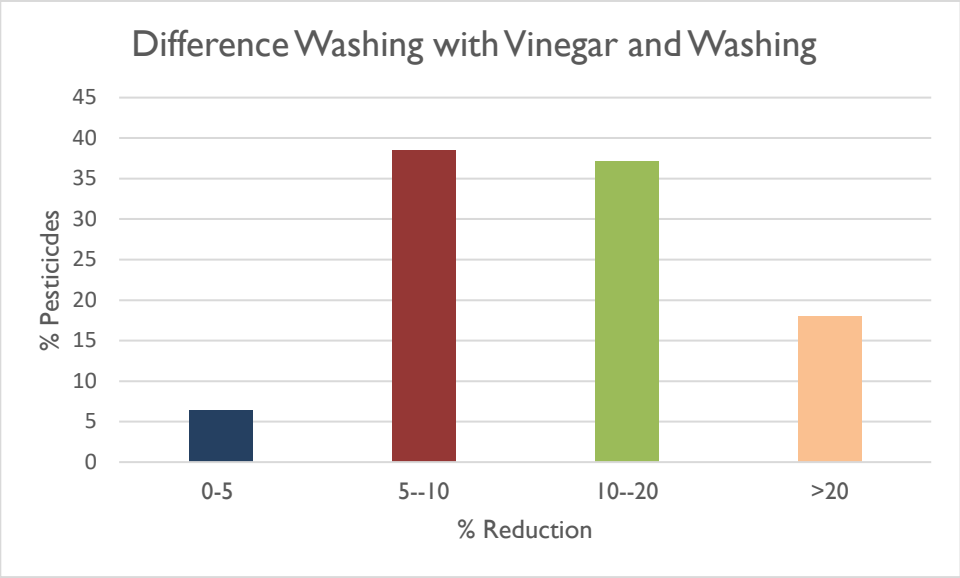

Figure 20. Difference (%) in the reduction of pesticides in rice samples between washing with vinegar and washing with just water.

Figure 21 shows the difference in pesticide concentration for the different samples: washed (1) and washed with vinegar (2), for Carbendazim.

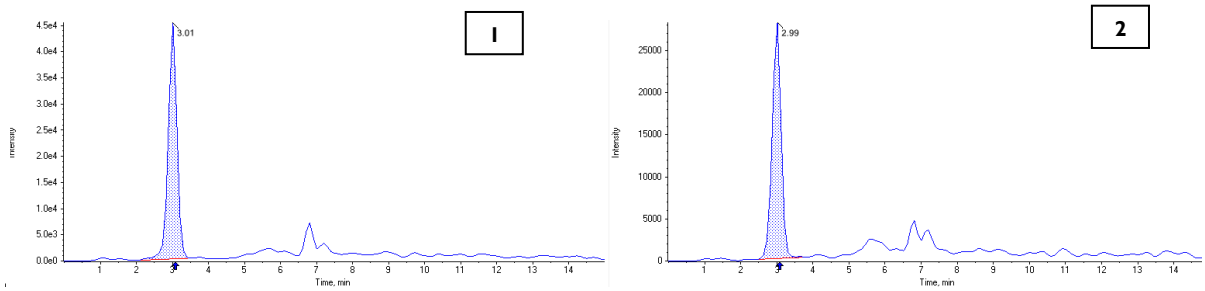

Figure 21. Chromatograms of different samples: washed (1) and washed with vinegar (2) for Carbendazim.

The results indicate that there was no correlation between chemical structure and the levels of residue removed by washing. For example, Diazinon has a small percentage of reduction compared to others form the same chemical group (Organophosphates), such as, Fosthiazate and EPN.

These results are in line with *Shakoori et. al.* (2018), because they have not found any correlation between chemical structure and the levels of residue removed by washing. In addition, they also proved that is no correlation between water solubility and residue removed by washing. For instance, the residues of oxadiazon and spinosyn D with water solubility 0.70 and 0.33 g/mL were reduced by 88.1% and 57.6%, respectively, while phosphamidon and

monocrotophos were reduced by 25.7% and 26.7%, respectively, with water solubility 1.00E+06 and 8.18+05 g/mL (Shakoori *et al.*, 2018).

In other studies, *Cabras et al.* (1997) and *Walter et al.* (2000) indicated that water solubility is not a major factor for removing pesticide residues by washing (Cabras *et al.*, 1997; Krol *et al.*, 2000).

Table 11. Mean concentrations (±SD, n=2), mean values of processing factors (PF) and reductions (%) of the pesticides in unprocessed rice samples, after washing.

Pesticides	Unprocessed Samples	Washing			Washing with Vinegar		
	Concentration ± SD	Concentration ± SD	PF	Reduction (%)	Concentration ± SD	PF	Reduction (%)
Acetamiprid	19.15 ± 1.93	12.50 ± 1.87	0.65	34.7	< LOQ	-	>50
Azoxystrobin	32.89 ± 4.50	13.22 ± 2.82	0.40	59.8	11.33 ± 1.93	0.34	65.6
Bixafen	32.70 ± 0.54	24.26 ± 1.71	0.74	25.8	19.81 ± 0.23	0.60	39.4
Boscalid	27.97 ± 2.69	15.0 ± 1.01	0.54	46.4	11.71 ± 2.54	0.42	58.2
Bupirimate	35.23 ± 4.89	16.14 ± 2.31	0.46	54.2	11.58 ± 1.02	0.33	67.1
Buprofezin	34.38 ± 2.47	19.45 ± 1.38	0.57	43.4	15.18 ± 2.43	0.44	55.6
Cadusafos	34.54 ± 6.30	11.83 ± 0.91	0.34	65.8	9.56 ± 2.51	0.28	72.3
Carbaryl	25.42 ± 0.73	19.33 ± 1.35	0.76	24	13.15 ± 2.36	0.52	48.3
Carbendazim	24.18 ± 0.52	18.28 ± 2.36	0.76	24.4	12.10 ± 1.69	0.50	50
Carbofuran	14.59 ± 1.63	9.85 ± 1.15	0.68	32.4	<LOQ	-	>31
Carboxin	<LOQ	<LOQ	-	-	<LOQ	-	-
Chlorantraniliprole	22.75 ± 0.13	14.06 ± 0.02	0.62	38.2	9.53 ± 0.29	0.42	56
Chlorfenvinphos	36.08 ± 1.25	21.99 ± 2.28	0.61	34	18.59 ± 1.91	0.51	48.5

Pesticides	Unprocessed Samples	Washing			Washing with Vinegar		
	Concentration ± SD	Concentration ± SD	PF	Reduction (%)	Concentration ± SD	PF	Reduction (%)
Chlorpyrifos-methyl	23.96 ± 0.23	15.86 ± 0.65	0.66	33.8	14.77 ± 2.67	0.62	38.4
Coumaphos	<LOQ	<LOQ	-	-	<LOQ	-	-
Cyprodinil	30.00 ± 1.97	14.89 ± 0.85	0.50	50.4	11.48 ± 1.28	0.38	61.7
Diazinon	37.30 ± 0.34	31.67 ± 1.80	0.85	15.1	27.31 ± 0.00	0.73	26.8
Difenoconazole	32.70 ± 5.21	12.76 ± 1.14	0.39	61	10.34 ± 2.36	0.32	68.4
Diflubenzuron	37.59 ± 4.07	19.21 ± 0.92	0.51	48.9	17.82 ± 2.25	0.47	52.6
Dimethoate	13.34 ± 1.26	10.67 ± 1.44	0.80	20.01	6.63 ± 0.91	0.50	50.31
Dimethomorph	26.70 ± 2.54	14.81 ± 1.67	0.55	44.5	13.44 ± 1.45	0.50	49.7
Diniconazole	40.59 ± 3.53	26.53 ± 1.42	0.65	34.6	22.10 ± 0.06	0.54	45.5
DMST	24.93 ± 0.54	13.91 ± 0.17	0.56	44.2	11.61 ± 0.76	0.47	53.4
EPN	21.54 ± 5.47	<LOQ	-	>53.6	<LOQ	-	>53.6
Epoxiconazole	41.15 ± 4.64	21.27 ± 1.44	0.52	48.3	16.88 ± 0.61	0.41	59
Ethoprophos	27.28 ± 3.13	13.40 ± 1.60	0.49	50.9	9.67 ± 2.00	0.35	64.6

Pesticides	Unprocessed Samples	Washing			Washing with Vinegar		
	Concentration ± SD	Concentration ± SD	PF	Reduction (%)	Concentration ± SD	PF	Reduction (%)
Etrimfos	<LOQ	<LOQ	-	-	<LOQ	-	-
Fenamidone	36.31 ± 5.15	12.39 ± 1.22	0.34	65.9	9.83 ± 1.80	0.27	72.9
Fenamiphos sulfone	14.79 ± 0.59	11.31 ± 1.61	0.76	23.5	7.02 ± 0.25	0.47	52.6
Fenamiphos sulfoxide	16.77 ± 1.69	<LOQ	-	>40.4	<LOQ	-	>40.4
Fenarimol	39.57 ± 2.82	23.30 ± 1.51	0.59	41.4	20.04 ± 0.20	0.51	49.4
Fenoxycarb	35.71 ± 5.81	12.91 ± 1.66	0.36	63.8	10.36 ± 2.02	0.29	71
Fenpropidin	37.22 ± 4.15	18.35 ± 1.92	0.49	50.7	<LOQ	-	>73.1
Fenpropimorph	39.88 ± 2.73	19.12 ± 0.89	0.48	52	13.99 ± 0.28	0.35	64.9
Fenthion oxon sulfone	23.32 ± 3.60	9.88 ± 0.50	0.42	57.1	<LOQ	-	>57.1
Fenthion oxon sulfoxide	11.27 ± 1.15	<LOQ	-	>11.3	<LOQ	-	>11.3
Fenthion oxon	23.82 ± 1.07	<LOQ	-	>58	<LOQ	-	>58
Fenthion sulfoxide	23.19 ± 1.82	10.48 ± 0.06	0.45	54.8	<LOQ	-	>57
Fluopyram	32.00 ± 2.28	13.07 ± 1.24	0.41	59.2	10.40 ± 2.19	0.32	67.5

Pesticides	Unprocessed Samples	Washing			Washing with Vinegar		
	Concentration ± SD	Concentration ± SD	PF	Reduction (%)	Concentration ± SD	PF	Reduction (%)
Fluquinconazole	31.62 ± 0.03	19.35 ± 2.59	0.61	38.8	17.98 ± 1.66	0.57	43.1
Flutriafol	30.94 ± 3.60	13.21 ± 0.80	0.43	57.3	11.51 ± 1.57	0.37	62.8
Fonofos	<LOQ	<LOQ	-	-	<LOQ	-	-
Fosthiazate	16.92 ± 2.22	13.75 ± 0.65	0.81	18.7	<LOQ	-	>40.9
Hexythiazox	13.23 ± 3.31	<LOQ	-	>24.4	<LOQ	-	>24.4
Indoxacarb	25.32 ± 2.28	23.39 ± 4.73	0.92	7.7	18.77 ± 1.01	0.74	25.9
Iprodione	26.85 ± 1.95	21.84 ± 2.31	0.81	18.7	17.18 ± (2.08	0.64	36
Iprovalicarb	34.69 ± 5.22	15.46 ± 1.25	0.44	55.4	13.77 ± 3.89	0.40	60.3
Isoprocarb	22.60 ± 1.45	15.69 ± 3.30	0.69	30.6	10.79 ± 1.18	0.48	52.3
Isoprothiolane	<LOQ	<LOQ	-	-	<LOQ	-	-
Isoproturon	22.81 ± 2.63	10.94 ± 0.73	0.48	52.03	9.67 ± 1.07	0.42	56.2
Linuron	28.79 ± 2.17	21.09 ± 2.08	0.73	26.7	17.41 ± 1.28	0.60	39.5
Lufenuron	13.60 ± 3.86	7.57 ± 1.97	0.55	44.3	5.67 ± 0.16	0.42	58.3

Pesticides	Unprocessed Samples	Washing			Washing with Vinegar		
	Concentration ± SD	Concentration ± SD	PF	Reduction (%)	Concentration ± SD	PF	Reduction (%)
Mepanipyrim	38.44 ± 3.16	24.38 ± (2.02)	0.63	36.6	19.67 ± 1.84	0.51	48.8
Metaflumizone	<LOQ	<LOQ	-	-	<LOQ	-	-
Metalaxyl	13.89 ± 2.26	9.66 ± 0.51	0.70	30.4	4.87 ± 0.71	0.35	64.9
Metalaxyl-M	14.82 ± 2.36	9.20 ± 0.37	0.62	37.9	<LOQ	-	>32.5
Metazachlor	21.29 ± 0.79	15.91 ± 2.09	0.75	25.3	11.29 ± 0.91	0.53	47.0
Metconazole	37.82 ± 1.66	24.69 ± 2.31	0.65	34.7	21.47 ± 1.93	0.57	43.2
Methiocarb	30.71 ± 2.36	12.99 ± 2.49	0.55	44.7	15.22 ± 2.51	0.50	50.44
Metrobromuron	28.07 ± 3.95	16.04 ± 0.02	0.57	42.9	11.86 ± 0.94	0.42	57.74
Metribuzin	20.32 ± 1.00	10.37 ± 0.69	0.51	49.0	<LOQ	-	>50.8
Oxadixyl	12.78 ± 1.03	9.25 ± 2.52	0.72	21.8	<LOQ	-	>21.8
Paclobutrazole	38.64 ± 3.59	17.41 ± 1.25	0.45	54.9	13.91 ± 1.36	0.36	64
Parathion	23.04 ± 2.04	18.02 ± 1.56	0.78	21.8	15.28 ± 0.52	0.66	33.6
Parathion-methyl	16.29 ± 2.10	11.32 ± 0.59	0.69	30.5	9.54 ± 2.60	0.59	41.5

Pesticides	Unprocessed Samples	Washing			Washing with Vinegar		
	Concentration ± SD	Concentration ± SD	PF	Reduction (%)	Concentration ± SD	PF	Reduction (%)
Penconazole	40.64 ± 1.35	23.81 ± 2.14	0.59	41.4	20.95 ± 2.92	0.52	48.4
Pencycuron	29.08 ± 3.95	17.14 ± 1.22	0.59	41.1	15.10 ± 0.64	0.52	48.1
Phenthoate	34.75 ± 4.92	21.57 ± 3.00	0.62	37.9	18.90 ± 4.46	0.54	45.6
Phosphamidon	<LOQ	<LOQ	-	-	<LOQ	-	-
Pirimiphos-ethyl	<LOQ	<LOQ	-	-	<LOQ	-	-
Pirimiphos-methyl	32.85 ± 5.57	8.86 ± 1.27	0.27	73.0	6.48 ± 1.39	0.20	80.3
Prochloraz	22.00 ± 6.35	11.42 ± 1.16	0.52	48.1	12.93 ± 1.86	0.59	41.2
Profenofos	27.43 ± 2.58	13.56 ± 1.63	0.49	50.5	11.84 ± 1.98	0.43	56.8
Propiconazole	41.73 ± 5.79	14.45 ± 1.72	0.35	65.4	12.08 ± 2.14	0.29	71.1
Propyzamide	33.29 ± 0.03	20.28 ± 3.53	0.61	39.1	16.68 ± 2.95	0.50	49.9
Prothioconazole-desthio	39.75 ± 3.30	15.29 ± 0.70	0.38	61.5	12.56 ± 2.17	0.32	68.4
Pyrazophos	34.01 ± 1.85	23.38 ± 1.31	0.69	31.3	19.86 ± 2.62	0.58	41.6
Pyrimethanil	36.24 ± 2.45	23.64 ± 1.52	0.65	34.8	16.22 ± 1.52	0.45	55.2

Pesticides	Unprocessed Samples	Washing			Washing with Vinegar		
	Concentration ± SD	Concentration ± SD	PF	Reduction (%)	Concentration ± SD	PF	Reduction (%)
Quinoxyfen	12.93 ± 0.87	9.28 ± 0.42	0.71	28.3	8.21 ± 0.25	0.63	36.5
Rotenone	18.56 ± 1.10	<LOQ	-	>46.2	<LOQ	-	>46.2
SpinosadA	29.02 ± 1.15	13.61 ± 2.10	0.47	53.1	8.72 ± 0.73	0.30	69.9
SpinosadD	27.24 ± 0.91	14.83 ± 1.06	0.54	45.6	10.11 ± 1.66	0.37	62.9
Spiroxamine	37.77 ± 4.59	17.70 ± 1.31	0.47	53.1	8.82 ± 0.76	0.23	76.6
Tebuconazole	<LOQ	<LOQ	-	-	<LOQ	-	-
Tebufenpyrad	31.60 ± 3.48	19.91 ± 1.86	0.63	37	16.90 ± 2.50	0.53	46.5
Terbuthylazine	35.27 ± 0.40	23.94 ± 2.98	0.68	32.1	20.67 ± 2.70	0.59	41.4
Tetraconazole	39.48 ± 4.30	15.73 ± 1.88	0.40	60.1	13.85 ± 3.10	0.35	64.9
Thiabendazole	26.18 ± 6.52	12.75 ± 2.49	0.49	51.3	10.45 ± 2.49	0.40	60.1
Thiacloprid	28.11 ± 3.11	14.55 ± 1.85	0.52	48.2	12.35 ± 1.42	0.44	56.1
Triadimefon	37.60 ± 0.61	27.12 ± 3.25	0.72	27.9	23.21 ± 0.26	0.62	38.3
Triadimenol	38.75 ± 3.35	18.90 ± 2.42	0.49	51.2	15.80 ± 1.98	0.41	59.2

Pesticides	Unprocessed Samples	Washing			Washing with Vinegar		
	Concentration ± SD	Concentration ± SD	PF	Reduction (%)	Concentration ± SD	PF	Reduction (%)
Tricyclazole	26.55 ± 2.21	13.91 ± 1.01	0.52	47.6	11.18 ± 0.39	0.42	57.9
Triflumuron	31.98 ± 5.43	11.74 ± 1.52	0.37	63.3	9.15 ± 1.15	0.29	71.4
Zoxamide	27.49 ± 0.13	23.04 ± 1.87	0.84	16.2	19.80 ± 0.63	0.72	28
Fludioxonil	24.15 ± 1.64	24.10 ± 1.96	1.00	0.21	24.86 ± 0.06	1.03	+ 3

Legend: DMST- N,N-dimethyl-N'-p-tolysulphamide; EPN- O-ethyl O-4-nitrophenyl phosphonothiate.

2.2.3. Effects of Cooking

As with washing, an even greater reduction in the concentration of pesticides in cooking is also expected. Furthermore, even more pesticides were found below the LOQ.

The percentage of cooking reduction varied between 21.2 and 81.2%, for pesticides above their LOQ. The pesticide with the lowest reduction is Fenthion sulfoxide (Organophosphate), and the one with the highest reduction is Pirimiphos-methyl (Organophosphate). Once again, it is confirmed that the cooking effect has no correlation with the chemical structures of the pesticides. In cooking, more than 35% of pesticides reduce between 50 and 60%, and about 15% of pesticides reduce more than 70% (Figure 22).

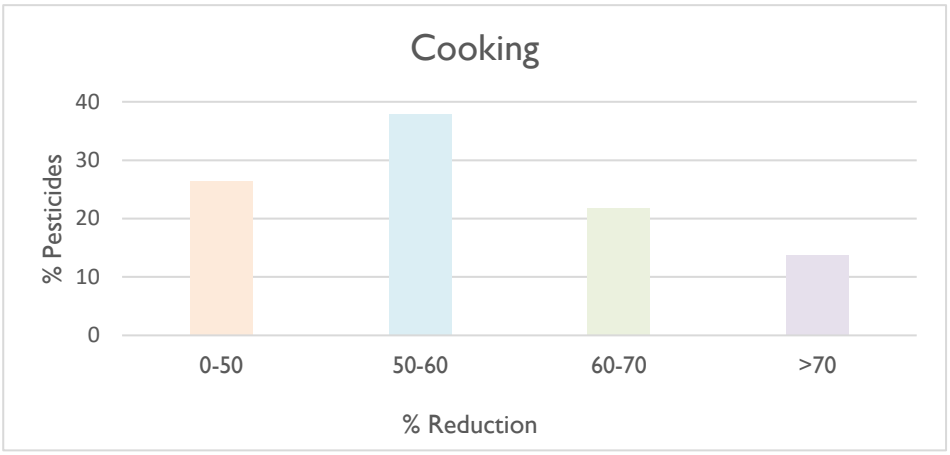

Figure 22. Effect of cooking on the pesticides' reduction in contaminated rice samples.

The percentage reduction of pesticides in steam cooking varies between 29.8 and 80%, for pesticides above their LOQ. The pesticide with the lowest reduction was Carbendazim (Benzimidazole), and the one with the highest reduction was Ethoprophos (Organophosphate). About 30% of the pesticides achieved a reduction of 60 to 70%, and approximately 25% of the pesticides had a reduction higher than 70% (Figure 23).

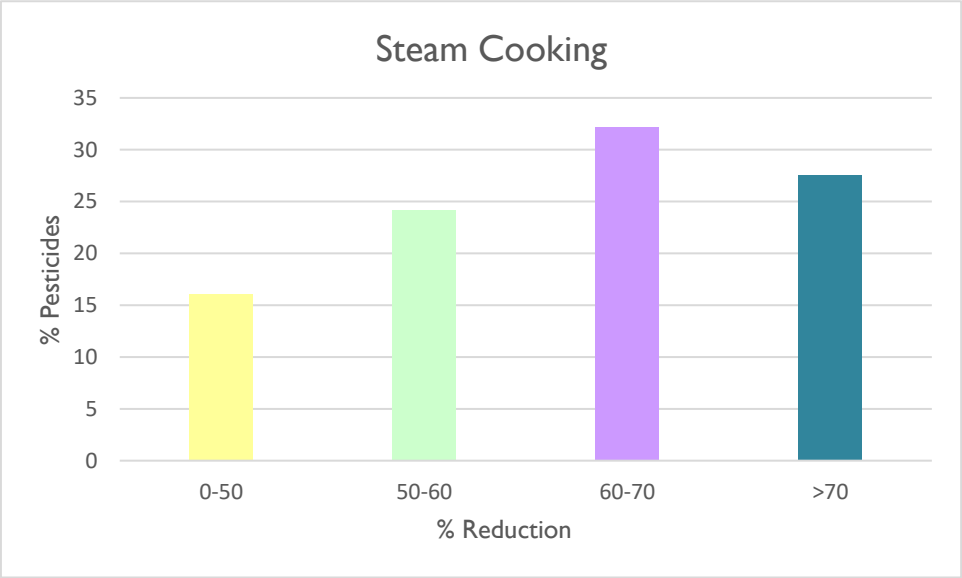

Figure 23. Effect of steam cooking on the pesticides' reduction in contaminated rice samples.

The percentage reduction of pesticides in rice after washing and conventional cooking varied between 29.8 and 75.8%, for pesticides determined above their LOQ. In this case, the pesticide that presented the highest reduction was Zoxamide (Benzamide), and the pesticide that presented the lowest reduction was Fenthion sulfoxide. In this case, 34 pesticides, were below their LOQ after being both washed and cooked. 45% of pesticides achieved a reduction between 60 and 70%, and 10% of pesticides achieved a reduction above 70% (Figure 24).

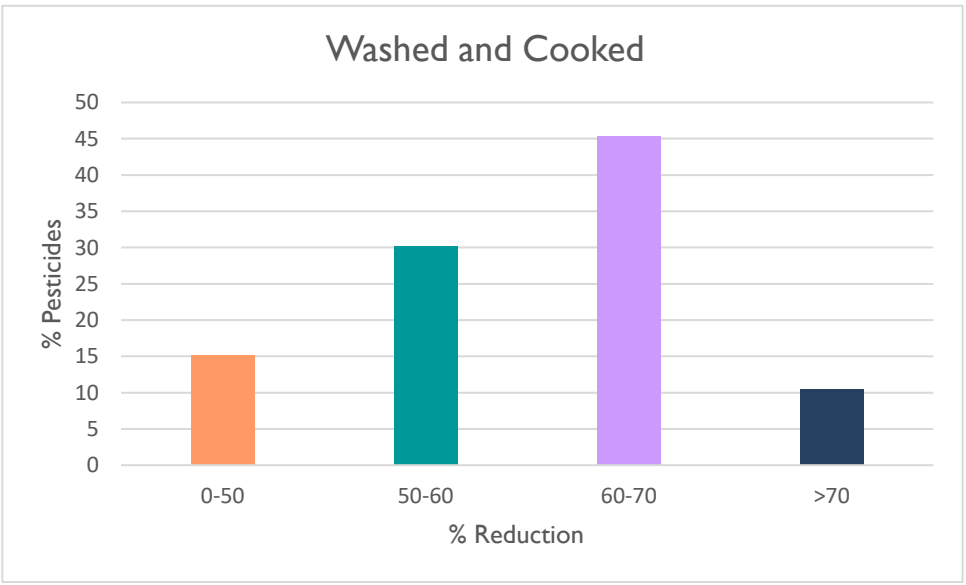

Figure 24. Effect of combination of washing and cooking on the pesticides' reduction in contaminated rice samples.

The percentage reduction of pesticides in rice samples after washing with vinegar and conventional cooking ranges from 49.3 to 85.8%, for pesticides determined above the LOQ. The pesticide with the lowest reduction was Fenthion sulfoxide (49.3%) and the one with the highest reduction was Spiroxamine (Spirocetalamide) (85.8%).

The combination of washing with vinegar and conventional cooking is the processing where more than half of the pesticides are below their LOQ, in a total of 62 pesticides. Also, the combination of these two methods allowed to obtain the highest percentage of pesticides (35%) with a reduction higher than 70% (Figure 25).

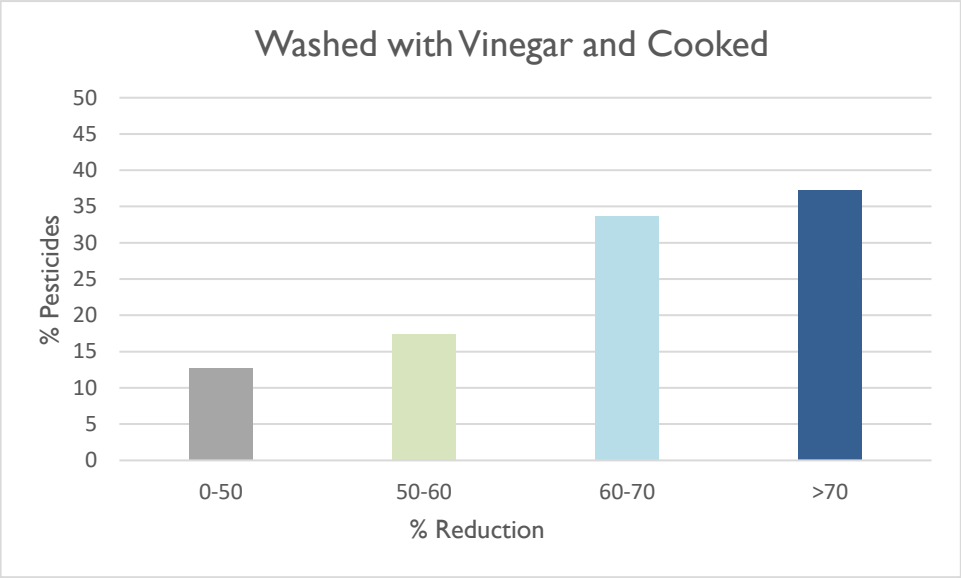

Figure 25. Effect of combination of washing with vinegar and conventional cooking on the pesticides' reduction in contaminated rice samples.

Comparing the cooking (conventional cooking) and steam cooking processing methods, there are variations depending on the pesticides; that is, some reduce more using conventional cooking and other using steam cooking. Some present no considerable differences between the two cooking methods. Also, some reduce more in steam cooking than in cooking. Figure 26 shows these differences.

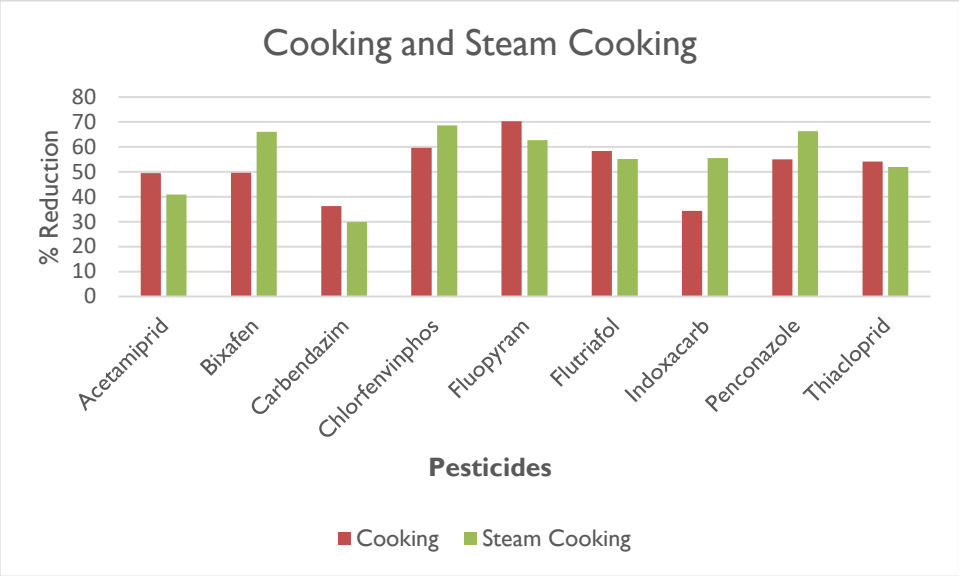

Figure 26. Effect of combination of washing and steam cooking on the pesticides' reduction in contaminated rice samples.

From the four processing methods evaluated and their combinations, it can be concluded that the one with the greatest positive impact on pesticide removal was washing with vinegar (5%, v/v) followed by conventional cooking. As seen in Figures 22, 23, 24, and 25, the combination of these two methods presented the higher percentage of pesticides with reduction above 70%. Furthermore, it is the combination of methods with the highest number of pesticides below its LOQ (Table 12).

Heating-related processes can speed up hydrolysis, volatilization, or other chemical degradation, which lowers residue levels (Shoeibi *et al.*, 2011). The removal of pesticide residues due to processing is affected by the degree of adsorption of pesticides by the cereals' grains, pesticide residues' solubility in water and heat-induced breakdown. Our findings are consistent with the literature, which shows that processing, particularly washing, and cooking, typically result in significant reductions in the levels of pesticide residues in the cooked product.

In fact, *Shakoori et al.* (2018), cooked rice by the Iranian method (Kateh), which consists of two principal steps: boiling and steam cooking, and their results are very consistent with our study. They found no relationship between the chemical group to which the pesticide belongs, and the reduction found. For instance, the level of edifenphos, diazinon, dicrotophos, and oxydemeton-methyl was reduced by 20.7%, 43.3%, 63.5%, and 100%, respectively, in the organophosphate group (Shakoori *et al.*, 2018).

In a study of three carbamates carried out by *Shoeibi et al.* (2011), no strong correlation was found between a single physicochemical property of each of the three pesticides and the percentage of their reduction after cooking, because carbaryl had a reduction of 78.47% and pirimicarb presented a reduction of 34.5% (Shoeibi *et al.*, 2011).

Figure 27 shows the difference in pesticide concentration for the different samples: cooked (1) and steam cooking (2), for Bixafen (B) and Carbendazim (C), as representative pesticides residues.

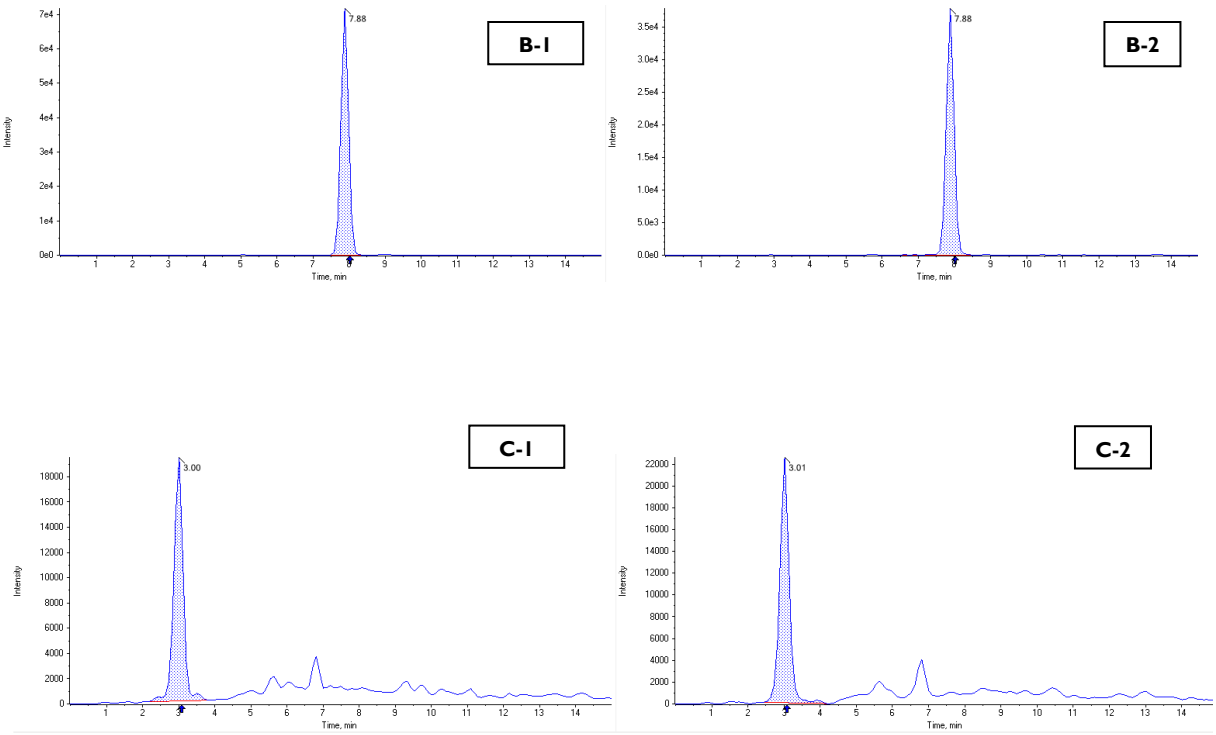

Figure 27. Chromatograms of different samples: cooked (1), and steam cooking (2) for Bixafen (B) and Carbendazim (C), as representative pesticides residues.

Figure 28 shows the difference in pesticide concentration for the different samples: unprocessed (1), washed (2), cooked (3) and washed and cooked (4), for Azoxystrobin and Bixafen, two representative pesticides residues.

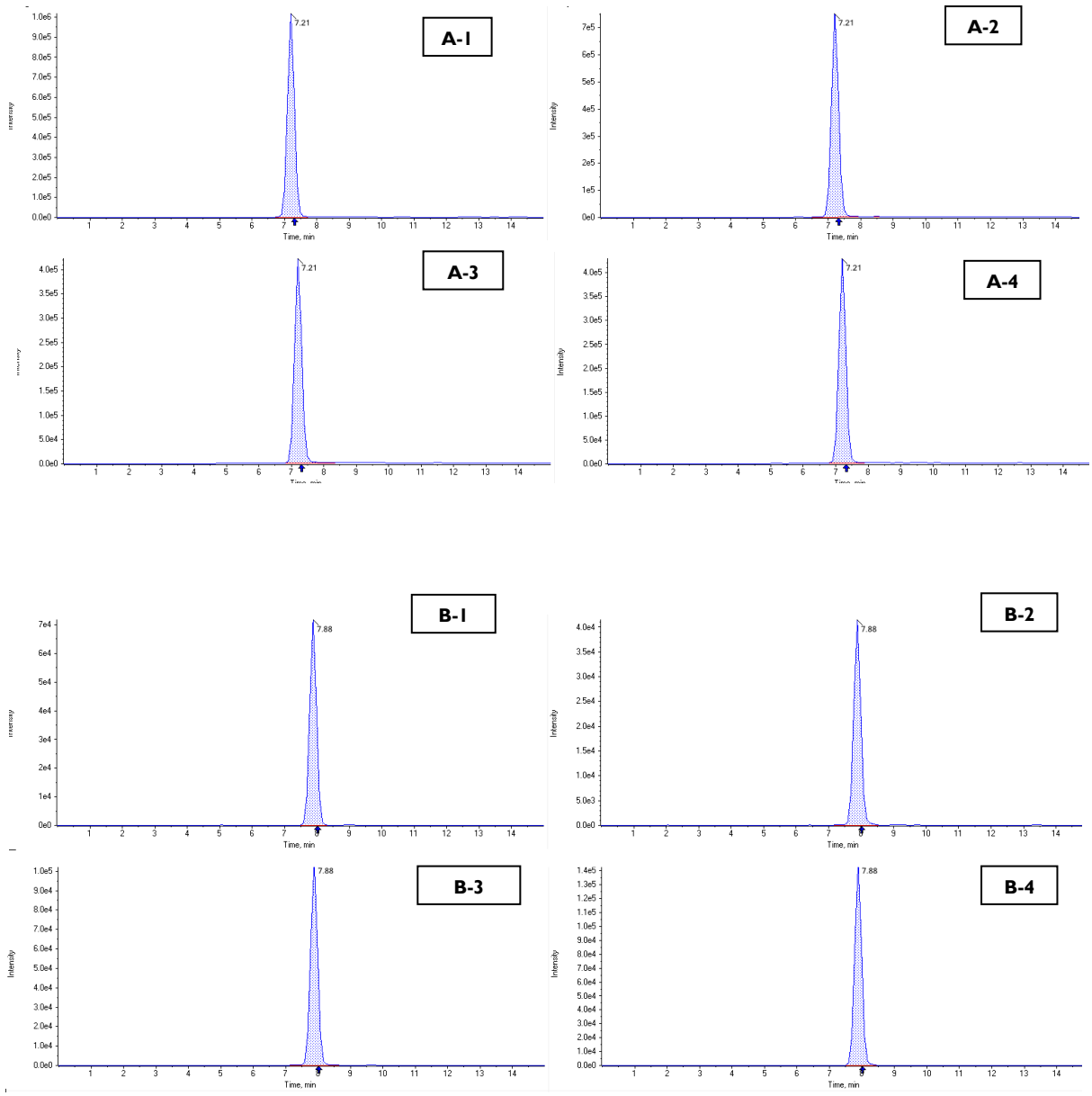

Figure 28. Chromatograms of different samples: unprocessed (1), washed (2), cooked (3) and washed and cooked (4) for (A)- Azoxystrobin and (B)- Bixafen as representative pesticides residues

Table 12. Mean concentrations (±SD, n=2), mean values of processing factors (PF) and reductions (Re, %) of the pesticides in unprocessed rice samples, after cooking (conventional cooking and steam cooking) and combination of washing and cooking.

Pesticides	Unprocessed Samples	Cooked			Steam Cooked			Washed and Cooked			Washed with Vinegar and Cooked		
	Concentration (Co) ± SD	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)
Acetamiprid	19.15 ± 1.93	9.67 ± 0.63	0.50	49.5	11.32 ± 0.89	0.59	40.9	<LOQ	-	>47.8	<LOQ	-	>47.8
Azoxystrobin	32.89 ± 4.50	13.10 ± 1.85	0.40	60.2	13.18 ± 1.54	0.40	59.9	12.73 ± 1.07	0.39	61.3	<LOQ	-	>84.8
Bixafen	32.70 ± 0.54	16.48 ± 1.86	0.50	49.6	11.09 ± 0.69	0.34	66.1	12.71 ± 0.39	0.39	61.1	10.61 ± 1.43	0.32	67.2
Boscalid	27.97 ± 2.69	12.72 ± 0.29	0.45	54.5	13.03 ± 0.11	0.47	53.4	11.33 ± 0.21	0.40	59.5	7.56 ± 2.11	0.27	73
Bupirimate	35.23 ± 4.89	12.11 ± 0.28	0.34	65.6	9.28 ± 1.52	0.26	73.7	12.07 ± 1.16	0.34	65.7	<LOQ	-	>71.6
Buprofezin	34.38 ± 2.47	15.40 ± 0.17	0.45	55.2	9.66 ± 0.65	0.28	72.0	14.08 ± 0.50	0.41	59.1	<LOQ	-	>70.9
Cadusafos	34.54 ± 6.30	9.12 ± 2.30	0.26	73.6	<LOQ	-	>85.5	9.86 ± 2.95	0.28	71.4	<LOQ	-	>85.5
Carbaryl	25.42 ± 0.73	10.66 ± 0.31	0.42	58.1	<LOQ	-	>60.7	<LOQ	-	>60.7	<LOQ	-	>60.7
Carbendazim	24.18 ± 0.52	15.39 ± 2.83	0.64	36.3	16.98 ± 0.56	0.70	29.8	<LOQ	-	>58.6	<LOQ	-	>58.6
Carbofuran	14.59 ± 1.63	<LOQ	-	>65.6	<LOQ	-	>65.6	<LOQ	-	>65.6	<LOQ	-	>65.6
Carboxin	<LOQ	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-
Chlorantraniliprole	22.75 ± 0.13	12.33 ± 0.30	0.54	45.8	<LOQ	-	>56.1	<LOQ	-	>56.1	<LOQ	-	>56.1
Chlorfenvinphos	36.08 ± 1.25	15.59 ± 0.38	0.40	59.6	11.28 ± 0.52	0.31	68.7	14.09 ± 0.89	0.39	60.9	10.20 ± 2.86	0.28	71.7

Pesticides	Unprocessed Samples	Cooked			Steam Cooked			Washed and Cooked			Washed with Vinegar and Cooked		
	Concentration (Co) ± SD	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)
Chlorpyrifos-methyl	23.96 ± 0.23	9.69 ± 1.10	0.40	59.6	<LOQ	-	>58.3	<LOQ	-	>58.3	<LOQ	-	>58.3
Coumaphos	<LOQ	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-
Cyprodinil	30.00 ± 1.97	11.70 ± 0.19	0.39	61.0	<LOQ	-	>66.7	9.98 ± 1.18	0.33	66.7	<LOQ	-	>66.7
Diazinon	37.30 ± 0.34	18.93 ± 1.51	0.51	49.2	<LOQ	-	>73.2	14.76 ± 0.00	0.40	60.4	13.11 ± 0.12	0.35	64.8
Difenoconazole	32.70 ± 5.21	9.78 ± 0.67	0.30	70.1	<LOQ	-	>69.5	9.79 ± 1.32	0.30	70.1	<LOQ	-	>69.5
Diflubenzuron	37.59 ± 4.07	14.93 ± 0.78	0.40	60.3	11.85 ± 0.82	0.31	68.5	14.49 ± 0.62	0.39	61.5	10.61 ± 3.05	0.28	71.8
Dimethoate	13.34 ± 1.26	6.89 ± 0.10	0.52	48.4	<LOQ	-	>62.5	5.54 ± 0.21	0.53	47	<LOQ	-	>62.5
Dimethomorph	26.70 ± 2.54	13.52 ± 0.10	0.51	49.3	11.41 ± 1.27	0.43	57.3	13.15 ± 0.48	0.49	50.8	<LOQ	-	>62.5
Diniconazole	40.59 ± 3.53	16.33 ± 2.24	0.40	59.8	10.89 ± 0.74	0.27	73.2	13.41 ± 0.81	0.33	67	10.14 ± 1.28	0.25	75
DMST	24.93 ± 0.54	12.42 ± 0.38	0.50	50.2	12.38 ± 0.03	50	50.4	11.53 ± 0.04	0.46	53.6	<LOQ	-	>59.9
EPN	21.54 ± 5.47	<LOQ	-	>53.6	<LOQ	-	>53.6	<LOQ	-	>53.6	<LOQ	-	>53.6
Epoxiconazole	41.15 ± 4.64	13.88 ± 1.28	0.34	66.3	11.57 ± 0.29	0.28	71.9	12.68 ± 1.35	0.31	69.2	<LOQ	-	>75.7
Ethoprophos	27.28 ± 3.13	8.51 ± 0.58	0.32	68.8	5.44 ± 0.40	0.20	80.0	8.98 ± 1.44	0.33	67.1	5.00 ± 1.09	0.18	81.7

Pesticides	Unprocessed Samples	Cooked			Steam Cooked			Washed and Cooked			Washed with Vinegar and Cooked		
	Concentration (Co) ± SD	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)
Etrimfos	<LOQ	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-
Fenamidone	36.31 ± 5.15	9.43 ± 0.75	0.26	74.0	<LOQ	-	>72.5	9.33 ± 1.45	0.26	64.6	<LOQ	-	>72.5
Fenamiphos sulfone	14.79 ± 0.59	8.09 ± 0.46	0.55	45.3	9.09 ± 0.20	0.62	38.5	6.84 ± 0.13	0.46	53.8	4.90 ± 0.96	0.33	66.9
Fenamiphos sulfoxide	16.77 ± 1.69	<LOQ	-	>40.4	<LOQ	-	>40.4	<LOQ	-	>40.4	<LOQ	-	>40.4
Fenarimol	39.57 ± 2.82	15.32 ± 2.68	0.39	61.3	11.55 ± 0.64	0.29	70.8	13.02 ± 0.18	0.33	67.1	<LOQ	-	>75
Fenoxycarb	35.71 ± 5.81	<LOQ	-	>72.0	<LOQ	-	>72.0	<LOQ	-	>72.0	<LOQ	-	>72.0
Fenpropidin	37.22 ± 4.15	13.00 ± 0.05	0.35	65.06	<LOQ	-	>73.1	11.17 ± 1.18	0.30	69.98	<LOQ	-	>73.1
Fenpropimorph	39.88 ± 2.73	15.16 ± 0.54	0.38	62	9.33 ± 1.35	0.23	76.7	13.73 ± 0.15	0.34	65.6	<LOQ	-	>74.9
Fenthion oxon sulfone	23.32 ± 3.60	10.72 ± 0.51	0.46	54	<LOQ	-	>57.1	<LOQ	-	>57.1	<LOQ	-	>57.1
Fenthion oxon sulfoxide	11.27 ± 1.15	<LOQ	-	>11.3	<LOQ	-	>11.3	<LOQ	-	11.3	<LOQ	-	>11.3
Fenthion oxon	23.82 ± 1.07	<LOQ	-	>58.0	<LOQ	-	>58.0	<LOQ	-	>58.0	<LOQ	-	>58.0
Fenthion sulfoxide	23.19 ± 1.82	16.89 ± 1.09	0.73	21.2	<LOQ	-	>56.9	16.28 ± 0.50	0.70	29.8	11.75 ± 2.44	0.51	49.3
Fluopyram	32.00 ± 2.28	9.49 ± 1.15	0.30	70.3	11.93 ± 1.88	0.37	62.7	11.02 ± 0.70	0.34	65.6	<LOQ	-	>68.8

Pesticides	Unprocessed Samples	Cooked			Steam Cooked			Washed and Cooked			Washed with Vinegar and Cooked		
	Concentration (Co) ± SD	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)
Fluquinconazole	31.62 ± 0.03	14.27 ± 0.20	0.45	54.9	9.30 ± 0.25	0.29	70.6	12.86 ± 0.20	0.41	59.3	9.96 ± 2.73	0.32	68.5
Flutriafol	30.94 ± 3.60	12.86 ± 0.47	0.42	58.4	13.91 ± 0.71	0.45	55.1	12.20 ± 2.23	0.39	60.6	7.40 ± 1.56	0.24	76.1
Fonofos	<LOQ	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-
Fosthiazate	16.92 ± 2.22	<LOQ	-	>40.9	<LOQ	-	>40.9	<LOQ	-	>40.9	<LOQ	-	>40.9
Hexythiazox	13.23 ± 3.31	<LOQ	-	>24.4	<LOQ	-	>24.4	<LOQ	-	>24.4	<LOQ	-	>24.4
Indoxacarb	25.32 ± 2.28	16.63 ± 2.78	0.66	34.3	11.24 ± 0.68	0.44	55.6	12.45 ± 0.34	0.49	50.8	11.72 ± 2.18	0.46	53.7
Iprodione	26.85 ± 1.95	11.14 ± 2.53	0.42	58.5	<LOQ	-	>62.8	<LOQ	-	>62.8	<LOQ	-	>62.8
Iprovalicarb	34.69 ± 5.22	<LOQ	-	>71.2	<LOQ	-	>71.2	<LOQ	-	>71.2	<LOQ	-	>71.2
Isoprocarb	22.60 ± 1.45	9.88 ± 0.28	0.44	56.3	<LOQ	-	>55.8	<LOQ	-	>55.8	<LOQ	-	>55.8
Isoprothiolane	<LOQ	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-
Isoproturon	22.81 ± 2.63	9.23 ± 0.26	0.40	59.5	<LOQ	-	>56.2	<LOQ	-	>56.2	<LOQ	-	56.2
Linuron	28.79 ± 2.17	14.39 ± 2.01	0.50	50	<LOQ	-	>65.3	10.83 ± 0.20	0.38	62.4	<LOQ	-	>65.3
Lufenuron	13.60 ± 3.86	<LOQ	-	>26.5	<LOQ	-	>26.5	<LOQ	-	>26.5	<LOQ	-	>26.5

Pesticides	Unprocessed Samples	Cooked			Steam Cooked			Washed and Cooked			Washed with Vinegar and Cooked		
	Concentration (Co) ± SD	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)
Mepanipyrim	38.44 ± 3.16	17.06 ± 0.84	0.44	55.6	11.68 ± 1.58	0.30	69.6	14.01 ± 0.40	0.36	63.6	10.21 ± 0.20	0.27	73.4
Metaflumizone	<LOQ	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-
Metalaxyl	13.89 ± 2.26	6.33 ± 0.26	0.45	54.5	7.20 ± 0.04	0.52	48.2	4.94 ± 0.28	0.36	64.4	<LOQ	-	>64.0
Metalaxyl-M	14.82 ± 2.36	<LOQ	-	>32.5	<LOQ	-	32.5	<LOQ	-	>32.5	<LOQ	-	>32.5
Metazachlor	21.29 ± 0.79	<LOQ	-	>53.0	<LOQ	-	>53.0	<LOQ	-	>53.0	<LOQ	-	>53.0
Metconazole	37.82 ± 1.66	17.70 ± 1.34	0.47	53.2	11.55 ± 0.62	0.31	69.5	14.11 ± 0.52	0.37	62.7	11.19 ± 1.76	0.30	70.4
Methiocarb	30.71 ± 2.36	14.53 ± 1.38	0.47	52.7	<LOQ	-	>67.4	10.17 ± 0.99	0.33	66.9	<LOQ	-	>67.4
Metrobromuron	28.07 ± 3.95	10.74 ± 0.23	0.38	61.7	<LOQ	-	>64.4	9.91 ± 1.30	0.35	64.7	<LOQ	-	>64.4
Metribuzin	20.32 ± 1.00	9.21 ± 0.36	0.45	54.6	9.75 ± 0.40	0.48	52.0	<LOQ	-	>50.8	<LOQ	-	>50.8
Oxadixyl	12.78 ± 1.03	<LOQ	-	>21.7	<LOQ	-	>21.7	<LOQ	-	>21.7	<LOQ	-	>21.7
Paclobutrazole	38.64 ± 3.59	13.74 ± 0.30	0.36	64.4	12.70 ± 0.64	0.33	67.1	12.89 ± 0.66	0.33	66.6	<LOQ	-	>74.1
Parathion	23.04 ± 2.04	9.65 ± 2.07	0.42	58.1	<LOQ	-	>56.6	<LOQ	-	>56.6	<LOQ	-	>56.6
Parathion-methyl	16.29 ± 2.100	<LOQ	-	>38.6	<LOQ	-	>38.6	<LOQ	-	>38.6	<LOQ	-	>38.6

Pesticides	Unprocessed Samples	Cooked			Steam Cooked			Washed and Cooked			Washed with Vinegar and Cooked		
	Concentration (Co) ± SD	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)
Penconazole	40.64 ± 1.35	18.27 ± 0.04	0.45	55	13.71 ± 0.37	0.34	66.3	16.11 ± 0.16	0.40	60.3	11.39 ± 3.09	0.28	72
Pencycuron	29.08 ± 3.95	<LOQ	-	>65.6	<LOQ	-	>65.6	<LOQ	-	>65.6	<LOQ	-	>65.6
Phenthoate	34.75 ± 4.92	13.43 ± 0.36	0.39	61.3	<LOQ	-	>71.2	14.03 ± 0.10	0.40	59.6	<LOQ	-	>71.2
Phosphamidon	<LOQ	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-
Pirimiphos-ethyl	<LOQ	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-
Pirimiphos-methyl	32.85 ± 5.57	6.16 ± 0.78	0.19	81.2	<LOQ	-	>84.8	<LOQ	-	>84.8	<LOQ	-	>84.8
Prochloraz	22.00 ± 6.35	9.23 ± 1.72	0.41	58	<LOQ	-	>54.5	<LOQ	-	>54.5	<LOQ	-	>54.5
Profenofos	27.43 ± 2.58	9.12 ± 0.29	0.33	66.7	<LOQ	-	>63.5	<LOQ	-	>63.5	<LOQ	-	>63.5
Propiconazole	41.73 ± 5.79	11.01 ± 0.40	0.26	73.6	11.29 ± 1.49	0.27	72.9	11.78 ± 2.42	0.28	71.8	<LOQ	-	>76.0
Propyzamide	33.29 ± 0.03	15.99 ± 0.85	0.48	52	11.64 ± 0.53	0.35	65	15.61 ± 1.09	0.47	53.1	10.56 ± 2.86	0.32	68.3
Prothioconazole-desthio	39.75 ± 3.30	11.41 ± 0.66	0.29	71.3	10.99 ± 1.05	0.27	72.4	12.45 ± 1.43	0.31	68.7	<LOQ	-	74.8
Pyrazophos	34.01 ± 1.85	16.61 ± 1.91	0.49	51.2	12.37 ± 0.51	0.36	63.6	13.91 ± 0.41	0.41	59.1	10.86 ± 2.16	0.32	68.1
Pyrimethanil	36.24 ± 2.45	13.94 ± 0.17	0.38	61.5	10.78 ± 0.56	0.30	70.2	13.53 ± 2.05	0.37	62.7	<LOQ	-	>72.4

Pesticides	Unprocessed Samples	Cooked			Steam Cooked			Washed and Cooked			Washed with Vinegar and Cooked		
	Concentration (Co) ± SD	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)
Quinoxyfen	12.93 ± 0.87	6.34 ± 1.08	0.49	51	<LOQ	-	>61.3	<LOQ	-	>61.3	<LOQ	-	>61.3
Rotenone	18.56 ± 1.10	<LOQ	-	>46.1	<LOQ	-	>46.1	<LOQ	-	>46.1	<LOQ	-	>46.1
SpinosadA	29.02 ± 1.15	12.42 ± 0.27	0.43	57.2	8.27 ± 0.45	0.29	71.5	9.74 ± 0.37	0.33	66.4	4.66 ± 1.11	0.16	83.9
SpinosadD	27.24 ± 0.91	10.64 ± 0.20	0.39	60.9	<LOQ	-	>63.3	9.21 ± 0.87	0.34	66.2	<LOQ	-	>63.3
Spiroxamine	37.77 ± 4.59	13.54 ± 0.34	0.36	64.1	6.88 ± 0.06	0.18	81.8	11.66 ± 0.58	0.31	69.1	5.34 ± 1.32	0.14	85.8
Tebuconazole	<LOQ	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-	<LOQ	-	-
Tebufenpyrad	31.60 ± 3.48	16.05 ± 1.01	0.51	49.2	11.56 ± 0.00	0.37	63.4	<LOQ	-	>68.4	<LOQ	-	>68.4
Terbuthylazine	35.27 ± 0.40	16.71 ± 1.02	0.47	52.6	11.35 ± 0.36	0.32	67.8	14.51 ± 0.31	0.41	58.8	11.54 ± 2.27	0.33	67.3
Tetraconazole	39.48 ± 4.30	11.79 ± 1.47	0.30	70.1	12.06 ± 1.35	0.30	69.5	11.87 ± 1.36	0.30	69.9	<LOQ	-	>74.7
Thiabendazole	26.18 ± 6.52	13.64 ± 2.19	0.52	47.9	<LOQ	-	>61.7	<LOQ	-	>61.7	<LOQ	-	>61.7
Thiacloprid	28.11 ± 3.11	12.91 ± 1.18	0.46	54.1	13.53 ± 1.04	0.48	51.9	12.59 ± 0.48	0.45	55.2	<LOQ	-	>64.4
Triadimefon	37.60 ± 0.61	21.20 ± 1.74	0.56	43.6	15.57 ± 0.27	0.41	58.6	16.78 ± 0.13	0.45	55.4	13.84 ± 2.14	0.37	63.2
Triadimenol	38.75 ± 3.35	<LOQ	-	>74.2	14.10 ± 0.35	0.36	63.6	<LOQ	-	>74.2	<LOQ	-	74.2

Pesticides	Unprocessed Samples	Cooked			Steam Cooked			Washed and Cooked			Washed with Vinegar and Cooked		
	Concentration (Co) ± SD	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)	Co ± SD	PF	Re (%)
Tricyclazole	26.55 ± 2.21	12.38 ± 0.89	0.46	53.4	14.41 ± 1.19	0.54	45.7	11.64 ± 0.16	0.44	56.1	<LOQ	-	>62.3
Triflumuron	31.98 ± 5.43	<LOQ	-	>68.7	<LOQ	-	>68.7	<LOQ	-	>68.7	<LOQ	-	>68.7
Zoxamide	27.49 ± 0.13	11.38 ± 0.60	0.41	58.6	<LOQ	-	>81.8	6.65 ± 0.18	0.24	75.8	>LOQ	-	>81.8
Fludioxonil	24.15 ± 1.64	18.62 ± 3.65	0.77	22.9	<LOQ	-	>58.6	13.36 ± 0.57	0.55	45.1	<LOQ	-	>58.6

Legend: DMST- N,N-dimethyl-N'-p-tolysulphamide; EPN- O-ethyl O-4-nitrophenyl phosphonothiate.

3. Pesticides Residues in Rice Commercial Samples

Four commercial rice samples, one long grain rice, one basmati, one brown rice and one *Carolino* rice (short grain rice) were analyzed regarding their content in the 121 pesticide residues included in the UHPLC-MS/MS methods validated earlier. Rice samples were collected in June 2023 in local supermarkets. Sample 1 corresponds to long grain rice, sample 2 to basmati rice, sample 3 to Brown rice and Sample 4 to *Carolino* rice. All samples are negative for pesticide residues, Melo *et al.* found Imidacloprid in 3 commercial rice samples, in rice sample 1: 0.0054 ± 0.0008 mg/kg, rice sample 2: 0.0125 ± 0.0005 mg/kg, and rice sample 3: 0.0658 ± 0.0018 mg/kg. Rice sample 1 corresponds to basmati rice, rice sample 2 corresponds to medium-grain rice, and the contaminated sample 3 corresponds to parboiled rice. The MRL for this pesticide is 1.5 mg/kg for rice so none of the samples exceeded this limit (Melo *et al*., 2020).

Tran-Lam et al. (2021), found on commercial rice samples collected in markets from Hanoi that 14 out of 20 samples were contaminated with at least one pesticide and insecticide was the most detected pesticide group in rice (Tran-Lam *et al*., 2021).

Tauseef et al., found in their study six rice samples contaminated with pesticides. Pesticides exceeding EU-MRLs were dimethoate, carbofuran, carbaryl, atrazine, triazophos, diazinon, bifenthrin, and hexaconazole (Tauseef *et al*., 2021).

Since 2015, several notifications have been reported through the Rapid Alert System for Food and Feed (RASFF). One notification is the presence of chlorpyrifos-methyl in rice from Pakistan found in Belgium, with a concentration of 0.039 mg/kg, where the MRL maximum is 0.01 mg/kg. Another one is the presence of thiamethoxam, tricyclazole (an unauthorized substance), and imidacloprid in rice from India found in Germany. With a concentration of 0.116 mg/kg, 0.207 mg/kg, and 0.026 mg/kg, respectively. And for which one the MRL is 0.01 mg/kg. And exist many more notifications of the presence of pesticide residues in both rice and other cereals.

So, it is increasingly important to carry out analyzes on commercial samples, for the safety of consumers.

4. Interlaboratory Assay organized by DTU National Food Institute (Denmark)

INIAV, I.P. is the national reference laboratory for pesticides in cereals and fruits and vegetables in Portugal. Therefore, it participates every year in two Interlaboratory Assays, one for cereals and other for fruits and vegetables. In 2023, the Interlaboratory Assay organized by the European Union Reference Laboratory for Pesticides in Cereals (DTU National Food Institute, Denmark) was CF17 and the selected matrix was wheat kernels. The samples of wheat kernels were received in laboratory and submitted to the validated methods (reported in section 1). The results of the z-scores of the pesticides residues obtain in this European Proficiency Test on Pesticide residues in Wheat kernels (CF17) are summarized in Table 13.

Table 13. Results of z-scores for the European Proficiency Test on Pesticide residues in Wheat kernels (CF17).

Pesticides	z-scores
Azoxistrobin	-1.1
Bixafen	-0.4
Cyprodinil	-1.2
Difenoconazole	0.9
Dimethomorph	-0.6
Fluopyram	-0.7
Metconazole	-0.5
Pyraclostrobin	-0.3
Pirimicarb	-0.4
Pirimicarb-desmethyl	-0.02
Pyriproxyfen	-0.9
Prothioconazole-destio	-0.3
Trifloxistrobin	0.9

Regarding the results, a |z-score| <2 is acceptable and a |z-score| <1 is good. As can be seen, most of the reported molecules have a |z-score| <1, so the test results are very good, showing the good analytical performance of our analytical method for pesticides residues determination.

CONCLUSIONS AND FUTURE PERSPECTIVES

Foods contaminated with pesticides residues and mycotoxins are associated with negative human health effects. It is therefore of utmost importance to develop simple and cost-effective analytical methodologies to enhance food safety and prevent the possible harms caused by contaminants, which may be present in foods, in particular in cereals like rice.

The present master's dissertation has met all the proposed objectives, managing to successfully validate two analytical methodologies, one for screening mycotoxins and other to quantify pesticides residues in cereals. In the first part of this dissertation a Biochip Array Technology was successfully validated for the multi-mycotoxins analysis of nine mycotoxins (AFB1, AFG1, OTA, ZEA, DON, FB1+FB2, and T-2+HT-2), although in two cases it detects and semi-quantifies the sum of two mycotoxins (FB1+FB2 and T-2+HT-2). This is a method of excellence for screening of multi-mycotoxins in cereals, offering significant advantages including high throughput (processing of a large number of samples simultaneously), and enables quick and cost-effective screening of multiple mycotoxins from feed and food cereal-based samples at different levels. In fact, this multi-analytes approach facilitates the screening process because only positive samples require confirmatory testing, by liquid chromatography with a mass spectrometry detector. Within the scope of the TRACE-RICE project, in the near future the mycotoxins in various types of rice will be assessed, before harvesting, during harvesting and post harvesting, namely in transport and storage.

The analysis of pesticides residues in foods is of great importance, but also very challenging as requires the analysis of many pesticides with different properties at very low levels in complex matrices. In the second part of this dissertation, a method to determine pesticides residues in rice using QuEChERS extraction followed by UHPLC-MS/MS was successfully validated. The validation parameters were acceptable for 121 pesticides residues according to the guidelines at established in the European Union (SANTE, 2021). The proposed method was found to be selective, sensitive, precise, accurate, and cost-effective.

Although the legislation only has MRLs for unprocessed samples, it is important to assess the presence of pesticides residues after processing. This study demonstrated that it is possible to considerably reduce pesticides by washing and cooking, and the level of reduction depends on the molecule. The results indicate that there was no correlation between chemical structure or class of pesticides and the levels of residue removed by washing (washing with water and

with vinegar 5%, v/v), cooking (conventional cooking and stream cooking) or their combinations.

The removal of pesticide residues due to processing is affected by the degree of adsorption of pesticides by the cereals' grains, pesticide residues' solubility in water and heat-induced breakdown. The rice samples submitted to both processing methods (wash with vinegar and cooking) presented higher reduction factors. In this line, it is recommended to use, at domestic and industrial levels, both methods in order to better guarantee food safety.

In the future it would be interesting to continue studying the pesticides residues in another types of rice, such as, basmati rice, brown rice, *Carolino* rice (short grain) and parboiled rice. It would be of interest to continue studying the effect of washing with vinegar in the reduction of pesticides residues in contaminated samples, using other concentrations of vinegar and other types of vinegar, such as fruit vinegar, white wine, balsamic and red wine. Moreover, instead of using acetic acid, the effect of citric acid (or directly lemon juice) could also be evaluated. In the case of cooking, it would be interesting in the future to evaluate the influence of the presence of olive oil and salt on the removal of pesticides residues in contaminated rice samples. It would also be interesting to carry out the study on other cereals (such as oats and rye), or even on pasta and also on pseudocereals such as amaranth, quinoa and buckwheat.

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APPENDIX

Table A1. Parameters for determination of pesticides residues in rice by HPLC-MS/MS in ESI+ mode. Transition 1: Quantification transition; Transition 2: Confirmation transition.

Pesticides	Transitions	Precursor ion (m/z ± 0.5)	Product ion (m/z ± 0.5)	DP (V)	CE (V)	CXP (V)
TPP		327	152	106	47	10
Acetamiprid	1	223	126	74	27	4
	2	225	128	61	29	8
Azoxystrobin	1	404	372	46	21	10
	2	404	344	46	35	20
Bixafen	1	414	394	76	21	24
	2	416	396	81	23	16
Bitertanol	1	338	99	51	21	6
	2	338	269	51	13	20
Boscalid	1	343	307	76	29	18
	2	343	140	116	27	6
Bupirimate	1	317	272	61	27	22
	2	317	108	91	41	6
Buprofezin	1	306	201	51	17	14
	2	306	116	56	23	20
Carbaryl	1	202	145	86	17	16
	2	202	127	86	28	14
Carbendazim	1	192	160	51	25	10
	2	192	105	61	51	18
Carbofuran	1	222	165	56	15	22
	2	222	123	56	25	14
Carbofuran-3-hydroxy	1	238	181	82	17	10
	2	238	163	82	21	10
Carboxin	1	236	143	46	23	8
	2	236	87	46	35	14
Chlorantraniliprole	1	484	99	51	21	16
	2	484	286	61	23	6
Chlorfenvinphos	1	359	99	75	41	6
	2	359	155	75	19	8
Chlorpyrifos-methyl	1	324	125	66	25	8
	2	322	125	66	29	6
Clofentezine	1	303	138	56	21	20
	2	303	102	56	59	18
Coumaphos	1	363	227	84	37	12
	2	363	307	84	23	7

Pesticides	Transitions	Precursor ion (m/z ± 0.5)	Product ion (m/z ± 0.5)	DP (V)	CE (V)	CXP (V)
Cymoxanil	1	199	128	46	35	14
	2	199	83	46	13	8
Cyproconazole	1	292	70	71	35	14
	2	294	125	16	35	4
Cyprodinil	1	226	91	81	49	16
	2	226	93	81	47	16
Demeton-S-methylsulfone	1	263	109	46	21	10
	2	263	169	76	37	20
Diazinon	1	414	394	76	16	6
	2	416	396	81	23	6
Dichlorvos	1	221	109	76	25	4
	2	223	109	66	23	18
Difenoconazole	1	406	251	81	37	4
	2	406	337	41	23	13
Diflubenzuron	1	311	158	81	19	18
	2	311	141	81	43	20
Dimethoate	1	230	199	61	13	8
	2	230	225	61	29	6
Dimethomorph	1	388	301	51	45	10
	2	388	165	51	29	18
Diniconazole	1	326	159	56	39	13
	2	326	70	56	45	13
DMST	1	215	106	100	10	13
	2	215	151	100	5	13
EPN	1	324	296	16	19	6
	2	324	157	16	29	6
Epoxiconazole	1	330	101	36	63	13
	2	330	121	36	27	13
Ethiofencarb	1	226	107	51	23	18
	2	226	164	51	13	10
Ethoprophos	1	243	97	71	41	4
	2	243	131	66	29	8
Etrimfos	1	293	125	64	33	8
	2	293	265	61	21	10
Fenamidone	1	312	65	81	71	4
	2	312	236	41	19	4
Fenamiphos sulfone	1	336	266	81	29	13
	2	336	188	81	41	13
Fenamiphos	1	320	233	86	35	12

Pesticides	Transitions	Precursor ion (m/z ± 0.5)	Product ion (m/z ± 0.5)	DP (V)	CE (V)	CXP (V)
sulfoxide	2	320	108	86	59	6
Fenarimol	1	331	268	86	31	10
	2	331	139	96	49	14
Fenitrothion	1	278	125	71	31	6
	2	278	109	71	25	6
Fenoxycarb	1	302	55	96	59	4
	2	302	97	91	35	6
Fenpropathrin	1	350	97	81	43	4
	2	350	125	76	19	6
Fenpropidin	1	274	147	51	37	7
	2	274	117	51	65	7
Fenpropimorph	1	304	147	74	39	7
	2	304	117	11	71	6
Fenpyroximate	1	422	366.1	76	23	6
	2	422	138	76	41	4
Fenthion oxon sulfone	1	295	217	96	27	12
	2	295	104	96	35	6
Fenthion oxon sulfoxide	1	279	264	56	27	18
	2	279	104	56	39	18
Fenthion oxon	1	263	231	66	23	14
	2	263	216	76	33	14
Fenthion sulfone	1	311	125	76	28	8
	2	311	279	76	27	16
Fenthion sulfoxide	1	295	280	76	25	16
	2	295	109	76	45	18
Fluopyram	1	397	145	81	79	8
	2	397	173	81	43	10
Fluquinconazole	1	376	307	56	33	7
	2	376	349	56	25	14
Flutriafol	1	302	123	41	39	13
	2	302	109	41	43	13
Fonofos	1	247	109	61	26	6
	2	247	137	61	15	6
Fosthiazate	1	284	104	11	27	6
	2	284	228	11	15	8
Hexythiazox	1	353	228	66	21	4
	2	353	168	66	37	14
Indoxacarb	1	528	150	26	31	18
	2	528	203	96	51	7

Pesticides	Transitions	Precursor ion (m/z ± 0.5)	Product ion (m/z ± 0.5)	DP (V)	CE (V)	CXP (V)
Iprodione	1	330	245	61	21	14
	2	332	247	61	21	14
Iprovalicarb	1	321	119	56	27	8
	2	321	186	56	17	16
Isoprocarb	1	194	95	65	19	14
	2	194	152	65	11	20
Isoprothiolane	1	291	231	51	17	14
	2	291	189	51	31	12
Isoproturon	1	207	72	61	35	12
	2	207	165	61	21	10
Kresoxim-methyl	1	314	267	75	5	13
	2	314	222	75	9	13
Linuron	1	249	160	61	25	18
	2	249	182	61	21	22
Lufenuron	1	511	158	79	27	4
	2	511	141	79	67	4
Malaoxon	1	315	99	71	31	4
	2	315	127	71	17	6
Malathion	1	331	127	64	17	4
	2	331	285	64	13	4
Mepanipyrim	1	224	106	66	35	7
	2	224	77	66	49	7
Metaflumizone	1	507	178	86	35	10
	2	507	89	86	123	4
Metalaxyl	1	280	192	61	25	10
	2	280	220	61	21	12
Metalaxyl-M	1	280	220	46	19	13
	2	280	160	46	31	13
Metazachlor	1	278	210	41	15	4
	2	278	134	41	29	8
Metconazole	1	320	70	76	45	7
	2	320	125	81	49	6
Methiocarb	1	226	169	101	13	20
	2	226	121	101	25	18
Methomyl	1	163	88	46	13	4
	2	163	106	46	15	18
Metobromuron	1	261	172	76	25	6.5
	2	261	148	76	21	4
Metribuzin	1	215	187	31	25	13

Pesticides	Transitions	Precursor ion (m/z ± 0.5)	Product ion (m/z ± 0.5)	DP (V)	CE (V)	CXP (V)
	2	215	84.1	31	29	13
Mevinphos	1	225	127	69	19	4
	2	225	193	69	13	4
Monocrotophos	1	224	127	86	21	6
	2	224	193	86	13	14
Oxadixyl	1	279	219	66	15	4
	2	279	132	66	41	4
Paclobutrazole	1	294	70	71	39	4
	2	294	89	71	81	4
Paraoxon-ethyl	1	276	220	61	19	6
	2	276	248	61	15	16
Paraoxon-methyl	1	248	202	96	25	7
	2	248	90	66	37	6
Parathion	1	292	236	81	21	6.5
	2	292	264	81	15	13
Parathion-methyl	1	264	125	76	25	7
	2	264	109	120	21	13
Penconazole	1	284	70	61	49	12
	2	284	159	61	47	14
Pencycuron	1	329	125	71	33	8
	2	329	218	71	23	14
Pendimethalin	1	282	212	41	17	12
	2	282	194	41	27	12
Phentoate	1	321	79	31	51	6
	2	321	247	16	13	10
Phosalone	1	368	182	66	19	14
	2	368	111	51	51	18
Phosmet	1	318.1	160	56	21	12
	2	318.1	77	56	69	13
Phosphamidon	1	300	127	66	27	6
	2	300	227	66	19	14
Phoxim	1	299	77	56	47	12
	2	299	129	56	17	12
Pirimiphos-ethyl	1	334	198	85	29	8
	2	334	182	85	31	8
Pirimiphos-methyl	1	306	108	71	43	6
	2	306	67	71	65	6
Prochloraz	1	376	308	16	17	13
	2	376	266	16	23	13

Pesticides	Transitions	Precursor ion (m/z ± 0.5)	Product ion (m/z ± 0.5)	DP (V)	CE (V)	CXP (V)
Profenofos	1	373	303	115	17	13
	2	375	305	115	17	13
Propiconazole	1	342	159	46	37	13
	2	342	89	86	99	4
Propoxur	1	210	168	58	11	24
	2	210	93	58	33	7
Propyzamide	1	256	173	56	31	8
	2	256	190	56	21	10
Prothioconazole-desthio	1	312	70	76	61	2
	2	312	125	76	49	6
Pyrazophos	1	374	222	81	29	6
	2	374	194	11	43	7
Pyrimethanil	1	200	107	71	35	8
	2	200	82	71	37	14
Quinoxyfen	1	308	197	61	43	7
	2	308	162	61	57	7
Rotenone	1	395	213	81	33	12
	2	395	192	91	31	12
SpinosadA	1	732.5	142	51	37	13
	2	732.5	98	51	75	13
SpinosadD	1	746.5	142	66	39	13
	2	746.5	98	66	79	13
Spiroxamine	1	298	144	41	27	10
	2	298	100	41	41	6
Tebuconazole	1	308	70	21	39	13
	2	308	125	21	47	13
Tebufenpyrad	1	334	117	86	57	6
	2	334	145	86	38	8
Terbuthylazine	1	232	176	81	25	7
	2	230	104	81	45	6
Tetraconazole	1	372	159	81	39	14
	2	374	161	76	35	8
Thiabendazole	1	202	175	79	35	4
	2	202	131	81	47	10
Thiacloprid	1	253	126	99	29	4
	2	255	128	76	31	6
Thidiocarb	1	355	88	31	19	12
	2	355	108	61	21	20
Triadimefon	1	294	225	61	19	14

Pesticides	Transitions	Precursor ion (m/z ± 0.5)	Product ion (m/z ± 0.5)	DP (V)	CE (V)	CXP (V)
	2	296	199	36	21	12
Triadimenol	1	296	70	41	37	12
	2	296	227	11	15	13
Tricyclazole	1	190	163	89	31	7
	2	190	136	91	36	7
Triflumuron	1	359	156	21	21	18
	2	359	139	21	45	18
Zoxamide	1	336	187	46	31	24
	2	336	159	46	55	20

Legend: DP- Declustering potential (V); CE- Collision energy (V); CXP- Collision cell exist potential (V); DMST- N,N-dimethyl-N'-p-tolysulphamide; EPN- O-ethyl O-4-nitrophenyl phosphonothiate

Table A2. Parameters for determination of pesticides residues in rice by HPLC-MS/MS in ESI- mode. Transition 1: Quantification transition; Transition 2: Confirmation transition.

Pesticide	Transitions	Percursor ion (m/z ±0.5)	Product ion (m/z ±0.5)	DP (V)	CE (V)	CXP(V)
DNC		301	137	-120	-16	-15
Fludioxonil	1	247	180	-65	-40	-9
	2	247	126	-65	-42	-7

Legend: DP- Declustering potential (V); CE- Collision energy (V); CXP- Collision cell exist potential (V)