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Development of a Methodology Based on QuEChERS-dSPE/UHPLC-PDA to Quantify Pesticide Residues in Potatoes

MASTER DISSERTATION

Débora Carina Freitas Reis
MASTER IN APPLIED BIOCHEMISTRY

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Dissertation submitted to the University of Madeira in fulfillment of the requirements for master's in Applied Biochemistry

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To my sweet Branquinho

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Abstract

Pesticides have been associated to human health hazards, ranging from headaches to cancer or, reproductive and endocrine system disruption. Inappropriate use of pesticides may cause damage to the environment, increase resistance in the target pest organisms and deleterious effects of non-target organisms. The European Union (EU) set directives for pesticides usage, establishing maximum residue limits (MRLs) in fruits and vegetables. It is very important to monitor if the pesticide residues are below the MRLs in food matrices and to evaluate if they pose a risk to the health of consumer and environment.

The purpose of this work was to develop a fast and sensitive analytical method to identify and quantify common pesticide residues in potatoes based on a quick, easy, cheap, effective, rugged, and safe (QuEChERS) procedure combined with ultra-high-pressure liquid chromatography tandem with photodiode array system (UHPLC-PDA).

The parameters that affect the QuEChERS/UHPLC-PDA efficiency, such as extraction solvent, buffered salts, stationary phases, gradient conditions, and eluents were optimized. The optimal parameters were 50% of acetonitrile (ACN) acidified with 0.1% phosphoric acid (PhA) and, magnesium sulfate: sodium chloride: disodium hydrogen citrate sesquihydrate: trisodium citrate dihydrate (1:1:1/2:1) ratio was used. Moreover, 30 min ultrasound time was added and for clean-up magnesium sulfate and primary secondary were used. The selected column was CORTECS with a gradient program combining an aqueous solution acidified with 0.1% PhA and ACN, a flow rate of 150 µL/min at 30 ºC.

After optimization, the method was validated according to IUPAC guidelines. The validated method showed to be selective for the studied pesticides, and showed satisfactory performance in terms of linearity, with correlation coefficient (r^2) higher than 0.997. The limits of detection (LOD) ranging from 0.005 (chlorpyrifos) to 2.581 (thiabendazole) mg/L, whereas the limit of quantification (LOQ) limit from 0.015 (chlorpyrifos) to 7.821 (thiabendazole) mg/L. In relation to the accuracy, the obtained values varied between 87.7 and 214.2 %, while in precision, the coefficients of variation remained in general below to 20 %. In terms of matrix effect, the values ranged between 87.6 and 185.8 %, which agreed with results reported by other studies.

Keywords: Pesticides, vegetables, QuEChERS, UHPLC, optimization, validation.

Resumo

Os pesticidas têm sido associados a diversos riscos para a saúde, que vão desde dores de cabeça até cancro ou ainda a distúrbios do sistema reprodutivo e endócrino. O uso inadequado de pesticidas pode não só causar danos ao meio ambiente como também, aumentar a resistência nos organismos-alvo da praga e ainda, exibir efeitos negativos em organismos não-alvo. Consequentemente, a União Europeia (UE) estabeleceu diretrizes para o uso de pesticidas, estabelecendo limites máximos de resíduos (LMRs) em frutas e vegetais. A monitorização dos resíduos é fundamental para avaliar se um determinado alimento representa um risco para a saúde do consumidor e, do meio ambiente.

O objetivo deste trabalho foi desenvolver um método analítico rápido e sensível para identificar e quantificar os resíduos de pesticidas mais utilizados em batatas. O método foi baseado num procedimento rápido, fácil, económico, eficaz, robusto e seguro (QuEChERS) seguido de cromatográfica em fase líquida de alta eficiência com detetor por arranjo de fotodiodos (UHPLC-PDA).

Os parâmetros que influenciam a eficiência da metodologia QuEChERS/UHPLC-PDA, nomeadamente solvente de extração, sais de partição, fases estacionárias, condições de gradiente, eluentes foram otimizados. As melhores condições extrativas foram obtidas usando 50% de acetonitrilo (ACN) acidificado com 0.1% ácido fosfórico (PhA) e, sulfato de magnésio: cloreto de sódio: citrato dissódico sesqui-hidratado :citrato trissódico di-hidratado (1: 1: 1/2: 1) como rácio dos sais de partição. A extração em fase sólida dispersiva (dSPE) foi efetuada usando sulfato de magnésio: amina primária secundária (PSA) como sorventes. A coluna selecionada foi a CORTECS, sendo usado como gradiente uma solução aquosa acidificada com 0,1% de PhA e ACN, fluxo de 150 µL/min a 30 ºC.

Após a otimização, o método foi validado de acordo com as diretrizes da IUPAC. Este mostrou-se seletivo para os pesticidas estudados e, apresentou um desempenho satisfatório em termos de linearidade, com coeficientes de correlação (r^2) maiores que 0,997. Os limites de deteção (LOD) variam de 0,005 (clorpirifós) a 2,581 (tiabendazol) mg/L, enquanto que o limite de quantificação (LOQ) varia de 0,015 (clorpirifós) a 7,821 (tiabendazol) mg/L. Em relação à exatidão, os valores obtidos variam entre 87,7 e 214,2%, mais ainda, na precisão, os coeficientes de variação permaneceram em geral abaixo de 20%. Em termos de efeito matriz, os valores variaram entre 87,6 e 185,8%, concordando com os resultados relatados por outros estudos.

Palavras-chave: Pesticidas, vegetais, QuEChERS, UHPLC, otimização, validação.

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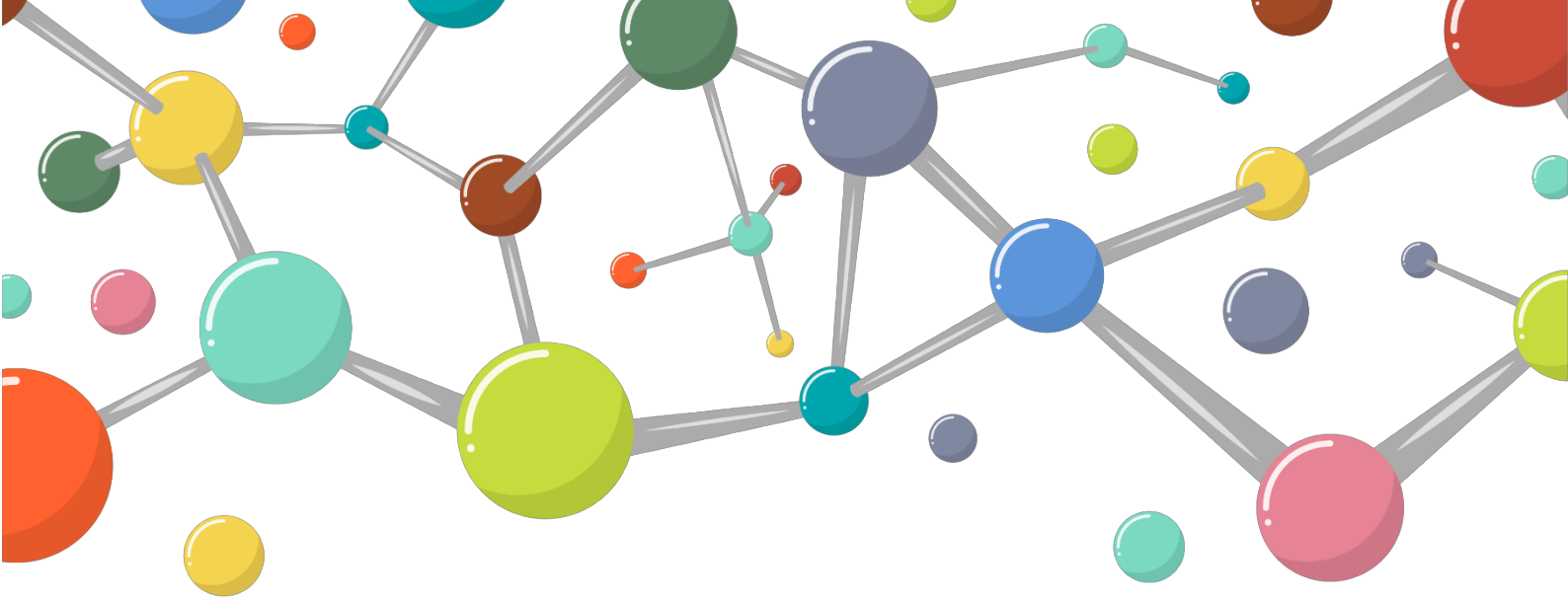
Abbreviations

AchE	Acetylcholinesterase
ASE	Accelerated solvent extraction
BEH	Ethylene Bridged Hybrid
C_{18}	Octadecylsilane
CEN	European Committee for Standardization
CNS	Central nervous system
CSH	Charged surface hybrid
dSPE	Dispersive solid phase extraction
EC	European Council
ECD	Electron capture detector
EFSA	European Food Safety Authority
EU	European Union
FA	Formic acid
FAO	Food and Agriculture Organization
FLR	Fluorescence detector
GAP	Good Agricultural Practice
GC	Gas chromatography
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
HILIC	Hydrophilic interaction liquid chromatography
HL	High level
HSS	High strength silica
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
LD_{50}	Median lethal dose
LL	Low level
LOD	Limit of detection
LOQ	Limit of quantification
MAE	Microwave-assisted extraction
MeOH	Methanol
ML	Medium level
MRLs	Maximum residue limit
MS	Mass Spectrometry
MSPD	Matrix solid phase extraction
NPD	Nitrogen phosphorus detector
OPs	Organophosphorus

PDA	Photodiode array detector
PhA	Phosphoric acid
PSA	Primary secondary amine
PTFE	Poly(tetrafluoroethylene)
QSM	Quaternary solvent manager
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
r^2	Correlation coefficient
RAM	Região Autónoma da Madeira
rpm	Rotation per minute
RSD	Relative standard deviation
RT	Retention time
SFE	Supercritical fluid extraction
UAE	Ultrasound-assisted extraction
UHPLC	Ultra high-pressure liquid chromatography
UHPLC-PDA	Ultra high-pressure liquid chromatography tandem with photodiode array system
UV	Ultraviolet
WHO	World Health Organization
λ_{max}	Maximum wavelength



Part 1.

Introduction

1. Introduction

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1.7. Objectives

1. Introduction

According to Food and Agriculture Organization (FAO), a pesticide is any chemical substance or mixture extensively used with the intent of eliminating, preventing, repelling, or decreasing any pest including vectors of human or animal diseases, unwanted species of plants or animals causing harm. Also, any chemical which interferes with the production, processing, storage, or marketing of food, agricultural commodities, wood and wood products, or animal feedstuffs, or which may be administered to animals to control pests on their bodies [1].

The term includes chemicals used as growth regulators, defoliants, desiccants, fruits thinning agents, agents to avoid premature fall of fruits, and also post-harvest substances which prevent deterioration during storage or transport [2]. Yet, the term is sometimes misunderstood and it does not include chemicals used as fertilizers, plant and animal nutrients, animal drugs or food additives [2].

Pesticide applications have rapidly increased mainly in agriculture since they offer undisputed benefits in providing a plentiful, low-cost supply of high-quality fruits and vegetables [3]. However, their excessive application may leave harmful residues which include metabolites and degradation products into the environment such as soil, water, plants and foods [4].

1.1. Historical background of pesticides in agriculture

The historical background of pesticides used in agriculture dated back to the beginning of agriculture itself with the use of sulfur to control insects and mites. However, it became more pronounced over time due to the growing pest population paralleled with decreasing soil fertility and the development of agricultural techniques [5].

Since they were introduced in the market in 1947, synthetic pesticides have been extensively used to reduce crop losses and improve production [6]. Food was cheaper because of the new chemical formulations and with the new pesticides, there were no documented cases of people dying by their use. There were some cases of harm from misuse of the chemicals. But the new pesticides seemed rather safe, especially compared to previous pesticides, the forms of arsenic that had killed people in the 1920s and 1930s [7].

One of the biggest agricultural developments of the last century was heralded in 1960 by Norman Borlaug, called the Green Revolution. It brought together a production of new varieties, coupled with the increased use of fertilizers, pesticides, and irrigation, provided many of the technological inputs required to feed the current global population in expansion [6,8].

Thus, the increase in productivity associated with the increment of fertilizers, pesticides, and irrigation, allowed a broad food production capable of feeding an expanding world population [6]. Several incorrect applications lead to some levels of contaminants in final products and environmental persistence [9].

As a consequence of the potential hazards and persistence of pesticides impact researches on this field has increased exponentially over the years [10]. Over the same period, scientific understanding of the effects of pesticides on human health and their mechanisms of action has also expanded rapidly, with studies revealing statistical associations between pesticide exposure and enhanced risks of developmental impairments, neurological and immune disorders and also some types of cancer [10].

1.2. Exposure pathways

The pathways of pesticide exposure include food, water, air, dust, and soil (Figure 1) [11]. Exposure can occur in agriculture by becoming airborne during application and can drift through great distances in the air. Also, people living in or near agricultural areas have high exposure to pesticides because of inhalation of pesticides spray drift. Besides, spraying pesticides for domestic pest control and in urban areas (parks, pavements, and playgrounds, for example) increases the inhalation of contaminated air [10].

In addition, pesticides are sprayed to food, especially to fruits and vegetables. They enter into soils and reach groundwater which can end up in drinking water. Then again, we are exposed to pesticides through the food we eat and the water we drink [12].

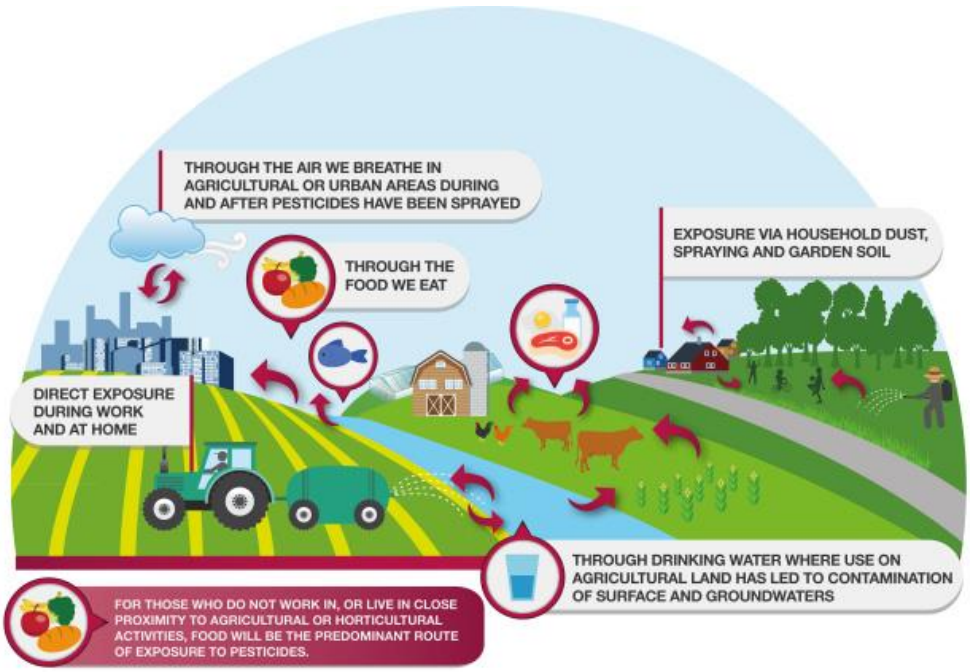


Figure 1. Pathways of exposition to pesticides [10].

1.2.1. Exposure risks

Pesticides can enter the human body by three ways: (i) through the skin (contact), (ii) the mouth (ingested), or (iii) the lungs (inhaled) (Figure 2) [12]. Pesticide exposure can lead either to acute or chronic toxicity [13].

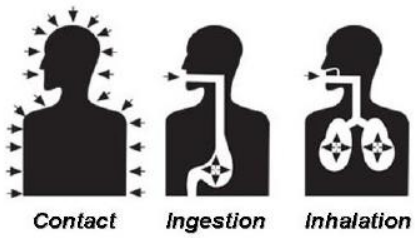


Figure 2. Routs of pesticides entry in the human organism [13].

Acute toxicity is developed by the capability of a substance to cause harmful effects after a short contact or exposure in a few hours or days by having a quick absorption and effect [14]. Having said that, the harmful effects resulting from a single exposure by any route of entry are named “acute effects”.

Pesticides drift from applied areas and intentional or unintentional exposure generally leads to several acute illnesses in humans [15]. Among the typical symptoms of poisoning in humans that are relatively easy to diagnose as acute pesticide poisoning are headaches, irritation of the nose, throat, eyes, skin, body aches, nausea, dizziness, impaired vision, cramps, panic attacks, salivation, muscular incoordination, inability to breath and in more severe cases unconsciousness, coma and death could occur due to acute poisoning [14,16].

On the other hand, chronic toxicity is developed by the capability of a substance to cause adverse health effects resulting from long term exposure to a pesticide [14]. Having said that, the harmful effects resulting from a long period of time exposure (years or decades) by any route of entry are named “chronic effects” [17].

The chronic toxicity of a pesticide is more difficult to determine. Not only farmers are at risk but also the general population due to contaminated food and water or pesticides drift from the fields [16]. Recent studies establish a link between long term exposure to pesticides and carcinogenesis, developmental and reproductive effects, immunological effects, neurotoxicity, cytogenic damage, and endocrine disruptive [15,16].

1.3. Pesticide effect on food production

According to the World Health Organization (WHO), the consumption of fruits and vegetables in Europe constituted over 30 % of consumer diet is an important part of a healthy diet and a valuable source of vitamins, minerals, fiber, carbohydrates and antioxidants [18].

Increasing consumption of fruits and vegetables is usually considered to have a large public health potential. Based on the literature, the importance of increased consumption of fruits and vegetables has been widely recognized, both for cancer and cardiovascular disease, for example. The available evidence is sufficient to justify public health education and promotion aimed at a substantial increase in the consumption of fruits and vegetables [19].

As the human population continues to grow, more and more crops are needed to meet this growing [20]. World's population grew from 2.5 billion in 1950 to 6.1 billion in the year 2000. It is estimated that by the year 2050 the world population will be around 7.7 and 10.6 billion [21]. Therefore, this requirement promotes the use of toxic substances as pesticides to guarantee and increase the production of food products and consequently commercialization as a quick response [22].

Thence, the world has known a continuous growth of pesticide usage, both in the number of chemicals and quantities, sprayed over the field. Pesticides are poisons intentionally added and their residues accumulate and remain in the crops, enter the food chain and are ingested by humans with foodstuffs and water [20]. Fruits and vegetables are the crops most likely to be contaminated by pesticides, mostly grapes, citrus, fruits, and potatoes [23].

The presence of pesticide residues in food constitutes a danger for final consumers being a global concern [22]. Regardless their unquestionable benefits, pesticides are potentially toxic to other organisms, including humans and for that reason, they need to be used safely without exceeding the quantities allowed under conditions of Good Agriculture Practice (GAP) and disposed of correctly [3].

1.3.1. Maximum residue limits

The use of pesticides for effective pests control raised several issues and concerns to public health and environmental pollution. These substances could have severe undesirable effects if they are not firmly regulated. They have been associated with a varied spectrum of human health hazards, ranging from headaches, nausea, to chronic impacts, endocrine system disruption, reproductive harm, and even cancer. In addition, inappropriate use of pesticides may cause damage to the environment, increase resistance in the target pest organisms and cause deleterious effects of non-target organisms [1,24]. Therefore, it is very important to control such contaminants in food and to asses if they pose any risk to public health and to the environment. Food safety thus has priority over plant protection [24].

In this context, the European Food Safety Authority (EFSA) restricted the use of pesticides establishing directives for tolerances or maximum residue limits (MRLs) for all

food and animal feed. The MRLs limit the types and quantity of pesticides that can be legally present on food or feed when pesticides are applied correctly (GAP), is determined by several regulatory bodies which minimize consumer exposure to harmful or unnecessary intake of pesticides.

1.4. Pesticides classification

Pesticides differ in identity, physical and chemical properties and for that, the reason is logical to have them classified and grouped under similar characteristics. Thereby, they can be classified in a few ways that provide useful information depending on the need. The common ways of grouping pesticides are: according to their chemical nature, chemical composition, target pest species and mode of action [25].

1.4.1. Classification of pesticides based on chemical nature

Traditionally, pesticides can be divided into two large groups: organic and inorganic. Organic ones are chemical compounds with a carbon-based skeleton who don't dissolve easily in water and who tend to be more complex than the inorganic ones. These second ones are much simpler compounds who have a crystalline, salt-like appearance, are environmentally stable and usually dissolve readily in water.

According to some literature, we know that the most primitive chemical pesticides were inorganic, for example, sulfur. Yet, most of the currently developed pesticides contain an organic chemical often with oxygen, phosphorus, and so on, in their molecules, in addition to their basic carbon structure [2].

Also, organic pesticides are subdivided into two other groups: (i) the natural organics and (ii) the synthetic organics. Natural organic pesticides usually named organics derived from naturally occurring sources such as plants or animals (e.g. nicotine). Synthetic organic pesticides, typically called synthetics are produced artificially by chemical synthesis. This group comprises most "modern" pesticides and includes glyphosate, for example [2].

1.4.2. Classification of pesticides based on the mode of action

Pesticides can also be classified based on the way they act to the wanted effect. Under this type of classification, pesticides can be separated as systemic or non-systemic (contact), stomach poisons, fumigants, and repellents (Figure 3).

(i) Systemic pesticides

Systemic pesticides are absorbed by plants and move through the plant vascular system to accomplish the desired effect and kill specific pests [25]. The movement of

pesticides in plant tissue can be either unidirectional or multidirectional. For instances, if a pesticide is applied to the root zone, it will travel along with the plant, on the other side, if another pesticide is applied to the leaves it will not move through the plant tissue [14].

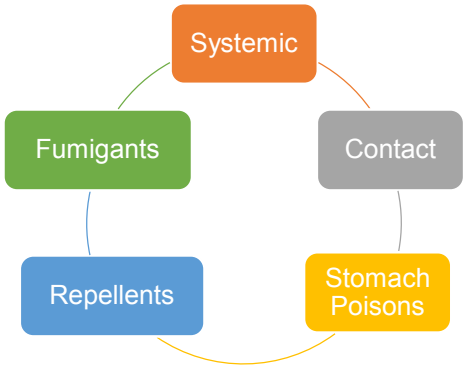


Figure 3. Pesticides classification based on chemical nature.

(ii) Non-systemic pesticides

The non-systemic pesticides are also called contact pesticides as it only exhibits an effect when they come into physical contact with the pest. Contact pesticides do not appreciably penetrate plant tissues and consequently not transported within the plant vascular system. The non-systemic pesticides will only bring about the desired effect when they enter the body of pests over the epidermis and causes death by poisoning [25].

(iii) Stomach poisoning

Stomach poisoning pesticide enters the body of pests over their mouth and digestive system, causing death by poisoning. They only bring about the desired effect after being applied to the leaves and other parts of the plant and then eaten by the pest [14].

(iv) Fumigants

Fumigants pesticides form toxic gases when applied. These pesticides in vapor form enter the body of pests through their respiratory system and cause death by poisoning. Some of the active ingredients are liquids or volatile liquids and change to gases when packaged under high pressure. Fumigants are used to remove the pest from stored fruits, vegetables, and grains. They are also very useful in controlling pests in soil [14].

(v) Repellents

Repellents do not effectively kill but are unsavory enough to maintain pests away from treated areas or commodities. They are also useful by interfering with the capacity of pests to locate crops [14].

1.4.3. Classification based on target pest species

In pesticides classification based on the target species, the names of pesticides arise from the Latin word *cide* (that means to kill or killer) and it is used as a suffix after corresponding name of pests they effetely kill, for example, insecticides, to kill insects (Table 1). However, not all the pesticides end with the word-*cide*. Some pesticides are just classified according to their function, for examples, defoliants, which cause plants to drop their leaves [14].

In addition, many pesticides have the capacity to control more than one type of target pests. For this reason, they are commonly grouped under the same name, such as acaricides, ovicides, and nematicides, often grouped together in insecticides as they are normally its main pesticidal function [2].

Table 1. Pesticide classification by target pest [14].

Pesticide	Target pest/Function
Insecticides	Kill insects and other arthropods
Fungicides	Kill fungi (including blights, mildews, molds, and rusts)
Bactericides	Kill bacteria or acts against bacteria
Herbicides	Kill weeds and other plants that grow where they are not wanted
Acaricides	Kill mites that feed on plants and animals
Rodenticides	Control mice and other rodents
Algaecides	Control or kill the growth of algae
Larvicides	Inhibits the growth of larvae
Repellents	Repel pests by its taste or smell
Desiccants	Act on plants by drying their tissues
Ovicides	Inhibits the growth of eggs of insects and mites
Virucides	Acts against viruses
Molluscicides	Inhibit or kill mollusc's *i.e.* snail's usually disturbing growth of plants or crops
Nematicides	Kill nematodes that act as parasites of plants
Avicides	Kill birds
Moth balls	Stop any damage to cloths by moth larvae or molds
Lampricides	Target larvae of lampreys which are jawless fish like vertebrates in the river
Piscicides	Act against fishes
Silvicides	Acts against woody vegetation
Termiticides	Kills termites

1.4.4. Classification based on the chemical composition

Pesticides can be categorized according to the chemical nature of the active substance. This type of classification it is one of the most valuable to general users and researchers in the field of pesticides because it provides knowledge of the efficacy, physical and chemical properties of the respective pesticides which give essential information for the application, as well as the precautions that need to be taken during their use and application rates [2]. In Table 2 are represented the most common chemical families of each pesticide.

Table 2. Usual chemical composition of pesticides.

Pesticide	Chemical Composition
Insecticide	Organophosphorus Carbamate Pyrethroid Neonicotinoid Organochlorine
Fungicide	Acetamide Benzimidazole Dithiocarbamate Thiazole & Triazole Inorganic (copper, mercury, sulphur)
Herbicide	Dicarboximide Oxazole Pyridine Uracil
Rodenticides	Coumarin Organofluorine Inorganic (arsenic, phosphorus, thallium)

1.4.5. Classification based on the target range

Under this classification type, pesticides can be broad-spectrum pesticides or selective pesticides [14]. The first ones are meant to kill a wide range of pests and other non-target organisms. They are not selective and pose a risk to reptiles, fish, pets, birds, and humans (e.g. chlorpyrifos). On the other hand, selective pesticides only kill a specific group of pests, being harmless to non-target organisms.

1.4.6. Classification based on the toxicity of pesticides

WHO developed a classification by the acute oral and dermal toxicity harmonized with the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). The GHS meets this requirement as a classification system with global acceptance following extensive international consultation [26,27]. This classification can distinguish between the more and the less hazardous forms of each pesticide [26,27].

Table 3. Acute toxicity hazard categories [26,27].

WHO classification	Toxicity level	LD_{50} for the rat (mg/kg body weight)	
		Oral	Dermal
Ia	Extremely hazardous	< 5	< 50
Ib	Highly hazardous	5 - 50	50 - 200
II	Moderately hazardous	50 - 2000	200 - 2000
III	Slightly hazardous	> 2000	
U	Unlikely to present acute hazard	≥ 5000	

For the effect, they conducted several experiments on rats and other laboratory animals by administering doses of pesticides and, afterward they evaluate the median lethal dose (LD_{50}) which causes death in 50% of the exposed animals [14,26]. The ranking class goes from the lowest to highest toxicity and it includes the extremely toxic, highly toxic, moderately toxic and slightly toxic categories (Table 3).

1.5. Groups of studied pesticides

Pesticides under study in this work belong to two big groups used very often in Região Autónoma da Madeira (RAM): insecticides and fungicides.

1.5.1. Insecticides

Insecticides are a class of pesticides formulated to kill, harm, repel or mitigate one or more species of insects or other arthropods [14]. Different insecticides formulations work in different ways, this means that some can damage the insect's exoskeleton, some act upon the nervous system, and others kill or control them by some other means. Most insecticides are available in various forms such as baits, sprays, or slow-release diffusion.

Insecticides have been claimed to be the biggest factor behind the advancement realized in agriculture and a large influence on 20th-century agricultural productivity. Therefore, they are mainly used to increase productivity and to avoid crop damage (Figure 4) [28]. Still, insecticides can be used in many settings and applications, for example in livestock, in public health programs by eliminating disease-carrying insects, for veterinary parasites and for household pests [29].

Figure 4. Damage leaf by potato leafhopper [28].

According to their chemical composition, insecticides under study in this thesis are classified as organophosphorus (OPs) and pyrethroids. The OP pesticide was chlorpyrifos. Deltamethrin, acrinathrin, and λ-cyhalothrin constitute the pyrethroids group [30].

Despite their uses and allied benefits, the currently used insecticides have a neurotoxic effect by acting on the nervous system of the target. OPs, like chlorpyrifos, is one of the most severe toxic pesticides used worldwide once they can inhibit the enzyme acetylcholinesterase (AchE). Pyrethroids are also neurotoxic, by blocking voltage-dependent sodium, chlorine and calcium channels [31,32].

Without ignoring the importance of pesticides in agricultural and in public health activities, are undeniable their toxic properties. Insecticides can pose a risk to non-target insects, pets, mammals, and the environment [33]. Nearly all insecticides have the potential to considerably alter ecosystems and some concentrate along the food chain.

(i) Organophosphorus

OPs are synthetic chemicals that belong to the organic esters or thiols derived from phosphoric, phosphonic or phosphoramidic acid [33,34]. The basic structure of OPs is exhibited in Figure 5.

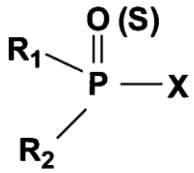


Figure 5. General structural formula of OPs.

Typically, R_1 and R_2 are aryl or alkyl groups bonded to the phosphorus atom directly (generating phosphinates) or through a sulfur or oxygen atom (generating, in this case, phosphorothioates or phosphates, respectively). In certain circumstances, R_1 is directly bonded to the phosphorus atom, and R_2 is bonded to an oxygen or sulfur atom (forming phosphonates or thiophosphonates). In phosphoramidates, at least one of these groups is a $-NH_2$ (may be un-, mono- or bi-substituted), and the atom double bonded to phosphorus is also oxygen or sulfur [33–36].

The X group, either called “leaving group”, is also binding to the phosphorus atom through an oxygen or sulfur atom and, it may belong to a wide range of halogen, aliphatic, aromatic or heterocyclic groups. This group is released from the phosphorus atom when the OP is hydrolyzed by phosphotriesterases (PTE) or upon interaction with protein targets [33,34]. Both in medicine and agriculture, the word “organophosphates” allude to a group of insecticides and nerve agents with the capacity to inhibit AChE [34,35].

The toxicity of OPs compounds is chiefly attributed to their capacity to phosphorylate irreversibly esterases in the central nervous system. The acute toxic effects are associated with the irreversible inactivation of AChE.

AChE is an enzyme that catalyzes the hydrolysis of the neurotransmitter acetylcholine (Ach). ACh is a molecule involved in the transmission of nervous signals

from nerves to muscles (so-called neuromuscular junctions) and between neurons in the brain (so-called cholinergic brain synapses). The neurotransmitter is released from the nerve ending as the neurohormonal transmitter in response to the nerve impulse and begins the excitation by reacting to the receptor [34–36]. In fact, OPs are substrate analogs to Ach, and like natural substrates they enter the active site covalently and bind to AChE, inhibiting it and consequently making it incapable to hydrolyze the neurotransmitter [34].

As the result of AChE inhibition by OPs, ACh molecules start to accumulate at the synapse, causing at the beginning excessive excitation and later leading to the blockage of the synaptic transmission [34–36].

OPs are not selective for insect species and they have the same mechanism of action for the warm-blooded organism including humans that can be intoxicated [34]. Most common symptoms of acute poisoning are agitation, muscle weakness, hypersalivation, sweating and also, in severe cases may lead to respiratory failure, unconsciousness, convulsion and possible death [34].

Poisoning normally results from the agricultural use of OP pesticides during the application or during subsequent work in the fields. As a matter of fact, OPs are one of the most common causes of poisoning worldwide occurring as a result of agricultural use (as reported before), suicide or accidental exposure [34,35]. These compounds can be absorbed into the human body through inhalation, ingestion, and likewise dermal absorption [35]. However, their toxicity is not restricted to the acute phase, once those chronic effects have been noted. Either repeated or prolonged exposure to OPs may lead to the same effects as acute exposure including impaired memory and concentration, disorientation, severe depressions, irritability confusion, delayed reaction times, etc [34].

At the present time, uncontrolled use of OP insecticides promotes their accumulation, environmental pollution, and acute and chronic poisoning events.

(ii) Pyrethroids

Pyrethroids insecticides are potent neurotoxicants which mimic the structure and insecticidal properties from the natural compounds named pyrethrins extracted from the flowers of *Chrysanthemums cinerariaefolium* [37,38]. Synthetic analogs have been developed to improve the specificity and activity of the natural insecticide while increasing their photostability [33,39]. The basic structure is displayed in Figure 6.

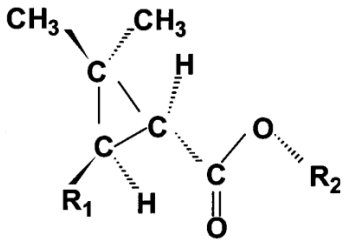


Figure 6. Basic structure of pyrethroids.

All pyrethroids contain some common features: an acid moiety, a central ester bond, and an alcohol moiety. They act on the central nervous system causing changes in the dynamics of the ionic channels in the membrane of the nerve cell, causing it to increase its opening time prolonging the ions current across the membrane in both insects or vertebrates [36]. Initial symptoms of pyrethroid poisoning include incoordination and locomotor instability (knockdown), followed by hyperexcitation, tremors, and convulsions. There are two groups of pyrethroids, types I and II, with distinctive poisoning symptoms, distinguished by the absence or presence, respectively, of an alpha-cyano group in their structure (Figure 7) [38].

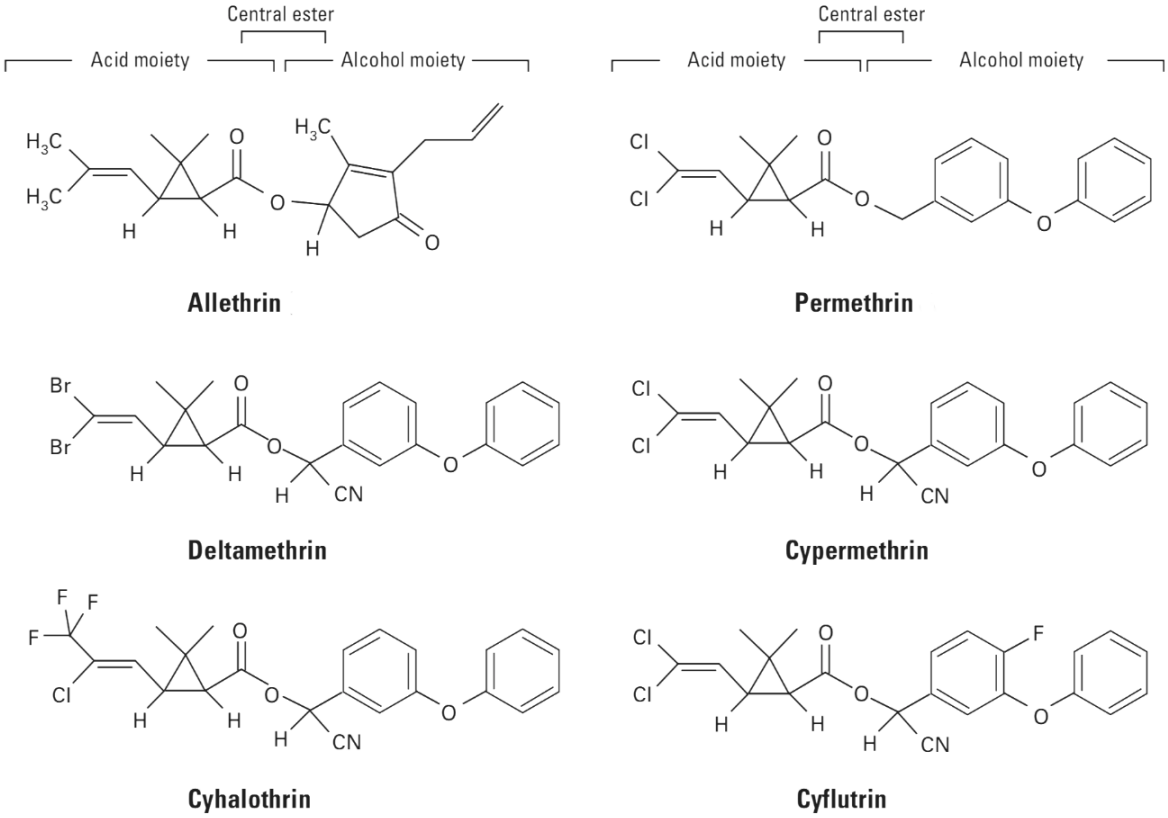


Figure 7. Type I and type II pyrethroids common features [37].

In one hand, those without an α-cyano group (type I pyrethroids, e.g., permethrin, cypermethrin, cyfluthrin), produce aggressive sparring, increased sensitivity to external stimuli, and fine tremor progressing to whole-body tremor and prostration. They exert their neurotoxicity primarily through interference with sodium channel function in the

nervous system [37,38]. On the other hand, those with the alpha-cyano group (type II, e.g., allethrin, deltamethrin, cyhalothrin) include salivation and chewing, burrowing, choreoathetosis, clonic and tonic seizures. These group can affect not only the sodium channel but also chloride and calcium channels [37–39].

Apart from their primary mechanism of action, pyrethroids in mammals can also decrease progesterone and estradiol production, causing estrogenic effects in females and antiandrogenic effects in males [38].

1.5.2. Fungicides

Fungicides, also named antimycotic, are a class of pesticides used to kill or inhibit the growth of fungi, fungal spores, and fungal infections by interfering with important cellular processes [40].

Fungicides are widely used to control diseases and to safeguard crop yield and its quality [41,42]. Diseases are a usual manifestation on plants, often having a significant negative economic impact. In this way, managing diseases is a fundamental component for improved crop production [40,43].

Typically, there are three reasons that motivate the use of agricultural and horticultural fungicides. The first one is to control diseases during the establishment and development of crops [44]. The second one is to increase productivity and to reduce blemishes. Blemishes (Figure 8) affect the leaves which are fundamental for photosynthesis and consequently crop development [45]. It also affects the edible part of the crop or, in case of ornamentals, their attractiveness which both influence their commercial value [46]. Finally, the third reason is to improve the storage life and maintain the quality of harvested tubers, fruits and vegetables [47].

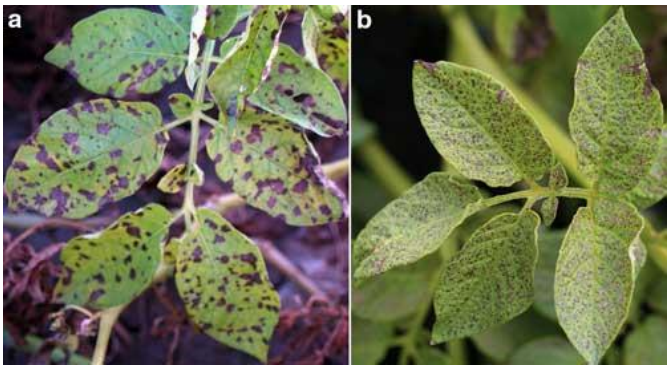


Figure 8. Affected leaves. a) symptoms of early blight on potato leaves; b) symptoms of brown leaf spot on potato leaves [46].

Fungicides are commercially available as sprays or dust. These products can have a systemic or non-systemic effect and can have protective, curative or eradicant properties. Protectant fungicides prevent infection and are active against spore germination, and growth. To be effective, these kinds of fungicides have to be re-applied

to eventual new plant tissues, leaves or needles expanding, for example, or even if the product washes off [47–49]. Curative or eradicant fungicides control the fungus after the infection. This means they have the ability to stop the disease after the infection has started. They are also xylem mobile, so they have the capacity to protect new foliage appearing after treatment and there's no need to be re-applied [44,47,48].

According to their chemical composition, fungicides under study in this thesis are classified as benzimidazole and cyanoacetamide. The benzimidazole pesticide was thiabendazole and cymoxanil constitute the cyanoacetamide fungicide [50].

Fungicides are an important element in the intensification of agriculture by boosting crops yields, improving quality and ensuring the stability of production [51]. However, the main constraint is related to resistance development to many of the most effective fungicides compromising disease control [42]. In addition, fungicides can pose a risk not only to target plant pathogens but may also influence populations or the activity of non-target microorganisms in soils [41]. Moreover, the newly developed chemicals have a low order of toxicity to mammals. Public concern has focused on its potential to cause reproductive and developmental toxicity in mammals because of their impact on endocrine systems [44,52].

(i) Benzimidazole

Benzimidazole is a heterocyclic aromatic organic compound. This is a bicyclic molecule that results of the fusion of benzene and imidazole rings [53]. A few synthetic methodologies are available for the synthesis of benzimidazole. Usually, the condensation of o-phenylenediamine with carboxylic acids and their nitrile, imidates and orthoester derivatives have been used [53]. However, the common synthesis of commercially available benzimidazole is achieved by the condensation of o-phenylenediamine with formic acid (FA) (Figure 9) [54,55].

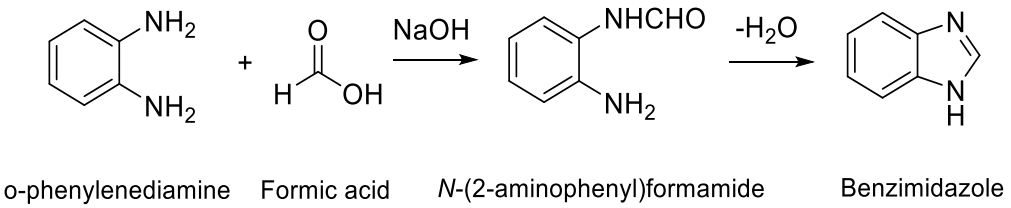


Figure 9. Synthesis of benzimidazole.

Modifications in position 2, 3, 5 and 6 of the molecule provide a number of different activity (Figure 10) [54]. The benzimidazole moiety is well established in the literature as a privileged substructure for drug design and it has been incorporated into many drugs

with several applications, including antifungal, antimicrobial, antiviral, antiparasitic, anticancer and anti-inflammatory agents, for example [54,56].

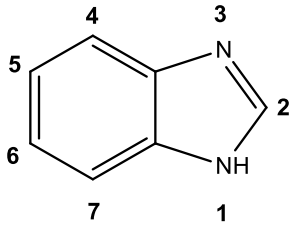


Figure 10. Benzimidazole structure.

Antifungal benzimidazoles are normally used for superficial and deep fungal infections. Their efficient activity is due to its mode of action where some studies show that these compounds are specific inhibitors of microtubule assembly which act by binding to their heterodimeric subunit, the tubulin molecule [57].

The main concern associated with benzimidazole fungicides usage and application is owed to its effects on the central nervous system (CNS) and hepatotoxic potential. Also, overexposure can cause nausea, vomiting, headache, weakness, drowsiness, and lack of appetite. Negative impacts of inhalation are exhibited by severe irritation on the upper respiratory tract. Additionally, overdosage may be related to disturbances of vision and psychic alterations [58].

(ii) Cyanoacetamide

Cyanoacetamide is an organic aliphatic compound. It is an acetic amide with a nitrile functional group. The basic structure of cyanoacetamide compounds is exhibited in Figure 11.

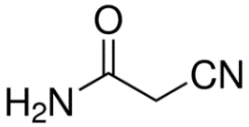


Figure 11. Basic structure of cyanoacetamide.

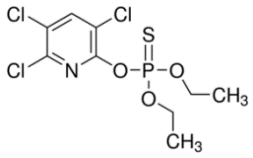
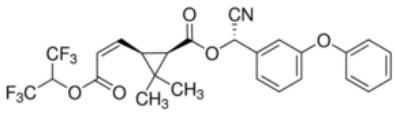
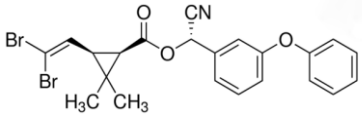
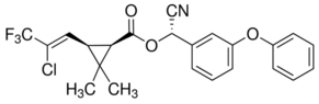
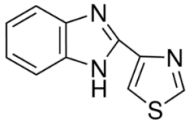
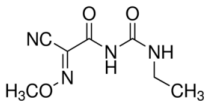
Cyanoacetamide fungicides are normally used in combination with other fungicides formulations to control foliar late blight and downy mildew of grapes, potatoes, and other vegetables by displaying a preventive, curative and penetrant activity on treated plants [59]. Typically, the combination with fungicides who differs in the mode of action helps to avoid fungicide-resistant strains [60].

The effectiveness of cyanoacetamide fungicides is due to their capacity to inhibit the synthesis of nucleic acids, amino acids, and other cellular processes in the target pathogen [59]. This compound has a low potential for leaching to groundwater and does not persist in soil and water systems. The main concern associated is the moderate

toxicity to mammals. The substance can be dermally absorbed or by inhalation causing skin and eye irritation [61,62]. The decomposition of organic nitriles in the body can lead to toxic cyanide [63]. However, the biggest concern was released when the International Agency for Research on Cancer determined that this compound is possibly carcinogenic to humans based on sufficient evidence of carcinogenicity in laboratory animals [61,62].

Tables 4 and 5 represented some brief characteristic of the studied pesticides. It is represented the most commonly used and useful information for researchers in the field of pesticides, specifically chemical family, mode of action and MRLs.

Table 4. Selected pesticides [64].

	Pesticide	Structure	Chemical family	Mode of action	
Insecticides	Chlorpyrifos $C_9H_{11}Cl_3NO_3PS$		Organophosphorus	Contact, Ingestion, Fumigation.	Acts on the nervous system as AchE inhibitor
	Acrinathrin $C_{26}H_{21}F_6NO_5$		Pyrethroid	Contact, Ingestion.	Acts on the nervous system by sodium channels modulation
	Deltamethrin $C_{22}H_{19}Br_2NO_3$				
	λ-Cyhalothrin $C_{23}H_{19}ClF_3NO_3$				
Fungicides	Thiabendazole $C_{10}H_7N_3S$		Benzimidazole	Systemic, Protective, Curative.	Inhibits mitosis and cellular division. Acts on βtubulin.
	Cymoxanil $C_7H_{10}N_4O_3$		Cyanoacetamide oxime		Inhibits the formation of cellular walls by an unknown mechanism.

The MRL established for each studied pesticide on the selected matrix are represented in Table 5 and can be found in the MRL database on the Commission website.

Table 5. MRL values for each pesticide on potatoes and respective regulation.

Pesticide	MRL[1] (potatoes)	Regulation (EC)[2] No
Chlorpyrifos	0.05	839/2008
Acrinathrin	0.05	839/2008
Deltamethrin	0.3	2016/1822
λ-Cyhalothrin	0.02	834/2013
Thiabendazole	15.0	149/2008
Cymoxanil	0.05	978/2011

1- Maximum residue level (mg/kg)

2- EC: European Council Directive

1.6. Methodologies for determination and quantification of pesticide residues

1.6.1. Extraction procedure

Typically, food matrices require a pre-treatment of the sample to turn it suitable for chromatographic analysis [65]. The determination of pesticide residues in food matrices is very challenging mainly because of the residuals quantities of analytes in the samples and because of the large amounts of interfering substances which can be co-extracted with the interest compounds [66].

As pesticides are a very assorted group of compounds ranging from biological and physicochemical properties, the trend in this kind of analysis is developing multi-residual methods that provide simultaneous extractions and determinations of a large variety of pesticides and also, that can be applied to samples with multiple origins (e.g. animal, vegetal, environment) [67].

Ideally, sample preparation should be as simple as possible (Figure 12). However, traditional extraction procedures (e.g. liquid-liquid extraction, Soxhlet extraction, among other) are time-consuming, labor-intensive, moreover, requires a large number of organic solvents and includes many steps, which increases the possibility of introducing contaminants [66,67].

As a result, modern sample procedures for pesticides extraction, such as microwave-assisted extraction (MAE) [69,70], accelerated solvent extraction (ASE) [71,72], matrix solid-phase dispersion (MSPD) [73,74], solid-phase microextraction (SPME) [75,76], supercritical fluid extraction (SFE) [77] and (quick, easy, cheap, effective, rugged and safe (QuEChERS) [78], have been developed to overcome the drawbacks of traditional approaches [67]. Nevertheless, QuEChERS methodology is the effective procedure for pesticide residues extraction in a large variety of matrices, including fruits and vegetables [9,78–81].

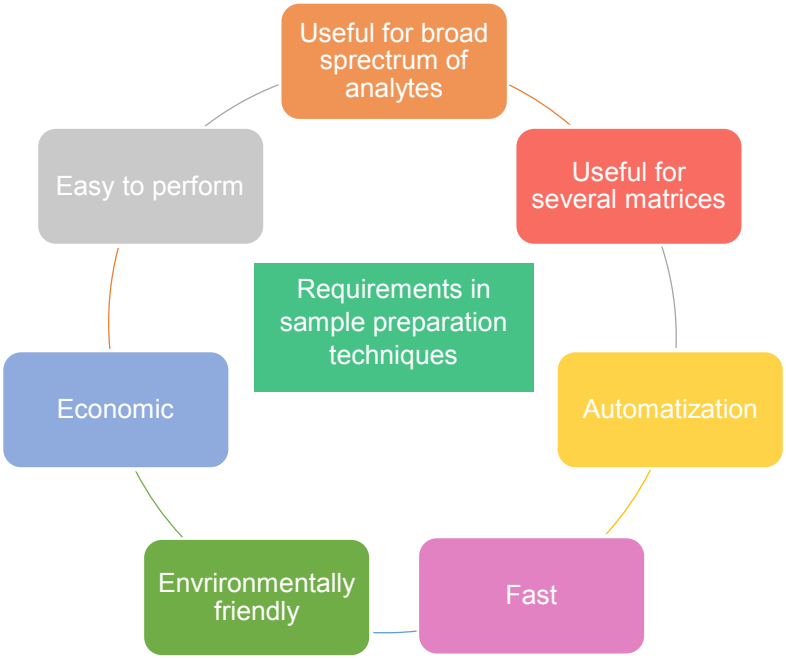


Figure 12. Trends in the development of sample preparation procedures. Adapted from [68].

QuEChERS approach was introduced in 2003 by Anastassiades et al. [82]. This sample preparation technique has gained special attention due to their suitability for multiclass analysis [65]. It is characterized by its high flexibility, which can, therefore, be adapted to a few applications, including not only the analysis of fruits and vegetables but also of biological fluids, water, sediments, and soil. It has shown efficient results on the extraction of both polar and non-polar compounds at once, it also allows the analysis of liquid and solid matrices and provides quality results with a quick, simple, easy and low-cost approach. The procedure comprehends only three steps before analysis: (i) Initial extraction with an organic solvent; (ii) Partitioning step with the addition of salt mixture and; (iii) Clean-up step of an aliquot of the extract (Figure 13).

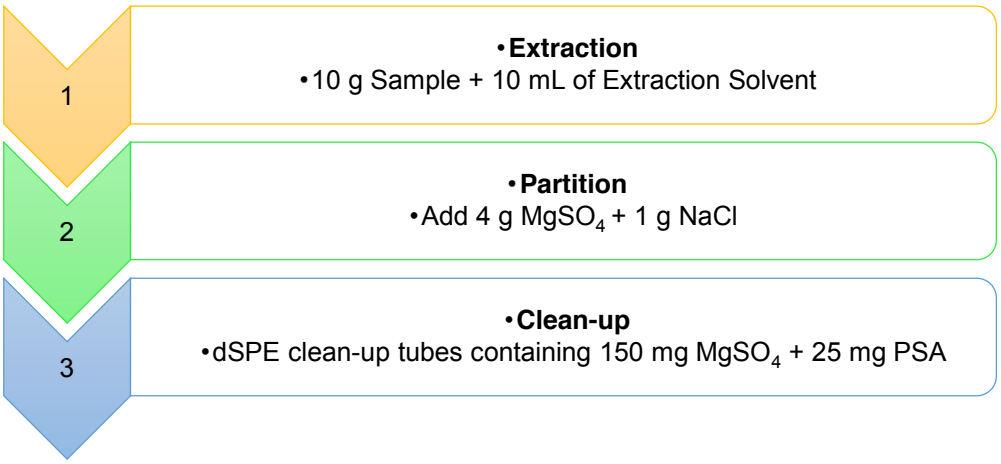


Figure 13. Schematic representation of QuEChERS-dSPE steps [82].

According to the original QuEChERS approach, the procedure involves initial single-phase extraction of 10 g sample with 10 mL acetonitrile (ACN), followed by liquid-liquid partition formed by the addition of 4 g anhydrous magnesium sulfate ($MgSO_4$) and 1 g of sodium chloride (NaCl). Removal of residual water and clean-up are performed simultaneously using a quick procedure named dispersive solid phase extraction (dSPE), where 150 mg of $MgSO_4$ and 25 mg primary secondary amine (PSA) sorbent are mixture with 1 mL of ACN extract. The dSPE effectively removes polar matrix compounds, like organic acids, pigments, sugar and lipidic compounds from the food matrices. In the final extract, we have an aliquot with low polarity by the precipitation of polar interferents [82,83].

Although the original method proved to be amenability high throughput and efficient for the recovery of a wide variety of pesticides in several different matrices, a few changes were tested and introduced in the procedure in order to achieve even better results [78,79]. The main changes for improvements in the procedure are related to both extraction and clean-up steps [84].

1.6.2. Chromatographic separation and identification of pesticide residues

Chromatography is the analytical technique used for separation of complexes mixtures allowing the identification of analytes in a sample. Hence, this methodology is performed based on the interaction of the analytes with a stationary phase (liquid or solid) while they are carried by a mobile phase (e.g. liquid, gas or supercritical fluid) [85]. According to the nature of the mobile phase, chromatographic techniques can be classified as liquid chromatography (LC), gas chromatography (GC) and supercritical chromatography (SFE) [85,86].

Normally, sample extracts are analyzed using high-performance or ultra high-performance liquid chromatography (HPLC or UHPLC) and/or gas chromatography (GC) [87]. In the last years, analysis of pesticide residues in vegetables has been performed using GC coupled with nitrogen phosphorus detector (NPD), electron capture detector (ECD) and mass spectrometry (MS) or MS/MS. However, GC methods were superseded by HPLC coupled to other less powerful detectors, that are, however, easier to acquire and use [88,89]. They are useful and preferable for polar, low volatile and thermolabile compounds that are not directly determinable by GC and not require a derivatization step [90]. Also, HPLC analysis promotes a shorter chromatographic run, allowing not only processing more samples but also reducing the solvent consumption, saving time and money [91]. Moreover, in the past few years, HPLC was replaced by UHPLC, the technique of choice for faster analysis with high resolution [92].

The main advantages of UHPLC over HPLC are the improved speed, better resolution, the narrower peaks (giving increased signal-to-noise ratio) and improved confirmation for the targeted pesticides in the analyses [93].

In recent years, UHPLC became the modern analytical separation technique applied to just about any sample, such as pharmaceuticals [94,95], food [92,96,97], nutraceuticals [98,99], cosmetics [100,101], environmental matrices [102], forensic samples [103], and industrial chemicals [104]. Using highly sophisticated equipment, it has the capacity to separate, identify, and quantify the compounds that are present in any sample and can be dissolved in a liquid [105]. The schematic of a UHPLC instrument typically includes a reservoir, an injection system, a chromatographic column, a detector and a data logger (Figure 14).

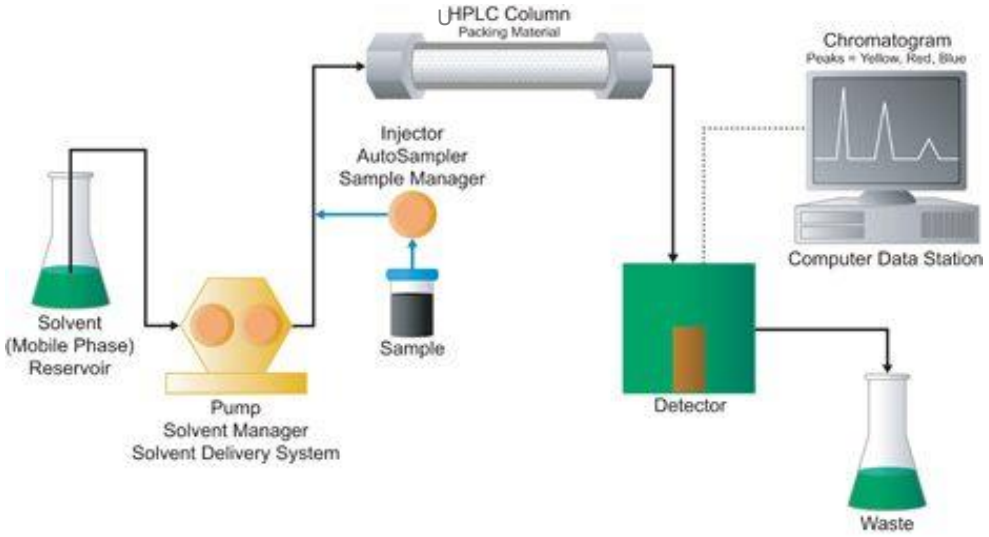


Figure 14. Schematic representation of UHLPC system.

This analytical technique is performed to achieve the separation of the compounds contained in a sample by means of two immiscible phases: a liquid mobile phase and a stationary phase contained in a column. Samples are dissolved in a suitable solvent and later analyzed in solution which is injected into the chromatographic system and is dragged by the mobile phase under elevated pressure along with the stationary phase. The stationary phase is packed into the column with sub 2 µm porous particles. The smaller the particle size, the higher column efficiency. It requires a higher pressure to work with so UHPLC systems need to be capable of operating above 6,000 PSI. The flow and the mobile phase can be rigorously altered during the chromatographic analysis process [85,86,105].

A detector is needed to see the separated compound bands as they elute from the UHPLC column. The mobile phase exits the detector and can be sent to waste, or collected, as desired, to further analysis. When the mobile phase contains a separated

compound band, the equipment offers the capacity to collect the interest fraction of the eluate containing the purified compound for later studies.

The detector is connected to the computer data station. This is a fundamental part of the system once it records the electrical signal required to generate the chromatogram allowing the identification and quantification of the sample constituents. The detector contains a flow cell that detects each separates compound band against a background of the mobile phase. An appropriated detector must be highly sensitive, selective, have a low limit of detection (LOD), good stability and reproducibility of the signal. Also, they should have a fast response to the sensor and be suitable for the analyzed compounds (Figure 15) [86,105,106].

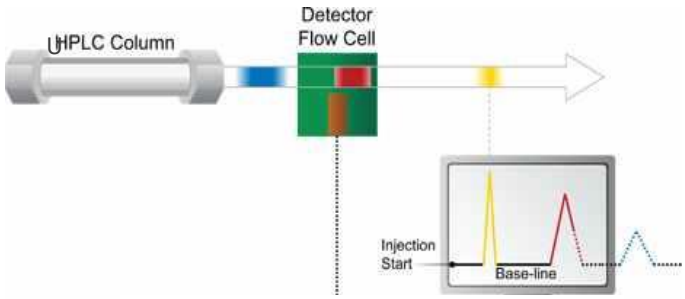


Figure 15. How peaks are generated.

The choice of a detector is made depending on the characteristics and concentrations of the compounds that are supposed to be separated and analyzed. For instances, if a compound has the capacity to absorb ultraviolet light a UV-absorbance detector is chosen, PDA [107]. In another hand, if the compound fluoresces, a fluorescent detector is used (FLR) and in this case, the intensity of the emitted light is controlled to quantify the concentration in the analyte [108]. Also, MS detector offers great sensitivity and selectivity and it is based on the fragmentation of molecules, being separated according to the mass and charge ratio of the fragmented molecule [109].

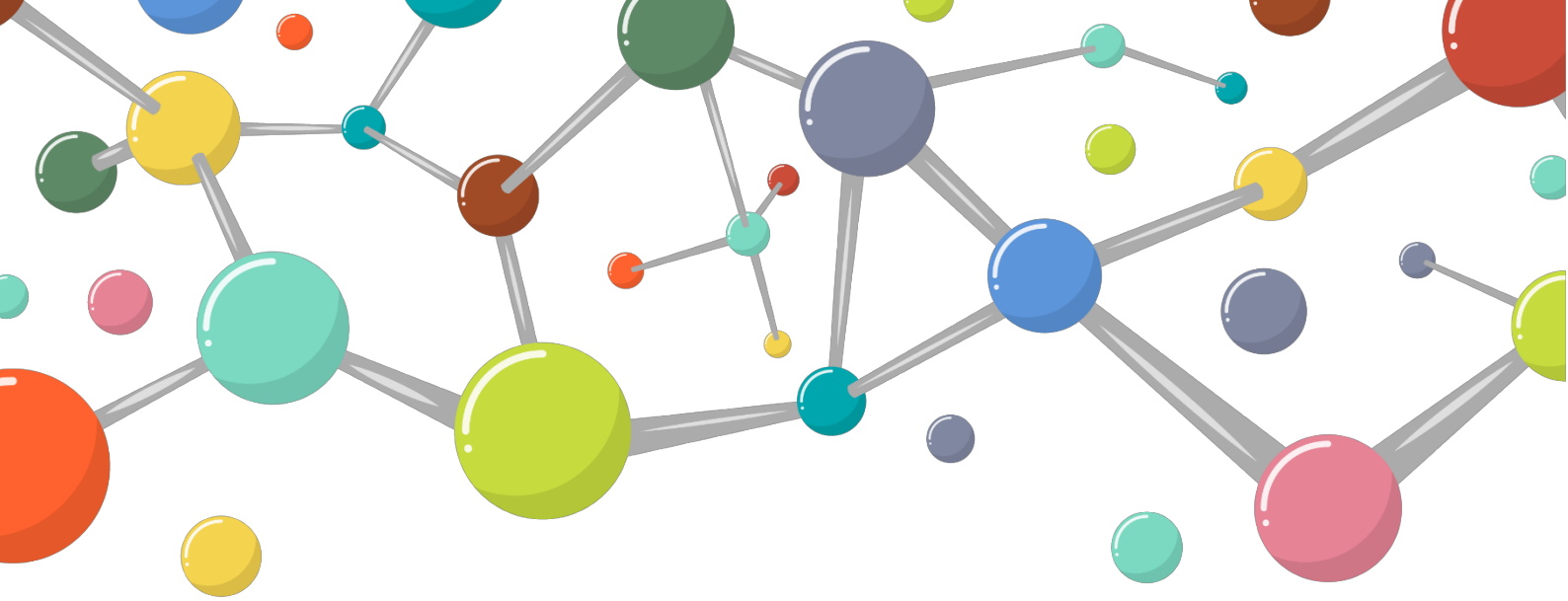
The practice of coupling a mass spectrometer to a UHPLC system is called LC-MS. Generally, LC-MS techniques are considered superior to LC-UV detection systems in terms of detection specificity and sensitivity. However, the high cost involved in the acquisition of LC-MS instrumentation does not allow its application in several analytical laboratories. Thus, methods for the analysis of pesticide residues in less costly but sensitive and selective instrumentation such as the UHPLC-PDA method are often required [89]. The detectors described above are commonly used for the determination of pesticides in several different matrices, including vegetables [110–112].

1.7. Objectives

The aim of this work was to develop and validate a multifactorial effective QuEChERS-dSPE/UHPLC-PDA methodology for determination of the most commonly used insecticides (e.g. chlorpyrifos, acrinathrin, deltamethrin, λ-cyhalothrin) and fungicides (e.g. thiabendazole, cymoxanil) in Madeira Island, Portugal. The selected matrix was its greater and economically profitable crop, namely potatoes (*Solanum tuberosum* L.).

The methodology was validated according to IUPAC guidelines, being evaluated the selectivity, linearity, accuracy, precision, matrix effect, limits of detection (LOD) and quantification (LOQ).

In this context, it was intended, that the validated methodology was used to quantify the most commonly used pesticides in Madeira Island. It would be performed in different anatomic parts of potatoes (epidermis, peeled potato, and potato with peel) produced in different regions of the Island.



Part 2.

Materials and methods

2. Experimental

2.1. Reagents and materials

2.2. Sample preparation

2.3. Preparation of standard solutions

2.4. QuEChERS procedure

2.4.1. Optimization of QuEChERS procedure

2.5. UHPLC-PDA conditions

2.5.1. Optimization of UHPLC conditions

2.6. Analytical method validation

2.6.1. Selectivity

2.6.2. Linearity

2.6.3. Limit of detection and quantification

2.6.4. Precision

2.6.5. Accuracy

2.6.6. Matrix effect

2. Experimental

2.1. Reagents and materials

All chemicals and reagents were of analytical quality grade. HPLC grade acetonitrile (ACN) and methanol (MeOH) were obtained from LabScan (Dublin, Ireland). Pesticide standards, chlorpyrifos (98.3%), acrinathrin (99.7 %), deltamethrin (99.9 %), ʎ-cyhalothrin (≥ 95.0 %), thiabendazole (≥ 98.0 %), and cymoxanil (99.9 %), were obtained from Sigma-Aldrich (St. Louis, MO, USA) with the highest available purity. Buffered salts for QuEChERS extraction: magnesium sulfate ($MgSO_4$), sodium chloride (NaCl), trisodium citrate dihydrate $C_6H_5Na3O_7.2H_2O$ and disodium hydrogencitrate sesquihydrate $C_6H_6Na_2O_7 \cdot 1.5H_2O$ were also purchased from Sigma-Aldrich. Phosphoric acid (PhA, ≥ 85%) was from BDH (Poole, England) and formic acid (FA, ≥ 99 %) was obtained from Merk (Darmstadt, Germany). Solvents were filtered with 0.22 µm membrane filters using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA, USA). Ultrapure water from a Milli-Q ultrapure water purification system (Millipore, Bedford, USA) was used for preparing the UHPLC mobile phase and other aqueous solutions. Filters of 13 mm with 0.22 µm PTFE membrane were used for filtration of the final extracts before analysis. Clean-up tubes with primary secondary amine (PSA) and $MgSO_4$ were obtained from Waters (Milford, MA, USA).

2.2. Sample preparation

Solid samples, as most of the food matrices, requires a previous complex and time-consuming stage. To ensure the maximum efficiency of the process, it is preferentially used the smallest amount of sample as long as it guarantees statistical representativeness in the final result [23]. In this case, for obtaining a solid homogeneous sample, three kg of potatoes were chopped in small pieces, followed by a lyophilization process. This procedure is normally used in foods that have high water content (potato is about 80% of water (H_2O)) [113,114].

All samples were stored in the freezer, under stable conditions (-20 ºC, in the dark) until further analysis.

2.3. Preparation of standard solutions

Stock solutions (1 g/L) of chlorpyrifos, acrinathrin, deltamethrin, ʎ-cyhalothrin, and cymoxanil were prepared in ACN. The single standard stock solution of thiabendazole was prepared in MeOH due to its insolubility in ACN. All the solutions were stored at 20 ºC and protected from light.

A standard multicomponent solution was prepared by diluting each primary standard solution with the chromatographic mobile phase (ACN) and was used for spiking the

target potatoes samples for calibration standard and linear dynamic range assessment of the UHPLC-PDA system. Stock solutions of chlorpyrifos and acrinathrin were constructed from 0.0125 to 0.1 mg/L; deltamethrin and λ-cyhalothrin from 0.025 to 0.5 mg/L; thiabendazole from 1.25 to 25 mg/L and cymoxanil from 0.25 to 5 mg/L.

2.4. QuEChERS procedure

A modified version of the QuEChERS CEN 15662 method was applied to the extraction of pesticide residues [82]. The procedure is described below and represented in Figure 16. In a centrifuge tube, 1.05 g of lyophilized sample was weighted, 5 mL of ACN was transferred into it and shaken vigorously for 1 min with a vortex mixer ensuring that the solvent interacted well with the entire sample. Then, 1 g of $MgSO_4$, 1 g of NaCl, 0.5 g of $C_6H_6Na_2O_7 \cdot 1.5H_2O$ and 1 g of $C_6H_5Na_3O_7.2H_2O$ was added to the homogenized mixture, and it was vigorously shaken again with the vortex mixer for 2 min. All centrifuge tubes were submitted to ultrasounds for 30 min followed by centrifugation at 4000 rpm for 5 min, completing the partition step and consequent separation of phases (H_2O and ACN phase). After, to promote the clean-up, 1 mL of supernatant extract was transferred into a 2 mL PTFE dSPE clean-up tubes containing 25 mg of PSA (removes various polar compounds, such as organic acids, pigments, sugars, and fatty acids) and 150 mg $MgSO_4$. The mixture was shaken in a vortex and centrifuged for 2 min at 3000 rpm. Finally, all extracts were filtered through a 0.22 µm PTFE filter membrane before the UHPLC-PDA analysis.

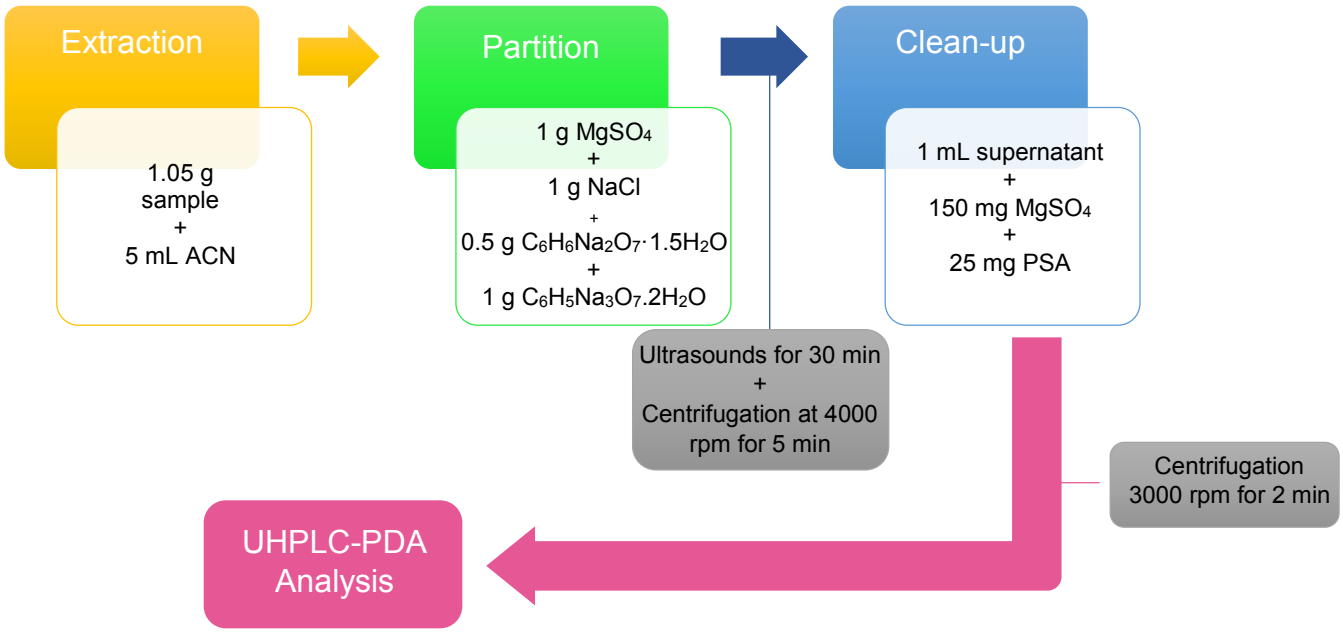


Figure 16. Schematic representation of the QuEChERS-dSPE extraction procedure.

2.4.1. Optimization of QuEChERS procedure

The optimization of the QuEChERS method was one of the fundamental steps in this work. In this study, modifications to the traditional QuEChERS procedure were tested to evaluate the better extraction conditions. Due to the low concentration of analytes in the sample, the different chemical proprieties and to the complexity of matrices, the determination of pesticide residues requires a previous sample preparation step. The main purpose of this stage is to promote the extraction, the enrichment of analytes and to remove untargeted compounds.

The optimal extraction efficiency was chosen through a full factorial design process. It means that we combine all the possible variables and study the effect of each factor on the response variable, as well as the interaction effects between the factors. To guarantee the best extraction efficiency, we evaluated several factors, including the percentage of extraction solvent in solution, the addition of acid during the extraction and the ratio of partition salts, for the first part (Table 6).

Table 6. Influence of extraction solvent percentage, acidification, and partition salts ratio (Full Factorial Design 3^3).

Run	Acid	% ACN	Salts[a]
1 2 3	None	50	1:1:1/2:1 2:1:1/2:1 3:1:1/2:1
4 5 6		75	
7 8 9		100	
10 11 12	0.1% FA	50	1:1:1/2:1 2:1:1/2:1 3:1:1/2:1
13 14 15		75	
16 17 18		100	
19 20 21	0.1% PhA	50	1:1:1/2:1 2:1:1/2:1 3:1:1/2:1
22 23 24		75	
25 26 27		100	

[a]$MgSO_4$: NaCl: $C_6H_6Na_2O_7$: $C_6H_5Na_3O_7.2H_2O$

Then, we tested the effect of preconcentrating and time of ultrasounds (Table 7).

Table 7. Evaluation of ultrasound extraction time and preconcentration step (Full Factorial Design $1^2/1^3$.)

Run	Preconcentration (%)	Ultra-sound time (min)
1	0	0
2		15
3		30
4	100	0
5		15
6		30

Finally, we tested the sorbent and C_{18} effect for clean-up (Table 8).

Table 8. Effect of sorbents PSA and C_{18} for clean-up procedure (Full Factorial Design 2^2.)

Run	$MgSO_4$ (mg)	PSA (mg)	C_{18} (mg)
1	150	-	-
2		25	-
3		-	25
4		25	25

Overall, this technique entails a few steps for optimization, but it provides the best conditions for QuEChERS methodology and subsequently best extraction results. All the experiments were done in triplicate in CORTECS column.

2.5. UHPLC-PDA conditions

The separation, identification and quantification of pesticide residues were performed on a Waters Ultra Pressure Liquid Chromatographic Acquity system (UPLC, Acquity H-Class) (Milford, MA, USA) combined with a Waters Acquity quaternary solvent manager (QSM), an Acquity sample manager (SM), a column heater, a 2996 PDA detector, and a degassing system (Figure 17).

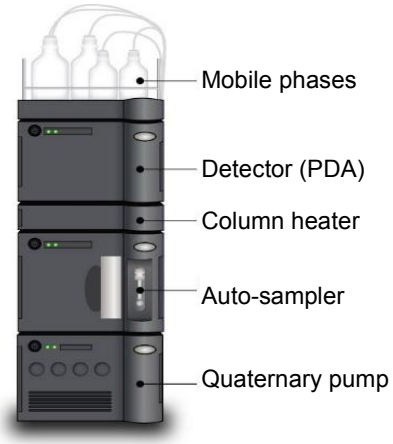


Figure 17. UHPLC-PDA system from Waters. Acquity UPLC H-Class model**.**

The whole configuration was driven by Empower software v2.0 from Waters Corporation. A high strength silica Acquity CORTECS analytical column (2.1 mm × 100 mm, 1.8 m particle size) packed with a trifunctional C_{18} alkyl phase, kept at 30 ◦C, was used for the separation of the selected pesticides. A binary mobile phase with a gradient program was used, combining solvent A (H_2O 0.1% PhA) and solvent B (ACN) as described in Table 9.

Table 9. Gradient program used in the separation of pesticides.

Time (min)	%A	%B
0	95	5
2	95	5
3	60	40
9	30	70
29	25	75
30	95	5

The flow rate was 150 µL/min, gave a maximum back pressure of 6000 psi, which is within the capabilities of the UHPLC. The injection volume, of both the standard solutions and sample extracts, was 10 µL. After each injection, the needle was rinsed initially with 400 µL of wash solution with H_2O: ACN (90:10, v/v) and after with 200 µL of H_2O: ACN (10:90, v:v) solution. The samples were kept at 15 ºC during the analysis. The system was re-equilibrated with the initial composition for 3 min, prior to the next injection. The target compounds elution and the additional equilibration at the initial mobile phase composition resulted in a total analysis time of 30 min. The UV detection wavelength was set to the maximum range of absorbance for the target compounds, 190-310 nm.

The identification of pesticide residues in the sample was based on the comparison of the retention times (RT) and PDA spectra of their peaks in samples with those previously obtained by the injection of pure pesticide standard.

2.5.1. Optimization of UHPLC conditions

The optimal separation conditions for UPLC analysis were also chosen through a full factorial design (Table 10). Different factors were combined and studied, namely different eluents (H_2O, H_2O with FA and H_2O with PhA), column flows (150, 250 and 350 µL/min) and temperature (30, 40 and 50 ºC). All the conditions were tested same way in triplicate in the five available columns namely, CORTECS, BEH (Ethylene Bridged Hybrid), HSS (High strength silica), CSH (Charged surface hybrid) and HILIC (Hydrophilic interaction liquid chromatography).

Table 10. Optimization of different instrumental (UHPLC) conditions (Full Factorial Design $2^2/3^3$.)

Run	Eluent A	Flow (µL/min)	Temperature (ºC)
1 2 3	H_2O	150	30 40 50
4 5 6		250	
7 8 9		350	
10 11 12	0.1% FA	150	30 40 50
13 14 15		250	
16 17 18		350	
19 20 21	0.1% PhA	150	30 40 50
22 23 24		250	
25 26 27		350	

2.6. Analytical method validation

Analytical method validation is performed to certify that an analytical method is accurate, specific, reproducible and robust on the specified range that an analyte will be analyzed [115]. Validation of a method provides an assurance of reliability during normal use and can be mentioned as the process of providing documented evidence that an analytical method is suitable for its planned purpose [115,116]**.**

In general, validated methodologies must include studies on selectivity, linearity, accuracy, precision, matrix effect, and LOD and LOQ [116].

2.6.1. Selectivity

The selectivity of the method was assessed by the absence of interfering peaks at the RT of the analytes. This parameter refers to the extent to which a method can determine a particular analyte in a complex mixture without interference from other components in the mixture [117].

2.6.2. Linearity

Linearity is the ability of the method to verify if the samples solutions are in a concentration range where the analyte response is directly proportional to concentration [115,118]. This kind of study is usually performed by preparing standard solutions at different concentrations that do not include the zero point in the curve, due to the possible associated errors [116].

2.6.3. Limit of detection and quantification

The LOD is frequently defined as the lowest concentration of analyte that can be reliably distinguished from zero or that can be detected but not necessarily quantitated [117,118]. On the other hand, LOQ is commonly defined as the lowest amount of analyte that can be determined quantitatively with acceptable precision and accuracy under the sated operational conditions of the method [115,117].

The LOD and LOQ calculations were based on the standard deviation(s) of calibration curve interception and the slope of a regression curve, which were calculated using Equations 1 and 2, respectively.

$$\text{LOD} = 3.3\ x\ \frac{s}{slope} \qquad \text{Equation 1}$$

$$\text{LOQ} = 10\ x\ \frac{s}{slope} \qquad \text{Equation 2}$$

2.6.4. Precision

The precision of an analytical method can be defined as the closeness of agreement between the values obtained under stipulated conditions [117]. Precision was performed at two different levels: repeatability precision (intra-run precision) and intermediate precision (inter-run precision) [118].

Repeatability is achieved with the same method on identical test items, in the same laboratory by the same operator using the same equipment within short intervals of time [118]. The assessment of intermediate precision is obtained with the same method on identical test items in the same laboratory but by different analysts, using different equipment over an extended period of time [118]. The precision is normally expressed as the percentage of relative standard deviation (% RSD) [115].

2.6.5. Accuracy

Accuracy is the nearness of agreement among the experimental value obtained by the analytical method and the true value [115,117,118]. The accuracy was determined through the percentage of the recovery. The recovery was obtained by comparing the known theoretical concentration added to sample ($C_{theoretical}$) with the experimental

concentration ($C_{experimental}$) of each pesticide in the sample spiked at low (LL), medium (ML) and high (HL) concentrations levels (Table 11). The $C_{experimental}$ was calculated by the difference between the peak areas of the analytes in the spiked sample and the peak areas from the sample without spiking [118]. The recovery was calculated using the equation below [118]:

$$\%\ \text{recovery} = \left(\frac{C_{experimental}}{C_{theoretical}}\right) x\ 100 \qquad \text{Equation 3}$$

Table 11. Experimental concentrations of each pesticide in a sample at high (HL), medium (ML) and low levels (LL).

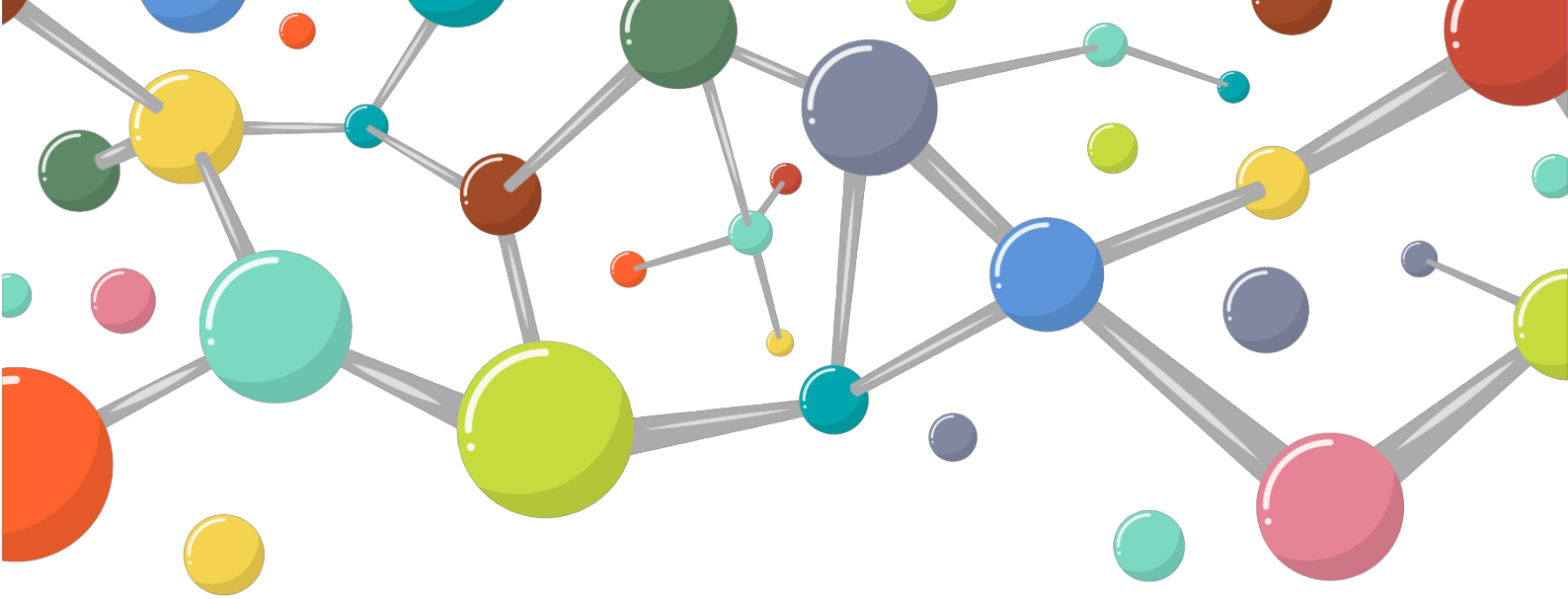
Pesticide	HL	ML	LL
Chlorpyrifos	0.1	0.05	0.0125
Acrinathrin	0.1	0.025	0.013
Deltamethrin	0.5	0.125	0.025
λ-Cyalothrin	0.5	0.125	0.025
Thiabendazole	25.0	6.25	1.25
Cymoxanil	5.0	1.25	0.250

2.6.6. Matrix Effect

The matrix effect is most pronounced in complex sample analyzes. The matrix effect was determined by applying the method of "standard additions" to a sample under study, which was calculated by comparison of the slopes obtained by calibration curves of pesticides in the sample and solvent-based matrix. The calibration functions for both matrixes were constructed in a similar way, the standard calibration curves were prepared with different concentrations of analytes.

The matrix effect was calculated using the following Equation 4.

$$\text{Matrix effect} = \left(\frac{\text{Slope}_{\text{Sample}}}{\text{Slope}_{\text{Solvent}}}\right) x100 \qquad \text{Equation 4}$$



Part 3.

Results and Discussion

3. Results and Discussion

3.1. Optimization of QuEChERS extraction parameters

3.1.1. Evaluation of influence of extraction solvent percentage, acidification, and partition salts ratio

3.1.2. Evaluation of ultrasound extraction time and preconcentration step

3.1.3. Effect of $MgSO_4$ and sorbents PSA and C_{18} for clean-up procedure

3.2. Determination of instrumental parameters

3.3. Analytical method validation

3.3.1. Selectivity

3.3.2. Linearity

3.3.3. Limits of detection and quantification

3.3.4. Precision

3.3.5. Accuracy

3.3.6. Matrix Effect

3.4. Applicability

3. Results and Discussion

3.1. Optimization of QuEChERS extraction parameters

The development of a reliable analytical method, associated with simplicity and quickness, is still a hard task to undertake. In method development, several parameters must be tested and optimized, and numerous difficulties can be found especially in the sample preparation step. In the present work, all the optimized parameters were done in triplicate, in potato samples.

3.1.1. Evaluation of the influence of extraction solvent percentage, acidification, and partition salts ratio

The complexity of sample treatment is linked to the potential matrix interferences and the physicochemical properties of the analyte [50]. The selection of the extraction solvent is one of the fundamental points in the development of a multiresidue extraction method. Many aspects should be considered, among them: the ability to extract a wide spectrum of pesticides with different polarities, selectivity for interest compounds during extraction, partition, and clean-up, compatibility with different chromatographic techniques, low cost, safety and, environmental sustainability.

The most commonly used solvents for multi-residue extraction of pesticides are ethyl acetate, acetone, and ACN, each of which has advantages and disadvantages. Ethyl acetate has been shown to be a solvent with universal characteristics since it has the capacity to extract pesticides of different classes in several types of samples, however, the recovery rates of some pesticides are low because of degradation problems. In those cases, the addition of NaOH is necessary for an increase in these percentages. Acetone and ACN are miscible with water and promote extraction in a single phase when in contact with the matrix. When the extraction is carried out with acetone there is a need for the addition of non-solvent solvents so that the separation between the organic and aqueous phases occurs, this is not necessary when ACN is used since the addition of salts to the extract causes such separation [119]. Meanwhile, the use on ACN allows the extraction of a small number of lipophilic co-extractives from the sample, such as waxes, fats, and pigments, and provides the extraction of a wide range of pesticides with different polarities with higher capacity and selectivity [119]. Another great advantage is that ACN is more suitable for LC than acetone or ethyl acetate.

Consequently, we decided to extract our target pesticides using ACN. In addition, to guarantee the best extraction efficiency and to reduce hazardous organic solvent consumption as much as possible, we evaluated the percentage of ACN in solution (50, 75 and 100%). Also, its acidification to provide extraction of a wide range of pesticides with different polarities (FA and PhA) and lastly, the effect of partition salts ratio in the

partition step (1, 2 or 3 mg $MgSO_4$) once it is reported that hydration of $MgSO_4$ is a high exothermic process, causing the sample extract to get hot during the extraction/partition step, reaching temperatures as high as 40-45 °C. The heat is beneficial for extraction, especially in the case of less polar pesticides [82]. The results are exhibited in Figures 18 and 19.

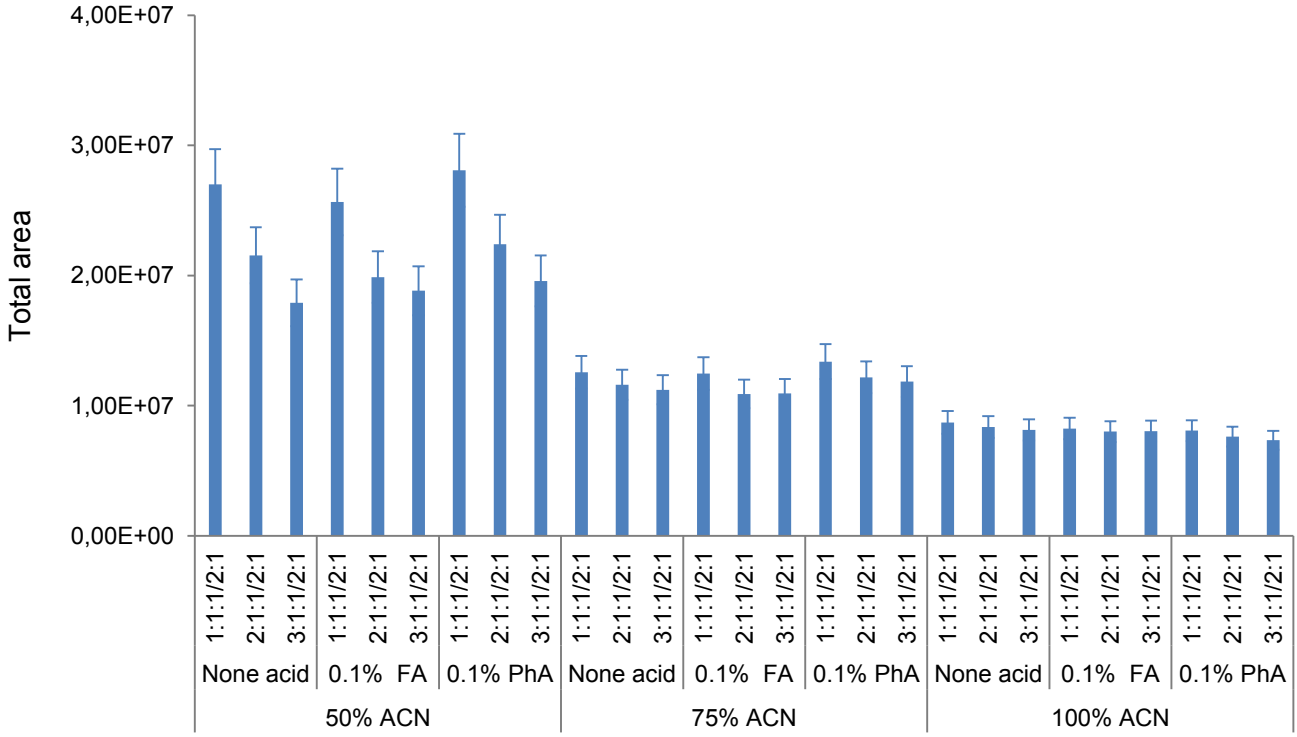


Figure 18. Influence of ACN, acidification and partition salts ratio in the extraction step.

Overall, better extractions were achieved with the lowest percentage of ACN (50%) providing higher total peak areas for most studied pesticides. The pesticides total peak areas decreased in the following order of ACN percentage in extraction solution: 50 > 75 > 100%. These results were expected according to literature were water is important to allow the extraction solvent access into the dry samples increasing the efficiency [120]. The first comprehensive study by Mol et al. [121] introduced a simple approach based on shaking of respective sample of plant or animal origin with an ACN:H_2O:FA (75:24:1, v/v/v) mixture providing acceptable performance characteristics for a wide range of analytes [121]. Years later, a study conducted by Romero-González et al. [121] utilized an extraction mixture consisted of ACN: H_2O (80:20, v/v) and sonication. The method was able to extract most analytes successfully with good recoveries. Additionally, according to the obtained results, we can infer, as expected from the literature, that the extraction of pesticides with 100% ACN was not efficient enough traducing the lowest values of peak area [121].

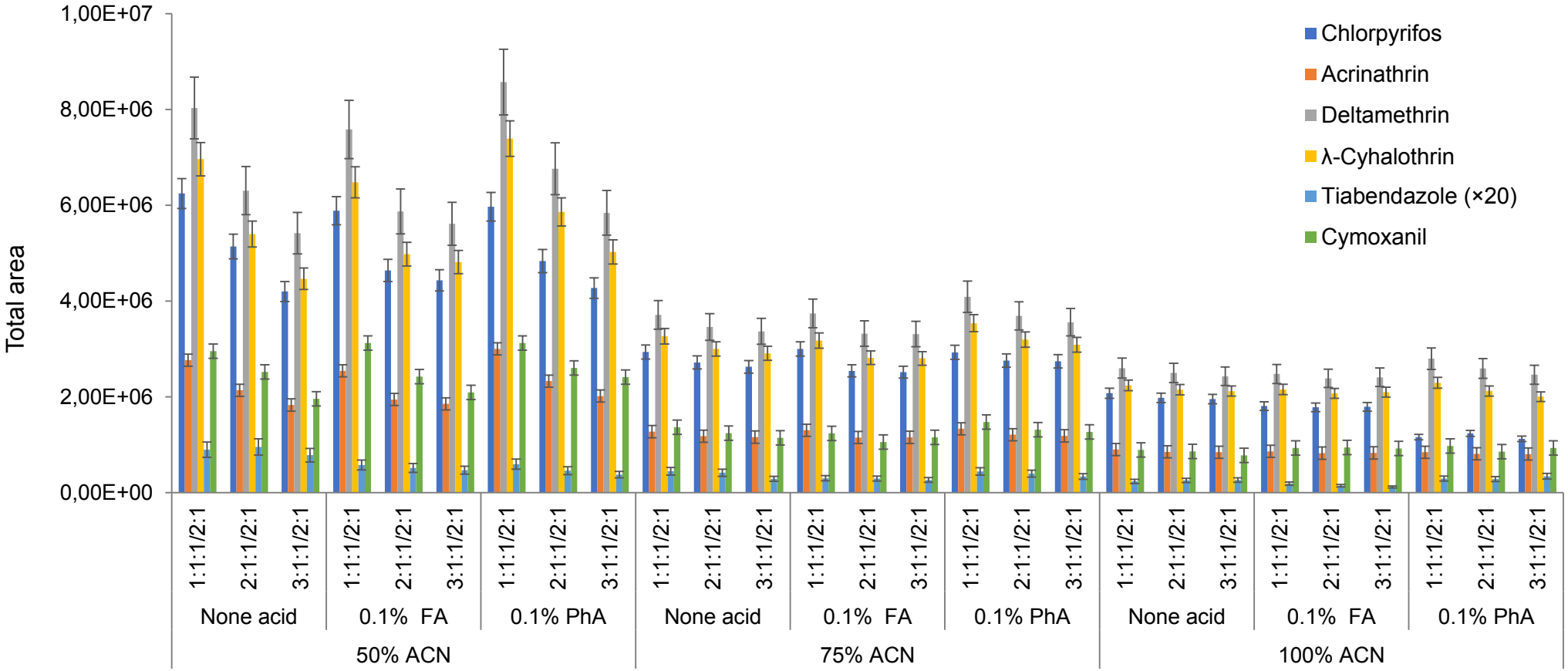


Figure 19. Influence of ACN, acidification and partition salts ratio in the extraction step in each studied pesticide.

In this study, the choice of acidified ACN was to provide extraction of a wide range of pesticides with different polarities [122]. According to Figure 19, the addition of an organic (FA) or inorganic acid (PhA) increases the extraction of pesticides in all different conditions. It is reported that ACN when acidified, promoted satisfactory recoveries of pesticides which generally present stability problems and also minimize degradation [123]. The acid addition not also show great influence on the peak areas but also in their shapes. The enhanced total areas were achieved when using the inorganic acid due to its higher capacity to protonate analytes and reducing its hydrophilicity.

Concerning the use of salts to induce phase separation, the 1:1:1/2:1 ratio shown to be most effective in selectivity and capacity to separate aqueous and organic phases, maintaining high peak values and low co-extraction of interferences in every test. Other ratios did not show to be so effective maybe because of the effect of temperature on the extraction of thermally-labile analytes. Some pesticides can be degraded with the addition of $MgSO_4$ anhydrous due to its exothermic hydration reaction [50,124].

The addition of $MgSO_4$ promotes partitioning of pesticides into the organic layer [82]. The combination of other salts, such as sodium, besides helping to promote the salting-out effect, allows, together with the 1% (v/v) acid added in ACN, the buffering of the medium. This enables a better extraction of pesticides that usually have stability problems [122,123]. For these reasons, 50% of ACN acidified with 0.1% PhA and 1:1:1/2:1 ratio was used.

3.1.2. Evaluation of ultrasound extraction time and preconcentration step

Ultrasound-assisted extraction (UAE) is usually an important stage of any extraction method. UAE is considered a good alternative for pesticides extraction from several different matrices, including vegetables [125]. Therefore, we decided to include this extra-step to the traditional QuEChERS methodology, and we evaluated it in order to obtain higher extraction efficiency. So, different ultrasound extraction times were tested, namely 0, 15 and 30 min.

Also, preconcentration is an important step for extracting pesticides from numerous matrices such as soil, water, animal or plant tissue, and foods [126]. Thus, we tested the preconcentration step by evaporating the extract until dryness under a stream of N_2. The obtained results are represented in Figure 20.

According to the obtained results, we can observe that the preconcentration step did not show coherent and satisfactory results once we obtained high RSD values, specifically above 35%, which cannot be considered. We also could not obtain reproducible values for each pesticide area under evaporation until dryness conditions. These values can be explained by the precipitation of matrix co-extractives occurring

during evaporation till dryness [127]. Accordingly, we decided not to consider these data and only use the results obtained without the preconcentration step.

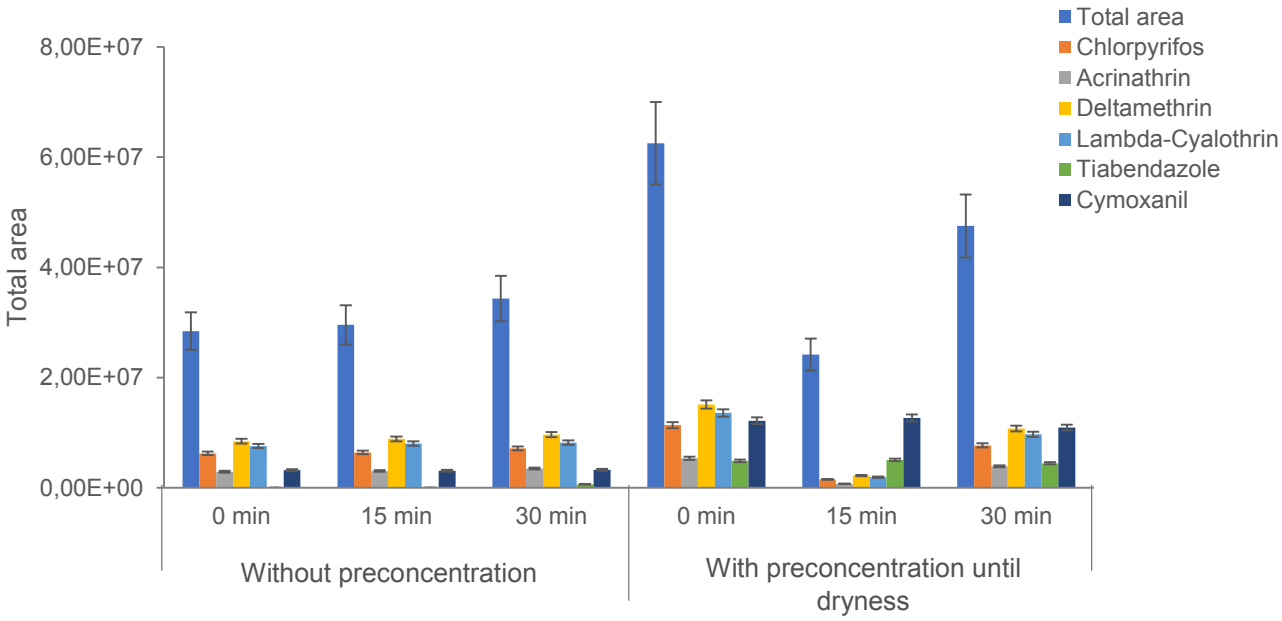


Figure 20. Influence of ultrasound time and preconcentration until dryness.

In the analysis without the preconcentrating step, the UAE revealed very pleasing results by increasing the extraction efficiency. We can see a relation between different ultrasound time and better extractions. As long as we increase UAE time, we observed better total areas. In fact, it was already expectable once that the increasement of both pressure and temperature improves penetration, transport, solubility, and diffusivity, of the extraction solvent resulting in better extraction of pesticides [128]. It either reduces coextracted compounds, with subsequent reduction or elimination of needing time-consuming clean-up steps [129,130]

Thence, according to the obtained results, for improved pesticide extraction we decided to not preconcentrate the extract and submit it to ultrasound for 30 min.

3.1.3. Effect of $MgSO_4$ and sorbents PSA and C_{18} for clean-up procedure

Clean-up step is essential to promote robustness and reliability to the results obtained by the chromatographic system [131]. Due to the presence of co-extractives in the extracts, it became necessary to evaluate the effectiveness of the most commonly used chemicals for this step. We tested the efficiency of $MgSO_4$, PSA and C_{18}. The results are presented in Figure 21.

We evaluate the potential to clean of $MgSO_4$, $MgSO_4$ + PSA, $MgSO_4$ + C_{18} and lastly, $MgSO_4$ + PSA + C_{18}. In the procedure, $MgSO_4$ is used to separate water from the

organic solvent, on the other hand, PSA removes many organic acids, polar pigments, some sugars, and fatty acids. Finally, C_{18} removes non-polar interfering substances [66].

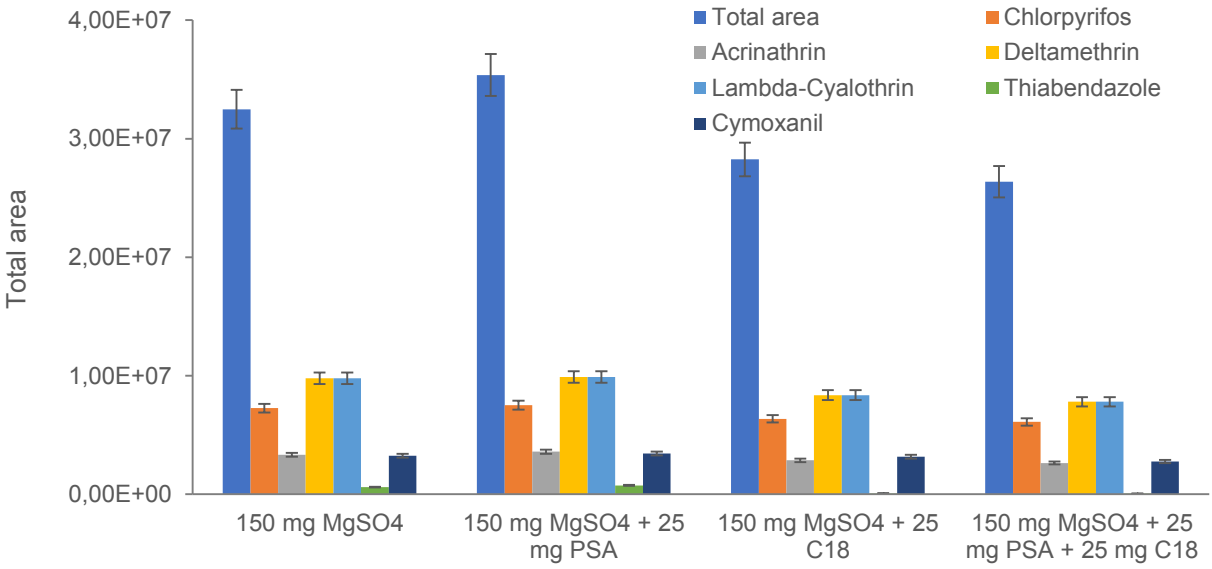


Figure 21. Influence of commonly used clean-up salts to evaluate their effectiveness.

The cleaner chromatogram and enhanced total areas were obtained using $MgSO_4$ + PSA. Removal of residual water and clean-up are performed simultaneously by using dSPE, in which 150 mg $MgSO_4$ and 25 mg PSA sorbent are simply mixed with ACN extract. Together, they effectively remove many polar interferent components from the potato extract [78,79,131]. Combining C_{18} to clean-up procedure did not positively influence the cleaning of the extracts because it is normally used for fatty matrices (e.g. milk, eggs, olive oil, avocado), efficiently removing interfering substances in those cases. It is reported that it also removes certain pesticides like thiabendazole, as shown in Figure 21, and other planar-ring pesticides [66]. Thus, it was decided to use 150 mg $MgSO_4$ and 25 mg PSA in the clean-up step.

3.2. Determination of instrumental parameters

Chromatographic conditions were optimized in order to achieve a suitable compromise between separation, resolution and analysis time. A Full Factorial Design was applied being evaluated the following parameters: elution solvent (H_2O with FA and H_2O with PhA), column flow (150, 250 and 350 µL/min) and column temperature (30, 40 and 50 ºC) for each column under study, namely the CORTECS, BEH, HSS, CSH and HILIC. The results are presented in Figure 22.

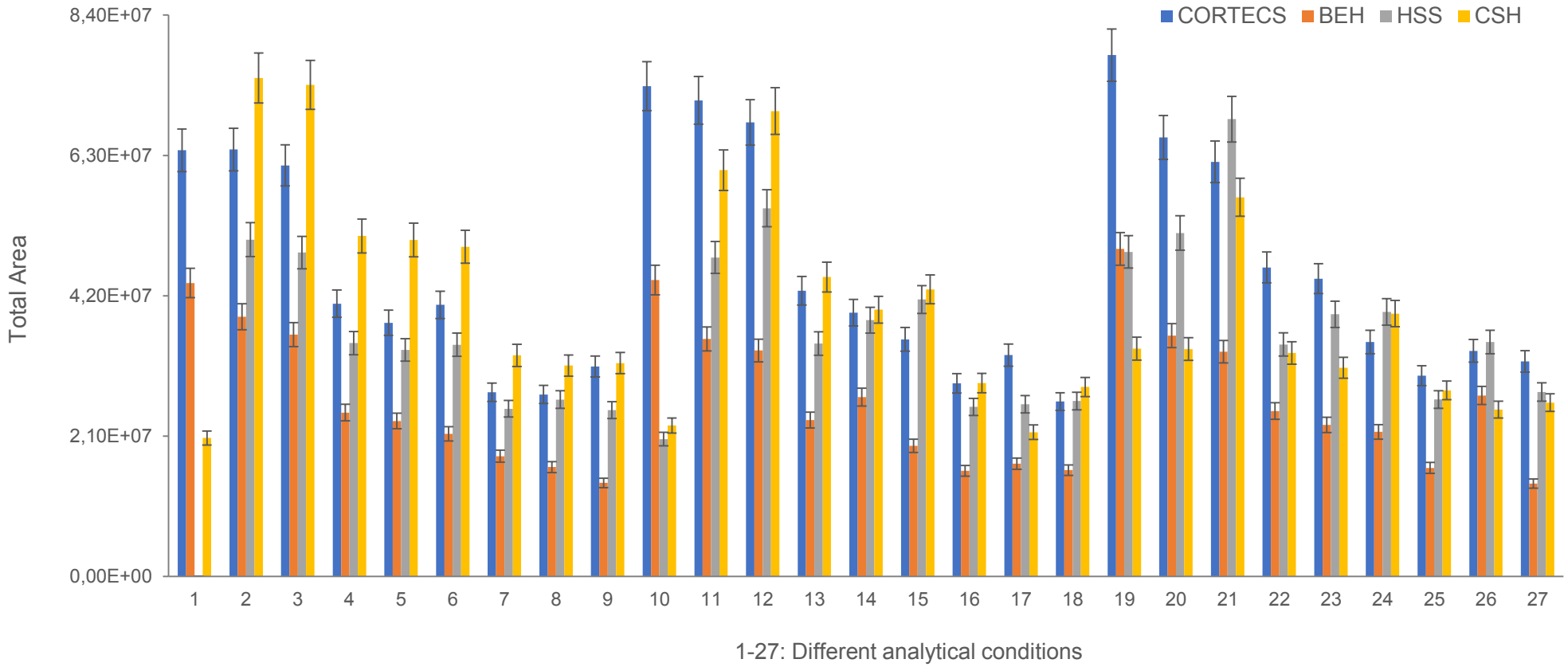


Figure 22. Optimization of chromatographic conditions in CORTECS, BEH, HSS and CSH columns. Conditions described in Table 10.

Regarding the chromatographic separation of pesticides, the stationary phase most commonly used is C_{18} [92]. Although, most columns were capable of separating pesticides under study. Chromatographic efficiency differed greatly depending on the column type. The pesticides total peak areas decreased in the following order CORTECS > CSH > HSS > BEH > HILIC. The high efficiency of CORTECS it is explained by small particle core-shell diameter (1.6 µm) than BEH, HSS and CSH (from 1.7 up to 1.8 µm), increasing the surface absorption and higher retention of analytes. These separations were based on reversed-phase through C_{18} surface. Moreover, HILIC values are not presented due to their poor results. They can be explained since it was a silica phase column used under hydrophilic interaction to provide de retention of polar compounds.

The most two types of eluents used are the aqueous phase with the addition of some additives and, the organic phase based on MeOH or ACN [92]. The tested eluents interfered mostly with peak resolution. We studied the addition of the same acids as during extraction, specifically formic and phosphoric acid. These additions are shown improved peak resolutions for most pesticides

From the results, it was possible to verify that higher total peak areas were influenced by the column flow rate. Better results were obtained with the lowest flow rate, namely 150 µL/min (conditions 1, 2, 3, 10, 11, 12, 19, 20, 21). Flow rates above, 250 µL/min (conditions 4, 5, 6, 13, 14, 15, 22, 23, 24) and 350 µL/min (conditions 7, 8, 9, 16, 17, 18,25, 26, 27), influenced directly the decrease of resolution and total peak areas in all studied columns. Furthermore, we can observe that different tested temperatures were not determinant for enhanced results. Small differences were observed between the three tested temperatures (30, 40, 50 ºC), being at 30 ºC the slightly better results.

Accordingly, the optimum instrumental parameters for improved results were obtained with CORTECS column on condition 19. More specifically, with ACN and H_2O with PhA as eluents, a flow rate of 150 µL/min at a temperature of 30 ºC.

3.3. Analytical method validation

Validation is an important step for the development of an analytical method. It is the process of evidencing that an analytical method is accepted for its intended purpose [116]. Also, the use of validated methods is important to show their qualification and competency [118]. The good performance of any analytical technique depends crucially on two parameters: the quality of the instrumental measurements and the reliability of the calculations involved in its processing [132]. This procedure was validated according to IUPAC guidelines, in terms of the selectivity, linearity, accuracy, precision, matrix effect, LOD, and LOQ in potato matrix.

3.3.1. Selectivity

The capacity of an analytical method to differentiate and quantify any particular compound instead of other interference from other components in a sample complex mixture is defined as selectivity [133]. Therefore, selectivity was assessed by the absence of interfering peaks at the RT and maximum wavelengths (λ_{max}) of studied pesticides. The evaluation was made by checking the peaks of a solution of analytical standards following the optimized QuEChERS procedure in potato sample. Figure 23 shows a representative example of a chromatogram for a potato sample matrix fortified with pesticides analytical standards (Annexes A1, A2, A3, A4, A5, and A6 – chromatograms of single standard pesticide).

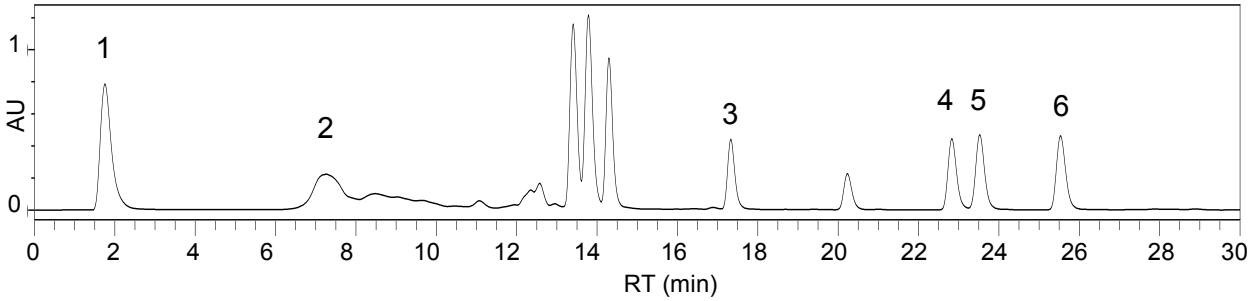


Figure 23. QuEChERS-dSPE/UHPLC-PDA chromatogram at 224 nm for a potato matrix fortified with pesticides analytical standards solution: 1. Thiabendazole; 2. Cymoxanil; 3. Chlorpyrifos; 4. λ-Cyhalothrin; 5. Deltamethrin; 6. Acrinathrin.

Observing the presented chromatograms, it was observed that no significant interference was found at the retention time and maximum wavelengths of each analyte under study in the potato matrix, thus evidencing the selectivity of the method. Besides, Table 12 summarizes the results obtained from the chromatographic analysis of each pesticide, distinguishing the RT and their λ_{max}.

Table 12. Retention time (RT) and maximum wavelength (λ_{max}) observed for each pesticide.

Pesticide	RT (min)	λ_{max} (nm)
Thiabendazole	1.98	301
Cymoxanil	7.59	242
Chlorpyrifos	17.53	197
λ-Cyhalothrin	23.26	193
Deltamethrin	23.99	192
Acrinathrin	26.02	235

3.3.2. Linearity

A linearity study shows the ability of an analytical method to provide directly proportional instrumental results to the analyte concentration, in a concentration range [134]. This study was performed by preparing calibration curves for each six target

analytes, with eight calibration points. Concentrations ranges were different according to pesticide response. Calibrations curves were constructed by plotting the pesticide signal obtained against the concentration of each target analytes in a solvent-based matrix. The obtained results for linearity tests are summarized in Table 13.

Table 13. Linearity study with respective regression equation obtained for each pesticide, correlation coefficient (r^2) and, obtained LOD and LOQ for pesticides under study with the developed method.

Pesticide	Concentration range (mg/L)	Regression equation	r^2	LOD (mg/L)	LOQ (mg/L)
Thiabendazole	25 - 1.25	y = 906812x + 1E+06	0.9997	2.581	7.821
Cymoxanil	5 - 0.25	y = 673205x + 32133	0.9976	0.424	1.286
Chlorpyrifos	0.1 - 0.0125	y = 1E+06x - 2668.5	0.9983	0.005	0.015
λ-Cyhalothrin	0.5 - 0.025	y = 3E+06x - 57640	0.9985	0.026	0.078
Deltamethrin	0.5 - 0.025	y = 2E+06x - 38245	0.9980	0.029	0.088
Acrinathrin	0.1 - 0.0125	y = 1E+06x - 10991	0.9968	0.008	0.023

Once gotten the regression equation, the linearity of the method can be easily demonstrated. In this sense, the slope of the line must have a value statistically different from zero, the ordinate at the origin should not be statistically different from zero and the coefficient of the line of calibration should not be significantly different from one [135]. From a practical point of view, the sensitivity of the method is defined by the slope of the calibration line and depends basically on the nature of the analytes and the detection technique used. This way, the greater the slope of the regression equation the better the sensitivity of the analytical method [135].

According to the obtained results, it was verified that the method is linear in the concentration range tested for each pesticide. The correlation coefficient, in the linear function model, were higher than 0.9968, thereby ensuring an adequate adjustment of the values to the calibration curve.

3.3.3. Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ calculations were based on the standard deviation (s) of calibration curve interception and the slope of a regression curve. Both limits are expressed in units of concentration and were calculated using equations 1 and 2, respectively. The results are displayed in Table 13.

Concerning to the obtained results, the developed method shows a very pleasing capability to detect and quantify the pesticides under study. LOD wide-ranged between 0.005 and 2.581 mg/L and the LOQ between 0.015 and 7.821 mg /L. According to the legislated MRLs for each pesticide in potato samples, most of them can be identified and

quantified with the present method. Only λ-cyhalothrin, which has an MRL of 0.02 mg/kg should not be quantified with this method because its LOQ value is slightly higher than the maximum allowed. All the other compounds could perfectly be quantified under the described method, traducing good results bellow their MRLs.

3.3.4. Precision

The precision of an analytical method is the closeness of agreement between the values obtained under stipulated conditions [117]. Precision can be estimated by measurements of dispersion (standard deviation, variance or coefficient of variation). In the present work, precision was performed at two different levels: repeatability precision (intra-run precision) and intermediate precision (inter-run precision). This parameter was evaluated in three levels of concentration namely, low (LL), medium (ML) and high (HL) concentration levels, in the linear interval of the analytical curve. Lastly, for the study of precision, the coefficients of variation are considered adequate when are equal to or, less than 20% [136]. The results are exhibited in Table 14.

Table 14. Obtained results for the study of analytical method precision. Obtained results for accuracy, accessed through recovery (%).

Pesticide	Concentration range (mg/L)		Precision		Accuracy
	Theoretical	Experimental	Repeatability (% RSD)	Intermediate Precision (% RSD)	Recovery (%)
Thiabendazole	25	24.092	0.3	1.6	96.4
	6.25	6.815	0.7	2.3	109.0
	1.25	2.678	0.9	2.5	214.2
Cymoxanil	5	4.973	0.6	3.1	99.5
	1.25	1.311	1.7	1.1	104.9
	0.25	0.327	0.5	0.4	130.6
Chlorpyrifos	0.1	0.103	7.6	2.1	103.1
	0.05	0.049	7.6	2.8	97.7
	0.0125	0.014	15	14.6	115.3
λ-Cyhalothrin	0.5	0.694	0.9	1.5	138.9
	0.125	0.152	2.9	6.0	121.6
	0.025	0.022	12.7	7.9	87.7
Deltamethrin	0.5	0.608	0.9	1.0	121.5
	0.125	0.151	6.7	3.4	121.1
	0.025	0.028	13.8	13.6	112.2
Acrinathrin	0.1	0.099	5.1	3.2	99.1
	0.025	0.023	18.7	29.1	93.6
	0.0125	0.013	11.6	34.7	105.1

From the results in Table 14, we can conclude that most of the pesticides revealed satisfactory RSD values, specifically below 20 %. According to the acceptance criteria defined for this study, only for the ML and LL of acrinathrin were not so attractive by revealing higher RSD values for the intermediate precision

3.3.5. Accuracy

The degree of agreement between the experimental value obtained by the analytical method and the true value is denominated accuracy [117]. This parameter was accessed through the percentage of recovery by comparing the known theoretical concentration added to sample with the experimental concentration of each pesticide in the sample spiked at low, medium and high concentrations levels. The recovery (%) was calculated according to Equation 3. The obtained results are described in Table 14.

Among the tested concentration levels, most of the obtained values were satisfactory being within acceptable recovery limits from literature guidelines (70 – 125%) [135].

3.3.6. Matrix Effect

The matrix effect was calculated by comparison of the slopes obtained by calibration curves of pesticides in the sample and solvent-based matrix. The matrix effect is shown in Table 15.

Table 15. Obtained results for matrix effect of the analytical method.

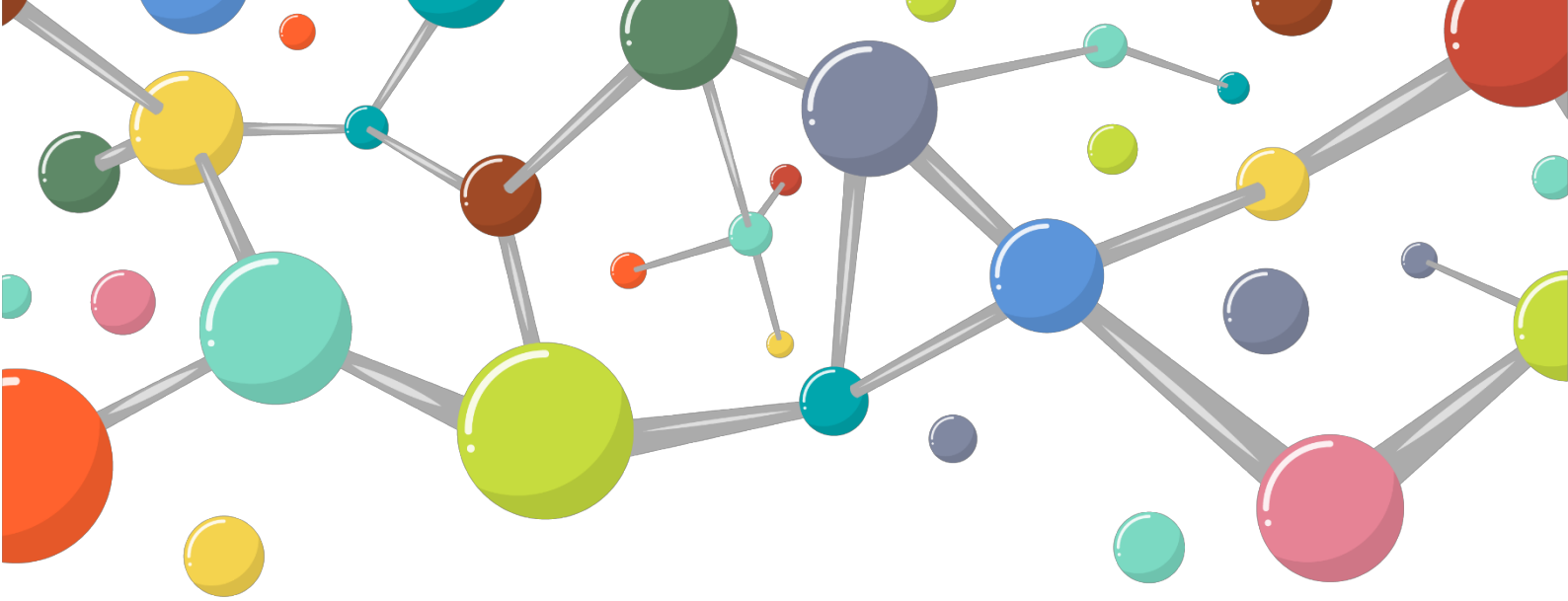
Pesticide	Concentration range (mg/L)	Matrix Effect (%)
Thiabendazole	25-1.25	87.6
Cymoxanil	5-0.25	95.2
Chlorpyrifos	0.1-0.0125	113.5
λ-Cyhalothrin	0.5-0.025	150
Deltamethrin	0.5-0.025	100
Acrinathrin	0.1-0.0125	185.8

According to the obtained results, no matrix effect was verified for chlorpyrifos, deltamethrin, thiabendazole, and cymoxanil, wherein values ranging between 87.6-113.5%. However, λ-cyhalothrin and acrinathrin were influenced by the potato matrix where their values range from 150 and 185.8%, respectively. These values were not acceptable from literature guidelines (70–125%) [137].

3.4. Applicability

In order to test the developed and validated methodology, different potatoes samples produced at different places at Madeira Island should have been analyzed and quantified. For the purpose of this study, the samples would be separated into three parts, epidermis, peeled potato, and potato with the peel.

Unfortunately, successive malfunctions of the equipment made the third part of the work unrealizable, not being applied in a commercial sample as we predicted.



Part 4.

Conclusions and Future Work

4. Conclusions and Future Work

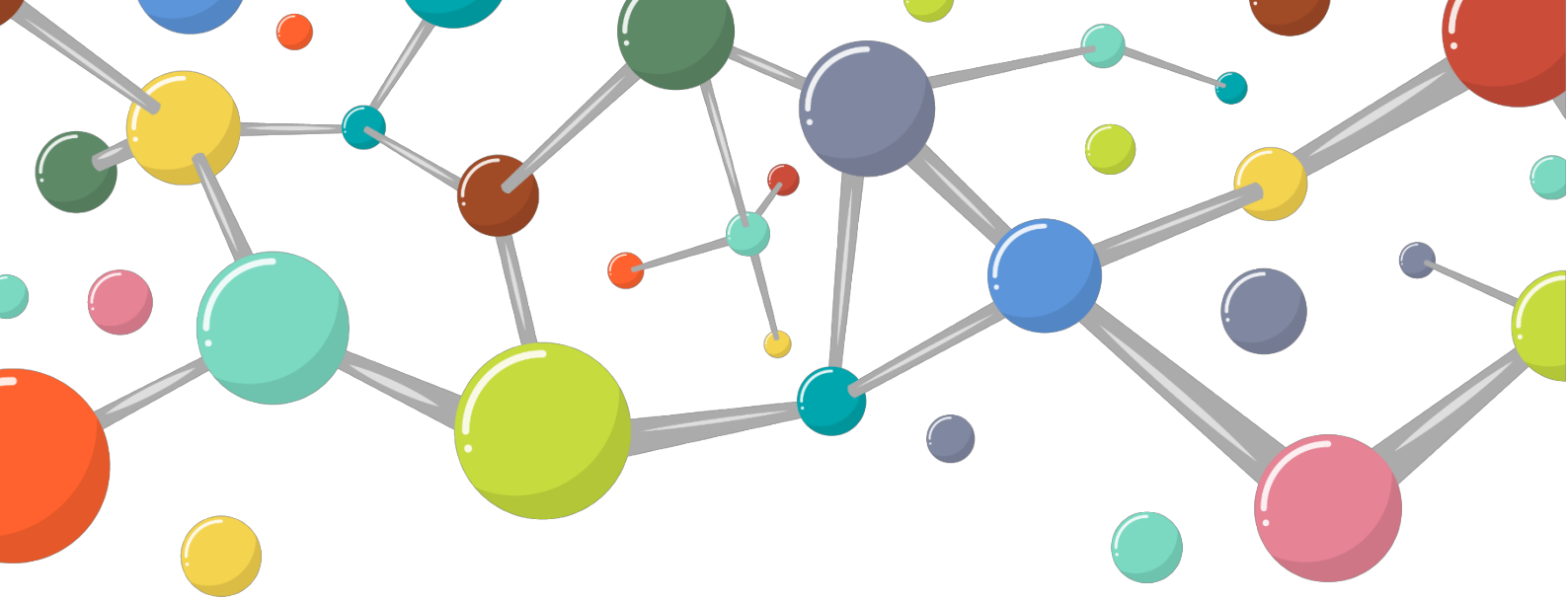
The developed and validated QuEChERS-dSPE/UHPLC-PDA methodology demonstrated that is a simple, fast and suitable strategy for identification and quantification of pesticide residues in potatoes. The methodology can be extended to the determination of them in other food products.

After careful selection of the chromatographic conditions, it was demonstrated that the separation of pesticides with different properties could be achieved with good chromatographic resolution. In addition, PDA has proven to be an economical alternative when compared to more sophisticated and expensive equipment, such as a mass spectrometry detector, making this methodology more accessible to laboratories.

After optimizing several variables that affect QuEChERS performance, the optimal parameters were 50% of ACN acidified with 0.1% PhA, and magnesium sulfate: sodium chloride: disodium hydrogencitrate sesquihydrate: trisodium citrate dihydrate (1:1:1/2:1) ratio were used. Therewithal, the small amount of hazardous organic solvent consumption assembly makes this technique not only more economical but also environmentally friendly. Moreover, 30 min ultrasound time additional step and magnesium sulfate: PSA for clean-up was chosen. The selected column was CORTECS with a gradient program combining H_2O 0.1% phosphoric acid: ACN, a flow rate of 150 µL/min at a temperature of 30 ºC.

The methodology was validated according to IUPAC guidelines being evaluated in terms of selectivity, linearity, limits of detection and quantification, accuracy, accuracy, and extraction efficiency. The validated method showed reliable results in terms of selectivity for the studied pesticides, linearity by presenting correlation coefficient (r^2) values higher than 0.997. extraction efficiency, precision, and accuracy. The limits of detection (LOD) ranging from 0.005 (chlorpyrifos) to 2.581 (thiabendazole) mg/L, whereas the limit of quantification (LOQ) limit from 0.015 (chlorpyrifos)to 7.821 (thiabendazole) mg/L. Moreover, obtained results for accuracy ranged between 87.7 and 214.2 %, while in precision, the coefficients of variation remained in general below to 20 %. Furthermore, matrix effect values ranged between 87.6 and 185.8 %, which agreed with results reported by other studies.

Lastly, applicability should have been performed in order to verify the practical use and advantages of the presented methodology. In the future, this work should be done to complement the developed work and also to monitor the most commonly applied pesticides due to safety concerns of consumer health.



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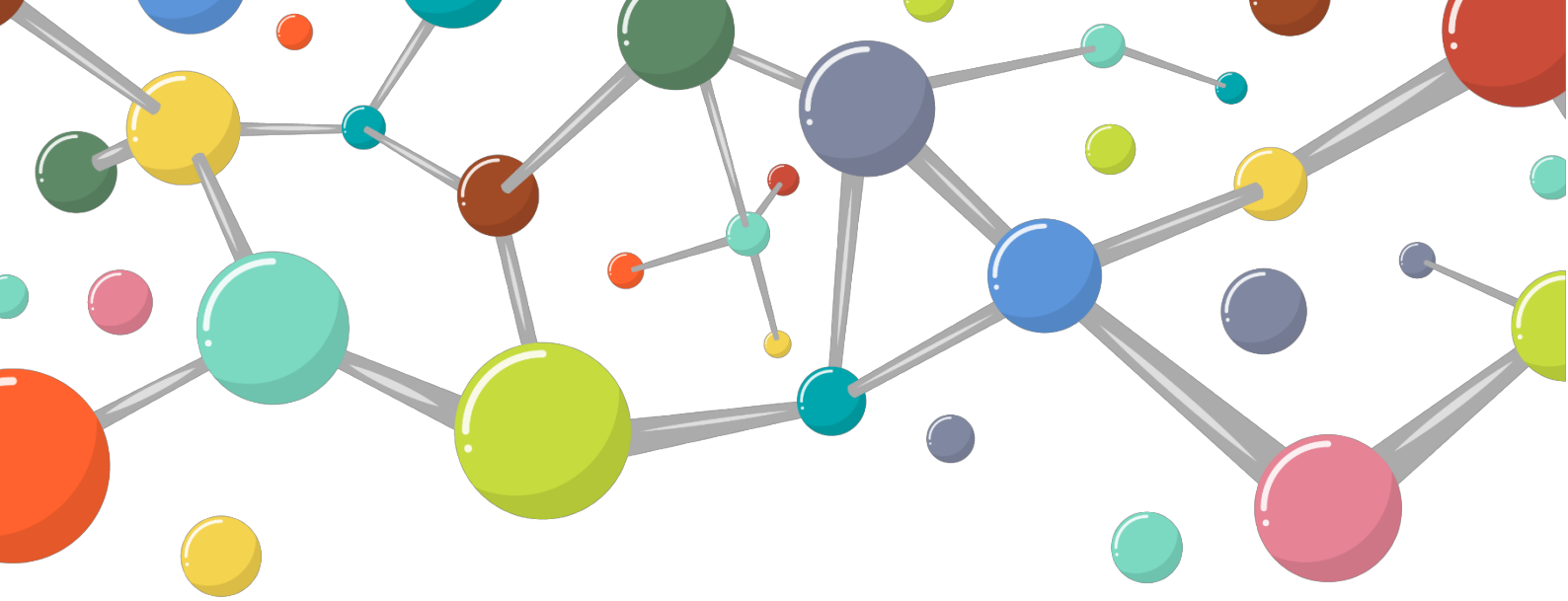
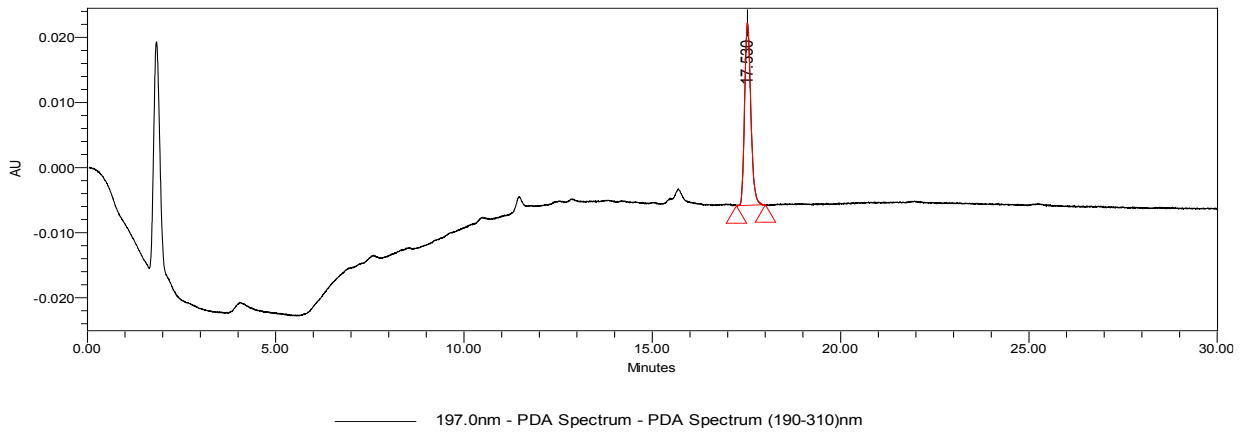
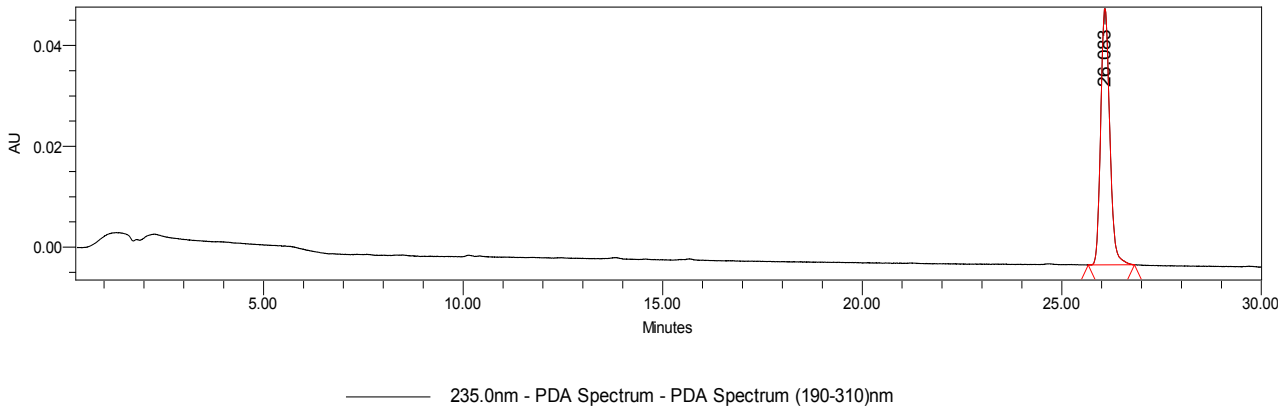


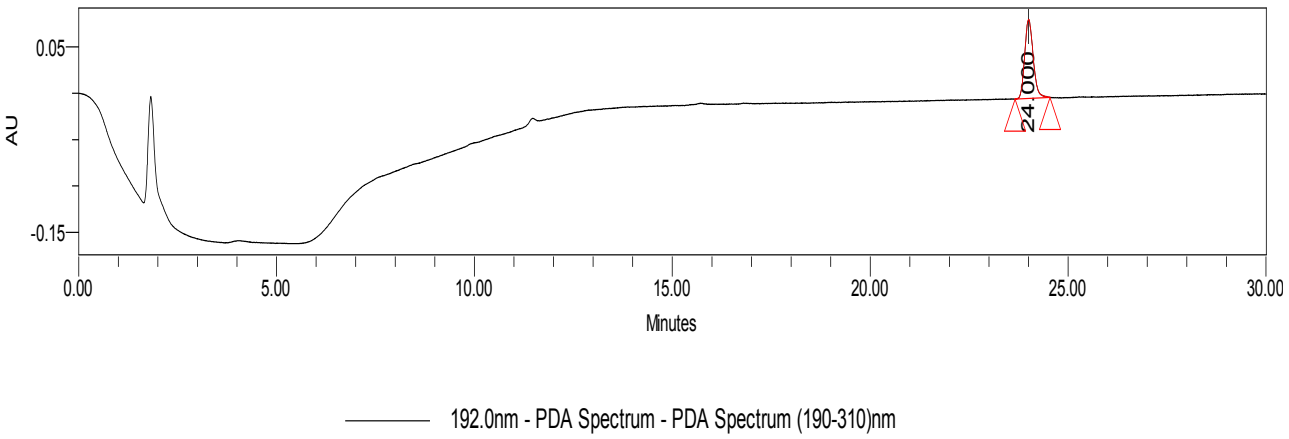
Annexes



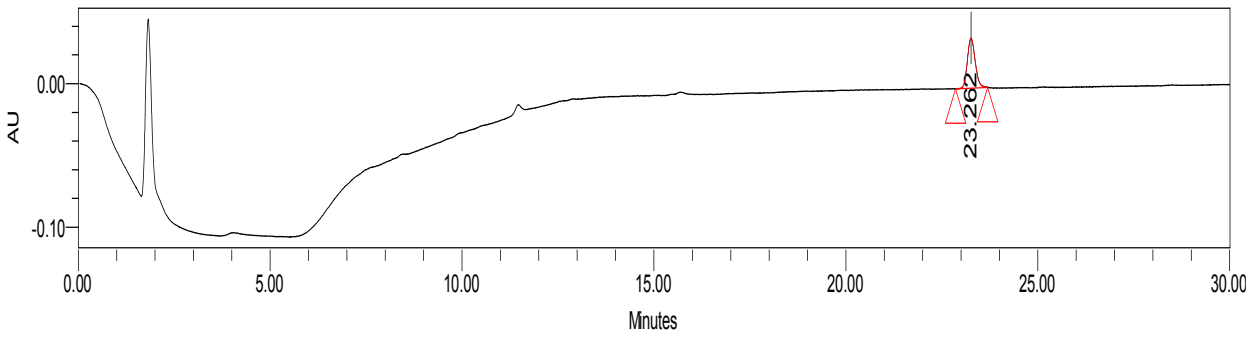
A1: Chromatogram of pure standard chlorpyrifos in ACN.



A2: Chromatogram of pure standard acrinathrin in ACN.

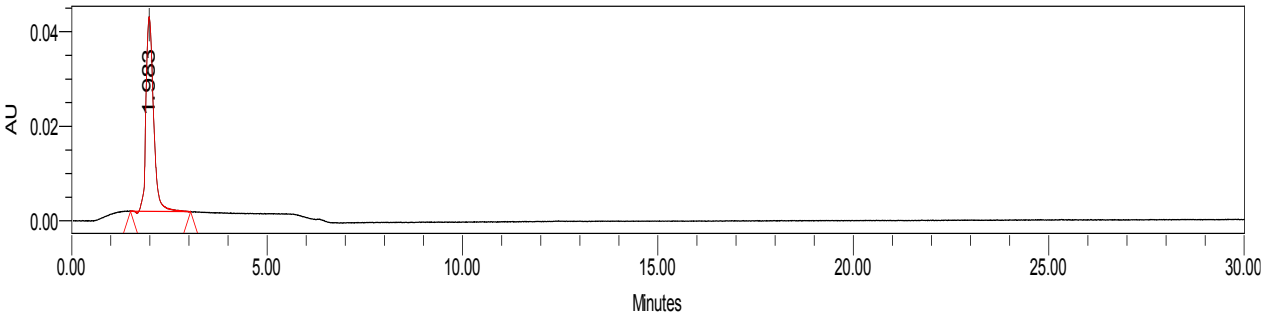


A3: Chromatogram of pure standard deltamethrin in ACN..



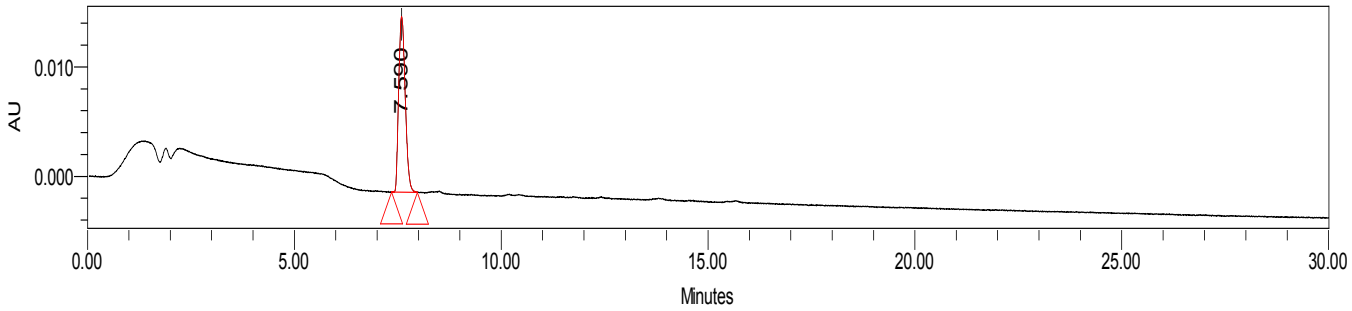
193.0nm - PDA Spectrum - PDA Spectrum (190-310)nm

A4: Chromatogram of pure standard Λ-cyhalothrin in ACN.



301.0nm - PDA Spectrum - PDA Spectrum (190-310)nm

A5: Chromatogram of pure standard thiabendazole in ACN.



242.0nm - PDA Spectrum - PDA Spectrum (190-310)nm

A7: Chromatogram of pure standard cymoxanil in ACN.

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