OPTIMIZATION OF A SPME/GC-MS ANALYTICAL METHOD USING RESPONSE SURFACE METHODOLOGY FOR PESTICIDES MONITORING IN AQUEOUS MATRICES

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ABSTRACT

In this work, is presented the development of an analytical methodology for monitoring six pesticides used in Portugal, namely acetochlor, alachlor, dimethoate, heptachlor, metolachlor and terbuthylazine.

The experimental methodology is based on the optimization of the solid-phase microextraction (SPME) technique which allows the extraction and concentration of the six pesticides from aqueous matrices. After extraction, the detection and quantification of pesticides is made by gas chromatography with mass spectrometry detection (GC-MS).

The extraction step is optimized by using a response surface methodology (RSM) based on an experimental planification defined using a Box-Behnken Design (BBD). Extraction temperature in a range from 50 °C to 70 °C, extraction time from 40 min to 80 min, pH value from 2 to 6 and salt addition (NaCl) from 0% to 20% were studied. The affinity of the compounds for the polyacrylate (PA) and polydimethylsiloxane/divinylbenzene (PDMS/DVB) coated SPME fibers was also studied using a standard mixture of 250 µg/L. The data treatment was carried out using a quadratic equation model which proved to be significant.

The optimum value for each parameter was selected after applying the Box-Behnken Design and considering the maximum total area response value, corresponding to the sum of the areas of each pesticide under study obtained in MS detector under FullScan mode. The optimized extraction conditions were obtained using a polyacrylate (PA) coated SPME fiber with direct immersion of the fiber in the sample mixture with 20% NaCl, a pH value of 2, at 70 °C for 80 min. The temperature of desorption in the GC port was done at 250°C for 4 min.

The operating conditions for the detection and quantification step (GC-MS) were selected based on previous works. The temperature of the column was programmed as follows: initial temperature of 120 ºC being at this temperature 2 min, then from 120 to 190 °C at 15 ºC.min^{-1} being 4 min at 190 °C and then from 190 to 227 ºC at 10 ºC.min^{-1}, with final time of 2.33 min at this temperature.

The mass spectrometry was operated in electron ionization mode (EI). The ion source temperature and the interface temperature were 200 °C and 270 °C, respectively.

Three samples taken from different rivers located in Bragança were analysed in order to implement the developed experimental methodology. This samples were from rivers Onor, Sabor and Fervença. None of the studied pesticides were detected in all the collected river samples.

Keywords: Pollutants, Emerging pollutants, Pesticides, Water contamination, Solid-phase microextraction, Gas chromatography with mass spectrometry.

RESUMO

Neste trabalho é apresentado o desenvolvimento de uma metodologia analítica para a monitorização de seis pesticidas utilizados em Portugal, designadamente acetocloro, alacloro, dimetoato, heptacloro, metolacloro e terbutilazina.

A metodologia experimental baseia-se na otimização da técnica de microextração em fase sólida (SPME) que permite a extração e concentração dos seis pesticidas a partir de matrizes aquosas. Depois da extração, a deteção e quantificação foi feita por cromatografia gasosa com espectrometria de massa (GC-MS).

A etapa de extração é otimizada utilizando uma metodologia de superfície de resposta (RSM) baseada numa planificação experimental definida utilizando um desenho Box-Behnken (BBD). Foram estudados a temperatura e o tempo de extração num intervalo de 50 °C a 70 °C e 40 min a 80 min respetivamente, o valor de pH de 2 a 6 e a adição de sal de 0% a 20%. Foi também estudada a afinidade dos compostos para as fibras para SPME revestidas de poliacrilato (PA) e polidimetilsiloxano/divinilbenzeno (PDMS/DVB) utilizando uma mistura standard de concentração 250 µg/L. O tratamento dos dados foi realizado utilizando um modelo de função quadrática que provou ser significativo.

O valor ótimo para cada parâmetro foi selecionado após a aplicação da ferramenta Box-Behnken Design, considerando o valor máximo da área total, correspondente à soma das áreas de cada pesticida em estudo obtidas no detetor MS no modo FullScan. As condições de extração ótimas foram obtidas utilizando uma fibra para SPME revestida de poliacrilato (PA), com imersão direita da fibra na mistura da amostra com 20% de NaCl, valor de pH de 2, a 70 °C durante 80 min. A temperatura de dessorção na porta GC foi feita a 250 °C durante 4 min.

As condições de funcionamento para a etapa de deteção e quantificação (GC-MS) foram selecionadas com base em trabalhos anteriores. A temperatura da coluna foi programada da seguinte forma: temperatura inicial de 120 °C ficando nesta temperatura 2 min, depois de 120 a 190 °C a 15 °C.min^{-1} ficando

4 min a 190 °C e depois de 190 a 227 °C a 10 °C.min^{-1}, com tempo final de 2,33 min a esta temperatura.

A espetrometria de massa foi operada em modo de ionização de eletrões (EI). A temperatura da fonte de iões e a temperatura da interface foram de 200 °C e 270 °C, respetivamente.

Foram analisadas três amostras retiradas de diferentes rios localizados em Bragança, a fim de implementar a metodologia experimental desenvolvida. Estas amostras foram recolhidas nos rios Onor, Sabor e Fervença. Em todas as amostras analisadas não foi detetada a presença dos pesticidas em estudo.

Palavras-chave: Poluentes, Poluentes emergentes, Pesticidas, Contaminação da água, Microextração de fase sólida, Cromatografia gasosa - espectrometria de massa.

RESUMEN

En este trabajo se presenta el desarrollo de una metodología analítica para el seguimiento de seis plaguicidas utilizados en Portugal, llamados acetocloro, el alacloro, el dimetoato, el heptacloro, el metolacloro y la terbutilazina.

La metodología experimental se basa en la optimización de la técnica de microextracción en fase sólida (SPME) que permite la extracción y concentración de los seis pesticidas a partir de matrices acuosas. A continuación, la detección y cuantificación fue realizada por cromatografía de gases con espectrometría de masas (GC-MS).

La etapa de extracción se optimiza mediante una metodología de superficie de respuesta (RSM) basada en un diseño experimental definido mediante un diseño Box-Behnken (BBD). Se estudiaron la temperatura y el tiempo de extracción en un intervalo de 50 °C a 70 °C y 40 min a 80 min respectivamente, el valor del pH en un rango entre 2 y 6 y la adición de sal entre 0 % y 20 %. También se estudió la afinidad de los compuestos por las fibras SPME recubiertas de poliacrilato (PA) y polidimetilsiloxano/divinilbenceno (PDMS/DVB) utilizando una mezcla standard de 250 µg/L. El tratamiento de los datos se llevó a cabo utilizando un modelo cuadrático el cual demostró ser significativo.

El valor óptimo de cada parámetro se seleccionó tras aplicar la herramienta Box-Behnken Design y, en base al valor máximo del área total, correspondiente a la suma de las áreas de cada pesticida bajo estudio obtenidas en el detector MS en modo FullScan. Las condiciones de extracción óptimas fueron obtenidas utilizando una fibra SPME recubierta de poliacrilato (PA), la cual se sumergió directamente en la mezcla de la muestra con un 20% de NaCl, con un valor de pH de 2, a 70 °C durante 80 min. La temperatura de desorción en el puerto GC se hizo a 250 °C durante 4 min.

Las condiciones operativas para la etapa de detección y cuantificación (GC-MS) se seleccionaron basándose en trabajos anteriores. La temperatura de la columna se programó de la siguiente manera: temperatura inicial de 120 °C permaneciendo a esta temperatura durante 2 min, luego de 120 a 190 °C a 15

°C.min^{-1} permaneciendo 4 min a 190 °C y luego de 190 a 227 °C a 10 °C.min^{-1}, con un tiempo final de 2,33 min a esta temperatura.

La espectrometría de masas funcionó en modo de ionización de electrones (EI). La temperatura de la fuente de iones y la temperatura de la interfaz fueron de 200 °C y 270 °C, respectivamente.

Se analizaron tres muestras tomadas de diferentes ríos situados en Bragança para aplicar la metodología experimental desarrollada. Estas muestras se recogieron en los ríos: Onor, Sabor y Fervença. En los que no se detectó la presencia de los plaguicidas objeto de estudio.

Palabras clave: Contaminantes, Contaminantes emergentes, Plaguicidas, Contaminación del agua, Microextracción en fase sólida, Cromatografía de gases-espectrometría de masas.

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List of Abbreviations

AOP	Advanced oxidation process
BBD	Box-Behnken design
CAS	Chemical abstracts service
CMR	Carcinogenic, mutagenic, toxic to reproduction
DAD	Diode array detector
ECD	Electron capture detector
EDC	Endocrine disrupting chemicals
EP	Emerging pollutants
ESI	Electrospray ionization
FAOSTAT	Statistics division of the food and agriculture organization of the United Nations
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HPV	High production volume chemicals
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
PBT	Persistent, bio accumulative, toxic
PFD	Photometric flame detector
PID	Photoionization detector
POP	Persistent organic pollutant
PPCP	Pharmaceuticals and personal care products
RSM	Response surface methodology
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
TCD	Thermal conductivity detector
UHPLC	Ultra-High-performance liquid chromatography
UV	Ultraviolet
vPvB	Very persistent, very bioaccumulative
PA	Polyacrylate
PDMS/DVB	Polydimethylsiloxane/divinylbenzene
EUROSTAT	European Statistical Office

1. INTRODUCTION

1.1 Motivation

By 2050, the world's population is expected to grow to almost 10 billion, boosting agricultural demand, requiring commensurate shifts in output and adding pressure on natural resources like water, a limited and crucial resource (Food and Agriculture Organization of the United Nations [FAO], 2017). Therefore, the preservation of fresh water is one of the most important challenges for humans, for the care of the environment and living beings.

To achieve this increase in the production of foods derived from agriculture, synthesis of various chemical substances is required and along with the increase in these products, the number of compounds considered potentially dangerous for the environment and health has also increased (Patiño *et al*., 2014). Many of these compounds end up in different water-resources such as lakes, rivers and groundwater, which are sources of freshwater.

The contamination of aqueous matrices with chemical compounds, most of them used in agriculture, is one of the main problems. These pollutants have diverse origins and chemical nature and can be found in a range from nanograms to micrograms per litre, because of these low concentrations they are called micropollutants. Due to the low concentration, great part of them have passed inadvertent in recent decades, and therefore are not regulated by any environmental legislation. Despite this, these contaminants can represent a significant damage to aquatic life and to human health. The World Health Organization determined that 80% of the world's diseases are caused by the unsanitary conditions of water and its inappropriate quality (Carocci *et al*., 2016; Ohoro *et al*., 2019).

Therefore, developing and validating appropriate methods to detect, quantify and remove micropollutants from freshwater is crucial for the well-being of the population and ecosystems (Schwarzenbach *et al*., 2006).

1.2 Objectives

The main purpose of this work is the optimization of a SPME/GC-MS method for the fast monitoring of pesticides in water sources.

The analytical methodology includes the optimization of the solid-phase microextraction (SPME), using a response surface methodology (RSM) based on an experimental planification using a Box-Behnken Design (BBD). After optimization of the complete analytical methodology (SPME/GC-MS), the method developed is applied by monitoring six of the most used pesticides in northeast of Portugal (acetochlor, alachlor, dimethoate, heptachlor, metolachlor and terbuthylazine).

1.3 Report Outline

The report is divided in 7 chapters, including the present one, where it is presented the motivation for the study of pesticides monitoring, the main objectives to be fulfilled and the proposed organization to present and discuss the developed studies.

The first part of this document (chapters 2 to 4) is roughly used to provide an introduction to water contamination and to collect the relevant bibliography that can help to develop and optimize a method for monitoring pesticides, a specific class of pollutants, in aquatic matrices.

In chapter 2, is presented the global problem of water pollution, and inside this issue the general classification of pollutants. Also, the definition of emerging contaminants is presented and some classes of emerging contaminants are detailed with some studies made to monitoring them.

Pesticides are presented in this chapter, as one of the main pollution problems faced by the actual society. The main characteristics and classification of pesticides are revised as well as an overview of worldwide production to show the importance of pesticides for human life.

In chapters 3 is presented a diagram detailing the sources through which pesticides are introduced to water sources, also, the actual removal and degradation methodologies used for pesticides in aqueous matrices. Farther, a

detailed state of the art for the extraction techniques and separation and detection used to monitoring of pesticides, is presented.

In chapter 4, is presented a literature review on the extraction and quantification of some of the most used pesticides.

In chapter 5, the chemicals and materials, as well as the equipment and experimental methodology employed in this study are described. In chapter 6, the experimental results are presented and discussed.

Finally, in chapter 7, the main conclusions of this thesis are discussed. Additionally, some suggestions for future work, are indicated in order to improve and complete the studies developed and presented in this thesis.

2. WATER POLLUTION

For humans and for other living beings, water is a fundamental resource and it is the most abundant compound in human body. All living beings, on average, are 70 percent of water. The stability of the workings of living beings and their ecosystem will be diminished without this resource (Stefanakis and Becker, 2016). While water covers 71 percent of the earth's surface, only 2.5 percent is fresh water that can be used for human needs, and only 0.025 percent is drinkable and sufficient for human consumption (Gleick and Schneider,1996).

It is necessary to highlight, in addition to the shortage of fresh water suitable for use, the alarming rise in the degree of pollution of the various sources of this resource. To a large degree, this is due to human activities, with industrial activity, agriculture and human settlements that have grown due to the increased population growth, being the main causes of water contamination. Fresh water pollutants include, on the one hand, microorganisms that are bacterial, infectious, fungal, and parasitic, and, on the other hand, simple or complex chemicals. Heavy metals, hazardous contaminants, insecticides, fertilizers, petroleum products, harmful industrial residues, soaps, legal and illegal drugs, among others, are among the chemical substances that can pose the greatest risk to the population's health (Carocci *et al*., 2016).

In terms of water pollution, there are various forms of contamination, such as mechanical, thermal, radioactive, bacteriological and chemical; the latter two being the most prominent and common. Although there are many factors that lead to increasing the issue of pollution of bodies of water, it is of interest for this analysis to focus primarily on chemical contamination. This type of contamination comes from sources of different nature, such as organic, through the presence of phenols, solvents, pesticides, which contribute to the presence of compounds that affect the pH of water bodies, and can also be toxic, such as heavy metals (Boelee *et al*., 2019).

2.1 Emerging pollutants

Exponential population growth, bring with it a substantial increase in industrial and agricultural activity, activities that add their waste to that generated by the growth of large urban centres, thus generating a concentration of different types of contaminants, which makes difficult to handle such waste properly (Boelee *et al*, 2019). Domestic, industrial, and agricultural emissions in water lead to a polluted state with thousands of substances and their residues, including nutrients, heavy metals, pesticides (insecticides, herbicides, fungicides), and pharmaceuticals. In general, pollutants from urban areas and industry pollute the atmosphere as point sources, while agricultural chemicals can produce diffuse emissions, requiring somewhat different approaches to risk management. Effluent treatment is insufficient in many countries for example, in countries in Africa and Asia, where 10 of the most polluted rivers are concentred, including the Nile (Egypt) and the Yangtze (China) (Boelee *et al*., 2019). Persistent and bio accumulative chemicals will build up in the food chain, especially in water-related food, such as, fish and seafood, and might pose health effects through food consumption (Schwarzenbach *et al*., 2010).

Environmental responses involve ecosystem conservation, whereby water supplies and their functions are harmonized. Intensive monitoring of water quality, where possible, may help the detection of areas for wastewater treatment, for example at industrial sites or community level. Driver-focused interventions include green development programs to reduce environmental impacts, better spatial planning and efficiency-enhancing infrastructure investments.

Because of the great concern that their effect on the environment creates, the emerging contaminants have recently become the subject of research for environmental and science organizations (Besbes *et al.*, 2018). Emerging pollutants are those pollutants to which no particular attention was previously given, since many of them escape the regulatory frameworks established by environmental control agencies, causing their presence and concentration in bodies of water to increase, which cannot continue to be ignored by society as a whole. The use of pollutants in agriculture as pesticides facilitates their

bioaccumulation in flora and fauna. In the long run, this effect not only harms the specimen where accumulation occurs, but also interferes with others by migration through the food chain (Mostafalou, 2016).

Emerging pollutants, depending on the origin, are divided into several types: pharmaceuticals, personal care products (PPCPs), endocrine disrupting chemicals (EDCs), hormones and steroids, surfactants and surfactant metabolites, flame retardants, pesticides, industrial additives, nanomaterials and gasoline additives, industrial additives and agents, perfluoronated compounds, antiseptics (Patel *et al*., 2019). The emerging contaminants are categorized relatively to their properties (Stefanakis and Becker, 2016):

- CMR: carcinogenic, mutagenic, toxic to reproduction.
- EDC: endocrine disrupting chemicals.
- PBT: persistent, bioaccumulative, toxic.
- vPvB: very persistent, very bio accumulative.
- POP: persistent organic pollutant (based on their environmental properties).
- PPCP: pharmaceuticals and personal care products.
- Priority pollutants (regulated).
- Xenobiotics, exotics (foreign vs endogenous pollutants).
- Toxicants, toxins, toxics (based on the overall toxicity).
- HPV: high production volume chemicals.

The following table shows the results of some published studies carried out for emerging micropollutants in different aqueous matrices.

Table 1. *Some studies on emerging micropollutants found in the literature.*

Class	Pollutant	Matrix	Method of extraction/analysis	Average concentration (ng/L)	Year	Reference
PPCP	Azithromycin	Surface water	SPE/UHPLC-MS-MS	30	2018	(Barbosa *et al*., 2018)
	Diclofenac	Sewer water	SPME/GC-MS	300	2018	(López-Serna *et al*., 2018)
EDC	Bisphenol A	Treated Water	SPE/HPLC-DAD	5	2013	(Santana, 2013)
POP	Atrazine	Treated Water	SPE/HPLC-MS/MS	2.65	2013	(Santana, 2013)
		Surface Water	SPE/UHPLC-MS/MS	557	2018	(Barbosa *et al*., 2018)

It is possible to observe in Table 1, that diverse classes of micropollutants were found in the last few years in different water matrices.
On the other hand, the solid-phase extraction was the method selected for the extraction step and the methods most used for the separation and quantification step were liquid or gas chromatography with mass spectrometry.

2.2 Pesticides

According to the Food and Agriculture Organization of the United Nations (FAO) database, from 1996 till 2016, the use of pesticides worldwide increased by 46% due to the human population increase (FAO, 2017). Pesticides can be synthetic chemical or natural compounds, and they are mainly used in the control of diseases, pests and weeds (Stefanakis and Becker, 2016). There are more than 800 types of pesticides that are used to control these issues in the agriculture production process. Depending on the physicochemical properties of the compounds, degradation products, and the characteristics of the soils most of these substances degrade although in other cases some traces affect the whole ecosystem by polluting the soil, air, and water resources and also can be transferred to humans by entering in the food chain, being potentially harmful to human health (Jurado *et al.*, 2012; Holt, 2013; Kaur, 2020). According to the World Health Organization as many as three million people are poisoned by pesticides per year.

2.2.1 Characteristics and classification

Pesticides are classified according to some of their main characteristics, such as toxicity (through median lethal dose or median lethal concentration), half-life (permanent, persistent, moderately persistent and non-persistent) and chemical structure (organochlorines, organophosphates, inorganics). Another way most used to classify them is according to the type of pest they control, differing mainly in herbicides, fungicides or insecticides as can be seen in Tables 2, 3 and 4 (Ramírez and Lacasaña, 2001).

Tables 2, 3 and 4 presents some properties of different herbicides, fungicides and insecticides respectively.

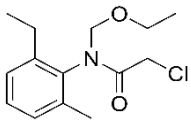
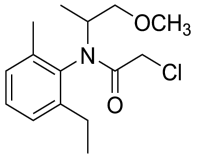
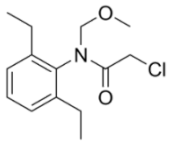
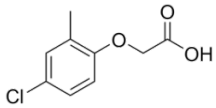
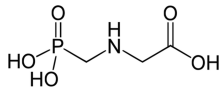
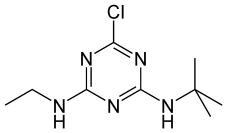
The octanol-water partition coefficient, K_{ow}, represents the tendency of a chemical to partition between an organic phase (due to n-octanol is similar in polarity and hydrophobicity to the major components of the cell membrane) and an aqueous phase, and provides information in an indirect way on the solubilization and distribution in a living organism or soils. Pollutants with high Log K_{ow} values (> 4-5) are more likely to be absorbed into solid-phase such as soils (López, 2007; Badii *et al*., 2005).

Another parameter presented (see Table 2) is solubility, the higher the water solubility, the greater the tendency to remain dissolved. Higher water-soluble compounds are likely to enter the aquatic environmental through run-off; substances with an aqueous solubility more than 500 mg/L are highly mobile in soils and other ecosystems. On the other hand, those with lower aqueous solubility tend to precipitate, to partition to soils. Substances with < 25 mg/L are very insoluble (López, 2007; Badii *et al*., 2005).

2.2.1.1 Herbicides

Herbicides are a class of pesticides used in agriculture to eliminate weeds or to reduce its density, promoting the growth of desirable species (Holt, 2013). Table 2 presents some properties of the most used herbicides, such as solubility in water at 20°C, log K_{OW} and boiling point at 1 atm.

Table 2. *Chemical structure and psychochemical properties of some herbicides.*

Name	Chemical structure and formula	Molar Mass (g/mol)	Solubility in water at 20°C (mg/L)	Log K_{ow}[(1)]	Boiling point (°C) at 1 atm	CAS Number
Acetochlor	 $C_{14}H_{20}ClNO_2$	269.8[a]	282[a]	4.14[a]	172[a]	34256-82-1[a]
Metolachlor	 $C_{15}H_{22}ClNO_2$	283.8[a]	530[b]; 530[a]	3.4[a]	-	51218-45-2[a]
Alachlor	 $C_{14}H_{20}ClNO_2$	269.8[a]	240[c]; 240[a]	3.09[a]	100[a]	15972-60-8[a]
MCPA	 $C_8H_6Cl_2O_3$	200.6[a]	-	-0.81[a]	Decomposes before boiling (290°C)[a]	94-74-6[a]
Glyphosate	 $C_3H_8NO_5P$	169.1[a]	10500[a]	-3.2[a]	Decomposes before boiling (200°C)[a]	1071-83-6[a]
Terbuthylazine	 $C_9H_{16}ClN_5$	229.7[a]	5[d]; 6.6[a]	3.4[a]	Decomposes before boiling (224°C)[a]	5915-41-3[a]

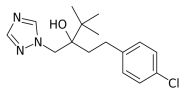
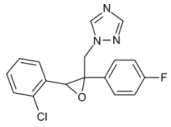
Note.[a] Lewis *et al*. (2016). [b] Wauchope *et al*. (1992). [c] Chesters *et al*. (1989). [d] Yalkowsky *et al*. (2016).

2.2.1.2 Fungicides

Some pesticides are used to prevent the growth of fungi or fungal spores, these compounds are named as fungicides. They protect crops as well as fruits,

vegetables and tuberculous during storage (Singh *et al*., 2019). Table 3 presents some properties of the most used fungicides.

Table 3. *Chemical structure and psychochemical properties of some fungicides.*

Name	Chemical structure and formula	Molar Mass (g/mol)	Solubility in water at 20°C (mg/L)	Log K_{ow}	Boiling point (°C) at 1 atm	CAS number
Tebuconazole	 $C_{16}H_{22}ClN_3O$	307.8[a]	36[b]; 36[a]	3.7[a]	Decomposes before boiling (350°C)[a]	107534-96-3[a]
Epoxiconazole	 $C_{17}H_{13}ClFN_3O$	329.8[a]	7.1[a]	3.3[a]	310[a]	135319-73-2[a]

Note.[a] Lewis *et al*. (2016). [b] Tomlin (2006).

2.2.1.3 Insecticides

Insecticides are a class of pesticides used to control insect pests. It has been demonstrated that in many cases the insects generate a resistance to the insecticides, like with the mosquito which transmit malaria agents to humans (Connor *et al*., 2011). A list with the chemical structure and physicochemical properties of some of the most used insecticides is presented at Table 4.

Table 4. *Chemical structure and psychochemical properties of some insecticides.*

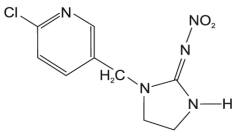
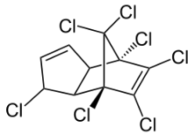
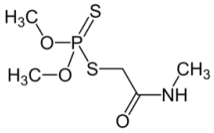
Name	Chemical structure and formula	Molar Mass (g/mol)	Solubility in water at 20°C (mg/L)	Log K_{ow}	Boiling point (°C) at 1 atm	CAS number
Imidacloprid	 $C_9H_{10}ClN_5O_2$	255.7[a]	610[b]; 610[a]	0.57[a]	Decomposes before boiling (230°C)[a]	138261-41-3[a]

Table 4. *Chemical structure and psychochemical properties of some insecticides (Continuation).*

Name	Chemical structure and formula	Molar Mass (g/mol)	Solubility in water at 20°C (mg/L)	Log K_{ow}	Boiling point at (°C) 1 atm	CAS number
Heptachlor	 $C_{10}H_5Cl_7$	373.3[a]	0.06[a]	5.44[a]	135[a]	76-44-8[a]
Dimethoate	 $C_5H_{12}NO_3PS_2$	229.3[a]	25000[c]; 25900[a]	0.75[a]	Decomposes before boiling (113 °C)[a]	60-51-5[a]

Note. [a] Lewis *et al*. (2016). [b] Tomlin (2006).[c] Fernandes (2014).

2.2.2 Overview of the pesticides production worldwide

It is public knowledge that the pesticides industry is a widely spread business with enormous profitability globally. In Figure 1, it can be appreciated that 43,2 % of world consumption of pesticides corresponds to Europe and America and the other 50 % to China that is the country which uses the highest quantity of pesticides. Every year more than 2 million tons of pesticides are employed to take care of the crops worldwide (Sharma *et al*., 2019; FAO, 2020).

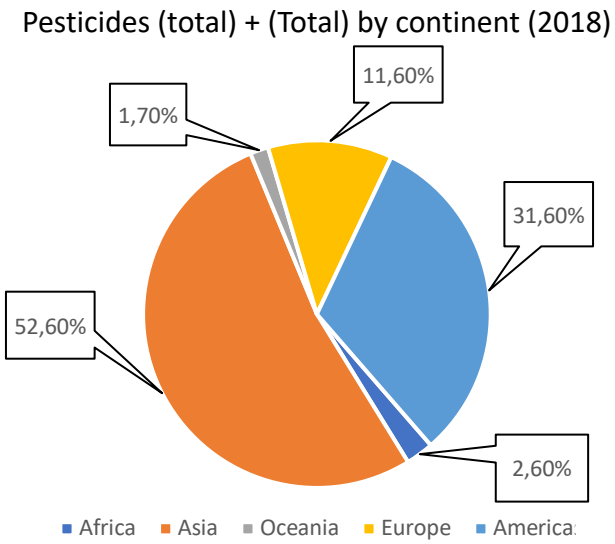


Figure 1. *Pesticide's consumption worldwide in 2018.*

Note. Adapted from *Pesticides (total) + (Total) by continent (2018)*, Food and Agriculture Organization of the United Nations, 2020, FAO.

(Source: http://www.fao.org/faostat/en/?#data/RP/visualize).

The pesticide industry is made up of a number of factories which have process plants around the world, some of the competitors are Syngenta International AG, Bayer AG, BASF SE, DowDuPont Inc., Monsanto Company, among others. This type of industry requires a large investment in technology and it is a very competitive industry due to the appearance, in pests, of several species that are resistant to one product, making the development of others necessary. The need of a more effective product against a pest, displaces, in a short time, another established because the cost/performance ratio is decisive. Without the use of pesticides, 50% of the crops would be lost. That, is why this sector, together with the pharmaceutical sector, has the highest research costs (up to 8% of gross income).

According to the FAOSTAT, in 2018 the export value of pesticides was concentrated between the Europe and Asia, with 48% and 34% respectively (FAO, 2018). In addition, according to the European Statistical Office (EUROSTAT), between 2011-2018, in the EU, more two out of three parts of pesticide sales were made by France, Spain, Italy and Germany. Also, these are the principal agricultural producers in the EU, counting with 51% of the total EU

farming area (total area taken up by arable land, permanent grassland, permanent crops and kitchen gardens) and 49% of the total EU arable land (land worked regularly, generally under a system of crop rotation) (EUROSTAT, 2020).

3. REMOVAL OF PESTICIDES IN WASTEWATERS TREATMENT PLANTS

3.1 Importance of pesticides detection in water

Despite of the numerous practical applications for pesticides, they present several long-term harmful effects on living organisms. It should be noted that agrochemical products generate metabolites, products of degradation, which interact with organic matter and under the conditions of the ecosystem they can be potentially toxic (Vidal *et al*., 2000). They are also mobile and stable, so they can persist in the environment for long time being capable of bioaccumulation (Biziuk *et al*., 1996; Tankiewicz *et al*., 2010). Figure 2 shows the different ways in which pesticides are introduced in aqueous systems, such as:

I. Inefficient operation of wastewater treatment plants.
II. Industry untreated sewage.
III. From agriculture.
IV. Landfill sites, hazardous waste disposal sites.
V. Atmospheric precipitation, dusts and aerosols.

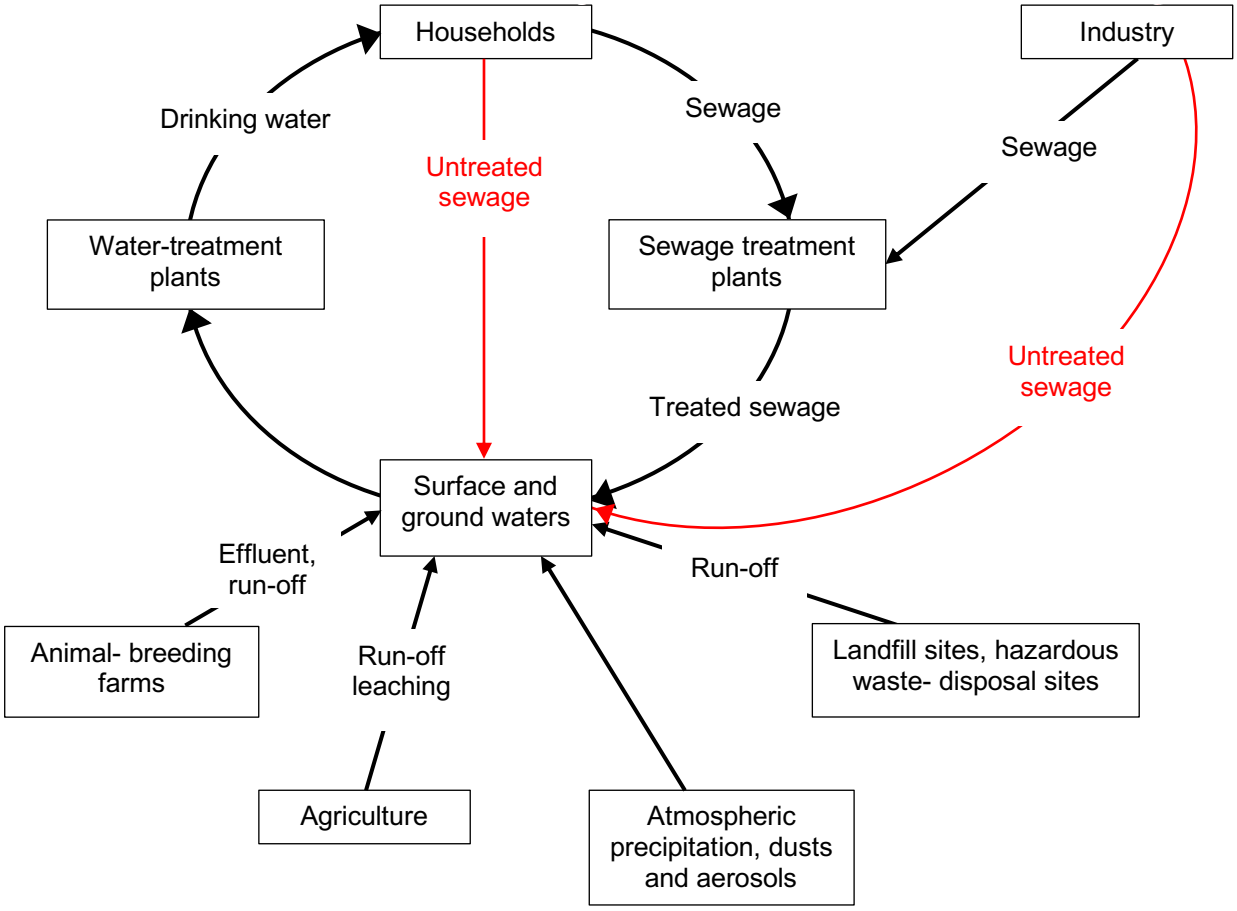


Figure 2. *Sources and fates of pesticides in the aquatic environment.*
Note: Adapted from Biziuk *et al*., 1996.

Although emerging pollutants concentration in the environment may be small, they present long-term effects after continuous exposure.

Wastewater treatment plants have been designed for the removal of certain types of organic pollutants, especially those specified in official regulations. However, the study of new pollutants such as the emerging contaminants, which have a wide range of chemical properties, require advanced treatments for the safe incorporation of wastewater into the environment.

The degree to which a compound can be removed during water treatment is influenced not only by the chemical and biological properties of the compound, but also by the characteristics of the water, operating conditions, and treatment

process type used. Several methods for removal emerging pollutants (EP) from water are briefly discussed below.

3.2 Removal by physical adsorption processes

Adsorption is a physical process which can be applied efficiently in the removal of organic matter in aqueous solution. Adsorption is a preferred process due to its high efficiency and profitability, as well as the availability of various adsorbents (activate carbon, agricultural activated waste adsorbents, industrial waste adsorbents, inorganic natural adsorbents, graphene and graphene oxide, carbon nanotubes, among others), as it is versatile and widely used for the treatment of municipal and industrial wastewater and for making potable water (Flores-Cano *et al*., 2013).

3.3 Removal by biological degradation processes

This method uses microorganisms capable of digesting pesticides, but due to the toxicity of pesticides on different bacteria and fungi, it is not always possible to digest pesticides using microorganisms (Goodwin *et al*., 2017). The advantages are low cost and mild operational conditions. For water treatment it can be used pure cultures, mixed cultures, activated sludges, among others.

3.4 Removal by chemical processes

To remove EP by chemical processes, a variety of chemical reactions are involved in the hydrolysing contaminants into safer chemicals. Coagulation and advanced oxidation processes (AOPs), including ozonation and Fenton treatments, are the major chemical methods. In conjunction with photo-catalysis and/or membrane techniques, chemical treatments are typically used (Salimi *et al*., 2017; Lin *et al*., 2020).

3.5 Pesticides monitoring in waters

3.5.1 Extraction procedures and analytical methods for pollutants

Effective analytical methods capable of detecting traces of these compounds, in complex environmental matrices, are needed in order to properly track organic pollutants in the aquatic environment and to allow for risk assessment (Maldaner and Jardim, 2012).

Usually, the analytical chain involves the following five steps: 1) sampling; 2) preparation of the sample, including pre-treatment, extraction, concentration and cleaning; 3) separation, generally chromatographic; 4) detection, which may include optical, spectrometric, electrochemical, radiochemical, immunoassay, etc.; 5) data processing and its interpretation (Stashenko and Martínez, 2011).

3.5.1.1 Extraction procedures

The extraction is the first step and the objective is to prepare the sample. The sensitivity of the analysis method can be improved and the minimum detection/quantification levels of the respective system can be reached by removing compounds that can interact with the target compounds, having the sample “clean”, and thus allow these compounds to be concentrated. This step is important when the compounds to be analysed are in the trace concentration levels. There are different types of extraction processes and the selection of the more suitable technique depends of a large range of parameters like the nature of the matrix (origin, aggregation state, stability), the objective of the analysis and the physicochemical properties of the compounds in study (Stashenko and Martínez, 2011).

In liquid-liquid extraction, the sample is dispersed or divided between two immiscible solvents in which the target pollutant and matrix have different solubilities. The selection of the solvents is really important since the selectivity and efficiency are key parameters for the efficiency of the extraction process (Fernández Castro, 2018). Today, several methods of sample preparation, especially for the analysis of pesticide residues in different matrices, are designed

to minimize the use of solvents in accordance with the theory of green chemistry because these organic compounds produce issues related to pollution, sometimes are expensive and the procedures are time consuming.

Purge and trap are commonly used to remove volatile organic compounds, followed by gas chromatography, from aqueous samples. The technique involves the injection of an aqueous sample into a glass sparging vessel (normally 5 mL). At a given flow-rate and time, the sample is then purged with high purity nitrogen (volatile species move from the aqueous to the vapour phase). The volatile organics extracted are then moved to a pit at ambient temperature. This is accompanied by the process of desorption. The trap is easily heated in this step to desorb the trapped volatile organic compounds in a narrow band. Desorbed compounds are transferred to the injector of a gas chromatograph for separation and detection through a heated transfer line (Fernandez Villarrenaga *et al.,* 2006).

Solid-phase extraction (SPE), includes bringing a solid-phase or sorbent into contact with a liquid or gaseous sample where the analyte is selectively adsorbed onto the solid-phase surface. The solid-phase is then separated and other solvents (liquids or gases) are applied to it. It used a first solvent to make a wash to eliminate possible adsorbed matrix components; and the eluting solvent is finally brought into contact with the sorbent to selectively desorb the analyte. This solvent is then collected for analysis. However, the low breakthrough volumes for more hydrophilic analytes, the need to pre-filter the real-life samples to prevent clogging and eventual loss of analytes, and the probability of interferences such as plasticizers present in the sorbent material may be some of the problems encountered when using SPE (Żwir-Ferenc and Biziuk, 2006).

As an alternative to conventional sample preparation techniques, solid-phase microextraction (SPME) has been introduced because it provides a simple, efficient, solvent-free, and sensitive pre-treatment process which can be easily combined with different separation techniques. The mechanism involves the equilibrium sorption of analytes onto the surface of a coated-silica fiber or stationary phase (polyacrylate, polydimethylsiloxane, etc.), the coat is a hydrophobic polymer. A needle-like device is used to fix the fiber. This device is connected to a stainless-steel tube used to provide greater mechanical resistance. The extraction is carried out by immersing the fiber in the gaseous or

liquid medium or by sampling the analytes above the examined medium from the headspace. According to their affinity for the solid-phase, analytes come into balance with the fiber. This equilibrium is reached between the concentration of the analyte in the sample and the amount of analyte adsorbed on the fiber, depending on the distribution coefficient. This step is followed by the desorption of the analytes into an effective separation and quantitation instrument. Solid-phase microextraction coupled with chromatographic techniques is gaining wide applicability as an analytical technique. Commercially available SPME fibers are expensive and have limited lifetime, since they tend to degrade with increased usage. The difference in length and thickness of SPME fiber coatings may result in variation of analyte enrichment from fiber to fiber (Chang *et al*., 2016).

3.5.1.2 Separation and detection methods

Chromatography is a stablished method used to separate complex samples into their individual components. It is classified into two main types based on the physical state of the mobile phase used, liquid chromatography (LC) and gas chromatography (GC).

The separation of the individual compounds in GC is based on their distribution between the stationary phase, which might be liquid or solid adsorbent (alumina, silica, among others), and the mobile phase (an inert gas called carrier gas). There are two general types of columns of gas chromatography, the capillary columns and the packed columns. The first type is the most used due to their great efficient. For the study of thermostable, volatile or semi-volatile, non or medium polar compounds and for compounds that are non-volatiles but are easily derivable compounds, GC is used preferentially to LC (Pássaro Carvalho *et al*., 2016).

There are different types of liquid chromatography such as, Ultra High-Performance Liquid Chromatography (UHPLC) and High-Performance Liquid Chromatography (HPLC) both are separation techniques for mixtures of low or non-volatile products. In LC the sample is introduced into the injection port where it is drawn by a solvent or a mixture of solvents (mobile phase) towards a chromatographic column. The different interaction of the analytes with the mobile

phase and the packing of the column allows the separation of the components of the mixture for detection, characterization and quantification using different types of detectors. These phases determine the type of liquid chromatography, such as liquid/liquid chromatography, adsorption chromatography, ion chromatography, exclusion chromatography, among others. When the polarity of the solid-phase is modified the process is called reverse phase chromatography. HPLC is suitable for almost all compounds soluble in organic solvents. Liquid chromatography is often preferred over gas chromatography because it does not require derivatization of the compounds, the mobile phase and the stationary phase are actively involved in chromatographic separation in HPLC, while the mobile phase only acts as a carrier gas for the analytes in GC. More selective interactions are therefore possible with the sample molecules, also, in HPLC, milder temperatures are used in the process of separation and there is a wide variety of stationary phases available on the market, also different types of packaging, which allows a wide range of specificity (Costa, 2015; Rohner Hernández, 2018).

Once the components of the sample have been separated by the column, at the outlet, a detector is available, capable of signalling the elution of a component and providing a signal proportional to the amount of substance that passes through it. The detection systems differ according to the type of chromatography. The most common detectors in liquid chromatography are the ultraviolet (UV) detector and the fluorescence detector (Mitra et al., 2018).

In gas chromatography, there are different types of detectors, them are classified by mechanism as ionization-based: flame ionization (FID), thermionic ionization, electron-capture (EDC), photoionization (PID), and barrier discharge; bulk physical property: thermal conductivity (TCD); optical: flame photometric, chemiluminescence, atomic emission; and electrochemical: electrolytic conductivity detectors. For example, the ECD is used for halogen detecting such as organochlorine pesticides (Poole, 2021)

Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are increasingly used for the identification and structural determination of compounds since with the use of others detectors, normally is not possible to identify and describe the structure of the compounds precisely.

• Mass spectrometry

With mass spectrometry it is possible to measure the mass/charge ratio (m/z) of a series of ions in the gas phase. The mass spectrometric detection and determination process comply the following steps: 1) ionization of the sample and creation of ions in the gas phase, 2) separation of the ions as a function of the m/z ratio, 3) measurement of the number of ions for each m/z value. MS can be coupled to either a liquid or gas chromatography system (Caño Carrillo, 2020).

The analyser is one of the most important elements of the mass spectrometer, enabling the various ions produced to be categorized according to their m/z ratio. The analysers can be categorized into four groups: electrical or magnetic analyser, time of flight (TOF), quadrupole (Q) and ion traps (Caño Carrillo, 2020).

• Tandem mass spectrometry

As an analytical tool to classify and measure compounds in complex mixtures, tandem mass spectrometry (MS/MS) is one of the best techniques (Dass, 2007).

The word tandem comes from the English word tandem, whose initial meaning is “two-seat bicycle”, it also has the meaning of “a set of two elements that complement each other”.

An MS/MS kit is made up of six basic components: 1) sample introduction system; 2) ionization source; 3) first mass analyser (MS1); 4) collision chamber or cell; 5) second mass analyser (MS2); 6) detector (Dass, 2007).

To carry out the method, a characteristic ion (precursor ion) of the substance to be analysed has to be selected. The method follows the following steps: 1) the sample is introduced, then it undergoes a gentle ionization; later the components are separated and ordered according to their m/z in a MS1. After that the ions pass through the collision cell where fragments are generated as a product of a collision with Argon; the generated fragments pass through the MS2, which can be correlated with the intact molecules produced in the MS1. The results generated are recorded (Dass, 2007; Fernández Lainez, 2009).

4. EXTRACTION TECHNIQUES AND ANALYTICAL METHODS USED FOR PESTICIDES

A literature review on the extraction and quantification of some pesticides is presented in this section.

In Table 5 are presented some studies carried out in different aqueous matrices, on the presence of pesticides.

Table 5. *Some studies on pesticides concentration in water found in the literature.*

Class	Name	Methods of detection and analysis	Concentration found µg/L	Country	Reference
Herbicides	Acetochlor	SPE/GC-ECD	0.02-0.41	Serbia	(Lazič *et al*., 2013)
	Metolachlor	SPME/GC-MS	17.0	Portugal	(Batista *et al*., 2002)
		SPME/GC-MS	3.7	Portugal	(Hmida, 2019)
	Alachlor	SPME/GC-MS	8.0	Portugal	(Batista *et al*., 2002)
		SPE/GC-ECD	0.05-0.78	Serbia	(Lazič *et al*., 2013)
	Terbuthylazine	SPE/GC-MS	0.02–1.65	Portugal	(Azevedo *et al*., 2000)
		SPME/GC-MS	26.6	Portugal	(Hmida, 2019)

Table 5. *Some studies on pesticides concentration in water found in the literature (Continuation).*

Class	Name	Methods of detection and analysis	Concentration found µg/L	Country	Reference
Fungicides	Tebuconazole	Without pre-concentration and/or clean up UPLC-ESI-MS/MS	0.38	Brazil	(Assalin *et al*., 2017)
	Epoxiconazole	SPE/HPLC-MS	0.006	Brazil	(Welter, 2018)
Insecticides	Imidacloprid	Without pre-concentration and/or clean up UPLC-MS/MS	1.5	Brazil	(Assalin *et al*., 2017)
		SPE/UHPLC–MS/MS	Below the LOQ	Portugal	(Barbosa *et al*., 2018)
	Dimethoate	SPE-/GC-MS	1.2	Portugal	(Batista *et al*., 2002)
		SPME/GC-MS	58.7	Portugal	(Hmida, 2019)

In the following sections a detailed description of the techniques used for the extraction and analysis of the pesticides named above is made.

4.1 Acetochlor and alachlor

Lazič *et al*., in 2013, carried out the extraction of the herbicides, acetochlor and alachlor, using SPE. Before the extraction, the cartridge was preconditioned with 5 mL of methanol, followed by 5 mL of ultrapure water, at a flow rate of 2 $mL.min^{-1}$. Then, vacuum was applied to filter the solution at rate of 10 $mL.min^{-1}$. After this, the cartridges were dried, and the elution of the pesticides was

performed with 6 mL of dichloromethane/n-hexane (40:60, v/v) and evaporated to dryness. The extract was dissolved in 1 mL of methanol, ultrasonically homogenized and analysed by GC-ECD. The GC volume of injection was 3 µL and a splitless injection mode was used. The carrier gas was helium was at a flow rate of 1 $mL.min^{-1}$. Another extraction method and analysis of alachlor is presented by Batista *et al*., (2002), in the section 4.2.

4.2 Metolachlor and dimethoate

For the extraction of alachlor, metolachlor, dimethoate and other pesticides, Batista *et al*., (2002) used a six-station manifold extraction disk apparatus from 3M Empore and membrane extraction disks, with 47 mm diameter and 0.5 mm thick. They placed each cartridge in the SPE apparatus and 5 mL of a solvent mixture prepared with ethyl acetate and dichloromethane (1:1, v/v), then the cartridge was conditioned with 5 mL of methanol and 10 mL of deionized water. The water sample (1 L) was loaded and the SDB-phase was rinsed with deionized water (10 mL). After that, vacuum was used during 20 min to dry some residual water that could cause degradation of some pesticides. Elution tubes were used to collect the pesticides trapped in the cartridge (after sample extraction) using 3 x 5 mL of solvent mixture made of ethyl acetate and dichloromethane (1:1, v/v) for elution. Extracted residues were transferred to a graduate tube and evaporated to a final volume of 200 mL for GC-MS analysis, after evaporation of the solvent to 1 mL using a stream of nitrogen.

To perform the GC-MS analyses, a Varian ChromPack CP-3800 Gas Chromatograph coupled with a Saturn 200 GC/MS equipped with an Ion Trap detector and a J&W DB-5MS 30 m x 0.25 mm Low Bleed/MS column was used. The carrier gas employed was helium at 12 psi. The temperature of the injector, interface and mass spectrometric detector were, respectively, 270, 260 and 190 °C and the temperature of the column was programmed from 50 to 170 °C at $10°C.min^{-1}$, from 170 to 180 °C at 1 $ºC.min^{-1}$, from 180 to 220 °C at 5 $°C.min^{-1}$, remaining 6 min at this temperature and then from 220 to 240 °C at 15 $°C.min^{-1}$, remaining at 240 °C more 4 min. Ionization mode was done by Electronic Impact (EI).

4.3 Terbuthylazine

Azevedo *et al*., in 2000, reported that terbuthylazine can be extracted by SPE and analysed by GC-MS. For the extraction, the cartridges were conditioned sequentially with 6 mL of dichloromethane, 6 mL of acetonitrile and 6 mL of water at a flow rate of 30 $mL.min^{-1}$. After, an aliquot of 200 mL of sample was taken and passed through the cartridge at a flow-rate of 6 $mL.min^{-1}$ and then washed with water. The elution step was carried out with 2.5 mL of acetonitrile and dichloromethane (1:1) and then by 3.2 mL of dichloromethane at a flow rate of 1 $mL.min^{-1}$. A stream of nitrogen was used to evaporate the solvent in order to obtain the initial mobile phase conditions for the injection into the GC-MS. Helium was used as the carrier gas and the following conditions were used: fused-silica column HP-5MS (30 m length x 0.25 mm internal diameter x 0.25 µm film). The temperature program was 60 ºC for 1 min, an increase to 175 ºC at 6 $ºC.min^{-1}$, then from 175 to 240 ºC at 3 $ºC.min^{-1}$ for 5 min, A new step from 240 to 300 ºC at 7 $ºC.min^{-1}$, for 1 min, splitless mode, the temperature of interface was 270 ºC, the source and injector temperatures were 200 ºC and 250 ºC, respectively.

4.4 Tebuconazole

Assalin *et al*., in 2017, determined the fungicide tebuconazole and other micropollutants like imidacloprid without a previous concentration and/or clean-up. For the study, was used a filter of 0.22 µm pore to retain impurities in the samples and then 20 µL of an aqueous formic acid solution 5% (v/v) was added to an aliquot of 1 mL of sample, directly into the injection vial. For the analysis of the components an ultra-efficiency liquid chromatograph coupled to a triple quadrupole mass spectrometer with electrospray ionization source was used. The collision gas was Argon (99.8%) with a flow of 0.2 $mL.min^{-1}$. As mobile phase, water containing formic acid 0.1% (A): ethanol (B) in the initial proportion 70% (A), in relation to the flow-rate of the mobile phase (0.30 $mL.min^{-1}$), reducing to 10% (A) in 10 min, restoring the initial balance after 10.2 min totalling 10.3 min of running. Capillary 3KV, extractor 3.0 V, source temperature 120ºC, temperature

and flow of desolvation (N_2) 400 ºC and 500 $L.hr^{-1}$ were the parameters used respectively.

The column used was a BEH C18 (100 mm x 2,1 mm x 1,7 µm) and the volume of injection was 20 µL. The mass spectrometer was operated in MS/MS mode.

4.5 Imidacloprid

Barbosa *et al*., in 2018, performed the extraction and analysis of the target micropollutant using SPE-UHPLC-MS/MS respectively.

Before SPE, a filter of 1.2 µm glass-fiber (47 mm GF/C, Whatman™; Maidstone, United Kingdom) was used to retain impurities in the samples and the pH was adjusted to 3 using sulfuric acid.

The Oasis® HLB cartridges were conditioned with 4 mL of ethanol and 4 mL of ultrapure water at a flow rate of 1 $mL.min^{-1}$. With a vacuum manifold unit, 500 mL of the samples were loaded at a constant flow-rate of 10 $mL.min^{-1}$. The cartridges were washed with 4 mL ultrapure water and then dried under vacuum for 45 min. The extracts were evaporated to dryness after the elution step which was performed at a flow-rate of 1 $mL.min^{-1}$ with 4 mL of ethanol. After that, were reconstituted in 250 µL of ethanol, and the resulting solutions were filtered through 0.22 µm polytetrafluoroethylene syringe filters. The analysis was performed by UHPLC-MS/MS, consisting of an UHPLC coupled to a triple quadrupole mass spectrometer. Analytical separation occurred along a Kinetex™ XB-C18 100 Å column (100 mm x 2.1 mm x 1.7 µm). The mobile phase consisted of 0.1% formic acid aqueous solution (A) and methanol (B) operated in gradient mode. The set temperature of the column oven and autosampler was 35 °C and 4 °C, respectively, and the injection volume was 5 µL.

Another extraction method and analysis of imidacloprid was presented by Assalin *et al*., (2017), in the section 4.4.

4.6 Epoxiconazole

Welter, in 2018, used solid-phase extraction as a technique to prepare the samples. To perform the SPE, aliquots of 250 mL from each sample were taken and acidified to pH 3 with phosphoric acid. The C18 cartridge was conditioned with 3 mL of methanol, 3 mL of ultrapure water and 3 mL of ultrapure water acidified with phosphoric acid to a pH value of 3.

Then, 250 mL of the sample was percolated through the cartridge at flow rate of 10 $mL.min^{-1}$, allowing the compounds of interest to be adsorbed. Ultrapure water was used in the washing process. Following this, the analytes were pre-concentrated in 2 mL in methanol (to allow the liquid chromatography technique analysis).

A LC-MS was used to analyse and quantify the pesticides, coupled to a quadrupole mass spectrometer with electrospray ionization source. A Varian® analytical column with C18 (50 mm x 2.0 mm i.d. x 2.8 μm) was used for the chromatographic separation. The mobile phase was ultrapure water (A) and methanol (B), both with formic acid 0.1% and ammonium formate 5 $mmol.L^{-1}$, and the column oven temperature was 30 °C. The elution mode was set with 80% B and 20% A, at a flow rate of 0.1 $mL.min^{-1}$. The total running time was 11 min.

5. MATERIALS AND METHODS

5.1 Chemicals and materials

The pesticides used in this work, acetochlor, alachlor, dimethoate, heptachlor, metolachlor and terbuthylazine (Fig. 3), were analytical standards with high-purity (98%), and were supplied by Sigma-Aldrich (Merck, Darmstadt, Germany).

To prepare the individual standard solutions, for direct injection (GC-MS analysis without SPME) was used methanol HPLC grade as dissolvent, supplied by Carlo Erba (Val de Reuil, France). Six individual stock solutions were prepared with the concentrations presented in Table 6, due the availability of the analytical standards and stored in the freeze at -18 °C. A microspatula was used for the manipulation of solid compounds and a micropipette for liquid compounds.

Table 6. *Concentration of individual stock solutions.*

Pesticide	Concentration (mg/L)
Acetochlor	45
Alachlor	50
Dimethoate	50
Heptachlor	22
Metolachlor	50
Terbuthylazine	50

To prepare the dilutions for SPME extraction, HPLC water grade, supplied by Carlo Erba, sodium chloride +98% and HCl with a concentration of 1.2 mol/L, both analytical grade, were used.

The analytical balance used for mass measurements was an ADA 210/C, ±0.0002 g, Adam Equipment.

Figure 3. *Sigma-Aldrich pesticides standards (Acetochlor, Alachlor, Dimethoate, Heptachlor, Metolachlor and Terbuthylazine).*

5.2 Equipment

For SPME extractions was used a hotplate stirrer M6, CAT Equipment, a thermometer for temperature control, a fiber holder for manual sampling from Supelco the Polyacrylate (PA) and Polydimethylsiloxane/Divinylbenzene-Coated fibers (PDMS/DVB), as presented in Fig. 4.

Figure 4. *SPME extraction using a manual holder, agitation and heating plate with a thermometer for temperature control and 4 mL amber vials.*

To carry out the detection and quantification analysis, a Shimadzu GC-MS system, model QP2020 equipped with an AOC-20i autosampler and a Rxi-5ms Low Bleed capillary column (30m length x 0.25 mm I.D. and 0.25 µm film thickness) supplied by Restek (Bellefonte, USA) was used, see Fig. 5.

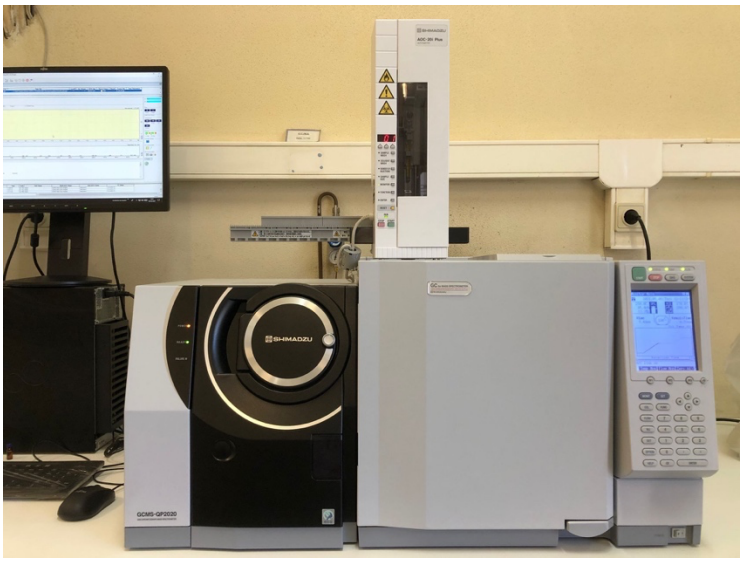


Figure 5. *Shimadzu GC-MS system equipped with an autosampler.*

5.3 Experimental methodology

The main objective of this work is the optimization of solid-phase microextraction (SPME), using a response surface methodology (RSM) based on an experimental planification defined using a Box-Behnken Design (BBD).

5.3.1 Optimization by design of experiment technique

Design of experiments (DOE) is a statistical technique for optimizing system performance with known input variables fast. The response surface methodology (RSM) is one of the most frequent experimental optimization tools.

The RSM has the advantage of a minimal number of runs, making the simultaneous optimization of several parameters less time consumption and more cost efficient when compared to on-variable-at-a-time methodologies, which monitor the influence of only one parameter at a time while the others remain constant. This method is based on a collection of mathematical and

statistical techniques aimed at fitting a non-linear equation to the experimental data in such a way that the equation can represent the relationship between the selected parameters and the objective function response and make statistical predictions.

The methodology can be based on an experimental planification defined using a Box-Behnken Design. This tool proposes a method for selecting points from a three-level factorial arrangement, that allows the efficient estimation of the mathematical model´s first- and second-order coefficients. According to the specifications of such design, the factors must be fixed in three levels (-1, 0 and 1), evenly spaced (Bezerra *et al*., 2008).

Equation (1) is the general formula for the mathematical model that describes the relationship between each parameter and the main response

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} {X_i}^2 + \sum_{j<1} \beta_{ji} X_j X_i \qquad (1)$$

Where Y is the response, in this case the total area, β_0 is the intercept coefficient, βi are the linear terms, βii the quadratic terms, βji the interaction terms and Xi and Xj are the independent factors displayed in Table 14. The values for each coefficient can be obtained by multiple linear regression and by maximizing the equation it is possible to obtain the optimal conditions for each of the responses separately.

The analysis for the total area was evaluated using different statistical tools. The analysis of variance (ANOVA) compares the variation in the response due to treatment, which is defined as a change in the level of the variables, with variation owing to random mistakes that are inherent to the response measurement. It is feasible to determine whether the suggested regression is adequate while taking into account the experimental defects related with the process (Bezerra *et al*., 2008).

The squares of the deviations of each observation from the mean are calculated to create the ANOVA table. The total sum of squares (S_{total}) derives from the sum of squares for all deviations, which may be broken down into two parts: the sum of squares owing to the regression (S_{model}) and the sum of squares due to residuals ($S_{residuals}$) created by the model. Since centre points are replicated, it is possible to estimate pure errors associated with response

measurement and, as a result, to divide the sum of squares of the residuals into the sum of squares owing to pure error (S_{pe}) and the sum of squares due to lack of fit (S_{lof}) (Bezerra *et al*., 2008).

The square media (MS) is given by divide the sums of squares by its respective degree of freedom.

To determine the significance of the regression it is necessary the F-value. The F-value is determined by dividing the MS of the regression (MS_{model}) by the MS of the residual ($MS_{residual}$). By taking into consideration the degrees of freedom from both the regression and the residual, F-value must be compared to F-value tabulated (F test) obtained from Fisher´s distribution tables for the degrees of freedom and a significant level value of α=0.05 (see Appendix B for SPME optimization). The regression is statistically significant and therefore, the model is well fitted to the data, if the calculated value is higher than the tabulated one.

The optimized methodology (SPME/GC-MS) is then applied for monitoring six pesticides in northeast of Portugal (acetochlor, alachlor, dimethoate, heptachlor, metolachlor and terbuthylazine) in water matrices (Hmida, 2019).

Due to the fact that the development of this new experimental methodology has a lot of points to consider and to narrow down the topic, it was decided to fix in the extraction technique those parameters considered less influential according to the bibliography consulted in previous section, and to vary those considered more important as extraction temperature, extraction time, pH and ionic strength.

The experimental methodology planification includes the following steps:

I. According to bibliography, determination of the most suitable operating conditions for GC-MS operation. In the case of gas chromatography, oven temperature program, injection temperature and mode of injection (split/splitless). For mass spectrometry, to allow the clearly identification of pesticides and maximize the total detector signal it is necessary to select the best detection mode (Full Scan or Single Ion Monitoring).
II. Optimization the most important parameters of the SPME, extraction temperature, extraction time, pH of the sample and ionic strength, using a response surface methodology (RSM) based on an experimental planification defined using a Box-Behnken Design (BBD) and stablished

of the values of other parameters, such as temperature in the gas chromatography injector port and desorption time.

III. Validation of the developed methodology by determination of the most important statistical parameters, the calibration curves for the six pesticides and the limits of detection and quantification.

IV. Application of the methodology to monitoring the pesticides in study in samples taken from different water sources such as rivers, lakes, in northeast of Portugal.

5.3.1.1 Determination of GS-MS operating conditions

After a literature review and considering the previous results obtained by Raed Ben Hmida in 2019, that studied different oven temperature programs, it was decided to operate the GC oven using the temperature ramp presented in Table 7:

Table 7. *GC oven temperature profile.*

Rate (°C/min)	Final Temperature (°C)	Hold Time (min)
-	120	2
15	190	4
10	227	2.33

It is important to select rigorously the oven temperature profile, because this step allows the identification of the elution order of each compound and the total analysis time need for each run. Considering that in the SPME experiments the solvent used was water for HPLC, to improve safety during the GS-MS analysis, the initial oven temperature was set to 120°C, in order to avoid the possibility of condensation of residual water inside the column, which could harm the stationary phase.

Other parameters to fix are the injection temperature and the mode of injection, in this study were set in 250°C during all the analysis and in splitless mode for 4 min followed by a split ratio 1:10, respectively (for analysis without SPME), the injection volume was fixed in 2 µL.

Finally, for mass detection, it was decided to study both modes, the ion monitoring “SIM” and the full scan mode “FullScan”, to select the one that allows to obtain the highest detector signal (sum of the signal of all the analysed compounds). The MS acquisition was performed in the range of 35-450 (m/z), the ion source temperature was 200 °C and the interface temperature was 270 °C.

At the beginning of each day the column was cleaned, making an injection with pure methanol using the same GC-MS conditions of the analysis.

5.3.1.2 Optimization of SPME main parameters

In this step, once more following the same procedure adopted in previous work from Raed Ben Hmida in 2019, the initial chosen SPME parameters were the sample agitation (1000 rpm), desorption time in the GC injection port (4 min), injector desorption temperature (250°C), the volume of the sample (3 mL in a vial of 4 mL) and direct immersion as extraction mode. The most important parameters considering in this step were extraction temperature, extraction time, pH of the sample and ionic strength. These parameters were optimized by using a response surface methodology based on an experimental planification defined using a Box-Behnken Design, a tool that requires 3 levels for each factor (-1, 0 and 1), requiring a total of 27 measurements. The level values selected for each factor were:

- Extraction temperature: 50, 60 and 70 °C
- Extraction Time: 40, 60 and 80 min
- pH value of the sample: 2, 4 and 6
- Ionic strength of the sample (or salt addition): 0, 10 and 20% NaCl

Considering that NaCl was the substance used to increase the ionic strength of the sample, the dilution of the standards was done in HPLC grade water, due the lower solubility of the sodium chloride in methanol (14.9 g/L at 25 °C) compared to water (359 g/L at 25 °C).

It is important to highlight that it is not advisable to use a salt addition greater than 20% because this can damage the fiber coating and also it is still advisable to clean the fiber after extraction. In this work it was decided to clean

the fiber after extraction for 2 min in HPLC water at the same temperature of extraction without agitation to remove the excess of salt. To adjust the pH of the sample it was used a 1.2 mol/L of hydrochloric acid.

To carry out the SPME, 3 mL of solution with all the pesticides were placed in a 4 mL vial. The solutions were prepared by taking 500 µL of each individual stock solution and diluting with HPLC water using a volumetric flask of 100 mL for the optimization of the main parameters and selection of the fibers. Before starting with the extraction technique, the fibers were conditioned following the supplier's instructions.

5.3.1.3 Monitoring of pesticides in three different water samples of northeast Portugal

The developed methodology was implemented by taking three samples from three different rivers located in the northeast Portugal, precisely in Bragança in "Onor", "Sabor" and "Fervença" rivers, with the following GPS coordinates respectively: (418034118, -6.6968261), (41.8041655, -6.6941267) and (41.8037228, -6.7558448). The samples were collected in well washed clear plastic laboratory bottles, then transferred in the laboratory to amber glass bottles and stored in the refrigerator at 4°C.

6. RESULTS AND DISCUSSION

6.1 Determination of GC-MS operating conditions

6.1.1 Identification of the elution order of the six pesticides

The objective of this first step is to identify the elution order of the six pesticides, by direct injection (analyses without SPME extraction). To prepare the individual stock solutions, the following masses of each pesticide were weighed, see Table 8, and transferred to 100 mL volumetric flasks using pure methanol as solvent. Then 500 µL of each solution was taken and diluted in 5 mL volumetric flasks with methanol. The diluted solutions were transferred to 4 mL amber vials and stored in freeze at -18 °C until the moment of analysis.

Table 8. *Concentration of individual stock solutions for direct injection.*

Pesticide	Mass (mg)	First dilution concentration (mg/L)	Second dilution concentration (mg/L)
Acetochlor	45	450	45
Alachlor	50	500	50
Dimethoate	50	500	50
Heptachlor	22	220	22
Metolachlor	50	500	50
Terbuthylazine	50	500	50

After, a standard mixture of the six pesticides was prepared by measuring 500 µL of each volumetric flask with the first dilution and transferred to a 5 mL volumetric flask with methanol.

The direct injection was made considering the oven temperature profile described in Table 7, and with the GC-MS conditions presented in Table 9.

Table 9. *GC-MS operating conditions.*

GC	
Oven initial temperature	120 °C
Injector temperature	250 °C
Injection volume	2 µL
Split mode	1:10
MS	
Mode	FullScan
m/z	35-450
Ion trap temperature	200 °C
Transfer line temperature	270 °C

The obtained results are presented in Figure 6, where the numbers represent the order of elution of the different pesticides being the number 1 with the shortest retention time and corresponding to dimethoate, 2 for terbuthylazine, 3 for acetochlor, 4 for alachlor, 5 for heptachlor and the one with the longest retention time 6 is for metolachlor.

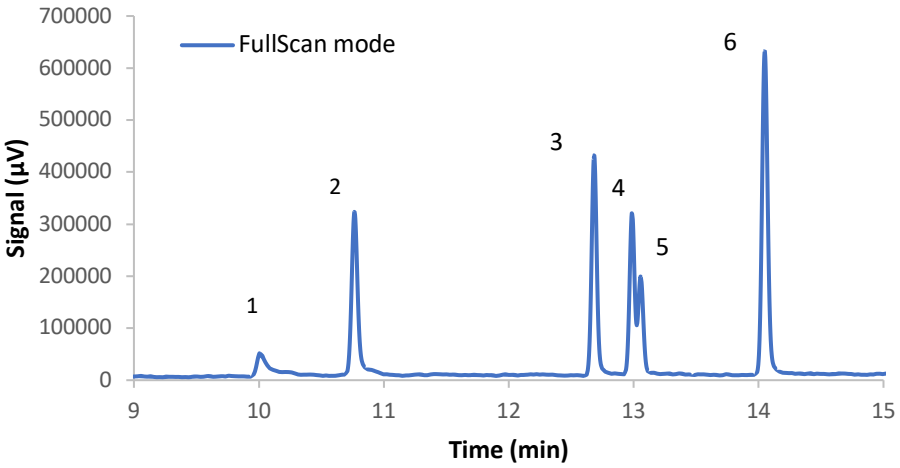


Figure 6. *GC-MS analysis of the mixture of the six selected pesticides.*

The identification of each pesticide was done by comparing the experimental MS chromatogram with the MS chromatogram available in the MS spectrum data base present in the equipment software (NIST). In table 10 is presented for each compound the experimental retention time and the obtained

total chromatogram area (TIC) obtained automatically by the equipment data treatment software.

Table 10. *Identification of the elution order and signal intensity (TIC) for each pesticide.*

Elution order	Pesticide	Retention time (min)	Area (counts)
1	Dimethoate	10.012	164615
2	Terbuthylazine	10.766	1017238
3	Acetochlor	12.684	1222580
4	Alachlor	12.988	890366
5	Heptachlor	13.056	526589
6	Metolachlor	14.05	1972617

It is possible to observe a partial overlay between alachlor and heptachlor elution profiles. The overlay of these two chromatographic peaks causes integration problems for individual quantification. In this study, the objective is to optimize the GC-MS operating parameters that improve the detection of the all pesticides, in others words, to find the values that allow to obtain the major total area (total sum area for the 6 pesticides). So, it was considered one only area as the sum of the areas of these two pesticides (alachlor and heptachlor).

6.1.2 Study of MS analysis modes

While the FullScan mode is used for the identification of the compounds present in a sample using their respective mass spectrum, the SIM mode is used for a selective scanning analysis of pre-selected ions. So, in order to carry out the study using single ion monitoring mode (SIM mode), it is necessary to collect from the literature and from de NIST MS library present in the Shimadzu software of the equipment, the m/z values or target ions, for this it was considered the mass spectrum of each pesticide (see Appendix A). These ions were determined according to the mass spectrum of the individual analysis of each pesticide, where the most abundant ions and those that stood out for each of the compounds were evaluated. In this study the target ions were selected as:

- Acetochlor: 59, 146 and 223.

- Alachlor: 45, 160 and 188.
- Dimethoate: 87 and 125.
- Heptachlor: 65, 100 and 272.
- Metolachlor: 162 and 238.
- Terbuthylazine: 43, 173 and 214.

Both studies, by SIM and FullScan, were done considering the same conditions previously summarized in Table 9.

In Figure 7, are presented (overlayed) the experimental GC-MS chromatograms using both FullScan and SIM modes of operation.

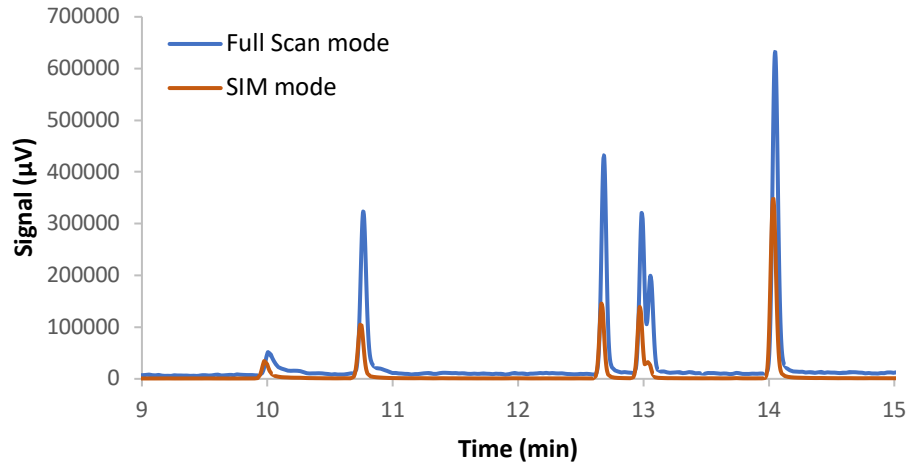


Figure 7. *Comparison between GC-MS chromatograms obtained using FullScan and SIM modes.*

The obtained retention times and areas for both modes and for all the 6 pesticides are presented in Table 11.

Table 11. *Identification of the elution order and signal intensity for each pesticide.*

Elution order	Pesticide	Retention time (min)	Area (counts) FullScan	Area (counts) SIM
1	Dimethoate	10.012	164615	120389
2	Terbuthylazine	10.766	1017238	337785
3	Acetochlor	12.684	1222580	402818
4	Alachlor	12.988	1416955	473032
5	Heptachlor	13.056		
6	Metolachlor	14.050	1972617	1097879

Because the detector in SIM mode only considers the selected target ions for each pesticide, the baseline produced is cleaner (without contaminants) than the one that is obtained using FullScan mode.

However, the FullScan mode presents a bigger detector signal than the SIM mode, this happens because were considered the mass/charge ratio of a small quantity of fragments of the whole mass spectrum of each pesticide. Because of this, it was decided to continue the experimental analysis using FullScan mode.

6.2 Optimization of SPME main parameters

6.2.1 SPME preliminary study

6.2.1.1 Selection of the fiber

Before starting the optimization of the selected parameters, it was decided to select the type of SPME fiber that maximizes the extraction efficiency.

The fibers were supplied by Supelco, a Sigma-Aldrich Corporation subsidiary. In Table 12, a list with some characteristics taking from the Supelco´s guides of the most used fiber for SPME of pesticides is presented.

Table 12. *Characteristics of the most used fibers for extraction of pesticides supplied by Supelco*.

Fiber core	Thickness	Bond phase	pH range	Recommended desorption temperature (°C)	Analyte type	Recommended use	Pesticide
Polydimethylsiloxane (PDMS)	100 µm	Non-bonded	2-10	200-280	Non polar volatile	GC/HPLC	-
	7 µm/30 µm	Bonded	2-11	220-320/200-280	Non polar semivolatile	GC/HPLC	
Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)	65 µm	Partially crosslinked	2-11	200-270	Polar volatile Amines Nitroaromatic	GC	Alachlor Heptachlor Metolachlor Terbuthylazine
Carboxen/Polydimethylsiloxane	75 µm	Partially crosslinked	2-11	250-310	Trace-level volatile	GC	Dimethoate
Polyacrylate (PA)	85 µm	Partially crosslinked	2-11	220-280	Polar semivolatile	GC/HPLC	Alachlor Heptachlor Metolachlor

Considering the properties of the compounds it was decided to study the efficiency in the extraction of the PA and PDMS/DVB fibers to determine which is the most appropriate for the extraction of the compounds selected under the best conditions studied in the master degree project by Raed Ben Hmida, in 2019. The selected operating conditions for GC-MS were done considering the same conditions summarized in Table 9 and the selected for the SPME are presented in Table 13. The method of preparation of the solutions for this step is explained in section 5.3.2.

Table 13. *SPME operating conditions.*

SPME	
Extraction time	80 min
Extraction temperature	60 °C
pH	2
Addition of NaCl	10 %
Agitation	1000 rpm

Two analyses for each fiber were made and the comparison of the results is presented in the Figure 8.

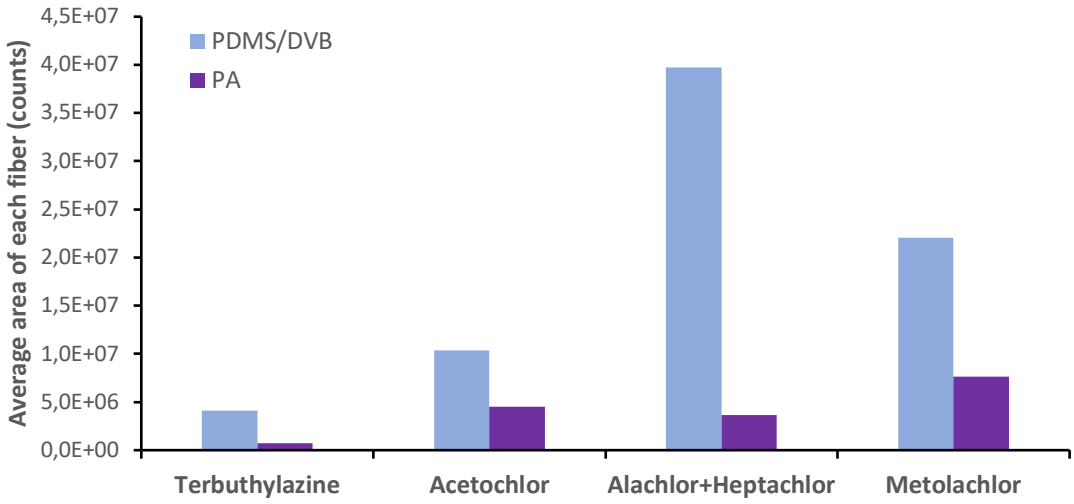


Figure 8. *Comparison between GC-MS areas obtained using PDMS/DVB and PA fibers.*

It is possible to notice the absence of the dimethoate in the Figure 8, this can mean a possible degradation of the compound. Also, the alachlor and heptachlor were considered together due to both compounds have similar elution times and their areas have overlapping. Another observation is that the PDMS/DVB shows bigger areas than the PA fiber being the first one the selected for continue the experimental work.

However, when starting to optimize the parameters selected, the available PVMS/DVB fiber was broken. This can be attributed to several factor, among them, all the fibers have a limited number of uses and could suffered wear and/or that when working with salt crystals have become embedded and when the fiber was exposed and retracted, they have obstructed the passage, damaging it. To avoid this problem in the future measurements it was decided to clean the residues of NaCl by exposing the fiber for 2 minutes in water HPLC at a range of 50-70°C, after extraction and before insert the fiber in the GC-MS.

6.2.2 Optimization using Response Surface Methodology (RSM)

The optimization of SPME was done using the Response Surface Methodology (RSM) based on an experimental planification defined using a Box-Behnken Design (BBD).

For this work, the model was built studying four parameters using three levels, requiring a total of 27 measurements. The studied main parameters were, extraction temperature, extraction time, pH value and ionic strength (salt addition). Table 14 describes the selected three levels used for each studied parameter.

Table 14. *Summary of studied factors and selected levels in the BBD.*

Factor	Code	Levels		
		-1	0	1
Extraction temperature (°C)	A	50	60	70
Extraction time (min)	B	40	60	80
pH	C	2	4	6
Addition of NaCl (%)	D	0	10	20

Table 15 describes the parameters selected and the conditions applied in each run and the obtained response (total area), which represents the sum of the areas of the pesticides under study, obtained from the Box-Behnken Design tool.

Table 15. *Experimental design, real conditions and experimental responses.*

Run	Experimental design				Experimental design				Experimental response
	Extraction Temperature (°C)	Extraction time (min)	pH	Addition of NaCl (%)	Extraction temperature (°C)	Extraction time (min)	pH	Addition of NaCl (%)	Total area
	A	B	C	D	A	B	C	D	
1	0	1	-1	0	60	80	2	10	16447081
2	0	0	-1	1	60	60	2	20	18842103
3	0	-1	0	1	60	40	4	20	13432733
4	0	0	0	0	60	60	4	10	12745574
5	-1	0	0	-1	50	60	4	0	4281965
6	-1	0	1	0	50	60	6	10	4901716
7	0	-1	1	0	60	40	6	10	7829340
8	0	0	0	0	60	60	4	10	13393704
9	-1	-1	0	0	50	40	4	10	7054733
10	0	0	1	1	60	60	6	20	6629241
11	0	1	0	-1	60	80	4	0	12402834
12	0	0	0	0	60	60	4	10	8593632
13	0	0	1	-1	60	60	6	0	5598822
14	0	-1	-1	0	60	40	2	10	6868399

Table 15. *Experimental design, real conditions and experimental responses. (Continuation).*

Run	Experimental design				Experimental design				Experimental response
	Extraction Temperature (°C)	Extraction time (min)	pH	Addition of NaCl (%)	Extraction temperature (°C)	Extraction time (min)	pH	Addition of NaCl (%)	Total area
15	-1	0	-1	0	50	60	2	10	4229305
16	1	0	0	-1	70	60	4	0	9489513
17	1	0	0	1	70	60	4	20	35292617
18	0	1	0	1	60	80	4	20	29041252
19	-1	0	0	1	50	60	4	20	12270951
20	0	1	1	0	60	80	6	10	13981297
21	0	0	-1	-1	60	60	2	0	5467626
22	1	0	1	0	70	60	6	10	15923133
23	1	-1	0	0	70	40	4	10	13418285
24	0	-1	0	-1	60	40	4	0	5088336
25	1	1	0	0	70	80	4	10	28275370
26	-1	1	0	0	50	80	4	10	13622702
27	1	0	-1	0	70	60	2	10	13729381

6.2.2.1 Data treatment using the analysis of variance (ANOVA)

The values obtained for the current experimental work applying a quadratic model are presented in Table 16.

Table 16. *ANOVA for quadratic model for total area response.*

Source	Sum of squares	Df	Mean square	F-value	Tabulated F-value	p-value
Model	1.493E+15	14	1.067E+14	11.47	2.637	<0.0001
Residual	1.116E+14	12	9.298E+12			
Lack of fit	9.801E+13	10	9.801E+12	1.44	19.396	0.4770
Pure error	1.357E+13	2	6.783E+12			
Cor total	1.605E+15	26				

Df=Degree of freedom

Checking for lack of fit is another technique to assess the model. The lack of fit should be evaluated similarly to the regression fit, by comparing the calculated F-value to the tabulated one. The degrees of freedom of the lack of fit and the pure error must be considered to obtain the tabulated value. In this experience, the value 1.44 in comparison with 19.396 means that the lack of fit is not significant relative to the pure error. In other words, the model mistakes are caused by the system´s random and inherent errors rather that a problem with the fit model.

The p-value is related to the F-value, the p-values less than 0.0500 indicate model terms are statistically significant. Values greater than 0.1000 indicate the model is irrelevant or that model terms are not significant (don´t influence the response). If there are many insignificant model terms, model reduction may improve the model. In this case, the current model is statistically significant and, the lack of fit is not.

Another statistical tool to assess the model fit is the regression coefficient. The regression coefficient which represents the percentage of variation in the response that is explained by the model was 0.9305. On the other hand, the Predicted R^2 which is used to determine how well the model predicts the response

for new observations, where at larger predicted R^2 values better predictive ability, was 0.6292 and the Adjusted R^2 which represents the percentage of the variation in response that is explained by the model, adjusted for the number of predictors in the model relative to the number of observations, was 0.8493 and was not to close to the Predicted R^2 as one might normally expect; the tool suggests a different less than 0.2 between each other. The different in this study was 0.2201. This may indicate a possible problem with the model and/or data. So, some facts were checked. One of them were the residuals of runs, them should be normally distributed. Another was the mean of the residuals, them should be close to 0 and at least, the residues should be unrelated to the levels of any known variables (Bezerra *et al*., 2008).

By subtracting the observe response (or the experimental response) from the projected response, residuals are estimated of the error done. The normality of the residuals can be checked by looking at the normal plot of residuals in Figure 9. The data is expected to be normally distributed if all the runs fall inside a straight diagonal line, with no residual too far away from the line and no propensity to create a specific pattern, such as a “s” curve.

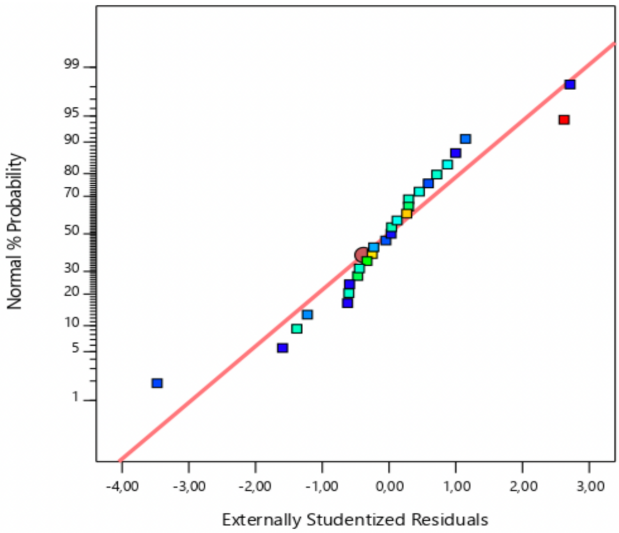


Figure 9. *Normal plot of residuals.*

In Figure 9 it is possible to observe that three points do not follow the trend line. Figure 10 shows the residuals versus expected plot, which may be used to see if the residuals are close to 0 and if them are unrelated to the variable levels.

The residual versus predicted values plot also aids in the detection of outliers, which are runs with extremely large residuals that must be excluded from statistical analysis. Any point outside of the red line should be treated as an outlier, and the experience or measurement should be redone.

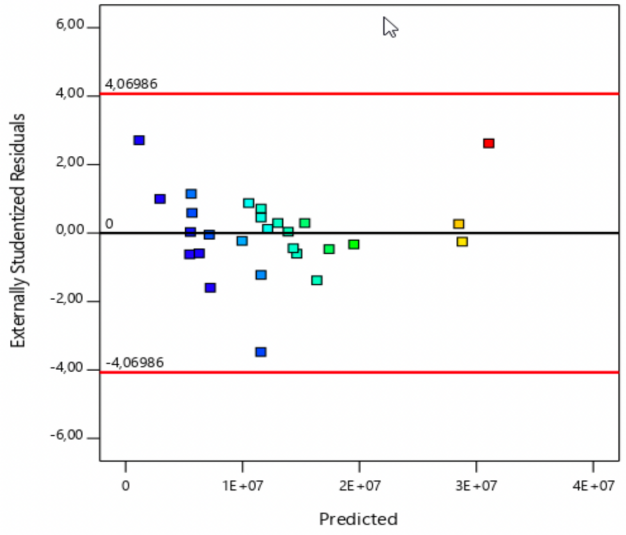


Figure 10. *Residuals versus predicted values.*

In Figure 10 there are residuals that are far away to the black line (line that indicates a deviation of 0), also there is a special pattern, like a funnel that forms, as the predict response increases.

From the analysis of both Figures 9 and 10, and since the difference greater than 0.02 between the predicted R^2 and adjusted R^2, it was decided to check which runs produced those errors. Looking at the report (see Appendix B for SPME optimization), it was observed that three points exceeded the limits of the influence of fitted values (difference in fits). And after identification, it is recommended to repeat these experiences, but due to this project was carried out in a pandemic context and with all the limitations that this entails, was decided to eliminate the runs number 10, 17 and 21.

After this step, the following results were obtained.

Table 17. *ANOVA for quadratic model for total area response for 24 runs.*

Source	Sum of squares	Df	Mean square	F-value	Tabulated F-value	p-value
Model	9.747E+14	14	6.962E+13	26.59	3.025	<0.0001
Residual	1.116E+14	9	2.619E+12			
Lack of fit	1.000E+13	7	1.429E+12	0.2106	19.353	0.9502
Pure error	1.357E+13	2	6.783E+12			
Cor total	9.983E+14	23				

Df=Degree of freedom

The new ANOVA table was built. The indicated values on Table 17 show that the model was significant, with a calculated F-value higher than the tabulated one and the lack of fit was not significant.

The new regression coefficient was 0.9764. The Predicted R^2 in this new study was 0.9397 and was close to the Adjusted R^2 of 0.8867 as one might normally expect; the tool suggests a different less than 0.2 between each other. The different in this study was 0.053, indicating a good regression.

To evaluate the factors effect on the response (total area) was taking into consideration the degrees of freedom of each factor and the degree of freedom of the residual. To investigate the impact of each factor, as well their interactions and quadratic effect on the response, can be also used the ANOVA table, as it can be seen on Table 18.

Table 18. *Parameters influencing the total area using ANOVA.*

Source	Sum of squares	Df	Mean square	F-value	Tabulated F-value	p-value
A-Extraction temperature	2.619E+14	1	2.619E+14	100.03	5.117	<0.0001
B- Extraction time	3.008E+14	1	3.008E+14	114.87	5.117	<0.0001
C- pH	9.590E+10	1	9.590E+10	0.0366	5.117	0.8525
D- Addition of NaCl	3.119E+14	1	3.119E+14	119.12	5.117	<0.0001
AB	1.718E+13	1	1.718E+13	6.56	5.117	0.0306
AC	5.786E+11	1	5.786E+11	0.2210	5.117	0.6495
AD	1.509E+13	1	1.509E+13	5.76	5.117	0.0399
BC	2.936E+12	1	2.936E+12	1.12	5.117	0.3173
BD	1.720E+13	1	1.720E+13	6.57	5.117	0.0306
CD	9.435E+12	1	9.435E+12	3.60	5.117	0.0901
A^2	6.696E+12	1	6.696E+12	2.56	5.117	0.1443
B^2	3.596E+13	1	3.596E+13	13.73	5.117	0.0049
C^2	3.540E+13	1	3.540E+13	13.52	5.117	0.0051
D^2	1.169E+12	1	1.169E+12	0.4462	5.117	0.5209

Df=Degree of freedom

For the following parameters, the calculated F-value was higher than the tabulated one: A (extraction temperature); B (extraction time); D (Addition of NaCl); AB; AD; BD; B^2 and C^2.

The ANOVA not only aids in determining whether a factor is statistically significant, but it also aids in determining how significant each one is. The p-value can be used to determine this. The lower it is, the more influence it has on the answer. In this way, the parameters A (extraction temperature), B (extraction time) and D (addition of NaCl) were the most relevant.

The response surface for different combination of pair of variables, as well as the interaction plots of those same variables and their influence on the total area, were studied and are shown in Figure 11.

Figure 11 presents the response surface of the influence of variables extraction time and pH and the interaction plot between them. In this case, the variables extraction temperature and addition of NaCl were fixed in 70 °C and 20% respectively. It is possible to notice that the pH variable does not have a significant importance for the total area, i.e., fixing a value for extraction time, for

instance 40 min, and moving along the pH axis, no a relevant change in the response is noticed. Nevertheless, by doing the same analysis for pH variable, and moving along the extraction axis value, for lower values of extraction time, lower values of total area and for greater values of the extraction time, greater the response.

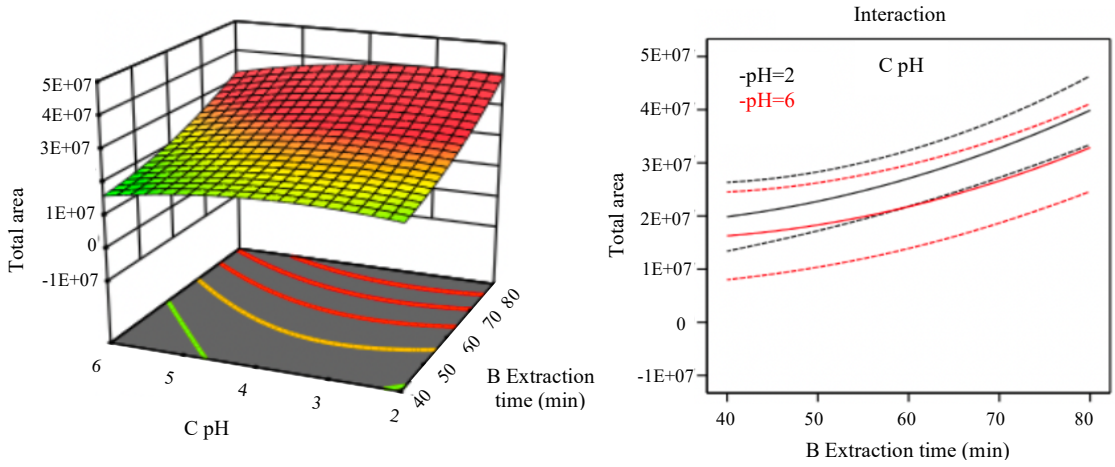


Figure 11. *Response surface for the total area being influenced by extraction time (B) and pH (C) and interaction plot of these variables.*

Figure 11 shows an interaction plot that can be used to see if the variables interact among them. The conclusion is that the effect of one component is independent of the level of the other factor if the interaction plot shows parallel lines (black and red superposed). If the lines are not parallel, it means that one factor´s effect is influenced by the level of the other, signifying that one variable influence not just the response but also the other variable, modifying the effect of the second variable on the response. It is evident from Figure 11 that the variables have no effect on one another.

The interaction plot displays two slight-parallel lines, meaning that these variables not influence each other. This is in agreement with the p-value of 0.3173 found for the interaction of these factors (BC).

6.2.2.2 Estimation of optimal conditions by ANOVA

As mentioned earlier, one of the benefits of using Box Behnken Design is that it allows us to estimate a quadratic equation (see equation (1)). As a result, it is possible to determine the best combination of the selected parameters. The coefficients estimated are presented in the Table 19 and the model equation as presented in equation (2).

Table 19. *Coefficients for the quadratic equation in terms of coded factors.*

Coded factor	Coefficient estimate
Intercept	1.158E+07
A	5.165E+06
B	5.007E+06
C	1.005E+06
D	6.443E+06
AB	2.072E+06
AC	3.803E+05
AD	2.507E+06
BC	-8.567E+05
BD	2.074E+06
CD	-3.135E+06
A^2	1.210E+06
B^2	2.805E+06
C^2	-3.096E+06
D^2	6.043E+05

$$Y = 1.158\text{E}07 + 5.165\text{E}06\text{A} + 5.007\text{E}06\text{B} + 1.005\text{E}06\text{C} + 6.443\text{E}06\text{D} \quad (2)$$
$$+1.210\text{E}06\text{A}^2 + 2.805\text{E}06\text{B}^2 - 3.096\text{E}06\text{C}^2 + 6.043\text{E}05\text{D}^2 + 2.072\text{E}06\text{A}$$
$$+3.803\text{E}05\text{AC} + 2.507\text{E}06\text{AD} - 8.567\text{E}05\text{BC} + 2.074\text{E}06\text{BD} - 3.135\text{E}06\text{CD}$$

It is feasible to calculate which values for the group of factors analysed would result in the largest total area, by maximizing equation (2). Also, it is possible to obtain the maximum total area by analysing the response surface plots. After this stage, the combination that allows to obtain the maximum area was: 80 min for the extraction time, 70 °C for the extraction temperature, pH 2 and addition of NaCl 20 %. In Figure 12 are presented different plots, where the two remaining variables were fixed at level 0 for each one.

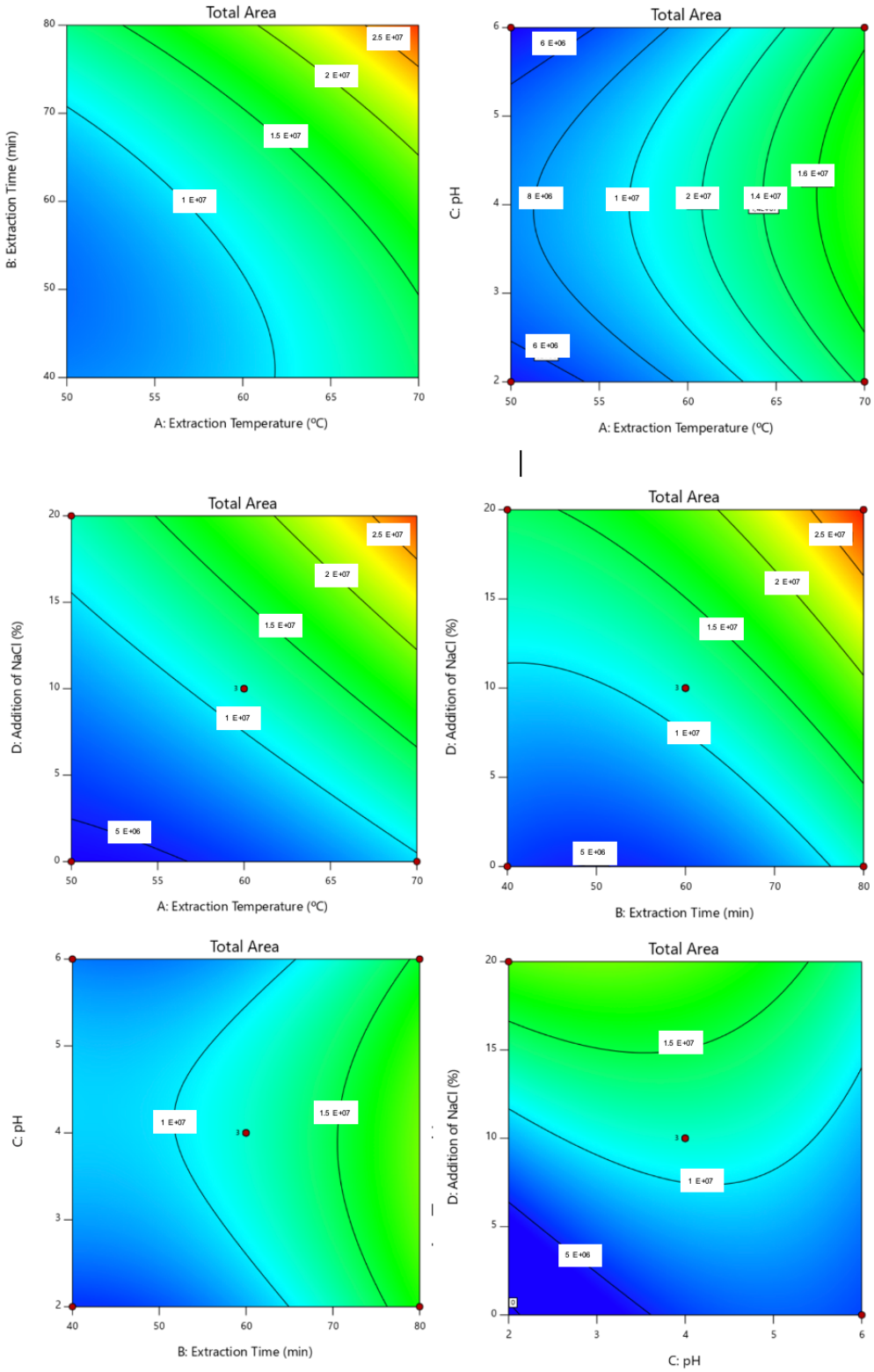


Figure 12. Six contour maps for the total area setting 2 variables at level 0.

It is possible to observe in Figure 12 that as the values of the influencing variables increase, the value of the response increases, reaching its maximum value when the relevant parameters are in the higher level. Also, it is possible to notice that the value of the pH does not have major influence on the response.

Due to the lack of time to carry out the measurements, it was decided to select for optimum values, an adsorption time of 60 min, a pH value of 2 and a NaCl content of 20%. These operation conditions are presented as it is represented in Figure 13.

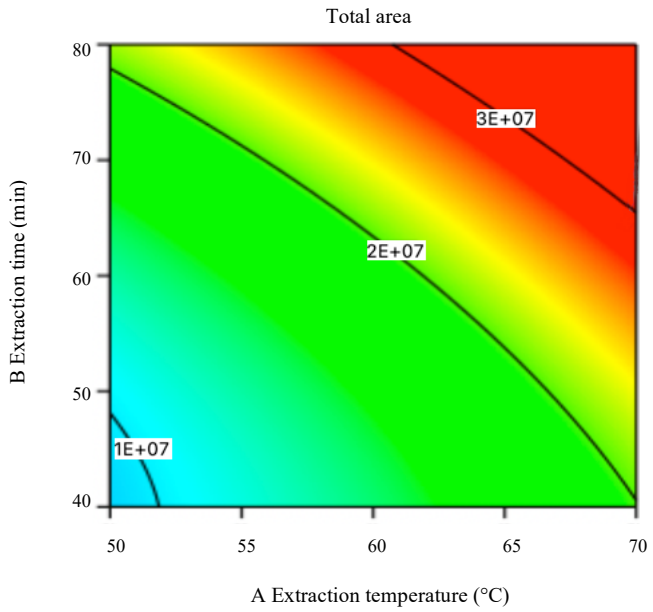


Figure 13. *Response surface for the total area with pH 2 and Addition of NaCl 20%.*

It is possible to notice that for an extraction time of 60 min the only possibility to maximize the response is to set an extraction temperature of 70 °C. Despite the fact that the pH has no major influence on the response, the values obtained for the optimized conditions reflect that to maximize the response, the value of pH has to 2. Table 20 presents the optimal values selected to maximize the total area value.

Table 20. *Optimal values selected for the total area.*

Factor	Code	Coded value	Value
Extraction temperature (°C)	A	1	70
Extraction time (min)	B	0	60
pH	C	-1	2
Addition of NaCl (%)	D	1	20

It is important to highlight that the values obtained are highly dependent on the region under consideration.

6.3 Monitoring of pesticides in three different water samples of northeast Portugal

Although after estimating the optimal conditions, the limit of detection (LOD), limit of quantification (LOQ) and percent recovery of the method should be calculated by analysing blanks and solutions spiked near the minimum calibration concentration before analyse real samples, this step could not be performed due to the pandemic context mentioned above.

After estimating the conditions, three samples from three different rivers from northeast of Portugal were analysed.

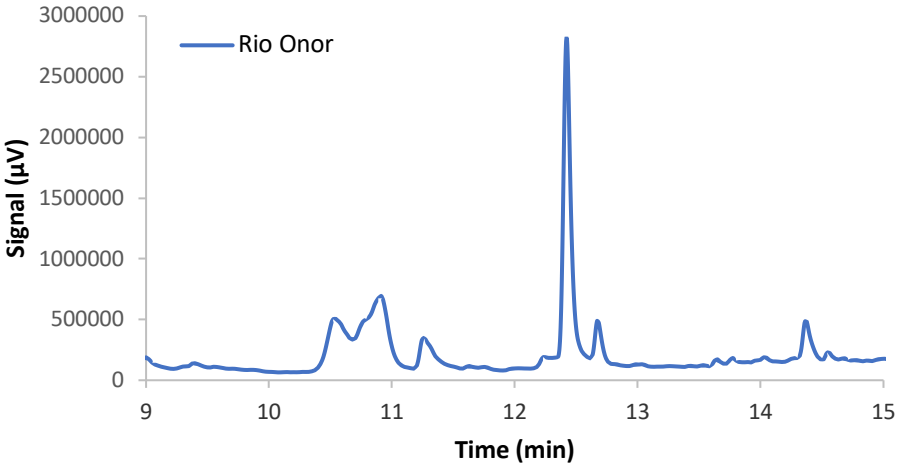
The final operating conditions and the chromatograms obtained are presented on Table 21 and in Figure 13 respectively.

Table 21. *SPME/GC-MS operating conditions for the analyses of three river samples.*

GC	
Oven initial temperature	120 °C
Injector temperature	250 °C
Injection volume	1 µL
Split mode	1:10

Table 21. *SPME/GC-MS operating conditions for the analyses of three river samples. (Continuation).*

MS	
Mode	FullScan
m/z	35-450
Ion trap temperature	200 °C
Transfer line temperature	270 °C
SPME	
Extraction time	60 min
Extraction temperature	70 °C
pH	2
Addition of NaCl	20%
Agitation	1000 rpm



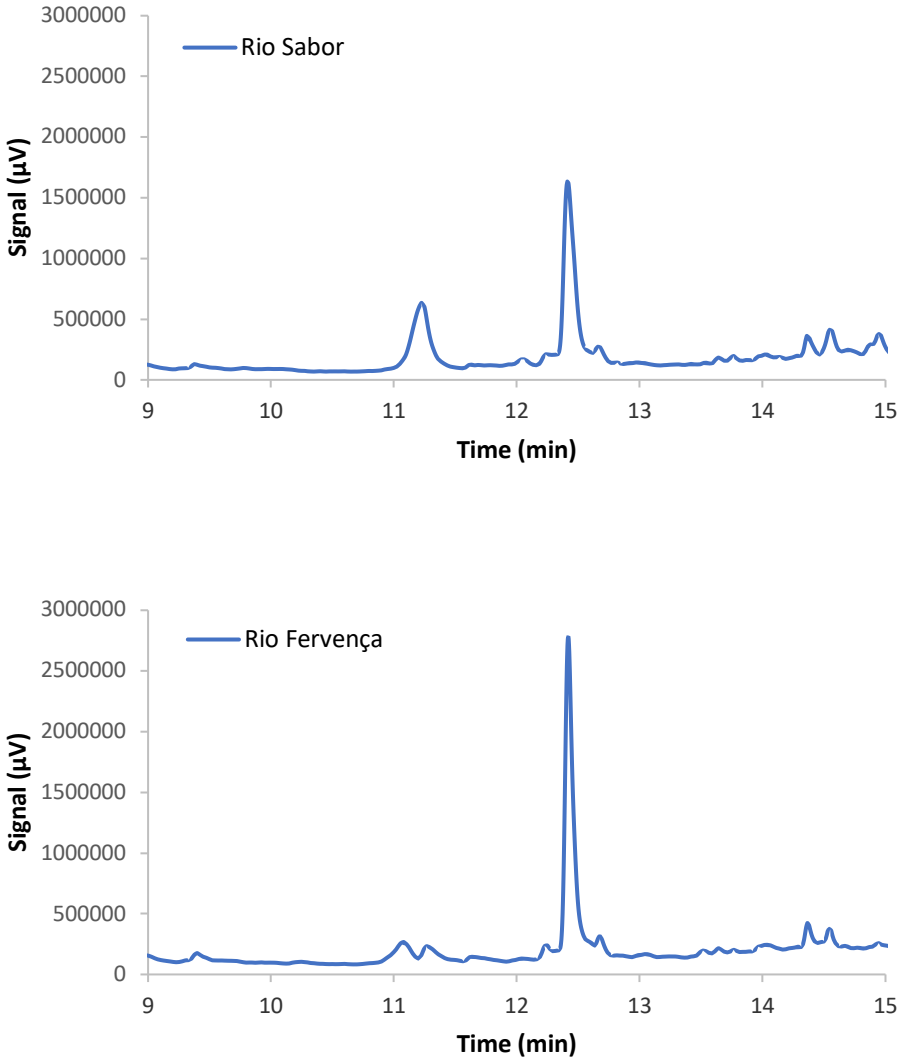


Figure 14. *Chromatograms of the analyses of three river samples by SPME/GC-MS.*

None of the pesticides under study were detected in the samples taken from the three rivers. However, the highest peak is common among the three analyses and indicate that morpholine (fungicide) is present in all the three samples.

7. CONCLUSIONS

Due to the great importance of pesticides in the production of food for human consumption and the consequences that their use entails at the environmental and health level. In this work, it was decided to study the optimization of solid-phase microextraction (SPME), using a response surface methodology (RSM), based on an experimental planification defined using Box-Behnken Design (BBD) for 6 pesticides used in northeast Portugal. Being the extraction and concentration of pesticides, an important step for their subsequent identification and quantification. After applying an ANOVA data treatment, the optimal values of the studied four parameters were defined as 70 °C for extraction temperature, 60 min for extraction time, pH 2 and NaCl addition 20%. Under these conditions, three real samples were analysed in which the pesticides under study were not detected. Beyond the results obtained in the analysis of the samples taken, this work provides the parameters for the determination of referred compounds.

It is important to note that some stages could not be adequately developed due to the pandemic context in which said project was located, which generated some laboratorial limitations.

Suggestions for future work

To completely assess the suitable of the optimization of the SPME methodology using RSM, more research is required. The following are some suggestions for future work:

- Analyse the resulting areas of each compound separately, in order to identify the optimal conditions for each family of compounds.

- Study the MS analysis using the Single Ion Monitoring (SIM) mode.

- Study the influence of other parameters that are involved in the extraction process such as sample agitation velocity and extraction by headspace.

- Study the effectiveness in the extraction of pesticides of other different types of fibers such as the CAR-PDMS or the PDMS/DVB.

- Make a preliminary study to corroborate the predicted results obtained from Box-Behnken Design for SPME with standard samples before applying the procedure to real samples. And make a repeatability study.

- Extend the list of pesticides for better monitoring of the water quality in the northeast Portugal.

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Appendix A. Mass spectrums

Figure A.1

Mass spectrum of acetochlor

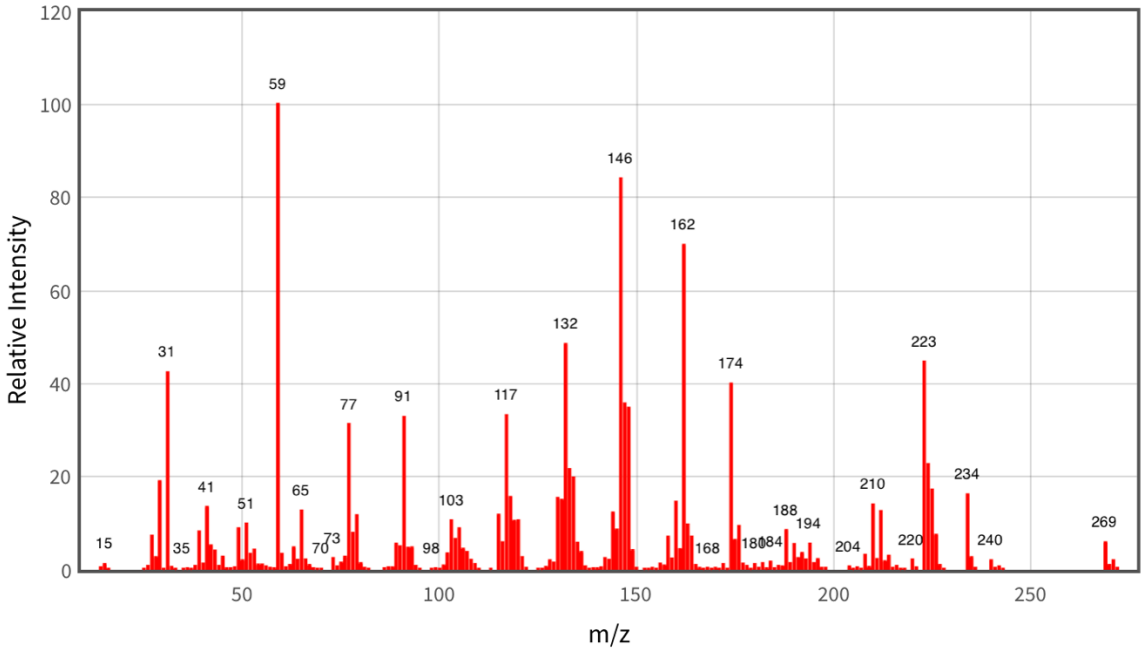


Figure A.2

Mass spectrum of alachlor

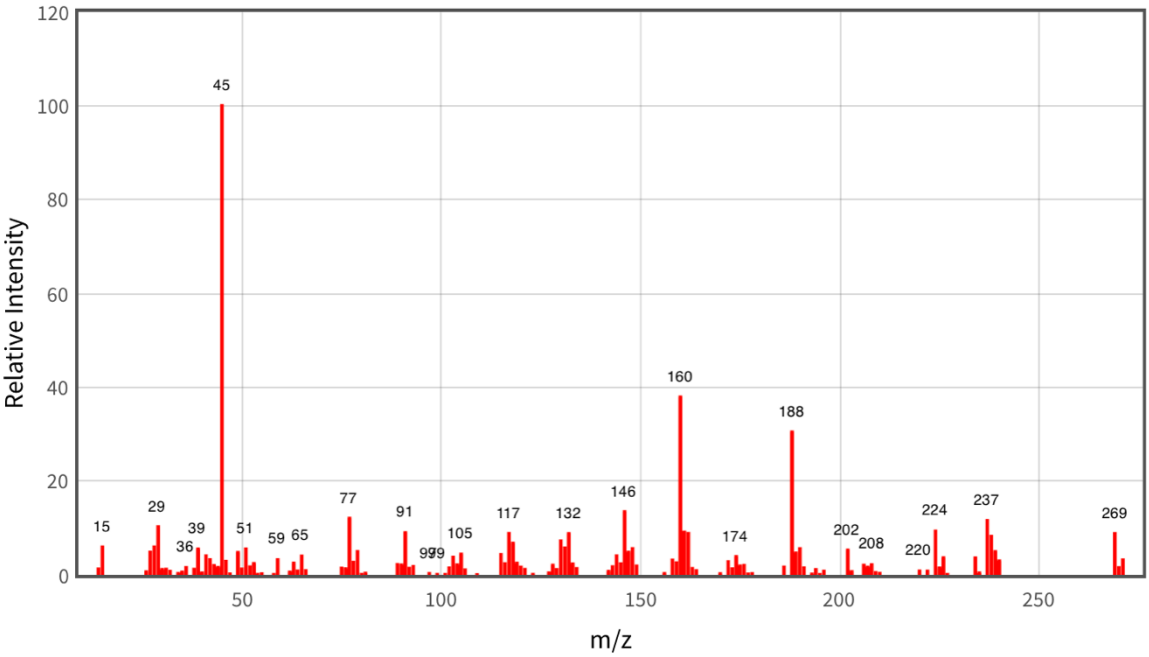


Figure A.3

Mass spectrum of dimethoate

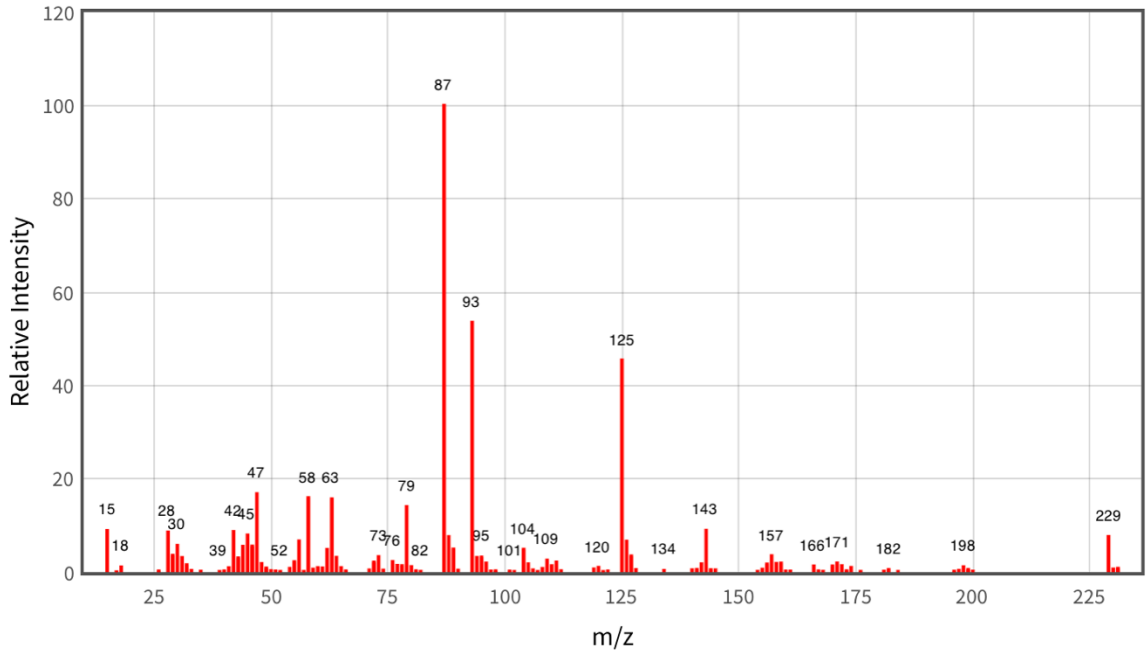


Figure A.4

Mass spectrum of heptachlor

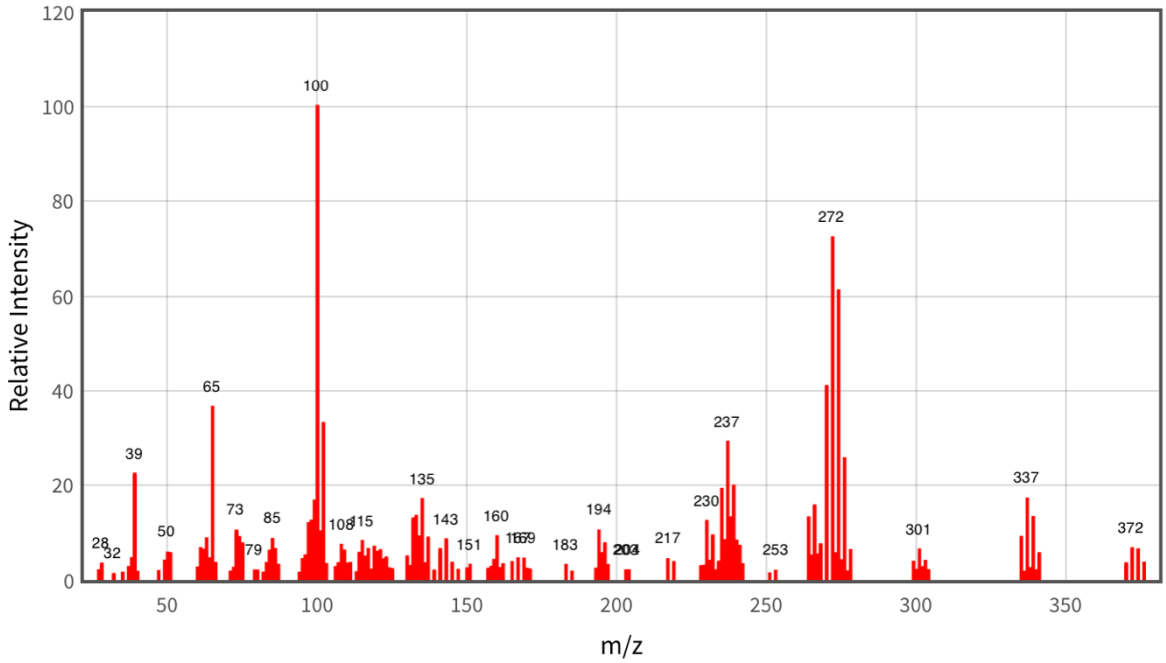


Figure A.5

Mass spectrum of metolachlor

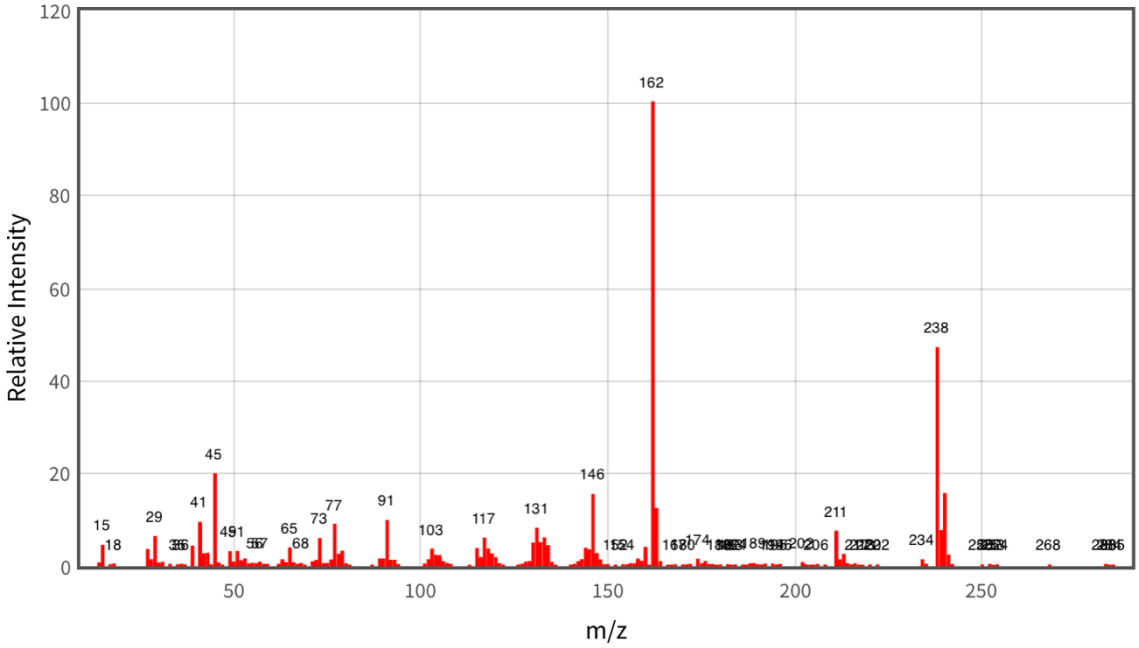
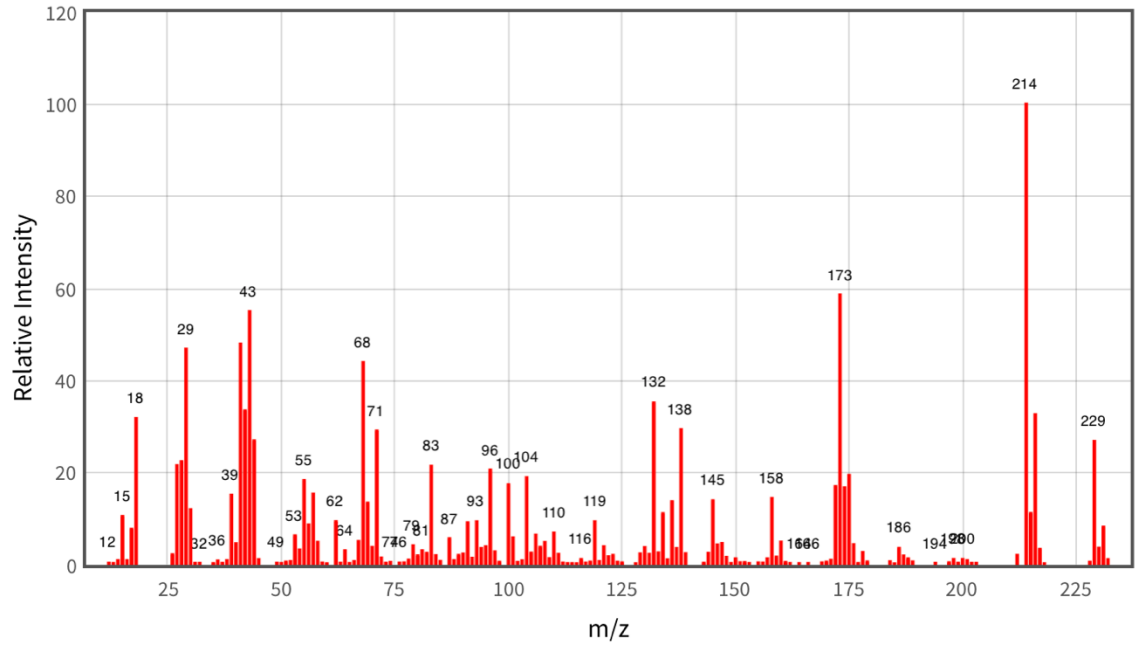


Figure A.6

Mass spectrum of terbuthylazine



Appendix B. SPME optimization

Figure B.1

Critical values of the F distribution (a=0.05). Fisher´s F distribution table.

Grados de libertad del Denominador	Grados de libertad del Numerador														
g.d.l	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	g.d.l
1	161,4	199,5	215,7	224,6	230,2	234,0	236,8	238,9	240,5	241,9	243,0	243,9	244,7	245,4	245,9	1
2	18,513	19,000	19,164	19,247	19,296	19,330	19,353	19,371	19,385	19,396	19,405	19,413	19,419	19,424	19,429	2
3	10,128	9,552	9,277	9,117	9,013	8,941	8,887	8,845	8,812	8,786	8,763	8,745	8,729	8,715	8,703	3
4	7,709	6,944	6,591	6,388	6,256	6,163	6,094	6,041	5,999	5,964	5,936	5,912	5,891	5,873	5,858	4
5	6,608	5,786	5,409	5,192	5,050	4,950	4,876	4,818	4,772	4,735	4,704	4,678	4,655	4,636	4,619	5
6	5,987	5,143	4,757	4,534	4,387	4,284	4,207	4,147	4,099	4,060	4,027	4,000	3,976	3,956	3,938	6
7	5,591	4,737	4,347	4,120	3,972	3,866	3,787	3,726	3,677	3,637	3,603	3,575	3,550	3,529	3,511	7
8	5,318	4,459	4,066	3,838	3,687	3,581	3,500	3,438	3,388	3,347	3,313	3,284	3,259	3,237	3,218	8
9	5,117	4,256	3,863	3,633	3,482	3,374	3,293	3,230	3,179	3,137	3,102	3,073	3,048	3,025	3,006	9
10	4,965	4,103	3,708	3,478	3,326	3,217	3,135	3,072	3,020	2,978	2,943	2,913	2,887	2,865	2,845	10
11	4,844	3,982	3,587	3,357	3,204	3,095	3,012	2,948	2,896	2,854	2,818	2,788	2,761	2,739	2,719	11
12	4,747	3,885	3,490	3,259	3,106	2,996	2,913	2,849	2,796	2,753	2,717	2,687	2,660	2,637	2,617	12
13	4,667	3,806	3,411	3,179	3,025	2,915	2,832	2,767	2,714	2,671	2,635	2,604	2,577	2,554	2,533	13
14	4,600	3,739	3,344	3,112	2,958	2,848	2,764	2,699	2,646	2,602	2,565	2,534	2,507	2,484	2,463	14
15	4,543	3,682	3,287	3,056	2,901	2,790	2,707	2,641	2,588	2,544	2,507	2,475	2,448	2,424	2,403	15
16	4,494	3,634	3,239	3,007	2,852	2,741	2,657	2,591	2,538	2,494	2,456	2,425	2,397	2,373	2,352	16
17	4,451	3,592	3,197	2,965	2,810	2,699	2,614	2,548	2,494	2,450	2,413	2,381	2,353	2,329	2,308	17
18	4,414	3,555	3,160	2,928	2,773	2,661	2,577	2,510	2,456	2,412	2,374	2,342	2,314	2,290	2,269	18
19	4,381	3,522	3,127	2,895	2,740	2,628	2,544	2,477	2,423	2,378	2,340	2,308	2,280	2,256	2,234	19
20	4,351	3,493	3,098	2,866	2,711	2,599	2,514	2,447	2,393	2,348	2,310	2,278	2,250	2,225	2,203	20
21	4,325	3,467	3,072	2,840	2,685	2,573	2,488	2,420	2,366	2,321	2,283	2,250	2,222	2,197	2,176	21
22	4,301	3,443	3,049	2,817	2,661	2,549	2,464	2,397	2,342	2,297	2,259	2,226	2,198	2,173	2,151	22
23	4,279	3,422	3,028	2,796	2,640	2,528	2,442	2,375	2,320	2,275	2,236	2,204	2,175	2,150	2,128	23
24	4,260	3,403	3,009	2,776	2,621	2,508	2,423	2,355	2,300	2,255	2,216	2,183	2,155	2,130	2,108	24
25	4,242	3,385	2,991	2,759	2,603	2,490	2,405	2,337	2,282	2,236	2,198	2,165	2,136	2,111	2,089	25
26	4,225	3,369	2,975	2,743	2,587	2,474	2,388	2,321	2,265	2,220	2,181	2,148	2,119	2,094	2,072	26
27	4,210	3,354	2,960	2,728	2,572	2,459	2,373	2,305	2,250	2,204	2,166	2,132	2,103	2,078	2,056	27
28	4,196	3,340	2,947	2,714	2,558	2,445	2,359	2,291	2,236	2,190	2,151	2,118	2,089	2,064	2,041	28
29	4,183	3,328	2,934	2,701	2,545	2,432	2,346	2,278	2,223	2,177	2,138	2,104	2,075	2,050	2,027	29
30	4,171	3,316	2,922	2,690	2,534	2,421	2,334	2,266	2,211	2,165	2,126	2,092	2,063	2,037	2,015	30
31	4,160	3,305	2,911	2,679	2,523	2,409	2,323	2,255	2,199	2,153	2,114	2,080	2,051	2,026	2,003	31
32	4,149	3,295	2,901	2,668	2,512	2,399	2,313	2,244	2,189	2,142	2,103	2,070	2,040	2,015	1,992	32
33	4,139	3,285	2,892	2,659	2,503	2,389	2,303	2,235	2,179	2,133	2,093	2,060	2,030	2,004	1,982	33
34	4,130	3,276	2,883	2,650	2,494	2,380	2,294	2,225	2,170	2,123	2,084	2,050	2,021	1,995	1,972	34
35	4,121	3,267	2,874	2,641	2,485	2,372	2,285	2,217	2,161	2,114	2,075	2,041	2,012	1,986	1,963	35
40	4,085	3,232	2,839	2,606	2,449	2,336	2,249	2,180	2,124	2,077	2,038	2,003	1,974	1,948	1,924	40
60	4,001	3,150	2,758	2,525	2,368	2,254	2,167	2,097	2,040	1,993	1,952	1,917	1,887	1,860	1,836	60
80	3,960	3,111	2,719	2,486	2,329	2,214	2,126	2,056	1,999	1,951	1,910	1,875	1,845	1,817	1,793	80
90	3,947	3,098	2,706	2,473	2,316	2,201	2,113	2,043	1,986	1,938	1,897	1,861	1,830	1,803	1,779	90
100	3,936	3,087	2,696	2,463	2,305	2,191	2,103	2,032	1,975	1,927	1,886	1,850	1,819	1,792	1,768	100
120	3,920	3,072	2,680	2,447	2,290	2,175	2,087	2,016	1,959	1,910	1,869	1,834	1,803	1,775	1,750	120
inf.	3,841	2,996	2,605	2,372	2,214	2,099	2,010	1,938	1,880	1,831	1,789	1,752	1,720	1,692	1,666	inf.

Nota. Adapted from Goos *et al.*, 2016.

Table B. 1

Report of ANOVA

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS
1	1,65E+07	1,74E+07	-9,55E+05	0,583	-0,485	-0,469	0,022	-0,555
2	1,88E+07	1,95E+07	-6,78E+05	0,583	-0,344	-0,331	0,011	-0,392
3	1,34E+07	1,43E+07	-9,05E+05	0,583	-0,46	-0,444	0,02	-0,525
4	1,28E+07	1,16E+07	1,17E+06	0,333	0,469	0,453	0,007	0,321
5	4,28E+06	7,24E+06	-2,96E+06	0,583	-1,502	-1,596	0,211	-1,889
6	4,90E+06	2,94E+06	1,97E+06	0,583	0,999	0,998	0,093	1,181
7	7,83E+06	5,60E+06	2,23E+06	0,583	1,131	1,146	0,119	1,356
8	1,34E+07	1,16E+07	1,82E+06	0,333	0,729	0,714	0,018	0,505
9	7,06E+06	7,15E+06	-97852,46	0,583	-0,05	-0,048	0	-0,056
10	6,63E+06	1,16E+07	-4,93E+06	0,583	-2,506	-3,475	0,586	-4,111(1)
11	1,24E+07	1,22E+07	2,49E+05	0,583	0,126	0,121	0,001	0,143
12	8,59E+06	1,16E+07	-2,98E+06	0,333	-1,199	-1,223	0,048	-0,865
13	5,60E+06	5,54E+06	62034,71	0,583	0,032	0,03	0	0,036
14	6,87E+06	5,68E+06	1,19E+06	0,583	0,606	0,589	0,034	0,697
15	4,23E+06	5,48E+06	-1,25E+06	0,583	-0,637	-0,621	0,038	-0,734
16	9,49E+06	9,96E+06	-4,70E+05	0,583	-0,239	-0,229	0,005	-0,271
17	3,53E+07	3,11E+07	4,23E+06	0,583	2,149	2,623	0,431	3,103(1)

18	2,90E+07	2,85E+07	5,44E+05	0,583	0,276	0,265	0,007	0,314

Table B. 2

Report of ANOVA (Continuation)

19	1,23E+07	1,05E+07	1,74E+06	0,583	0,885	0,877	0,073	1,037
20	1,40E+07	1,39E+07	79283,42	0,583	0,04	0,039	0	0,046
21	5,47E+06	1,15E+06	4,32E+06	0,583	2,193	2,712	0,449	3,209(1)
22	1,59E+07	1,53E+07	5,98E+05	0,583	0,304	0,292	0,009	0,346
23	1,34E+07	1,46E+07	-1,22E+06	0,583	-0,619	-0,602	0,036	-0,712
24	5,09E+06	6,29E+06	-1,20E+06	0,583	-0,609	-0,593	0,035	-0,701
25	2,83E+07	2,88E+07	-5,18E+05	0,583	-0,263	-0,253	0,006	-0,299
26	1,36E+07	1,30E+07	6,02E+05	0,583	0,306	0,294	0,009	0,348
27	1,37E+07	1,64E+07	-2,62E+06	0,583	-1,332	-1,381	0,166	-1,634

DFFITS= difference in fits

(1) Exceeds limits