TESE DE DOUTORAMENTO

USE OF HIGH RESOLUTION MASS/MASS SPECTROMETRY FOR THE INVESTIGATION OF FOOD SAFETY AND ENVIRONMENTAL IMPACT OF ORGANIC EMERGING CONTAMINANTS

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DE COMPOSTELA

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DE SANTIAGO
DE COMPOSTELA

INDEX

USC
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DE SANTIAGO
DE COMPOSTELA

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DE SANTIAGO
DE COMPOSTELA

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ABBREVIATIONS

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_____#

2-EHMC: 2-ethylhexyl methoxycinnamate
3-ADON: 3-acetyl deoxynivalenol
4-MBC: 4-methylbenzylidene camphor
15-ADON: 15-acetyl deoxynivalenol
α-ZOL: α-zearalenol
β-ZOL: β-zearalenol

_____A

ACN: acetonitrile
AF: aflatoxin
AND A: andrastin A
ANOVA: analysis of variance
AOH: alternariol
AOPs: advanced oxidation processes
au: arbitrary units

_____B

BEN: benalaxyl
BMDM: butyl methoxy dibenzoyl methane
BP1: benzophenone-1
BP2: benzophenone-2
BP3: benzophenone-3
BP4: benzophenone-4
BP6: benzophenone-6
BP8: benzophenone-8
BS: benzyl salicylate

_____C

CE: collision energy
CPA: cyclopiazonic acid
CYP: cyprodinil

D

DHHB: Diethylaminohydroxybenzoyl hexyl benzoate
DI: direct immersion
DIA: data independent acquisition
DIM: dimethomorph
DLLME: dispersive liquid-liquid microextraction
DON: deoxynivalenol
DRT: Drometrizole trisiloxane
DSPE: dispersive solid phase extraction

E

EFSA: European Food Safety Authority
EHPABA: ethylhexyl dimethyl p-aminobenzoic acid
EHS: ethylhexyl salicylate
EI: electron impact
ENN: enniatin
EPT: 12, 13-epoxytrichothec-9-ene
ESI: electrospray ionization
ETO: etocrylene

F

FAO: Food and Agricultural Organization
FB: fumonisin
FEN: fenhexamid
FUS X: Fusarenon X

G

GC: Gas chromatography

H

HESI: heated electrospray ionization
HMS: homosalate
HPLC: high performance liquid chromatography
HRMS: high resolution mass spectrometry
HS: headspace

I

IAMC: isoamyl methoxycinnamate
IARC: International Agency for Research on Cancer
IDLs: instrumental detection limits
INCI: International Nomenclature of Cosmetic Ingredients
IPR: Iprovalicarb
IPRO: iprodione
IUPAC: International Union of Pure and Applied Chemistry

K

KRE: Kresoxim-methyl

L

LC: liquid chromatography
LLE: liquid-liquid extraction
LPME: liquid-phase microextraction
LOD: limit of detection
LOQ: limit of quantification

M

MA: Menthyl anthranilate
MAC A: marcfortine A
MeOH: methanol
MET: metalaxyl

MOA: mode of action
MPA: mycophenolic acid
MS: mass spectrometry
MS/MS: tandem mass spectrometry
MW: molecular weight
MYC: myclobutanil
m/z: mass-to-charge ratio

N

NIV: nivalenol

O

OCR: octocrylene
OTA: ochratoxin A

P

PA: polyacrylate
PBSA: ensulizole
PCPs: personal care products
PDMS/DVB: polydimethylsiloxane/divinylbenzene
PEN A: penitrem A
PRO: procymidone
PSA: primary secondary amine
PTFE: polytetrafluoroethylene

Q

Q: quadrupole
QuEChERS: Quick, Easy, Cheap, Effective, Rugged and Safe

R

ROQ-C: roquefortine C
ROS: reactive oxygen species
RSD: relative standard deviation

S

SLE: solid-liquid extraction
S/N: signal to noise ratio
SPE: solid phase extraction
SPME: solid phase microextraction
SRM: selected reaction monitoring
STE: sterigmatocystin
SWATH™: Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra

T

TEB: tebuconazole
TOF: Time-of-flight

U

USAEME: ultrasound-assisted emulsification-microextraction
UV: ultraviolet
UV-Vis: ultraviolet-visible

W

WHO: World Health Organization
WWTP: wastewater treatment plants

Z

ZEA: zearalenone

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ABSTRACT

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The main objectives of this thesis were the development of analytical methodologies to determine organic contaminants of interest in environmental and food matrices and the evaluation of their occurrence and fate in these matrices. The first objective involves the determination of widespread used fungicides and UV filters in water and the analysis of mycotoxins in feed. The second aim was focused on the degradation of the previously mentioned fungicides and UV filters in the aquatic media. The analytical techniques employed were based in gas and, mostly, liquid chromatography. Apart from tandem mass spectrometry, the fundamental technique used, especially in the case of photodegradation studies, was high resolution mass spectrometry.

The present work is divided in six sections. **Section I** -justification and objectives- includes the explanation of the importance of the analytes selected and the problematic or potential hazard that they may present in the analysed matrices. **Section II** is an introduction of the different analytes studied, including their classification, physico-chemical properties, toxicity, and legal framework. Photodegradation processes in water are also considered in this chapter, as well as the different sample preparation and analytical techniques employed. In **Section III**, a brief description of the methodology used is displayed. **Section IV** includes the presentation and discussion of the experimental work and results obtained during this thesis.

The different studies carried out are compiled in four articles resulting in three different chapters:

Chapter 1. Determination and fate of fungicides in water

In the first publication included in this section, a methodology based on solid phase microextraction (SPME) followed by gas chromatography-tandem mass spectrometry (GC-MS/MS) was optimized for the determination of a number of widespread used fungicides in environmental waters.

The second work is focused in the photodegradation suffered by a selection of this family of compounds in water using UVC radiation. Kinetics evaluation was carried out using high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) and HPLC coupled to high resolution mass spectrometry (HRMS). The last one was also employed on the search of possible by-products formed after the radiation.

Chapter 2. Photodegradation of UV filters

This chapter is based in an article that includes the assessment of different photodegradation strategies to remove 21 multiclass organic UV filters from the aquatic media. Direct photolysis under UVA and UVC radiations and advanced oxidation processes (AOPs) based on heterogeneous UVA/TiO_2 photocatalysis and the UVC/H_2O_2 system were applied for the degradation tests. SPME-GC-MS/MS and HPLC coupled both with MS/MS and HRMS were used for the monitoring of the photodegradation processes. Tentative identification of by-products was also carried out.

Chapter 3. Determination of mycotoxins in feed

In this chapter, an exhaustive methodology for the determination of 26 mycotoxins from different genera in a complex feed matrix is presented. The different separation techniques used were solid phase extraction (SPE), dispersive solid phase extraction (DSPE) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction. Matrix effect was evaluated. HPLC-HRMS was employed for the unequivocal identification and quantification of the analytes.

Section V includes a general discussion putting into a cohesive context all studies presented in the previous section.

Finally, **Section VI** comprehends the general conclusions extracted from the work presented in this thesis.

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I. JUSTIFICATION AND OBJECTIVES

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In the last years, a growing presence of emerging contaminants, such as personal care products or pesticides was detected on the aquatic ecosystem and, by extension, in other environmental matrices like soils and sediments.

Pesticides are massively used in agriculture in order to prevent plagues. Fungicides are particularly employed, for example, in vineyards, often situated near rivers or other water bodies. Therefore, the entering of these organic contaminants in the water cycle can easily occur via lixiviation or filtration.

Likewise, UV filters are broadly employed, mostly in cosmetic formulations. These compounds have also an easy access to surface and groundwater both directly -bathing and recreational activities in rivers or beaches- and indirectly -wastewater treatment plants and industry discharge-.

Therefore, it is necessary to develop analytical methodologies for the determination of these families of compounds, as well as monitoring the possible transformations that can occur because of UV irradiation,

Hence, the environmental part of this thesis was developed based on the following objectives:

I. Development of analytical methodologies for the determination of fungicides in environmental samples at very low concentration levels (trace level).

   A method based on solid-phase microextraction (SPME), a miniaturized extraction technique which allows highly efficient results using very low amount of solvents, is proposed. Gas chromatography (GC) followed by tandem mass spectrometry (MS/MS) is the configuration of choice in order to achieve high selectivity and sensitivity (**Section IV, Chapter 1, *1.1***).

II. Monitoring of the photodegradation of organic emerging contaminants in water matrices and proposing efficient processes for the exhaustive elimination of these substances.

- Fungicides: a selection of 9 multiclass fungicides was made, considering those which are more broadly used (**Section IV, Chapter 1, *1.2***).

- UV filters: 21 multiclass organic UV filters, including the most employed in cosmetic formulations since there is no specific legislation for these compounds in environmental matrices (**Section IV, Chapter 2, *2.1***).

The photodegradation systems proposed consist of direct UV photolysis (UVA and UVC radiation), and advanced oxidation processes (AOPs), such as UVC/H_2O_2 and heterogeneous UVA/TiO_2 photocatalysis.

For the determination of the kinetic profiles of both families of analytes, HPLC-MS/MS configuration is proposed, because it enables the detection at low concentrations, which is the most realistic scenario working with real water samples.

III. Identification of degradation by-products of the studied contaminants in water.

After the photodegradation processes carried out, high resolution mass spectrometry (HRMS) is proposed for the non-target identification of possible by-products of both fungicides and UV filters (**Section IV, Chapter 1, *1.2*; Chapter 2, *2.1***).

Regarding food safety, some organic contaminants, such as mycotoxins are frequently found in food and feed matrices. These compounds present very prejudicial effects for both animal and human health. Several methodologies were proposed for the determination of mycotoxins in feed samples. However, most of them only consider one compound of a family of mycotoxins when the most common scenario is the co-occurrence of multiple toxins. Therefore, multianalyte

methodologies are recommended for a comprehensive analysis of the samples.

Analysis techniques so far were mostly based on chromatography coupled to tandem mass spectrometers (MS/MS). However, a high-resolution mass spectrometry approach, apart from the exact identification and quantification of the target analytes, offers the possibility of the detection of non-targeted mycotoxins present in the samples.

Taking these considerations into account, in the chapter dedicated to food safety, the objective was:

IV. The development and optimization of multianalyte methodologies for the accurate identification and quantification of mycotoxins in complex feed matrices. Application of simple, effective and "green" extraction techniques such as QuEChERS extraction, solid phase extraction (SPE) and dispersive solid-phase extraction (DSPE). HPLC-HRMS analysis is proposed, because it offers a precise identification and quantification of the analytes, while enabling the search for non-targeted mycotoxins and modified forms of the targeted analytes (**Section IV, Chapter 3, *3.1***).

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II. INTRODUCTION

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1. Fungicides

1.1. Introduction

The European Food Safety Authority (EFSA) defines pesticide as a substance used to kill or control pests, including disease-carrying organisms and undesirable insects, animals, and plants. The generic term "pesticides" includes, amongst others: herbicides, insecticides, rodenticides, grown regulators and, our topic of interest, fungicides.

Fungicides, also called antimycotics, are biocidal chemical compounds that can kill or inhibit the growth of fungi and their spores in plants, stored products, or soil. Fungicides are extensively used worldwide to prevent economical damage by fungi or mould infestation in agriculture, forestry, and horticulture practices, among others.

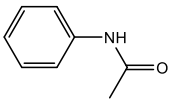
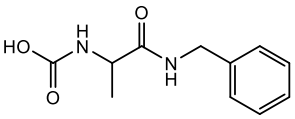
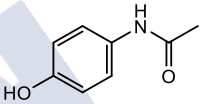
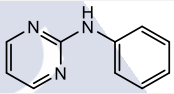
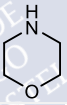
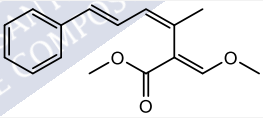
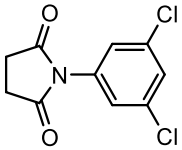
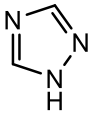
To assess their effectiveness, fungicides should have the following properties: low toxicity to the plant or animal but high toxicity to the specific fungus (specificity); ability to transform themselves by enzymes into toxic intermediates; capacity to penetrate fungal spores to reach the site of action; low ecotoxicity; and ability to form a protective deposit on the plant surface that can resist climatological adversities [1].

1.2. Classification

Fungicides can be classified according to:

- The chemistry family of their active ingredient. Chemical groups include amides, benzimidazoles, carbamates, dicarboximides, dithiocarbamates, imidazoles, morpholines, pyridines, pyrimidines, quinolines, strobilurins, thiazoles, triazoles, and ureas [2].

Table 1.1. Classification of the studied fungicides by their active ingredient structure.

Name	Chemical group	Active ingredient structure
Benalaxyl	Aromatic amide	
Metalaxyl		
Iprovalicarb	Carboxylic acid amide	
Fenhexamid	Hydroxyanilide	
Cyprodinil	Anilinopyrimidine	
Dimethomorph	Morpholine	
Kresoxim Methyl	Strobilurin	
Iprodione	Dicarboximide	
Procymidone		
Myclobutanil	Triazole	
Tebuconazole		

The structures of the studied fungicides are represented in **Figure 1.1**.

- Their hazard profile, following the World Health Organization (WHO) recommendation [3]. The value used to classify them is oral LD_{50}, which is a statistical estimate of the number of mg of toxicant per kg of bodyweight required to kill 50% of a large population of test animals. The selected fungicides studied in this thesis are labelled as follows:

Table 1.2. Classification of the studied fungicides in terms of hazard according to WHO [3]**.**

Name	LD_{50} mg/kg		Classification
Benalaxyl	4200	Class III	Slightly hazardous
Dimethomorph	3500	Class III	Slightly hazardous
Fenhexamid	>5000		Unlikely to present acute hazard in normal use
Iprodione	3500	Class III	Slightly hazardous
Iprovalicarb	>5000		Unlikely to present acute hazard in normal use
Metalaxyl	670	Class II	Moderately hazardous
Myclobutanil	1600	Class II	Moderately hazardous
Procymidone	6800		Unlikely to present acute hazard in normal use
Tebuconazole	1700	Class II	Moderately hazardous

- Their mode of action (MOA). There are three types of fungicides: **contact fungicides** create a barrier on the leaf surface after application and do not penetrate de tissue; **mesostemic fungicides** are strongly attached to the leaf surface, and small amounts penetrate the tissue giving the leaf coverage for both sides; and **systemic fungicides** are absorbed into the plant and require the plant to be actively growing to circulate through its system.

1.3. STRUCTURE AND PHYSICO-CHEMICAL PROPERTIES

The fungicides selected are listed in **Table 1.3**, including their identifiers: name (INCI name), abbreviation and CAS number. The molecular formula and molecular weight (MW) are also indicated, as well as the wavelength of maximum absorbance. All data were obtained from Scifinder ((Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (©1994-2018 ACD/Labs)) and from ChemSpider (Predicted data is generated using the US Environmental Protection Agency's EPISuite™).

Table 1.3. INCI name, CAS number, molecular formula, molecular weight, partition coefficient, solubility in water and the wavelength of maximum absorbance.

INCI name	CAS No.	Molecular formula	MW (g mol^{-1})	log K_{ow}*	Solubility in water (mol L^{-1})*	λ_{max} (nm)
Benalaxyl (BEN)	71626-11-4	$C_{20}H_{23}NO_3$	214.22	3.5	1.14×10^{-4}	258.6
Cyprodinil (CYP)	121552-61-2	$C_{14}H_{15}N_3$	246.22	4.0	5.76×10^{-5}	270.8
Dimethomorph (DIM)	110448-70-5	$C_{21}H_{22}ClNO_4$	228.24	2.6	7.41×10^{-5}	200
Fenhexamid (FEN)	126833-17-8	$C_{14}H_{17}Cl_2NO_2$	308.31	3.5	6.61×10^{-5}	203, 291
Iprodione (IPRO)	36734-19-7	$C_{13}H_{13}Cl_2N_3O_3$	228.24	3.0	4.01×10^{-5}	
Iprovalicarb (IPR)	140923-17-7	$C_{18}H_{28}N_2O_3$	228.24	3.3	5.5×10^{-5}	220
Kresoxim methyl (KRE)	143390-89-0	$C_{18}H_{19}NO_4$	244.24	3.4	6.42×10^{-6}	290
Metalaxyl (MET)	70630-17-0	$C_{15}H_{21}NO_4$	290.40	1.65	2.91×10^{-2}	206

Table 1.3 (cont.). INCI name, CAS number, molecular formula, molecular weight, partition coefficient, solubility in water and the wavelength of maximum absorbance.

INCI name	CAS No.	Molecular formula	MW (g mol^{-1})	log K_{ow}*	Solubility in water (mol L^{-1})*	λ_{max} (nm)
Myclobutanil (MYC)	88671-89-0	$C_{15}H_{17}ClN_4$	248.32	2.9	4.85×10^{-2}	219
Procymidone (PRO)	32809-16-8	$C_{13}H_{11}Cl_2NO_2$	250.33	3.0	1.34×10^{-5}	
Tebuconazole (TEB)	107534-96-3	$C_{16}H_{22}ClN_3O$	250.33	3.7	1.17×10^{-4}	221

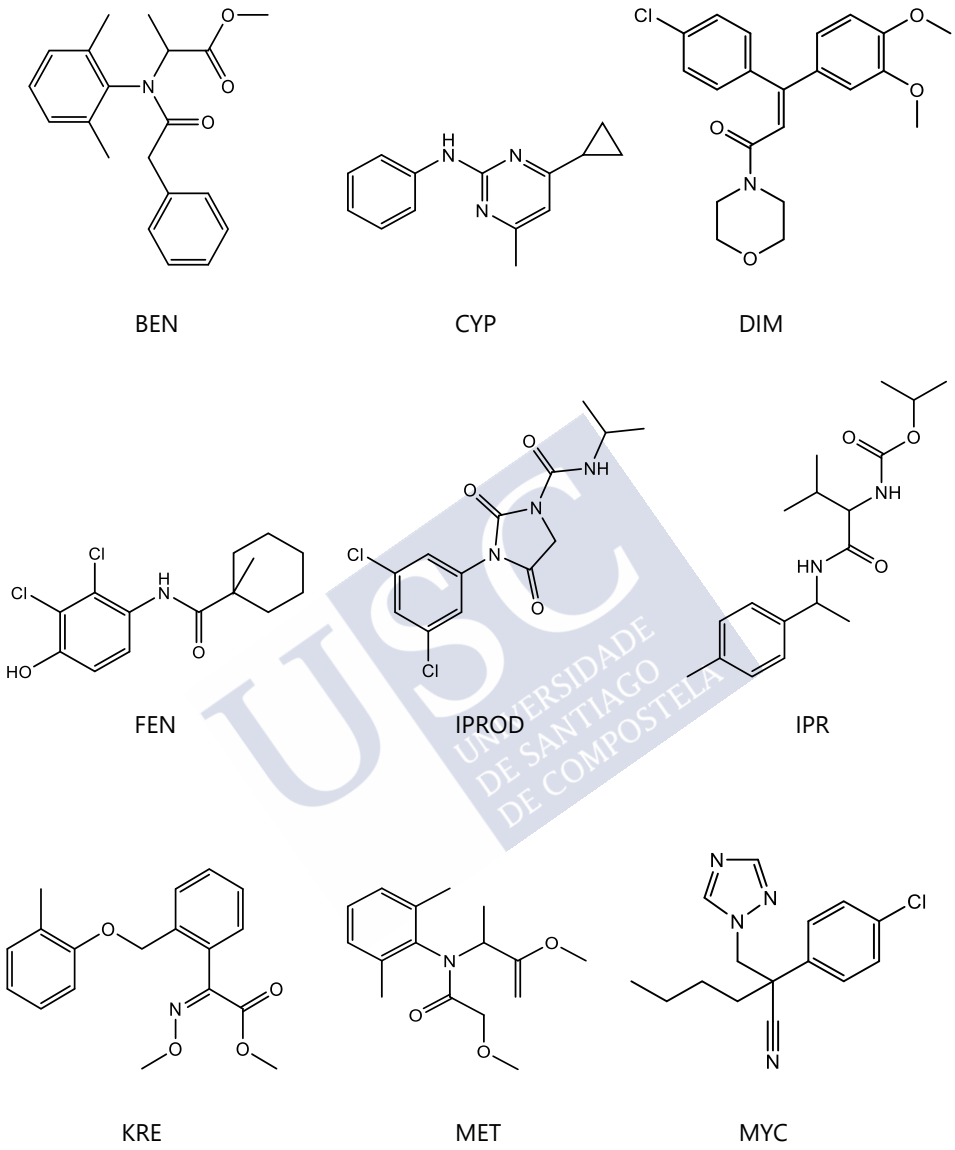
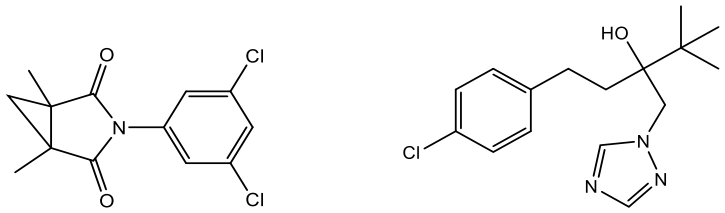


Figure 1.1. Fungicide structures



PRO TEB

Figure 1.1 (cont.). Fungicide structures

1.4. TOXICITY

As a result of their extensive use, fungicide residues can accumulate in vegetation and soil, leading ultimately to their entry in the surface water system. Fungicides can have access to the environment by storage, direct spraying, runoff, or factory wastewater discharge [4–7].

Contamination of surface waters with fungicides is more accused in areas where intensive agricultural activity take place or in vineyards, which usually are located near rivers or other water reservoirs [8].

Some fungicides have very stable chemical structures, so their degradation and transformation to less toxic products may take years. Also, the low solubility in water of some of the compounds can enhance the phenomenon of bioaccumulation (e.g., in fish tissue) and biomagnification, affecting also human health through the food chain [9–11].

The European Food Safety Authority published peer review reports of different fungicides evaluating, among other characteristics, the toxicological activity in surface and ground waters.

Table 1.4. Risk assessment by EFSA of the studied fungicides for the environmental compartments.

Fungicide		Soil	Ground water	Surface water and sediment
Benalaxyl	[12]	Moderated to high persistence	Pesticidal activity Toxicological relevance	Low ecotoxicological risk
Cyprodinil	[13]	Moderated persistence	Pesticidal activity Toxicological and ecotoxicological relevance	n.d.
Dimethomorph	[14]	Medium to moderated persistence	Pesticidal activity Toxicological and ecotoxicological relevance	n.d.
Fenhexamid	[15]	Very low to low persistence	Pesticidal activity Toxicological relevance	Low risk to aquatic organisms
Iprodione	[16]	Moderated to high persistence	Pesticidal activity Toxicological relevance	High risk to aquatic organisms in surface water
Iprovalicarb	[17]	Low to medium persistence	Pesticidal activity Toxicological relevance	Low ecotoxicological risk
Kresoxim Methyl	[18]	Very low to low persistence	Pesticidal activity Toxicological and ecotoxicological relevance	Very toxic to aquatic organisms
Metalaxyl	[19]	Low to medium persistence	Pesticidal activity Toxicological and ecotoxicological relevance	Low risk to aquatic organisms
Myclobutanil	[20]	High to very high persistence	Pesticidal activity Toxicological relevance Very toxic to aquatic organisms	Very toxic to aquatic organisms
Tebuconazole	[21]	Medium to moderated persistence	Pesticidal activity Toxicological relevance	Low risk to aquatic organisms

Besides the risk assessment for the precursor fungicides, it is key to studying the transformation products that may be formed due to the light exposure or to different kind of reactions that can take place in the environmental matrices (e.g., oxidation, reduction, hydrolysis, substitution, or loss of functional groups).

In the experimental part (**Section III**) of this thesis, photodegradation studies of the previously indicated fungicides are presented, including the tentative identification of transformation products in aqueous matrices.

1.5. LEGISLATION IN WATER

Taking into account the risks that the exposure of fungicides may pose, the European Parliament published Directive 2009/128/EC, establishing a framework for Community action to achieve the sustainable use of pesticides [22].

The continuous analysis and monitoring of risks of the authorized pesticides allowed to gather the sufficient evidence to ban many hazardous compounds that years ago were routinely used. These harmful substances have been replaced by safer pesticides, or even non-chemical methods.

To guarantee public drinking water quality, the European Union Directive on the quality of water intended for human consumption (98/83/EC) established a maximum allowed concentration of 0.1 μg L^{-1} for individual pesticides and of 0.5 μg L^{-1} for total pesticides in drinking water [23]. Hence, the determination of pesticide residues in environmental water is of general interest to the human health and environmental safety.

For instance, since the EFSA pointed out the possible contamination of ground waters by benalaxyl metabolites, the European Commission has recently implemented regulation 2020/1280 in which

is stated the non-renewal of the approval of benalaxyl active substance [24].

According to Directive 2013/39/EU [25] a watch list of substances for Union-wide monitoring in water was stablished in 2015. The objective was to make it easier to assess the risk from chemical substances found in surface waters. In august 2020, the European Commission updated the watch list, including tebuconazole [26].

Fungicides are generally present in environmental waters at very low concentrations, which is why is important the use of pre-concentration techniques prior to the analysis of these matrices to achieve higher sensitivity. Also, multi-analyte methodologies are preferred for the detection and monitoring of these compounds, because it is highly unlikely that only one fungicide is present in a water sample.

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2. UV Filters

2.1. INTRODUCTION

According to the European Regulation [27], UV filters are defined as "substances which are exclusively or mainly intended to protect the skin against certain UV radiation by absorbing, reflecting or scattering UV radiation".

UV radiation is the section of the electromagnetic spectrum situated between X-rays and visible light, and its most common form is solar radiation or sunlight. Considering wavelength range, UV radiation presents three differentiated regions: UVA (320-400 nm), UVB (290-320 nm) and UVC radiation (100-290 nm).

Earth's surface is not naturally exposed to UVC radiation because it is absorbed by atmosphere [28]. Thus, it does not represent a significant risk to human health. However, UVB and UVA radiation can reach the surface and middle layer of our skin, respectively. As a result, UVB radiation is mainly responsible for immediate reactions such as skin pigmentation, sunburns, inflammations (i.e. edema and erythema) and also immunosuppression and carcinogenesis [29]. UVA represents around 95% of the total UV radiation emitted and can reach the dermis. It can cause damages in the structure of proteins and nucleic acids, resulting in premature skin aging. Also, UVA exposure can produce photoallergic reactions, which may lead to immunosuppression and carcinogenesis [28,29].

Considering the proved effects of UV radiation exposure and the increasing awareness of the society, UV filters are massively used nowadays. Besides sunscreens, a wide range of PCPs (personal care

products) contain these substances in their formula to offer protection against the harmful effects of UV radiation. UV filters or UV blockers are also used in the manufacture of textile, plastic or coating materials to assure the stability of the product [30].

UV filters are increasing their presence in our everyday life, so it is crucial to make sure that the legislation on maximum allowed concentration of this substances is being followed.

2.2. CLASSIFICATION

UV filters can be classified depending on their nature in two types: organic and inorganic [31].

- **Inorganic or physical** UV filters are substances that protect from the solar radiation by reflection or scattering. These compounds can be metallic oxides such as zinc oxide (ZnO) and titanium dioxide (TiO_2), or minerals like talc.

Inorganic UV filters offer a higher protection than the organic ones. Due to their insolubility in water, these substances form a waterproof layer on the skin. This way, solar radiation cannot penetrate the dermis, avoiding the risk of DNA damage. However, physical UV filters are not as widely used in PCPs because of the thickness, antiperspirant, and whitening effect on the skin.

- **Organic or chemical** UV filters are organic molecules with aromatic structures that have a great absorbance capacity in the UVA-UVB region and transform this radiation into vibrational, fluorescent, or radical energy. These mechanisms can cause protein and DNA damage on the skin. Also, organic UV filters are known to produce some dermatologic adverse effects due to their capacity to penetrate the superficial layer of the skin.

Despite these proven risks, chemical UV filters are far more accepted and used than physical filters because their application is more comfortable, and they are perspirant and invisible.

Organic UV filters can be classified in different families depending on their chemical structure: benzophenone derivatives, p-aminobenzoic acid derivatives, cinnamates, salicylates, camphor derivatives, triazine derivatives, benzotriazole derivatives and benzimidazole derivatives, amongst others [32].

In the EU Cosmetics Directive, 25 out of the 26 allowed UV filters listed are organic UV filters. The only inorganic substance considered is TiO_2 [27].

In this thesis, 20 of these allowed organic UV filters will be studied. Their structure and physico-chemical properties are listed in the following section (**Section 2.3**).

2.3. STRUCTURE AND PHYSICO-CHEMICAL PROPERTIES

The UV filters selected are listed in **Table 2.1**, including their identifiers: name (INCI name), abbreviation and CAS number. The molecular formula and molecular weight (MW) are also indicated, as well as relevant physicochemical properties for the intended studies. The negative decimal logarithm of the acid dissociation constant K_a (pK_a), the decimal logarithm of the octanol-water partition coefficient (log K_{ow}) and the solubility in water of each compound is indicated. Also, the wavelength of maximum absorbance is specified. All data were obtained from Scifinder ((Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (©1994-2018 ACD/Labs)) and from ChemSpider (Predicted data is generated using the US Environmental Protection Agency‘s EPISuite™).

The structures of the studied UV filters are represented in **Figure 2.1**. These compounds generally present simple or multiple aromatic structures. Sometimes, they also have conjugated carbon-carbon double bonds and/or carbonyl groups.

Most organic UV filters are lipophilic, but some of these compounds have ionizable groups in their structures, like carboxylate (-COOH) or sulphonic ($-SO_3H$), which allows their solubility in water.

Table 2.1. INCI name, CAS number, molecular formula, molecular weight, and some physico-chemical properties of the studied UV filters

INCI* name	CAS No.	Molecular formula	MW (g mol^{-1})	pK_a	log K_{ow}	Solubility in water (mol L^{-1})	λ_{max} (nm)
Benzophenone-1 (BP1)	131-56-6	$C_{13}H_{10}O_3$	214.22	7.72±0.4	3.2±0.4	pH 1-5: 1.8×10^{-3} pH 7: 2.4×10^{-3} pH 10: 3.62	291
Benzophenone-2 (BP2)	131-55-5	$C_{13}H_{10}O_5$	246.22	6.98±0.4	3.1±0.4	pH 1-4: 4.0×10^{-3} pH 7: 0.013 pH 10: 4.06	287
Benzophenone-3 (BP3)	131-57-7	$C_{14}H_{12}O_3$	228.24	7.56±0.4	4.0±0.4	pH 1-4: 4.4×10^{-4} pH 7: 5.7×10^{-4} pH 10: 0.10	286, 325
Benzophenone-4 (BP4)	4065-45-6	$C_{14}H_{12}O_6S$	308.31	-0.7±0.5	0.9±0.4	pH 1: 2.72 pH 2-10: 3.24	288, 366
Benzophenone-6 (BP6)	131-54-4	$C_{15}H_{14}O_5$	228.24	6.8±0.4	4.8±0.4	pH 1-5: 2.4×10^{-4} pH 7: 7.0×10^{-4} pH 10: 3.15	284
Benzophenone-8 (BP8)	131-53-3	$C_{14}H_{12}O_4$	244.24	7.1±0.4	4.3±0.4	pH 1-4: 4.9×10^{-4} pH 7: 1.0×10^{-3} pH 10: 4.09	284
2-Ethylhexylmethoxy-cinnamate (2-EHMC)	5466-77-3	$C_{18}H_{26}O_3$	290.40	-	5.9±0.5	2.2×10^{-5}	311
Isoamyl methoxycinnamate (IAMC)	71617-10-2	$C_{15}H_{20}O_3$	248.32	-	4.5±0.2	2.4×10^{-4}	308

* International Nomenclature of Cosmetic Ingredients

Table 2.1 (cont.). INCI name, CAS number, molecular formula, molecular weight and some physico-chemical properties of the studied UV filters

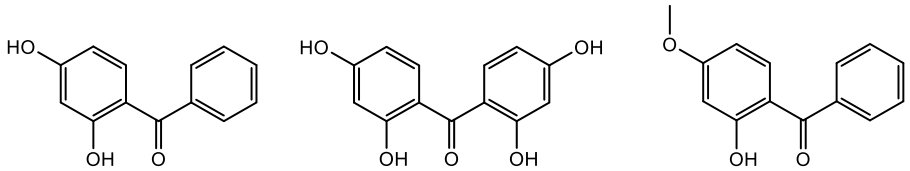
INCI* name	CAS No.	Molecular formula	MW (g mol^{-1})	pK_a	log K_{ow}	Solubility in water (mol L^{-1})	λ_{max} (nm)
Ethylhexyl salicylate (EHS)	118-60-5	$C_{15}H_{22}O_3$	250.33	8.1±0.3	5.9±0.2	pH 1-5: 6.1 x 10^{-5} pH 7: 6.6 x 10^{-5} pH 10: 4.4 x 10^{-3}	305
Benzyl salicylate (BS)	118-58-1	$C_{14}H_{12}O_3$	228.24	8.1±0.3	4.2±0.3	pH 1-6: 3.8 x 10^{-4} pH 10: 0.028	
Homosalate (HMS)	118-56-9	$C_{16}H_{22}O_3$	262.34	8.1±0.3	5.9±0.3	pH 1-6: 7.9 x 10^{-5} pH 10: 6.1 x 10^{-3}	306
Etocrylene (Eto)	5232-99-5	$C_{18}H_{15}NO_2$	277.32	-	4.0±0.3	9.5 x 10^{-5}	303
Octocrylene (OCR)	6197-30-4	$C_{24}H_{27}NO_2$	361.48	-	6.9±0.3	1.0 x 10^{-6}	303
4-Methylbenzylidene camphor (4MBC)	36861-47-9	$C_{18}H_{22}O$	254.37	-	3.4±0.3	6.5 x 10^{-5}	300
Ensulizole (PBSA)	27503-81-7	$C_{12}H_{10}N_2O_3S$	274.30	-0.9±0.4 4.2±0.1	-0.2±0.8	pH 1-3: 5.5 x 10^{-2} pH 5: 0.25 pH 7-10: 3.6	310

* International Nomenclature of Cosmetic Ingredients

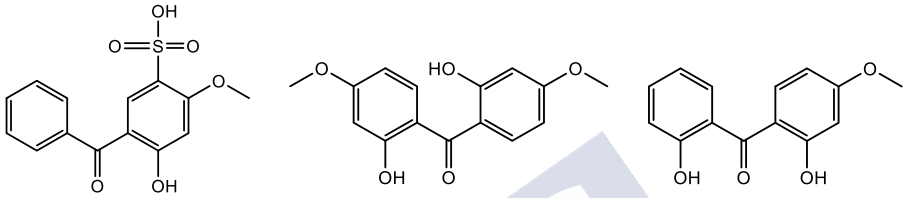
Table 2.1 (cont.). INCI name, CAS number, molecular formula, molecular weight and some physico-chemical properties of the studied UV filters

INCI* name	CAS No.	Molecular formula	MW (g mol^{-1})	pK_a	log K_{ow}	Solubility in water (mol L^{-1})	λ_{max} (nm)
Butyl methoxy dibenzoyl methane (BMDM)	70356-09-1	$C_{20}H_{22}O_3$	310.39	9.7±0.1	4.2±0.4	pH 1-8: 1.4×10^{-5} pH 10: 4.0×10^{-5}	360
Ethylhexyl dimethyl p-aminobenzoic acid (EHPABA)	21245-02-3	$C_{17}H_{27}NO_2$	274.30	2.4±0.1	5.4±0.2	pH 1: 4.1×10^{-4} pH 5-10: 1.7×10^{-5}	311
Diethylaminohydroxy benzoyl hexyl benzoate (DHHB)	302776-68-7	$C_{24}H_{31}NO_4$	397.51	7.6±0.5 2.7±0.4	6.9±0.4	pH 1: 2.5×10^{-7} pH 7: 8.1×10^{-7} pH 10: 1.6×10^{-4}	354
Drometrizole trisiloxane (DRT)	155633-54-8	$C_{24}H_{39}N_3O_3Si_3$	501.84	8.4±0.5 0.7±0.3	8.3±1.2	pH 1: 2.5×10^{-7} pH 7: 1.9×10^{-7} pH 10: 1.3×10^{-5}	303
Menthyl anthranilate (MA)	134-09-8	$C_8H_9NO_2$	275.39	2.2±0.1	6.1±0.3	pH 1: 4.1×10^{-4} pH 7: 6.6×10^{-5} pH 4-10: 1.3×10^{-5}	366

* International Nomenclature of Cosmetic Ingredients

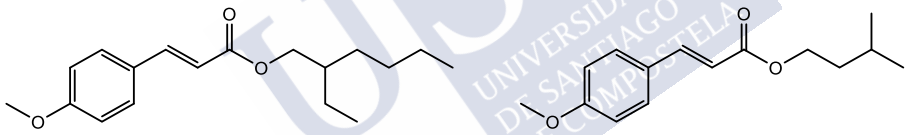


BP1 BP2 BP3



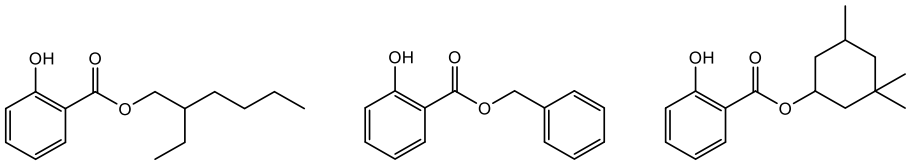
BP4 BP6 BP8

Figure 2.1.a. Benzophenone derivatives



2-EHMC IAMC

Figure 2.1.b. Cinnamates



EHS BS HMS

Figure 2.1.c. Salycilates

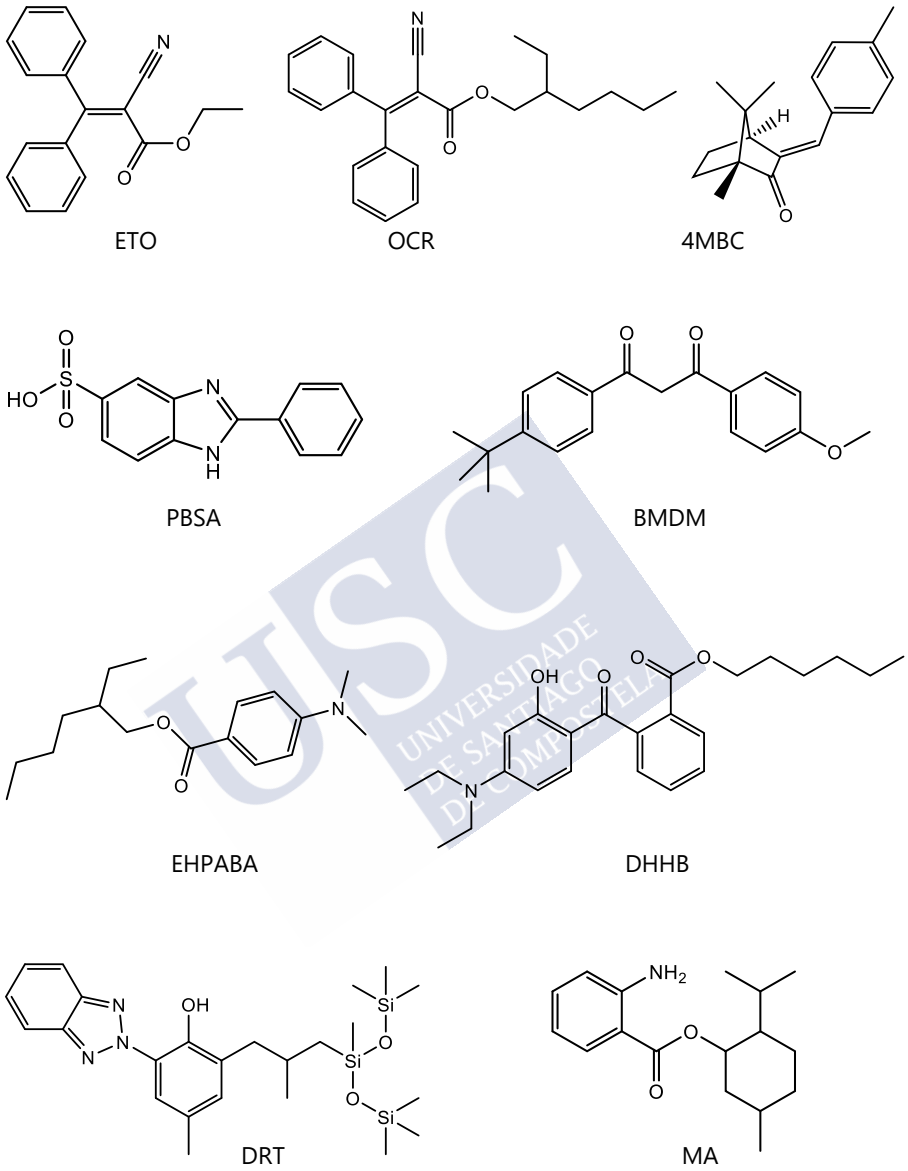


Figure 2.1.d. Others

2.4. TOXICITY

The use of UV filters in personal care products (PCPs), as well as in other industrial products and materials, continues increasing. As a result, these compounds may be accumulating in the aquatic environment through direct and indirect sources. Recreational use of natural waters, such as swimming and bathing in the sea, and wastewater discharges from industrial areas are the main examples of direct sources. The indirect contribution comes from wastewater discharge from wastewater treatment plants (WWTPs).

The presence of relevant concentrations of UV filters in natural waters are reported in many published studies [33–38]. Due to its persistence, UV filters are also found in sediments and sewage sludge [39–43], and there are also evidences of their potential bioaccumulation in fish and human tissues and fluids [44–48].

The main concern about UV filters toxicity is the fact that they present endocrine disrupting effects. Benzophenones, camphor and cinnamate derivatives are examples of estrogenic and androgenic disrupting substances [49,50].

An additional possibility to be considered is the formation of oxidative species like O_2^- and H_2O_2 (ROS, reactive oxygen species) due to exposure to UV radiation. This degradation process takes place mainly by photolysis, but can be interfered by chlorination, e.g., in the case of swimming pool water. Potential degradation products of UV filters generated by these processes may pose a higher risk than their precursors [51]. The degradation processes in water will be more extensively addressed in **Section 3.**

2.5. LEGISLATION IN WATER

To this day, UV filters are not still included in official monitoring programs and there is no legal framework concerning water matrices. Nevertheless, due to the enhanced interest in these compounds, 2-ethylhexylmethoxicinnamate (2-EHMC) was included in 2015 in an European Watch List of substances for further monitoring in water according to Directive 2008/105/CE of the European Parliament and of the Councill [52]. This directive does not set a maximum concentration allowed for the substances but proposes analytical methodologies for the analysis in water samples, as well as a maximum acceptable method limit of detection.

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3. Photodegradation processes

3.1. INTRODUCTION

As previously commented in **Section 2.1**, UV radiation is the sector of the electromagnetic spectrum sited between X-rays and visible light, and its most common form is solar radiation or sunlight. Regarding wavelength range, UV radiation can be divided into three differentiated regions: UVA (320-400 nm), UVB (290-320 nm) and UVC radiation (100-290 nm). UVA radiation can be divided into UV-I or short UVA (340-400 nm) and UVA-II or long UVA (320-340 nm).

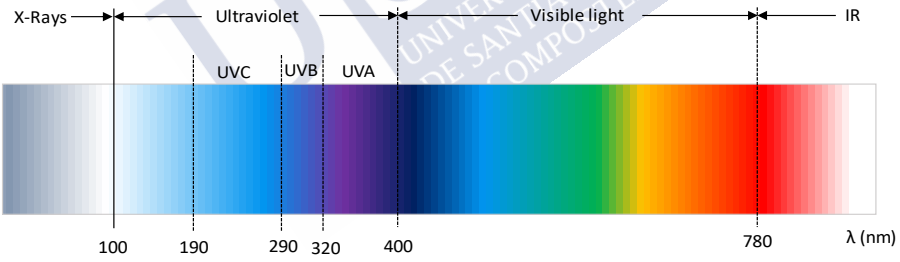


Figure 3.1. Electromagnetic spectrum

According to the International Union of Pure and Applied Chemistry (IUPAC), photodegradation is a "photochemical transformation of a molecule into lower molecular mass fragments taking place in an oxidation or reduction process" [53]. Sunlight exposure may cause photochemical reactions in some compounds, such as bond scission, cyclization, or rearrangement.

When the photochemical reaction takes place in aqueous solution, two different processes may have occurred:

- Direct photolysis: involves the transformation of a chemical resulting from the direct absorption of a solar photon [54].

- Indirect photolysis: the radiation is absorbed by a photosensitizer (photoactive compound), which acts as intermediate producing reactive transients that can prompt the transformation of the compound. This type of photolysis is common in seawater and it is relevant when some molecules are resistant to direct photolysis [55].

3.2. PHOTODEGRADATION KINETICS

Although degradation processes are typically described using first order kinetic equations, most photodegradation studies for UV filters and fungicides in water have shown that a pseudo-first order kinetic is more accurate to describe these processes [8,56–63].

In a pseudo-first order kinetic, one of the reactants (B) would have a significantly higher concentration, while the other reactant (A) would have a substantially lower concentration. In this way, the concentration of the reactant B can be assumed to remain constant and a new rate constant (k') can be described as k [B] and the rate of the reaction is:

$$Rate = k'[A] \qquad \text{(equation 3.1)}$$

A pseudo-first order reaction is described with the equation:

$$[A] = [A]_o\, e^{-k't} \qquad \text{(equation 3.2)}$$

In which $[A]_o$ is the initial concentration of A, k' is the pseudo-first order reaction constant, and [A] is the concentration of A at a time t.

$$\ln\left(\frac{A}{A_o}\right) = k't \qquad \text{(equation 3.3)}$$

The half-life time is defined as the time at which the concentration of a certain compound is reduce to half his initial concentration. For a pseudo-first order kinetic the half-life time is inversely proportionate to the concentration of B:

$$t_{1/2} = \frac{\ln 2}{k\,[A]_o} \qquad \text{(equation 3.4)}$$

Knowing the kinetic characteristics of a photodegradation reaction, such as half-life time and degradation rate, it is vital to study the photostability of the degraded compounds. Then, it would be possible to identify and monitor the appearance of degradation or transformation products and to propose viable pathways for their formation.

3.3. ADVANCED OXIDATION PROCESSES (AOPS)

Advanced oxidation processes (AOPs) were first proposed in the 1980s as a promising solution for potable water decontamination and treatment [64]. Later, AOPs have been used successfully for the elimination of naturally occurring toxins, pesticides, and emerging contaminants in wastewaters, substituting chlorine use.

AOPs were initially defined as the oxidative processes involving the generation of hydroxyl radicals (OH·) in sufficient quantity to affect water purification. With time, AOPs include several methods for generating hydroxyl radical and other reactive oxygen species, such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), or sulphate radicals ($SO_4^{\cdot-}$) [65]. Nevertheless, hydroxyl radical is still the most effective species in advanced oxidation processes.

Most organic compounds rapidly react with OH· due to its lack of selectivity, through various pathways: radical addition, hydrogen abstraction, electron transfer, and radical combination. The result is a carbon-centred radical (R· or R·-OH) that may react with molecular

oxygen (O_2) to form a peroxyl radical ($ROO\cdot$) that reacts further, generating oxidation products such as ketones, aldehydes, and alcohols. Alongside with these products, more reactive species such as H_2O_2 and $O2^{\cdot-}$ are formed, producing chemical degradation and even mineralization of the organic compounds [65,66]. Hydroxyl radicals can only be *in situ* produced during application because of their short lifetime. Oxidizing agents (such as H_2O_2 and O_3), irradiation (such as UV light or ultrasound), and catalysts (such as Fe^{2+}) or photocatalyst (TiO_2) can be used [67].

The interesting feature of these processes is that the oxidation products formed are usually less toxic and more vulnerable to bioremediation.

AOPs can be classified in two categories: photochemical and non-photochemical processes. The processes utilized in this thesis are photochemical: UV/H_2O_2 and heterogeneous photocatalysis.

3.3.1. UV/H_2O_2

UV/H_2O_2 is a homogeneous phase photochemical process in which UV photolysis and H_2O_2 oxidation occur simultaneously producing hydroxyl radicals, as represented in equation 3.5:

$$H_2O_2 + h\upsilon \longrightarrow 2\,HO\cdot \qquad \text{(equation 3.5)}$$

UV/H_2O_2 AOP usually uses medium-low pressure mercury vapor lamps, typically at 254 nm. By generating $HO\cdot$, a relatively non-selective oxidant that reacts with many compounds with nearly diffusion-limited rate constants, the UV/H_2O_2 AOP can degrade compounds that do not absorb UV light directly [68].

The use of hydrogen peroxide as an oxidant present various advantages, such as total miscibility with water, the stability and commercial availability of H_2O_2, no phase transfer problems, simplicity of operation, and relative low investment costs [69].

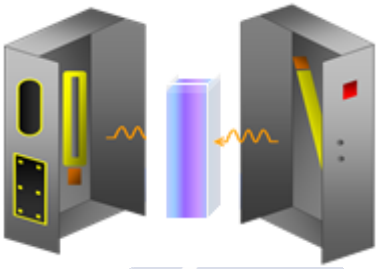


Figure 3.2. Example of a lab-scaled photoreactor with low-pressure Hg lamp

H_2O_2 photolysis has been successfully applied for the removal of different organic pollutants in the aquatic environment even at low concentration levels [68–71]. However, keeping in mind that some photoproducts may present higher toxicity than their precursors, it is essential the identification and monitoring of these products [72].

3.3.2. Heterogeneous photocatalysis

According to IUPAC, the definition of photocatalysis is "change in the rate of a chemical reaction or its initiation under the action of ultraviolet, visible, or infrared radiation in the presence of a substance, the photocatalyst, that absorbs light and is involved in the chemical transformation of the reaction partners" [53]. Photocatalysis processes can be labelled as homogeneous or heterogeneous, depending on the phase in which the catalysis is with respect to the reactants.

Heterogeneous photocatalysis can be described as the acceleration of photoreaction in the presence of a catalyst which is in a different phase from the reactants.

Heterogeneous photocatalysis is a process based on direct or indirect absorption of radiant energy (UV or visible) by a solid (heterogeneous catalyst). In the gap region between the excited solid and the solution, the reactions of destruction of contaminants take place, without the catalysts having chemically changed.

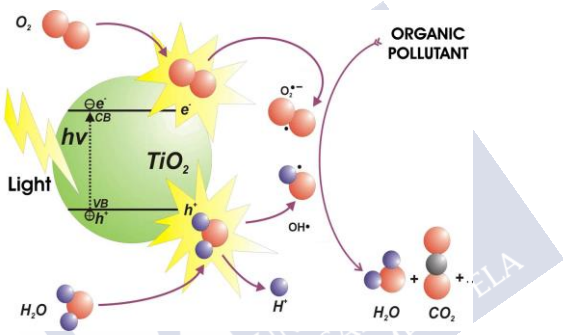


Figure 3.3. Schematic representation of semiconductor excitation by band gap illumination [73].

The most studied photocatalyst are broadband semiconductor metal oxides, especially TiO_2 because its high chemical stability and its capability of working in a wide range of pH.

TiO_2 and more recently TiO_2 nanoparticles, have been proven to effectively degrade organic contaminants such as carboxylic acids, phenolic derivatives, or chlorinated aromatics in water, producing harmless products (CO_2, H_2O, and mineral acids) [74–76].

4. Mycotoxins

4.1. INTRODUCTION

Mycotoxins (from the Greek *mykes* "fungus" and from latin *toxicum* "toxin") are toxic secondary metabolites naturally produced by various filamentous fungi, mainly belonging to *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* genera.

These fungi can grow on numerous foodstuffs, such as cereals, nuts, spices, or dried fruits. Several aspects may be taken into account when explaining why these fungi originated: climatological factors, genetic predisposition of the mould, and bad transportation and storage conditions [77]. This may lead to mycotoxin contamination in any step of the process: harvest, recollection, storage, transport, processing, and or/conservation. Most mycotoxins are chemically stable and survive food processing.

The presence of mycotoxins in feedstuff can diminish the nutritional value of the food/feed, which can also lead to harmful effects on both animal and human health. From the productive point of view, mycotoxins can cause lower efficiency in farm activity, resulting in great economical loses [78].

As it is a global concern, the Joint Food and Agricultural Organization/World Health Organization (FAO/WHO) Committee recognized the risk posed by mycotoxin contamination in food, considering these toxins to be the most important substances due to their daily intake [79].

Moreover, the Scientific Committee of the European Food Safety Authority (EFSA) published rules and recommendations that aim to regulate the maximum allowed concentration in food and feedstuff [80].

Although over 400 different mycotoxins have been identified so far, there are more metabolites that are being detected in feedstuff, so the total number is expected to be higher in the next years. The most observed and concerning mycotoxins are aflatoxins, ochratoxin A, fumonisins, zearalenone and nivalenol/deoxynivalenol.

4.2. CLASSIFICATION

The most relevant groups of mycotoxins found in food and feedstuff are produced by four fungal genera: *Fusarium*, *Aspergillus*, *Penicillium,* and *Alternaria*. The classification by genera of the mycotoxins selected in this thesis is shown in the following table.

Table 4.1. Classification of the studied mycotoxins by genera.

Mycotoxin	Genera
Deoxynivalenol	*Fusarium*
3-Acetyl-deoxynivalenol	*Fusarium*
15-Acetyl-deoxynivalenol	*Fusarium*
Enniatin B	*Fusarium*
Enniatin B_1	*Fusarium*
Fumonisin B_1	*Fusarium*
Fumonisin B_2	*Fusarium*
Toxin T-2	*Fusarium*
Toxin HT-2	*Fusarium*
Zearalenone	*Fusarium*
α-Zearalenol	*Fusarium*
ß-Zearalenol	*Fusarium*
Fusarenon X	*Fusarium*

Table 4.1 (cont.). Classification of the studied mycotoxins by genera.

Mycotoxin	Genera
Aflatoxin B_1	*Aspergillus*
Aflatoxin B_2	*Aspergillus*
Aflatoxin G_1	*Aspergillus*
Aflatoxin G_2	*Aspergillus*
Sterigmatocystin	*Aspergillus*
Ochratoxin A	*Aspergillus, Penicillium*
Cyclopiazonic acid	*Penicillium, Aspergillus*
Andrastin A	*Penicillium*
Marcfortine A	*Penicillium*
Roquefortine C	*Penicillium*
Mycophenolic acid	*Penicillium*
Penitrem A	*Penicillium*
Alternariol	*Alternaria*

4.3. STRUCTURE AND PHYSICO-CHEMICAL PROPERTIES

The studied compounds, their acronym, molecular formula, exact mass, and CAS numbers are summarized in **Table 4.2**.

Table 4.2. Target compounds: molecular formula, exact mass, CAS number, molecular ion, retention time and structure.

Mycotoxin	Acronym	Molecular formula	Exact mass	CAS number
3-Acetyl deoxynivalenol	3-ADON	$C_{17}H_{22}O_7$	338.1365	50722-38-8
15-Acetyl deoxynivalenol	15-ADON	$C_{17}H_{22}O_7$	338.1365	88337-96-6
Deoxynivalenol	DON	$C_{15}H_{20}O_6$	296.1259	51481-10-8

Table 4.2 (cont.). Target compounds: molecular formula, exact mass, CAS number, molecular ion, retention time and structure.

Mycotoxin	Acronym	Molecular formula	Exact mass	CAS number
Enniatin B	ENN B	$C_{33}H_{57}N_3O_9$	639.4094	917-13-5
Enniatin B_1	ENN B_1	$C_{34}H_{59}N_3O_9$	653.4251	19914-20-6
Fumonisin B_1	FB_1	$C_{34}H_{59}NO_{15}$	721.3884	116355-83-0
Fumonisin B_2	FB_2	$C_{34}H_{59}NO_{14}$	705.3935	116355-84-1
HT-2 toxin	HT-2	$C_{22}H_{32}O_8$	424.2097	26934-87-2
Marcfortine A	MAC A	$C_{28}H_{35}N_3O_4$	477.2627	75731-43-0
Ochratoxin A	OTA	$C_{20}H_{18}ClNO_6$	403.0822	303-47-9
Roquefortine C	ROQ-C	$C_{22}H_{23}N_5O_2$	389.1851	58735-64-1
Sterigmatocystin	STE	$C_{18}H_{12}O_6$	324.0633	10048-13-2
T-2 toxin	T-2	$C_{24}H_{34}O_9$	466.2202	21259-20-1
Cyclopiazonic acid	CPA	$C_{20}H_{20}N_2O_3$	336.1473	18172-33-3
Andrastin A	AND A	$C_{28}H_{38}O_7$	486.2617	174232-42-9
Alternariol	AOH	$C_{14}H_{10}O_5$	258.0528	641-38-3
Mycophenolic acid	MPA	$C_{17}H_{20}O_6$	320.1259	24280-93-1
Penitrem A	PEN A	$C_{37}H_{44}ClNO_6$	633.2857	12627-35-9

Table 4.2 (cont.). Target compounds: molecular formula, exact mass, CAS number, molecular ion, retention time and structure.

Mycotoxin	Acronym	Molecular formula	Exact mass	CAS number
α-Zearalenol	α-ZOL	$C_{18}H_{24}O_5$	320.1623	36455-72-8
β-Zearalenol	β-ZOL	$C_{18}H_{24}O_5$	320.1623	71030-11-0
Zearalenone	ZEA	$C_{18}H_{22}O_5$	318.1467	17924-92-4
Fusarenon X	FUS X	$C_{17}H_{22}O_8$	354.1314	23255-69-8
Aflatoxin B_1	AF B_1	$C_{17}H_{12}O_6$	312.0633	1162-65-8
Aflatoxin B_2	AF B_2	$C_{17}H_{14}O_6$	314.0790	7220-81-7
Aflatoxin G_1	AF G_1	$C_{17}H_{12}O_7$	328.0583	1165-39-5
Aflatoxin G_2	AF G_2	$C_{17}H_{14}O_7$	330.0739	7241-98-7

Mycotoxins can be classified by their chemical structure. The structures of the studied mycotoxins are represented in **Figure 4.1**.

- **Trichothecenes** are a family of more than sixty sesquiterpenoid metabolites. All trichothecenes have the core 12, 13-epoxytrichothec-9-ene (EPT) structure [81], which is characterised by a double bond between C9 and C10 and an epoxide group between C12 and C13.

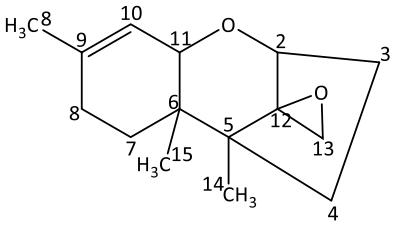


Figure 4.1. EPT structure

However, many analogues can be characterised by their different substitution combination. Mainly, *Fusarium* trichothecenes are classified as type A or type B.

Type A trichothecenes can present a hydroxyl group, an ester function at C8 of the EPT molecule or no functional group substitutions. T-2 toxin and HT-2 toxin, with an ester substitution at C8, are examples of this type.

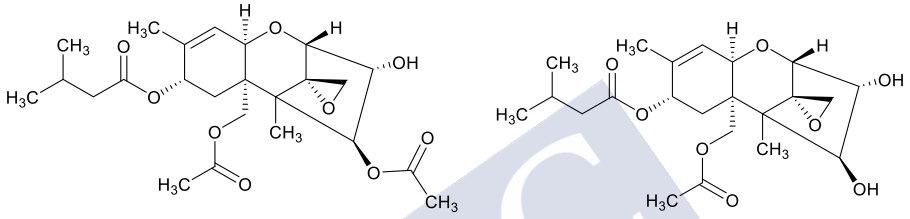


Figure 4.2. Type A trichothecenes

Type B trichothecenes are defined by the presence of a carbonyl functional group at C8 of the EPT structure and more hydroxyl groups around the molecule. Common examples of this type are deoxynivalenol (DON), its acetylated derivatives (3-ADON, 15-ADON, FUS X) and nivalenol (NIV).

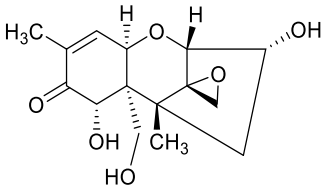
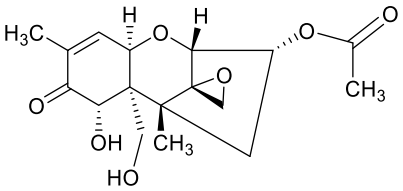
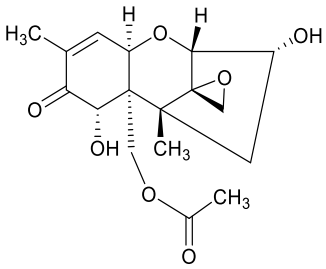


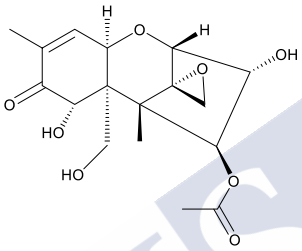
Figure 4.3. Type B trichothecenes



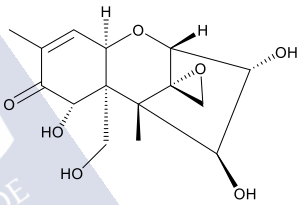
3-ADON



15-ADON



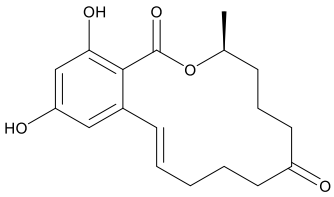
FUS X



NIV

Figure 4.3 (cont.). Type B trichothecenes

- **Zearalenone** (ZEA) has the chemical structure of a resorcylic acid lactone [77]. The reduction of the ketone group at C8 leads to the formation of its most frequent metabolites: α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL).



ZEA

Figure 4.4. Zearalenone and derivatives

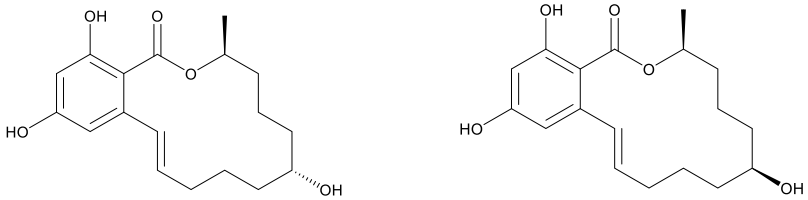


Figure 4.4 (cont.). Zearalenone and derivatives

- **Fumonisins** are primary amines with 2 tricarballylic groups, long chains, and various hydroxyl groups. Of more the 15 fumonisin analogues, the most prevalent are the B series (FBs): fumonisins B_1, B_2, and B_3. These toxins are diesters of propane-1,2,3-tricarboxylic acid (TCA).

Due to their non-aromatic structure, fumonisins have unique physical properties compared to the rest of mycotoxins. They are soluble in water, acetonitrile and methanol, thermally stable and not photosensitive [77].

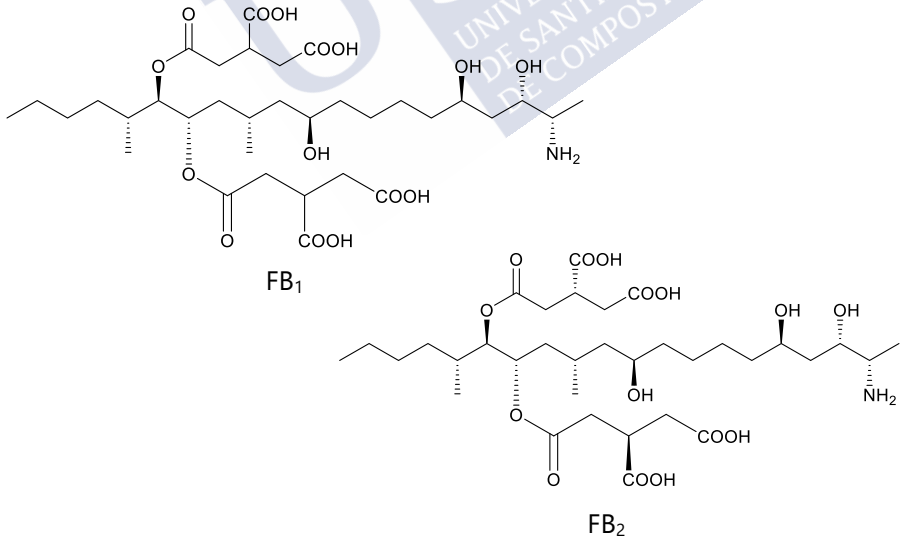
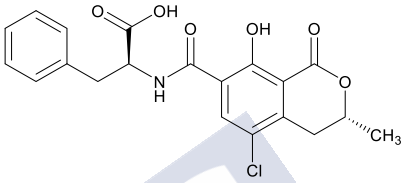


Figure 4.5. Fumonisins

- **Ochratoxin A** (OTA) is a phenylalanyl derivative of a substituted isocoumarin (R)-N-[5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-y1)-carbonyl]-L-phenylalanine.

There are two known OTA analogues, ochratoxin B (OTB) and ochratoxin C (OTC). However, the occurrence of OTB and OTC is rare [82].

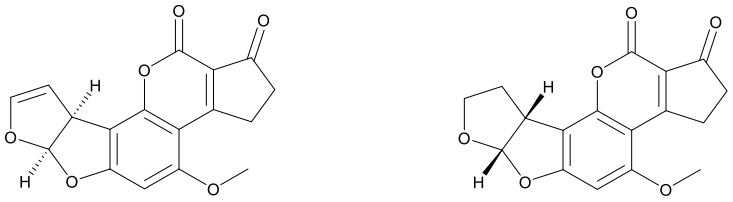


OTA

Figure 4.6. Ochratoxin A

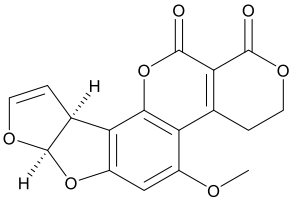
- **Aflatoxins** are difuranocoumarins, with lactone-type rings. Their structure consists of a bifuran ring attached to a coumarin nucleus with a pentenone ring (in B and M aflatoxins) or a six-membered lactone ring in G aflatoxins.

The four major aflatoxins are called B_1, B_2, G_1 and G_2 based on their fluorescent properties under UV light (blue and green) and relative chromatographic mobility [83]. Aflatoxins M_1 and M_2 are hydroxylated metabolites of AFB_1 and AFB_2, respectively. M_1 metabolite is of special concern to human safety due to its presence in milk and dairy products.

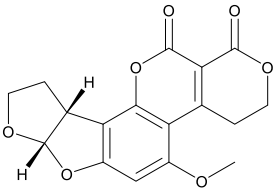


AF-B_1 AF-B_2

Figure 4.7. Aflatoxins



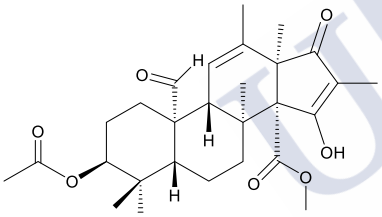
AF-G_1



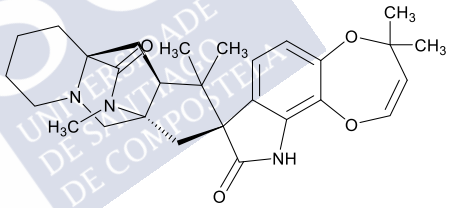
AF-G_2

Figure 4.7 (cont.). Aflatoxins

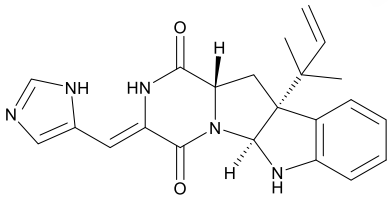
- ***Penicillium*** **genera mycotoxins** Andrastin A (AND A), marcfortine A (MARC A), roquefortine C (ROQ-C), mycophenolic acid (MPA), penitrem A (PEN A) and cyclopiazonic acid (CPA) are examples of mycotoxins produced by different *Penicillium* species.



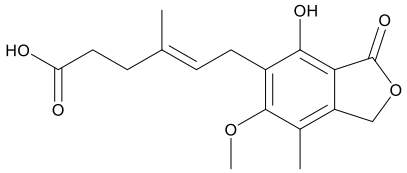
AND A



MARC A

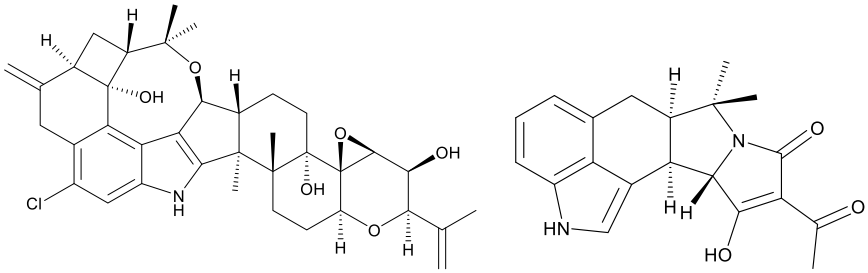


ROQ-C



MPA

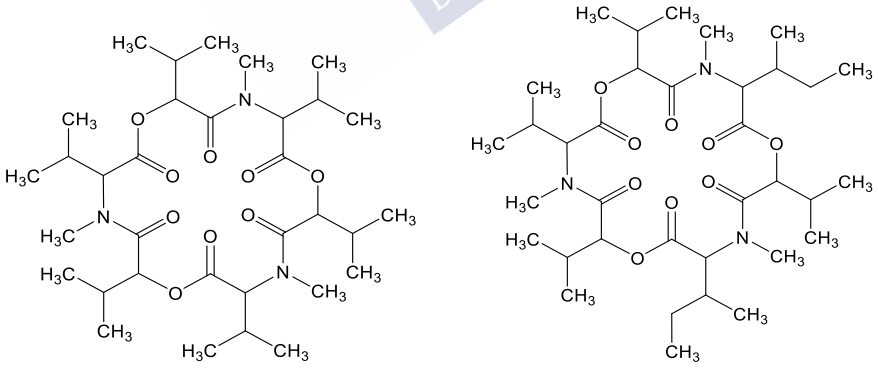
Figure 4.8. *Penicillium* genera



PEN A CPA

Figure 4.8 (cont.). *Penicillium* genera

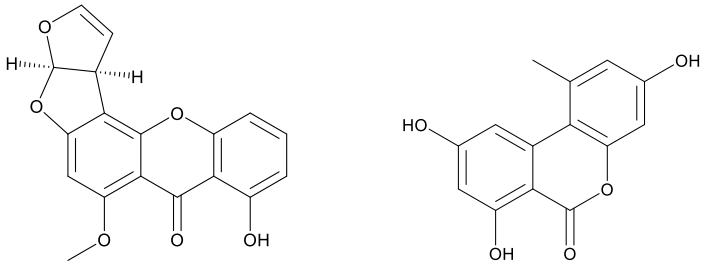
- ***Enniatin*** mycotoxins are cyclic hexadepsipeptides that consist of three N-methylated amino acids and three D-2-hydroxy isovaleric acids which form the branched chain by ester and peptide bonds. 27 different enniatins are described, however the most frequently found are enniatin B (ENN B) and enniatin B_1 (ENN B_1)[84].



ENN B ENN B_1

Figure 4.9. Enniatin B and B_1

Other mycotoxins studied are sterigmatocystin (STER) and alternariol (AOH).



STER AOH

Figure 4.10. Others

4. TOXICITY

The pathologies caused in humans and animals by exposure (ingestion, inhalation, or skin absorption) to mycotoxins are known as mycotoxicoses. The effects can be acute, when it is due to ingestion; or chronic, caused by prolonged exposure to these toxins.

The hazardous effects can be enhanced due to the co-occurrence of several mycotoxins, which is a common situation for zearalenone and deoxynivalenol, or ZEA/DON/FB_1 combination.

- **Trichothecenes**

Toxicity of DON and other trichothecenes depends on their polarity. Due to their small size and amphipathic character, they can cross the biological barriers and be easily absorbed by the gastrointestinal system. They can rapidly proliferate tissues, affecting the functions and viability of the cells [85]. Exposure to these toxins can predispose humans and animals to infectious diseases (from parasites, bacteria, and viruses) and cause immunological problems.

Even though T-2 toxin is not as frequently detected as the other trichothecenes, it is one of the most toxic mycotoxins present in wheat, maize, or rye. T-2 toxin can inhibit protein synthesis, which leads to secondary disruption of DNA and RNA synthesis [86]. HT-2 toxin is the major metabolite of T-2 toxin, and its toxicity is similar as its precursor.

- **Zearalenone**

ZEA, along with FBs and DON, is found to be the most relevant *Fusarium* mycotoxins in terms of animal health and, consequently, productivity and economic loss.

ZEA is considered to present low toxicity. However, it does have a resemblance to 17β-estradiol, the principal estrogen hormone in human ovaries. This means that ZEA competitively binds to estrogen receptors, causing alterations in the mammalian reproductive system [87]. Zearalenone is often classified as a nonsteroidal estrogen or mycoestrogen.

ZEA has also been shown to present immunotoxic and hepatotoxic properties [88].

- **Fumonisins**

Fumonisins affect animals by interfering with their sphingolipid metabolism. The consequences can be hepatotoxic effects, pulmonary edema, and brain lesions, such as leukoencephalomalacia [89].

In terms of occurrence and toxicity, fumonisin B_1 is the most significant. FB_1 has been classified as a 2B carcinogen (possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC) [90].

- **Ochratoxin**

Ochratoxin A form presents the most prevalence and toxicity in foods, due to its thermal stability. OTA is proved to be a nephrotoxin, and studies show that it can also produce liver toxicity, immunosuppression, as well as teratogenic and carcinogenic effects [83,91].

- **Aflatoxins**

Aflatoxins (AFs) are considered the most relevant group of mycotoxins in food and feedstuff because of their hepatotoxicity and carcinogenicity in humans.

AFB_1 is the most potent hepatocarcinogen known in mammals. Aflatoxin M_1 is an epoxide produced by metabolism of AFB_1 that can react to form DNA adducts and hence induce mutations and cancer [92]. AFM_1 can be found in milk samples.

- **Other mycotoxins**

Penicillium produced toxin cyclopiazonic acid (CPA) is considered a neurotoxin. In addition, it may generate gastrointestinal lesions.

Alternariol (AOH) has genotoxic effects causing inhibition of DNA relaxation and stimulation, and can induce alterations in mucosa cells associated to oesophageal cancer development [80].

4.5. LEGISLATION

Establishing limits and regulations for mycotoxins is a challenging subject because both scientific data, socio-economic and political aspects need to be taken into account.

In order to reduce the effects of mycotoxin ingestion, the European Union Commission Regulation established the maximum levels allowed in certain kinds of feed for the major mycotoxins, such as aflatoxin B_1 [93], deoxynivalenol, zearalenone, ochratoxin A, and fumonisins FB_1 and FB_2 [94] (**table 4.3**).

Table 4.3. Recommendations on the presence of DON, ZEA, OTA and fumonisins in products intended for animal feeding according to 2003/100/EC and 2006/576/EC.

Mycotoxin	Products intended for animal feed	Guidance value in mg/kg (ppm) relative to a feeding stuff with a moisture content of 12 %
Aflatoxin B_1	*Feed materials with the exception of:*	0.05
	- groundnut, copra, palm-kernel, cotton seed, babassu, maize and products derived from the processing thereof	0.02
	Complete feeding stuffs for cattle, sheep and goats with the exception of:	0.05
	- Dairy cattle	0.005
	- Calves and lambs	0.01
	Complete feeding stuffs for pigs and poultry (except young animals)	0.02

Table 4.3 (cont.). Recommendations on the presence of DON, ZEA, OTA and fumonisins in products intended for animal feeding according to 2003/100/EC and 2006/576/EC.

Mycotoxin	Products intended for animal feed	Guidance value in mg/kg (ppm) relative to a feeding stuff with a moisture content of 12 %
Aflatoxin B_1	Other complete feeding stuffs	0.01
	Complementary feeding stuffs for cattle, sheep and goats (except complementary feeding stuffs for dairy animals, calves and lambs)	0.05
	Complementary feeding stuffs for pigs and poultry (except young animals)	0.03
	Other complementary feeding stuff	0.005
Deoxynivalenol	*Feed materials*	
	▪ Cereals and cereal products except maize by-products	8
	▪ Maize by-products	12
	Complementary and complete feeding stuffs except:	5
	- Complementary and complete feeding stuffs for pigs	0.9
	- Complementary and complete feeding stuffs for calves (<4 months), lambs and kids	2

Table 4.3 (cont.). Recommendations on the presence of DON, ZEA, OTA and fumonisins in products intended for animal feeding according to 2003/100/EC and 2006/576/EC.

Mycotoxin	Products intended for animal feed	Guidance value in mg/kg (ppm) relative to a feeding stuff with a moisture content of 12 %
Zearalenone	*Feed materials*	
	- Cereals and cereal products except maize by-products	2
	- Maize by-products	3
	Complementary and complete feeding stuffs	
	- Complementary and complete feeding stuffs for piglets and gilts (young sows)	0.1
	- Complementary and complete feeding stuffs for sows and fattening pigs	0.25
	- Complementary and complete feeding stuffs for calves, dairy cattle, sheep (including lamb) and goats (including kids)	0.5
Ochratoxin A	*Feed materials*	
	- Cereals and cereal products	0.25
	Complementary and complete feeding stuffs	
	- Complementary and complete feeding stuffs for pigs	0.05
	- Complementary and complete feeding stuffs for poultry	0.1

Table 4.3 (cont.). Recommendations on the presence of DON, ZEA, OTA and fumonisins in products intended for animal feeding according to 2003/100/EC and 2006/576/EC.

Mycotoxin	Products intended for animal feed	Guidance value in mg/kg (ppm) relative to a feeding stuff with a moisture content of 12 %
Fumonisin B_1+B_2	*Feed materials*	
	- Maize and maize products	60
	Complementary and complete feeding stuffs for:	
	- pigs, horses, rabbits, and pet animals	5
	- *fish*	10
	- poultry, calves (<4 months), lambs and kids	20
	- adult ruminants (>4 months) and mink	50

According to 2013/165/UE [95], maximum indicative levels were also established for the sum of T-2 and HT-2 toxin in cereals and cereal-based products in food and feed. The indicative levels for T-2 and HT-2 in cereal products for feed and compound feed are indicated in **Table 4.4.**

Table 4.4. Recommendations on the presence of T-2 and HT-2 toxin in cereal and cereal products according to 2013/165/EU.

Mycotoxin	Products intended for animal feed	Indicative levels for T-2 + HT-2 (µg/kg) from which onwards/above which investigations should be performed
T-2+HT-2	*Cereal products for feed and compound feed*	
	▪ Oat milling products (husks)	2000
	- Other cereal products	500
	- Compound feed, with the exception of feed for cats	250

Modified forms of mycotoxins are also being considered by EFSA [96]. These modified mycotoxins are metabolites produced by chemical mechanisms such as hydrolysis or acetylation. They can be absorbed in the same way as their precursors, so they should be taken into account when measuring mycotoxin concentrations. Modified forms of fumonisins, zearalenone, T-2 and HT-2 toxins were examined and proved that the total amount of these mycotoxins can be increased from 10 to 100%.

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5. Sample preparation techniques

5.1. INTRODUCTION

Accurate determination of organic contaminants or any other compounds, especially when they are only present at trace levels, is a hard task. The difficulty is higher when it comes to a complex matrix, such as food or biological and environmental samples [97].

Therefore, an adequate sample preparation step prior to the analysis of any substance is key to achieve satisfactory results. The goals of this preparation stage are:

- The separation between the analytes of interest and possible interferents present in the matrix.
- The preconcentration of the analytes in the sample, in order to achieve lower detection and quantification limits.
- The conditioning of the sample and/or the analyte into a suitable form for the instrumental analysis.

Sample preparation techniques have evolved through the years. The first pre-treatment methods (e.g., Soxhlet extraction) were very time consuming and they required a large amount of solvent [98]. The trend in this area has been towards miniaturization, reducing the amount of sample and solvent needed, and also minimizing the extraction time, which are mainly the principles of Green Chemistry [99].

Therefore, the extraction procedures proposed in this thesis for the analysis of fungicides and UV filters in environmental waters are based on solid-phase micro extraction (SPME). For the analysis of

mycotoxins in feed samples, a modified Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) protocol was used.

5.2. SOLID-PHASE EXTRACTION (SPE)

5.2.1. Introduction

Solid phase extraction (SPE) is based on the partition of the analyte between a solid phase (sorbent) and a liquid phase (sample matrix or solvent with analytes). This technique evolved from batch-mode liquid-solid extractions (LSE), in which a liquid sample was put in contact with a bulk free-flowing solid extraction phase. The first application of packed sorbent SPE was described in 1951 by Braus et al. [100].

Alongside with liquid-liquid extraction (LLE), SPE is considered the most classical and commonly used methodology for sample preparation of trace amount of analytes. Also, nowadays it is widely used for pre-concentration and clean-up of liquid samples and extracts.

SPE is a convenient and simple sample preparation technique because it is very cost-effective, time-saving and requires small usage of organic solvents. One of the main benefits of SPE techniques is the availability of a variety of sorbents with different chemical characteristics, allowing multiresidue analysis for compounds with a wide range of polarities [101]. Also, SPE provides higher enrichment factors because it entails a pre-concentration of the analytes. In addition, SPE can be combined with different detection techniques, because it can be used off-line, on continuous or coupled on-line to a chromatographic technique [102,103].

5.2.2. SPE procedure

SPE cartridges operate as a chromatographic column. During the extraction process, the analytes are separated by their differential

adsorption strength between compounds and the stationary phase, and their solubility in the elution solvents. SPE extraction entails four steps:

- Cartridge conditioning: the solid phase needs to be prepared to properly interact with the analytes in the sample. MeOH is usually used for this matter, followed by the solvent of choice for the extraction.

- Sample loading: implies analyte selective retention into the solid phase.

- Column washing: a selective solvent passes through the cartridge, carrying the interferent substances while the analytes remain attached to the solid phase.

- Analyte elution: in this final step analytes are eluted using a solvent with higher polarity than the stationary phase.

At the end of the procedure, a concentrated and purified extract with the analytes is obtained.

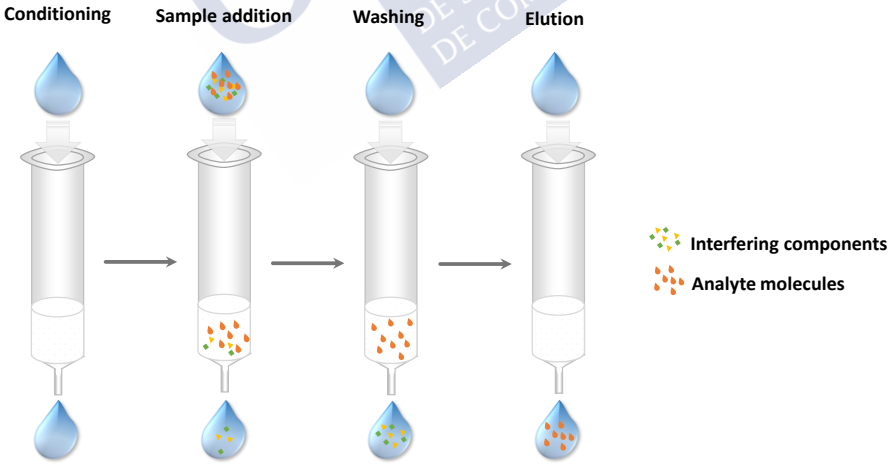

Figure 5.1. SPE schematic procedure

5.2.3. Factors influencing extraction efficiency

The principal factors to be considered are the matrix, the type of sorbent, the solvents used and of course, the analytes.

- Type of matrix. If the sample is a solid, it needs to be previously extracted with a proper solvent, which makes SPE useful more as a clean-up step than as an extraction technique. If the sample is a liquid, the only requirement would be a previous dilution, in the case of dense or viscous matrices (e.g., milk or honey samples).

- Type of sorbent. There are three main categories of sorbent material used: silica-based packaging, GCB (graphitized carbon black) and polymeric packaging. The most commonly used are silica, florisil or alumina cartridges when working with polar compounds (normal phase); and C_8 and C_{18} when the analytes are less polar or even apolar (reverse phase). In the case of polymeric packaging, cross-linked HLB (hydrophilic lipophilic balanced) cartridges are widely used [104].

- Solvent. Physico-chemical characteristics such as polarity, density, and specific volume of solvent particles affect the extraction.

- Analytes. The solubility of the target analytes in the solvent used in the different steps of the procedure, their polarity and affinity for both the sorbent and solvent are crucial factors.

5.2.4. Applications

Solid phase extraction is one of the most extensively used techniques for the determination of a variety of analytes (veterinary drugs, pesticides) in environmental samples, such as groundwaters, soil or sediments [105,106]. SPE is also commonly used in food sample treatment. As it is the case of pesticides in fresh fruits and vegetables [103], mycotoxins in feed [107], or veterinary drugs in milk samples [108].

Applications in biological samples treatment such as plasma [109], are also found in the literature.

5.3. DISPERSIVE SOLID PHASE EXTRACTION (DSPE)

Dispersive solid phase extraction (DSPE) is based on the dispersion of a sorbent, generally superficially modified silica (primary and secondary amine functions, PSA), in the sample solution. The nature of the sorbent is similar to the common phases in SPE.

The contact area between analytes and the sorbent is very high and, therefore, the extraction performance is improved, and the time is reduced. After the extraction step, the sorbent which contains the analytes is separated by centrifugation, and then again eluted with an appropriate solvent.

DSPE is frequently used as a clean-up step as well, providing rapid results, easy operation, low solvent volume and high recoveries.

5.4. QUECHERS EXTRACTION

5.4.1. Introduction

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method was first described in 2003 by Anastassiades et al. [110] as an alternative for the determination of pesticides in fruits and vegetables, which presented high water content. Due to its versatility, and the benefits literally described in its name, QuEChERS methodology was broadly implemented.

QuEChERS method is in line with the concept of green chemistry. It uses very small amounts of reagents and materials, which makes it also a safe and non-expensive methodology. Also, acetonitrile is

preferred rather than organochlorine solvents. ACN extracts are versatile and fit for injection both in gas and liquid chromatographs.

Flexibility is another advantage of QuEChERS because modifications can be made depending on the analyte properties, the matrix composition and the equipment available [110]. Thus, QuEChERS extraction is extensively used for the determination of polar, slightly polar, and non-polar analytes.

5.4.2. QuEChERS procedure

QuEChERS approach is based 3 general steps: an acetonitrile (ACN) extraction, followed by a liquid-liquid partitioning between an organic and an aqueous phase using buffered salts ($MgSO_4$, Na_2SO_4), and a final dispersive solid phase extraction (DSPE) clean-up step. **Figure 5.2** shows a schematic representation of QuEChERS methodology.

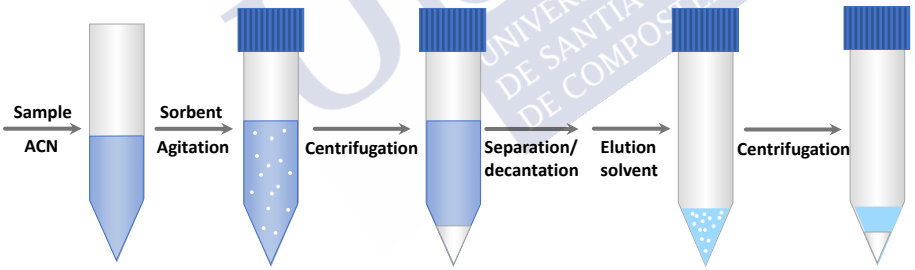

Figure 5.2. QuEChERS schematic procedure

5.4.3. Factors influencing extraction efficiency

Despite the simplicity of the methodology, several factors can be modified and optimized to achieve a higher efficiency.

- Sample conditions. QuEChERS extraction was initially developed for samples with a high water percentage. If the sample has low humidity, a proportion of water should be added initially for a more efficient extraction. Also, for the sake of reproducibility, samples must be properly homogenized.

- Addition of salts. The addition of salts induces the modification of the partition equilibrium of the analyte between the phases, favouring the migration to the organic phase. Anhydrous form of $MgSO_4$ is often used to help this process [111].

- Addition of sorbents. In the DSPE phase, PSA is the sorbent of choice for purifying the organic extract. However, the nature of the matrix and the analytes would determine the combination with other sorbents such as C18, used for eliminating non-polar interferences, or GCB for pigment removal.

5.4.4. Applications

Due to its versatility, QuEChERS methodology is a popular choice for multiple applications, apart from the original use in fruits and vegetables determination [112,113]. Pesticides, amongst other organic contaminats, are also extracted from different matrices using this technique, like environmental samples such as soil and water [114,115], and biological tissues [11,116,117].

Recently, it has been applied in the determination of mycotoxins and other contaminants in feed and cereals [118,119].

In **Section IV**, Chapter 3, a methodology using a modified QuEChERS extraction is proposed for the determination of a range of mycotoxins in feed.

5.5. SOLID PHASE MICRO-EXTRACTION (SPME)

5.5.1. Introduction

Microextraction techniques are a result of the miniaturization of conventional extraction modes, such as SPE, with the objective of reducing the volume of sample and solvent needed for the extraction.

Solid-phase microextraction (SPME) was first described by Pawliszyn et al. in 1990 [120] aiming for a rapid sample procedure which could be used in field work as well as in the laboratory.

SPME is based on a thin, chemically inert, and thermally stable fused-silica fibre coated with a layer of a polymeric ab/adsorbent as a stationary phase. This fibre is accommodated in a syringe-like device, and it is exposed to the sample the corresponding time, then it is retracted, and it can be directly desorbed into the injector. **Figure 5.3** shows a scheme of a commercial SPME device, which was first introduced by Supelco in 1993.

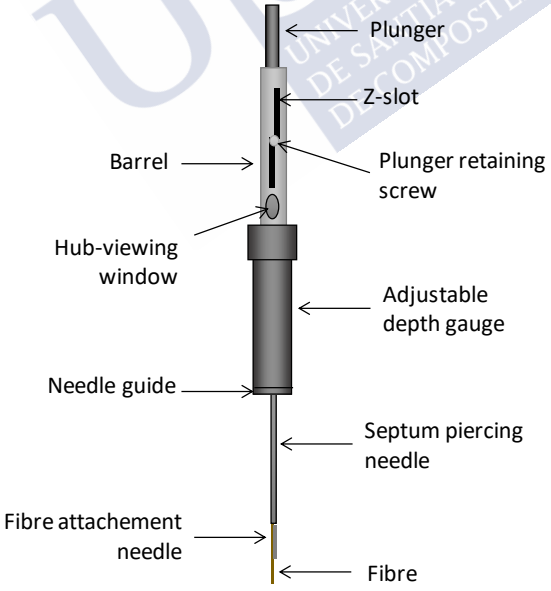

Figure 5.3. Commercial SPME device

SPME presents many advantages compared to other extraction techniques, such as the possibility of simultaneous sampling, pre-concentration, and extraction in a single stage. It requires a minimal amount of organic solvents, and it can be solvent-free for gas chromatography applications, which makes SPME a "green" sample preparation technique.

SPME also enables the full automation of the process, so it is simple, it presents a short extraction time, and it requires minimal supervision. It also allows the direct introduction of the analytes into the detection instrument, so the risk of analyte loss is minimized. Due to the concentration of the analytes in the fibre, the efficiency of the extraction is also enhanced.

5.5.2. SPME procedure

The SPME technique consists of two processes [121]:

- The analytes partition between the sample and the fibre coating when the fibre is exposed to the sample contained in a sealed vial. The extraction is completed when the analyte concentration reaches the distribution equilibrium between the sample matrix and the fibre coating.

- The concentrated analytes desorb from the coated fibre to an analytical instrument directly (gas chromatography) or indirectly (liquid chromatography).

Concerning the first step, there are two possible modes of fibre exposure (see **Figure 5.4**):

- Direct immersion extraction (DI-SPME): the fibre is introduced in the sample, allowing the direct migration of the analytes to the fibre. It is the preferred mode when working with slightly volatile analytes or with simple matrices.

- Headspace extraction (HS-SPME): the analytes are extracted from the gaseous phase above the sample. In this mode, the analytes passed first from the sample to the head space, and then to the fibre coating. This approach is indicated for the extraction of volatile and semivolatile compounds in complex matrices.

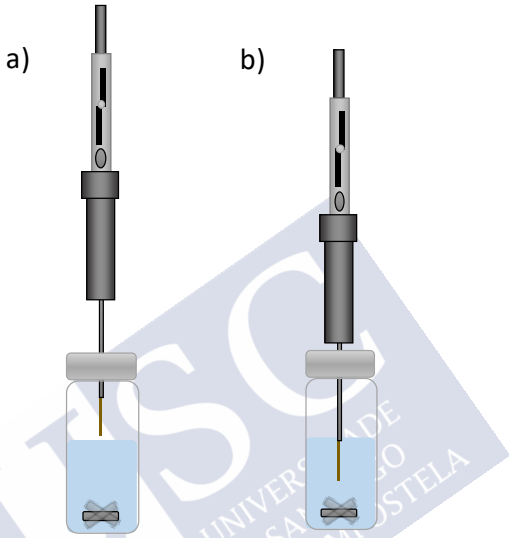

Figure 5.4. SPME extraction modes: a) head-space extraction; b) direct immersion

5.5.3. Factors influencing extraction efficiency

Several factors can affect the distribution equilibrium of the analytes between the sample and the fibre, therefore affecting the efficacy of the process [122]:

- Fibre coating. There are a range of coatings differing on their polarity, and also with different thickness of the phase. The chemical properties of the analyte determine the type of coating used, selecting generally the most similar in polarity.

- Extraction time. The highest analyte extraction is achieved after the necessary time to reach the equilibrium. Compounds with low distribution constants need more time to achieve equilibrium, so in these cases, shorter times can be selected. However, in order to accomplish reproducibility, exposure time of the fibre should be controlled.

- Extraction temperature. The diffusion of the analytes towards the fibre is favoured by higher temperatures. More so, when working in HS-SPME mode, the temperature helps the analytes transfer into the head space. However, being the absorption step an exothermic process, the increase of the temperature reduces the fibre/sample distribution constant of the analytes.

- Salting-out effect. The addition of salts, such as NaCl or KCl, induces an increasing in the ionic strength, hence diminishing the solubility of the analytes in water, and facilitating their transfer to the head space and the fibre.

- pH of the sample. In order to achieve the highest extraction efficiency, pH must be adjusted two units below the pK_a when working with acidic compounds, and two units above in the case of basic substances.

- Agitation of the sample. Agitation favours the diffusion of the analytes from a diluted sample to the fibre, accelerating the extraction kinetics.

5.5.4. Applications

SPME is the sample preparation technique preferred for a wide range of applications. It has been broadly used for the extraction of organic contaminants in environmental samples [123], including UV filters and other personal care products in water [33,34,124] and sand samples [43]; and fungicides in groundwater and grape juice samples [125].

SPME has also been used for monitoring the degradation processes of some organic contaminants in water matrices [126]. In **Section IV**, Chapters 1 and 2, SPME has been applied for the determination of photodegradation kinetics in water matrices of both fungicides and UV filters, and the tentative identification of their photoproducts.

6. Analysis

6.1. INTRODUCTION

Chromatographic separation is essential for the correct identification and quantification of the studied analytes. In the case of fungicides and UV filters, both liquid chromatography (LC) and gas chromatography (GC) were utilized. Because the concentration of these compounds in environmental water samples can be as low as ng L^{-1} level, a sensitive detection method like tandem mass spectrometry (MS/MS) must be used.

In the case of mycotoxin analysis in feed, LC is the preferred separation method. MS/MS is often used as the detection technique; however, high resolution mass spectrometry (HRMS) is a very powerful tool for the unequivocal identification of targeted compounds as well as for the inspection of un-targeted substances.

In the studies presented in **Section IV,** GC was always followed by tandem mass spectrometry (MS/MS), whereas LC was employed coupled to MS/MS and HRMS.

6.2. LIQUID CHROMATOGRAPHY

6.2.1. Introduction

Liquid chromatography (LC) is based on the partition of the analytes between a liquid mobile phase and a solid stationary phase (chromatographic column). It is the most broadly used separation technique because of its versatility and sensitivity. LC is often selected

for the determination of non-volatile, polar, or thermally unstable analytes in a wide range of fields.

High performance liquid chromatography (HPLC) can be classified according to the nature of the stationary phase used: normal-phase HPLC (polar stationary phase and a much less polar mobile phase), and reverse-phase HPLC (polar mobile phase and a non-polar stationary phase). The selection of the stationary phase would depend on the characteristic of the analytes.

When developing a methodology that involves HPLC analysis, several factors should be optimized to achieve a better separation of the analytes. Apart from the selection of the stationary phase, the composition of the mobile phase, the pH, and the temperature of the chromatographic column must be considered.

6.2.2. Mass spectrometry

Mass spectrometry (MS) is an analytical methodology that can provide both qualitative (structural) and quantitative information on molecules after their transformation into ions. MS presents numerous advantages, such as high selectivity, specificity, sensitivity and also a fast analysis time.

A mass spectrometer incorporates three fundamental elements: an ionization source, a mass analyser, and a detector. In a LC-MS coupling, the liquid sample must be vaporized to enter the mass spectrometer. The ionization source transforms the molecules in the solution from the HPLC column into ions in gas phase; then, the ions are filtered according to their mass-to-charge ratios (*m/z*) in the mass analyser before entering the detector [127].

Regarding the ionization source, the most commonly used are atmospheric pressure ionization interfaces, such as atmospheric pressure chemical ionization (APCI) and, especially, electrospray

ionization (ESI). ESI employs electrical energy to facilitate the transfer of ions from solution to the gaseous phase. This process involves three steps: (1) dispersion of a fine spray of charged droplets, (2) evaporation of the solvent, and (3) ion ejection from the highly charged droplets (Coulombic explosion) [128].

In the work presented in this thesis, both ESI and HESI (heated electrospray ionization) have been utilized.

Concerning the mass analysers, various MS can be coupled in a series to increase selectivity and sensitivity, resulting in tandem mass spectrometry (MS/MS).

6.2.2.1. Tandem mass spectrometry

MS/MS is a method that allows the mass spectrum of pre-selected and fragmented ions to be obtained. Triple-quadrupole mass spectrometer (QqQ) is the most common of these instruments. In QqQ spectrometers, the sample is first introduced into the ionization source; then, the ions generated are accelerated into quadrupole 1 (Q1), which act as a mass filter, considering the *m/z* of the ions. The selected speeded ions pass into quadrupole 2 (q2), which is a collision cell where fragmentation of the ions selected by Q1 takes place. Finally, quadrupole 3 (Q3) allows mass analysis of the product ions formed in the collision cell [128].

Figure 6.1 shows the schematic configuration of a triple-quadrupole mass spectrometer.

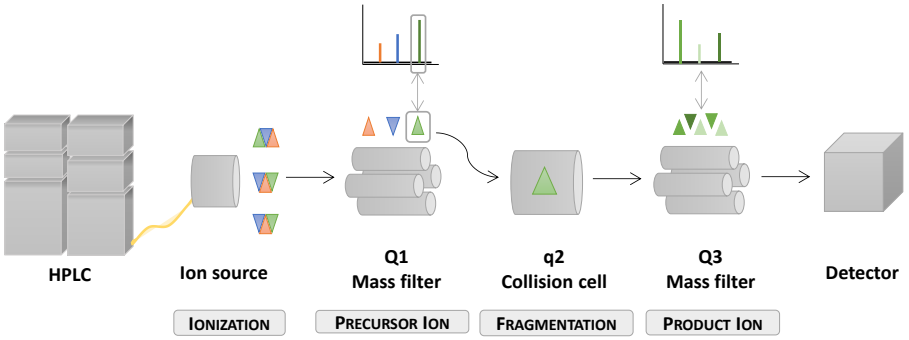

Figure 6.1. Triple quadrupole mass spectrometer (SRM mode)

Triple quadrupole systems can perform in different ion monitoring modes:

- Full scan: continuous filtration of ions, where all masses are registered without following *m/z* criteria.

- Selected Ion Monitoring (SIM): ions with a selected value of *m/z* are filtered.

- Selected Reaction Monitoring (SRM) or Multiple Reaction Monitoring (MRM): Q1 and Q3 work in SIM mode, selecting specific precursor ions in Q1 and, after fragmentation in q2, some specific product ions are filtered in Q3. This is a way of minimizing interferences, resulting in chromatograms with very low noise and higher selectivity and sensitivity.

LC coupled to MS/MS performing in MRM mode was the configuration of choice for the determination of the photodegradation kinetics of fungicides and UV filters presented in **Section IV**.

6.2.2.2. High resolution mass spectrometry

In order to achieve a higher resolution, quadrupoles can be combined with a time-of-flight detector (QqTOF). This configuration provides MS/MS spectra of high resolution, as well as accurate monoisotopic mass determinations, which is very valuable for confirmation of target compounds and for the identification of unknowns [129].

QqTOF configuration also provides additional confirmatory parameters, besides retention time and ion fragment ratio, such as the exact mass, isotope pattern and the possibility of spectral comparison against spectra libraries.

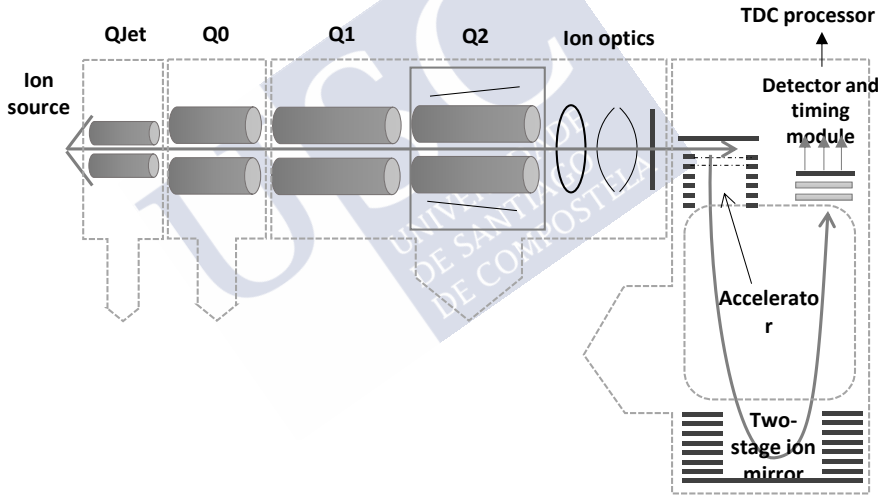

Figure 6.2. Scheme of a QqTOF spectrometer

In this thesis, a QqTOF (TripleTOF®) spectrometer was used performing in different ion monitoring modes:

- Full scan. Allows the continuous filtration of ions, while all masses are registered without *m/z* criteria.

- Sequential Windowed Acquisition of all Theoretical mass spectra (SWATH^{TM}). Independent data acquisition (IDA) in which MS/MS spectra of all precursor ions between a specified wide mass range are acquired by dividing the whole mass range into smaller *m/z* windows. This allows several repeat analysis of each window during the elution of a chromatographic peak; hence, for every detectable analyte in the sample that passes through Q1, a full MS and MS/MS spectra is acquired [130].

In applications where the approach is a non-targeted analysis, unknown compounds can be identified retrospectively via data re-interrogation without the need of re-acquisition.

LC coupled to high resolution mass spectrometry is largely implemented in proteomics and metabolomics [130,131]. It has also been used for the determination of pesticide residues [132,133] and toxicological analyses [134].

In **Section IV**, two studies are presented where LC-HRMS is employed for the monitoring of photodegradation processes and subsequently, the identification of unknown by-products.

6.3. GAS CHROMATOGRAPHY

6.3.1. Introduction

The principle of gas chromatography (GC) is the partition of the analytes between a carrier gas acting as a gaseous mobile phase, and a chromatographic column, which is the stationary phase. GC can accomplish the separation of volatile or semi-volatile compounds that are thermally stable.

For the analysis of polar and/or thermosensitive compounds, a previous step of derivatization is needed in order to improve the chromatographic resolution. Derivatization process consists of the

modification of a certain functional group of the molecule, obtaining a less polar or more volatile species [135].

6.3.2. GC-MS/MS

Gas chromatography can be more easily coupled to a mass spectrometer than LC since both systems operate in gas phase and the amount of sample needed for analysis is very small.

The sample is directly introduced from the chromatographic column into the mass spectrometer through a heated interface. Then the sample is ionized. Electronic impact (EI) is the most common ionization source, and it is based on the bombardment of the molecules with electrons from a highly charged filament, provoking the emission of outer shell electrons. This excess of energy causes the formation of neutral atoms and fragment ions.

The work developed in this thesis regarding the determination of fungicides an UV filters in water was carried out using a GC-MS/MS configuration with an EI ionization source and operating in MRM mode.

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III. METHODOLOGY

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The methodologies used in this thesis for the sample preparation stage were based on solid phase extraction techniques. For the environmental applications, a miniaturized solid phase extraction (SPME) was used. For the analysis of mycotoxins in feed samples, a modified QuEChERS procedure was utilized, followed by solid phase extraction (SPE) and dispersive solid phase extraction (DSPE) steps for the clean-up of the extract.

Chemometric tools such as multifactor experimental designs were employed for the optimization of the extraction efficiency of the sample preparation techniques used.

The analytical methods employed were based on chromatography coupled to mass spectrometry were used. Both liquid (LC) and gas chromatography (GC) were followed by tandem mass spectrometry (MSMS). Liquid chromatography coupled to high resolution mass spectrometry (HRMS) was also applied for targeted and non-targeted analyses using a TripleTOF equipment operating in SWATH acquisition mode.

Different photodegradation methodologies were tested for the elimination or organic contaminants in water samples. Starting from direct photolysis under UVA and UVC radiation using lab-scale photoreactors. Then, advanced photodegradation processes (AOPs), such as UVC/H_2O_2, and UVA/TiO_2 heterogeneous photocatalysis were employed to achieve the complete degradation of the most persistent compounds.

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IV. RESULTS AND DISCUSSION

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CHAPTER 1. DETERMINATION AND FATE OF FUNGICIDES IN WATER

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Fungicides are widespread used in agriculture for the prevention of fungi infection. This type of pesticides is applied in relative low concentrations, but several times a year and in most cases, nearby water sources. Therefore, the risk of these organic contaminants reaching the aquatic environment is something that needs to be evaluated. As commented in Section 1.4 of the introduction, fungicides can pose a hazard to aquatic organisms and also human health, due the phenomenon of bioaccumulation. Therefore, the evaluation of the persistence level of fungicides in natural waters is also a relevant matter.

In this chapter, two different works are compiled, concerning both the identification and quantification of fungicides in natural waters, and the evaluation of the photodegradation processes that might occur.

In the first study, a methodology for the determination of trace levels of multiclass fungicides in natural waters was optimized. SPME followed by GC-MS/MS was the proposed methodology. This protocol was also tested for the assessment of photodegradation kinetics of the studied fungicides at very low concentration levels.

The second study presented was based on the photodegradation of a group of broadly used fungicides using two different systems: direct UV photolysis and UVC/H_2O_2 advanced oxidation process. LC-MS/MS analysis was used for the monitoring of the degradation and LC-HRMS was applied for tentative identification of photoproducts.

These studies have been published and are presented next.

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1.1.

Simultaneous determination of trace levels of multiclass fungicides in natural waters by solid - phase microextraction-gas chromatography - tandem mass spectrometry

María Celeiro, Rocío Facorro, Thierry Dagnac, María Llompart

Analytica Chimica Acta, 1020 (2018) 51-61

ISSN: 0003-2670, Elsevier

DOI: 10.1016/j.aca.2018.03.014

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1.2.

Photodegradation of multiclass fungicides in the aquatic environment and determination by liquid chromatography-tandem mass spectrometry

María Celeiro, Rocío Facorro, Thierry Dagnac, Vítor J.P. Vilar, María Llompart

Environmental Science and Pollution Research, 24 (2017) 1-13

ISSN: 0944-1344 (Springer)

DOI: 10.1007/s11356-017-9487-2

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CHAPTER 2. PHOTODEGRADATION OF UV FILTERS IN WATER

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UV filters are a family of personal care products that are considered to be emerging contaminants because of their widespread used, mostly in cosmetic formulations. UV filters can reach the aquatic environment via bathing activities in recreational waters, or indirectly via wastewater treatment plants. As commented in Section 2.4, UV filters present hazardous estrogenic activity and potential bioaccumulation in animal and human tissues. Due to their structural characteristics, direct photolysis has been applied for the removal of UV filters from water. However, because of the high stability of some of the compounds, more effective processes are necessary. Moreover, most of the published studies only assess the removal of one compound at a time, and just a few 2-3 compounds simultaneously.

In this chapter, a methodology for the removal of 21 multiclass organic UV filters at low concentration level from water is presented. Direct photolysis under UVA and UVC radiation, and AOPs based on an UVC/H_2O_2 system and heterogeneous photocatalysis were carried out. Heterogeneous photocatalysis was assisted by TiO_2 nanoparticles immobilized onto inert structures acting as a catalyst.

The monitoring of the kinetic profile of the selected UV filters was evaluated using direct injection-LC-MS/MS and SPME-GC-MS/MS. Tentative identification of photodegradation by-products has been carried out using LC-HRMS.

The published work is displayed hereunder.

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2.1.

Photodegradation behaviour of multiclass ultraviolet filters in the aquatic environment: removal strategies and photoproduct identification by liquid chromatography-high resolution mass spectrometry

María Celeiro, Rocío Facorro, Thierry Dagnac, Vítor J.P. Vilar, María Llompart

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CHAPTER 3. DETERMINATION OF MYCOTOXINS IN FEED

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Mycotoxins, as exposed in **Section II.4**, are natural metabolites that can develop in crops and cereals during all stages of production, causing an important productivity decline. These substances also present hazardous effects in both animal and human health, since they have carcinogenic, estrogenic and immunotoxicogenic activity. Some mycotoxins are very stable under high temperatures, so they might not be eliminated during food or feed processing procedures. Therefore, methodologies for the selective and sensitive monitoring of mycotoxins in feed and food matrices are crucial. Also, due to the frequent co-occurrence of more than one mycotoxin in theses samples, multianalyte methodologies are recommended.

In the study presented below, a methodology for the simultaneous analysis of more than 20 multiclass mycotoxins in complex feed samples was developed. A simple modified QuEChERS extraction including a SPE and a DSPE clean up-step was successfully used for the extraction and elimination of matrix interferences. Due to the complexity of the matrix, quantification was also corrected by means of a matrix-matched calibration. An HPLC-QqTOF analytical methodology was optimized and validated for the accurate identification and quantification of the mycotoxins. Moreover, some forms of modified mycotoxins where later identified using a non-target approach.

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3.1.

Combined (d)SPE-QuEChERS Extraction of Mycotoxins in Mixed Feed Rations and Analysis by High Performance Liquid Chromatography - High-Resolution Mass Spectrometry

Rocío Facorro, Thierry Dagnac, María Llompart

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V. GENERAL DISCUSSION

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The studies carried out throughout this doctoral thesis can be divided into two different areas, as indicated in the title. On one hand, the environmental part, more precisely the analysis and monitoring of fungicides and UV filters in environmental waters; on the other hand, a section focused on food/feed safety, in which the screening and quantification of mycotoxins in complex feed matrices intended for dairy cows was performed.

The selection of the target organic contaminants was proposed according to their use and occurrence in the matrices of interest. Therefore, a selection of a group of extensively applied fungicides in agricultural practices as well as several families of organic UV filters, including the most utilized in personal care products (benzophenones, cinnamates or salicylates), was made.

The election of the mycotoxins of interested was also based on their occurrence in cereals and feed samples and on the current legislation. Those mycotoxins that were not relevant in the matrices analysed were not taken into account, as the case of aflatoxin metabolites M_1 and M_2, which are only found in dairy products.

In all the work presented in this thesis, the extraction and sample preparation stages play a very relevant part, because all matrices analysed in both the environmental and the food safety section, are complex and often present matrix interferences. Thus, this step is crucial in order to obtain the cleanest possible extracts, reducing the interferences. It is also important to note that the selection of the sample preparation technique has to be adequate for the analytes, the matrix and also the analysis techniques employed afterwards.

Therefore, in the environmental analysis section, the preferred extraction technique was the solid phase micro-extraction (SPME). This technique is widely employed for the analysis of water samples. It allows the reduction of the matrix interferences almost completely

while it is also a very sensitive and selective extraction method. In addition, SPME offers the advantage of the direct injection when coupled to gas chromatography. This sample preparation technique was employed both for the analysis of fungicides and UV filters, exclusively or supported by liquid chromatography analysis.

Because of its adequation to the matrix and the vast bibliography about food applications, a modified QuEChERS protocol was the selected methodology for the determination of mycotoxins in feed samples. The modifications implemented were made in accordance with the complexity of the matrices analysed. The objective was to reduce the suppressive matrix effect, which was very pronounced for various target analytes. Therefore, after the conventional QuEChERS protocol, additional clean-up steps were performed, including a solid phase extraction and a dispersive solid phase extraction step. Thereby, along with a matrix-matched calibration, the matrix effect was largely decreased, improving the identification and quantification parameters for the selected analytes.

All sample preparation techniques utilized during this thesis have some characteristics in common, starting from their simplicity. The protocols proposed presented few steps, an easy implementation and, in the case of SPME, also the possibility of automation. Apart from their suitability for the applications of interest, these extraction techniques also follow the principles of "green chemistry" due to the low amount of reagents and organic solvents involved.

Regarding the methodologies proposed for conducting the degradation of organic contaminants in the environmental compartment, the aim was to achieve high removal efficiency using simple and practical approaches. Photodegradation procedures were employed for the elimination of fungicides and UV filters in water matrices. Both families of compounds can be commonly found in environmental waters due to their massive use and their way of

application. Fungicides and UV filters are susceptible to enter the aquatic environment directly, or indirectly via waste-water treatment plants discharge. Precisely, with the aim of efficiently degrade these contaminants in waste-water treatment plants and therefore avoid their entering in the water cycle, degradation methods based on photolysis are proposed. For both families of organic contaminants, direct photolysis using UVA and UVC radiations was used and compared, giving UVC radiation the highest removal efficiency. Advanced oxidation processes such as UVC/H_2O_2 system were also tested and, in the case of UV filters, heterogeneous catalysis was likewise assessed for the elimination of the most recalcitrant compounds. The heterogeneous catalysis method was based on a UVA/TiO_2 system, in which TiO_2 nanoparticles were supported onto a monolithic structure. This approach represents an alternative as an environmentally friendly photodegradation process using solar energy and, because the nanoparticles are retained in the monolithic structure, they do not present the risk of being released into the environment.

The degradation kinetics of the targeted compounds were determined using the different photodegradation configurations proposed, allowing their comparison in terms of degradation efficiency. As well as the assessment of the behaviour of the contaminants, a monitoring of possible degradation products was carried out at different exposure times.

Analytical techniques utilized for all the studies included in this thesis were based on chromatography coupled to mass spectrometry. The type of chromatography, gas or liquid, was selected depending on the application and/or the target compounds. Gas chromatography was used for the analysis of fungicides and UV filters in environmental waters, due to the easy coupling with SPME technique and the compatibility with the analytes. However, liquid chromatography was

used for the determination of all families of compounds, both in the environmental and food compartments.

Detection instruments were based on low resolution (triple quadrupole detector) and, mainly, high resolution mass spectrometry (triple quadrupole-time of flight detector). In this thesis, emphasis is placed on the use of high-resolution mass spectrometry (HRMS) due to its added value for the exact and unequivocal identification and quantification of the analytes. HRMS offers additional identification parameters comparing to tandem mass spectrometry, such as the exact mass of the compounds, their isotopic profile, and the possibility of contrasting the experimental spectra obtained with spectrum libraries containing fragments with exact masses, as well.

The high-resolution mass spectrometry configuration utilized during this thesis was a SCIEX TripleTOF instrument performing in SWATH^{TM} (sequential windowed acquisition of all theoretical mass spectra) mode, which is an advanced data independent acquisition (DIA) mode. This approach is based on the selection of a m/z range in which all analytes are included, then the range is divided into numerous small m/z windows (typically between 20 and 30) and MS/MS spectra are registered for all detected peaks. In this way, a full scan of the sample and MS/MS spectra for all peaks are simultaneously obtained without having to introduce mass transitions. SWATH acquisition was proved useful as a sensitive and accurate method for both the detection and quantification of the analytes of interest in all samples analysed. However, the most important feature that this scan mode enables consists of the possibility of performing retrospective non-targeted searches using the same raw acquisition file. SWATH was then utilized for the identification of possible degradation products in the case of fungicide and UV filter photolysis, and for the detection of mycotoxins in feed samples that were not in the target list.

In the search for photodegradation products in the water samples analysed, the possibility of contrasting MS/MS spectra with those of a

library was very useful in order to propose photoproducts and link them to their corresponding precursors. Taking into account the molecular formula proposed for each exact mass detected and the MS/MS spectra confirmation, several degradation/transformation products were identified and associated to the initial compounds present in the sample. Then, a monitoring of these degradation compounds could be performed at different times in order to assess their persistence in the water samples.

Besides the work presented in this thesis, this SWATH protocol leaves the door open to future searches on the already performed analytics for other applications. For instance, the identification of other families of contaminants in the environmental waters analysed, or a more exhaustive search for metabolised and modified forms of mycotoxins in the hundred feed samples studied could be performed, showing that most of these forms originated from *Fusarium* fungi.

A common characteristic of all works presented is the multi-analyte methodology approach, in contrast with most studies published, especially regarding photodegradation experiments. Usually, the evaluation of the degradation kinetics and the proposal of photoproducts are carried out for a solely compound or for a class of compounds. However, co-occurrence of several contaminants in one sample is a very frequent circumstance. For instance, personal care products normally gather various UV filters in their formulations, and feed samples were proved to present co-occurrence of two or more mycotoxins in most samples.

Therefore, multi-analyte methodologies are more representative of the actual incidence of the contaminants of interest in real matrices, and they allow a rapid monitoring of several compounds in only one analytical run. In this thesis, a selection of 11 fungicides, 20 UV filters and 26 mycotoxins from different families was made for the development of multi-analyte methodologies.

The fact that these methods are multi-analyte makes the optimization of the sample preparation and the analysis more complex. For example, in the case of SPME applications, chemometric tools by means of a multifactor experimental design were utilized in order to obtain the optimal values for the evaluated factors, reaching a compromise between all analytes.

All the methodologies published and presented in this thesis were validated in terms of linearity and precision. Also, the techniques utilized showed very low limits of detection and quantification, allowing the detection of contaminants in water matrices at trace levels (low ppt).

The proposed methods were always evaluated using real samples. In the case of the photodegradation studies of fungicides and UV filters, different waters were analysed considering the matrices more potentially affected by each class of compounds (superficial waters, swimming pool water, etc.).

For the determination of mycotoxins, almost a hundred mixed ration samples were analysed, obtaining relevant data about the occurrence and the concentrations of the targeted analytes. While all these feed samples exhibited co-occurrence of two or more mycotoxins, (zearalenone and fumonisin B_1), the concentrations were much lower than those reported in previous studies conducted on maize based feed and silages.

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VI. CONCLUSIONS

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In this thesis, the main objectives initially proposed were achieved:

- Optimization of sample preparation procedures. A methodology for the determination of fungicides in environmental waters using microextraction techniques was developed. Also, in the food control field, a method for the extraction of mycotoxins in complex feed matrices was validated.

- Monitoring of photodegradation processes of fungicides and UV filters in water. UV direct photolysis and advanced oxidation processes where used for the degradation of the compounds.

- Tentative identification of degradation products was carried out using HRMS.

The sample preparation techniques used in this thesis are characterised by their simplicity, rapidness, and concordance with the principles of green chemistry due to their low consumption of organic solvents and other reagents. The methods used were a solid-phase microextraction (SPME) for the environmental applications; and a modified Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) extraction followed by a dispersive solid-phase extraction (DSPE) clean-up for feed analyses.

The optimization of these techniques was carried out by means of chemometric tools. Experimental designs were generated for the simultaneous optimization of several factors, also considering the interactions between them.

The analytical methods used were based on chromatography, both GC and LC, coupled to mass spectrometry for a comprehensive qualitative and quantitative analysis. Tandem mass spectrometry and, mainly, high resolution mass spectrometry configurations were used. HRMS was especially crucial for the identification of possible

transformation products in the photodegradation studies on fungicides and UV filters.

The quality parameters of the analytical methods were evaluated in terms of linearity, repeatability, reproducibility, precision and limits of detection and quantification, obtaining satisfactory results for all applications.

The general conclusions of each chapter are commented bellow:

Chapter 1. Determination and fate of fungicides in water

1.1. Determination of fungicides in water

- A simple and fast method based on SPME for the determination of multiclass fungicides was optimized. Extraction parameters such as temperature, type of fibre and extraction mode were examined.

- The extracts were analysed by GC-MS/MS and the methodology was successfully validated in terms of recovery, and repeatability. This method also proved to be suitable for detection of fungicides at low ng L^{-1} level in water.

- The validated SPME-GC-MS/MS method was applied to nine different water samples, demonstrating the absence of matrix effect. It also proved to be appropriate for the monitoring of fungicide photodegradation.

1.2. Photodegradation of fungicides in water

- Simultaneous degradation of multiclass pesticides in different types of real water samples was evaluated through direct photolysis under UVA and UVC radiation. UVC radiation showed a higher removal efficiency for most compounds, with mean values about 60% after 30 minutes of exposure.

- Initial concentrations of the analytes were proved to have no effect on the degradation efficiency under UVC radiation in most cases.

- UVC/ H_2O_2 advanced oxidation process (AOP) was tested in order to accelerate the degradation, resulting in the efficient removal (>82%) of all compounds after only 6 minutes of irradiation. This proved it to be a cheaper procedure suitable for its implementation in real treatment plants.

- The degradation monitoring was simultaneously assessed by the development of a direct-injection LC-MS/MS method which provided IDLs at ng L^{-1} levels, enabling a rapid detection of trace levels of the target fungicides in a short analysis time.

- HRMS allowed the determination of by-products after UVC photolysis. With UVC/H_2O_2 treatment only two transformation products could be detected. The abundance of the photoproducts compared to the analytes was considerably low.

Chapter 2. Photodegradation of UV filters in water

- The simultaneous photodegradation of 21 multiclass UV filters has been assessed by direct photolysis under UVA and UVC radiation, and by advanced oxidation processes based on heterogeneous UVA/TiO_2 photocatalysis and UVC/H_2O_2.

- UVC radiation showed higher removal efficiency for most of the target compounds than UVA, reaching percentages of elimination higher than 90% after 1 hour of irradiation.

- The extraction efficiency of UVA photolysis was improved by using TiO_2 nanoparticles supported onto inert structures acting as catalysts, hence presenting an environmentally friendly process.

- UVC photolysis showed its suitability for the elimination of most UV filters in different natural water matrices with efficiencies > 90% for most compounds in sea and river water. UVC/H_2O_2 system was successfully employed for the elimination of the most persistent compounds in recreational waters.

- Nineteen photodegradation by-products were tentatively identified by HRMS after UVC photolysis, four of them being detected in all water matrices.

Chapter 3. Determination of mycotoxins in feed

- An analytical methodology based on QuEChERS with (d)SPE clean-up and HPLC-HRMS analysis allowed the screening and quantitation of 26 mycotoxins in complex feed samples. Matrix effect was compensated by a matrix-matched calibration, and the methodology was successfully validated.

- Co-occurrence of two or more mycotoxins were corroborated in almost 100 analyzed samples, being the most frequent zearalenone and fumonisin B_1.

- SWATH^{TM} acquisition mode proved to be a very useful approach for both the accurate identification and the quantification of the studied mycotoxins in complex feed matrices. Non-targeted mycotoxins could also be identified on a retrospective analysis.

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Annex I: List of publications

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- María Celeiro, Rocío Facorro, Thierry Dagnac, Vítor J.P. Vilar, María Llompart, Photodegradation of multiclass fungicides in the aquatic environment and determination by liquid chromatography – tandem mass spectrometry, *Environmental Science and Pollution Research* 24 (2017) 1-13

  DOI: 10.1007/s11356-017-9487-2
  Impact Factor: 2.950 (2017)
  (Q2) in Environmental Chemistry
  Contribution: Experimental work, data treatment and draft co-writing

- María Celeiro, Rocío Facorro, Thierry Dagnac, María Llompart, Simultaneous determination of trace levels of multiclass fungicides in natural waters by solid-phase microextraction – gas chromatography – tandem mass spectrometry, *Analytica Chimica Acta* 1020 (2018) 51-61

  DOI: 10.1016/j.aca.2018.03.014
  Impact Factor: 5.41 (2018)
  (Q1) in Environmental Chemistry and Analytical Chemistry
  Contribution: Experimental work, data treatment and draft co-writing

- María Celeiro, Rocío Facorro, Thierry Dagnac, Vítor J.P. Vilar, María Llompart, Photodegradation behaviour of multiclass ultraviolet filters in the aquatic environment: removal strategies and photoproduct identification by liquid chromatography-high resolution mass spectrometry, *Journal of Chromatography A* 1596 (2019) 8-19

  DOI: 10.1016/j.chroma.2019.02.065
  Impact Factor: 4.049 (2019)
  (Q1) in Analytical Chemistry
  Contribution: Experimental work, data treatment and draft co-writing

- Rocío Facorro, María Llompart, Thierry Dagnac, Combined (d)SPE-QuEChERS extraction of mycotoxins in mixed feed rations and analysis by high performance liquid chromatography - high-resolution mass spectrometry, *Toxins* 12 (2020) 206-226

  DOI: 10.3390/toxins12030206
  Impact Factor: 3.531 (2019)
  (Q1) in Toxicology
  Contribution: Experimental work, data treatment and original draft preparation.

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Annex II: Resumo

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A presente tese céntrase no desenvolvemento e optimización de metodoloxías analíticas, principalmente baseadas na espectrometría de masas en tándem de alta resolución, para a determinación de contaminantes orgánicos emerxentes e o seu impacto ambiental, así como a investigación no ámbito da seguridade alimentaria.

Son varias as familias de compostos que se van estudar atendendo a estes dous ámbitos. Por un lado, preténdese investigar a presenza de funxicidas, así como de filtros ultravioleta en matrices ambientais, particularmente en mostras de augas naturais. Do mesmo xeito, levarase a cabo a análise do comportamento destas dúas familias de compostos nas devanditas matrices. En canto ao ámbito da seguridade alimentaria, levarase a cabo un estudo sobre a presenza dun amplo grupo de micotoxinas en mostras de alimentación animal.

Os pesticidas están definidos pola Autoridade Europea de Seguridade Alimentaria (EFSA) como substancias que se empregan para eliminar ou controlar pragas. Os funxicidas, tamén chamados antimicóticos, son unha clase de pesticidas que se encargan de eliminar ou inhibir o crecemento de fungos en plantas, en produtos de almacenaxe ou no solo. Estas substancias, que están consideradas contaminantes orgánicos, empréganse de forma intensiva en todo o mundo para a prevención das perdas económicas que poidan ser provocadas por infeccións fúnxicas en diversos ámbitos como na agricultura, nas actividades forestais ou na viticultura. Tamén é algo común que este tipo de actividades se desenvolvan relativamente preto de ríos e outras masas de auga.

Debido a este uso extenso que se fai dos funxicidas que, a pesares de empregarse en concentracións relativamente baixas, se aplican varias veces durante o ano, a acumulación destas substancias pode chegar a producirse. Deste xeito, os residuos de funxicidas poden chegar a introducirse no sistema de augas superficiais a través da lixiviación do solo, do tratamento de augas residuais, ou incluso por fumigación

directa. Diversos estudos publicados aseguran que certos funxicidas presentan actividade tóxica, non só para os organismos acuáticos, senón tamén para a saúde humana, posto que existen substancias cunha gran estabilidade química que pode favorecer os fenómenos de bioacumulación e biomagnificación.

É por isto que o estudo da ocorrencia destes contaminantes orgánicos en augas naturais é de gran interese. Así mesmo, é tamén relevante o comportamento que os funxicidas teñen fronte a elementos que poidan favorecer a súa degradación. Polo tanto, precísanse metodoloxías sensibles e selectivas para a determinación e a cuantificación destas substancias a baixos niveles de concentración en augas naturais. Do mesmo xeito, o desenvolvemento de metodoloxías que permitan unha eliminación eficaz dos residuos de funxicidas e os posibles produtos de degradación nestas matrices.

Os filtros ultravioleta ou filtros UV están definidos pola Regulación Europea como substancias que se destinan á protección da pel fronte a certa radiación UV a través de mecanismos como a absorción, o reflexo ou a dispersión desta radiación. Estes produtos son utilizados actualmente de forma masiva principalmente en produtos de coidado persoal, pero tamén na manufactura téxtil, de plásticos ou de conservantes industriais.

Debido a este continuo incremento no uso dos filtros UV, están considerados como contaminantes orgánicos emerxentes pola súa potencial acumulación nas augas ambientais. O acceso destas substancias ao ciclo da auga pódese producir de dúas maneiras; de forma directa a través de actividades recreacionais como o baño en praias ou ríos, ou de forma indirecta principalmente a través das descargas das plantas de tratamento de augas residuais. Isto supón un grave problema ambiental, pero tamén para a saúde, posto que algúns filtros UV como as benzofenonas presentan efectos disruptores endócrinos. Ao igual que o comentado anteriormente sobre os

funxicidas, os filtros UV tamén presentan evidencias de potencial bioacumulación en tecidos de animais acuáticos, así coma en tecidos e fluídos humanos.

A exposición destes compostos a radiación UV tamén pode dar lugar á formación de especies oxidativas reactivas tras o proceso de fotólise. Estes produtos de degradación poden presentar incluso un maior risco que os propios precursores. É por isto que, ademais do desenvolvemento e optimización de metodoloxías para poder detectar filtros UV en augas naturais a unhas concentracións baixas, é de vital importancia analizar tamén os produtos de fotodegradación que se poidan chegar a formar tras os procesos de fotólise.

A terceira familia de compostos que se estudou durante o transcurso desta tese, foron as micotoxinas. As micotoxinas son metabolitos secundarios tóxicos que son producidos de xeito natural por diversos fungos filamentosos. O crecemento destas substancias é frecuente en matrices alimentarias, como cereais, froitos ou especias, e pode producirse en calquera das fases do proceso, dende o cultivo ata o procesado ou a conservación. Moitas das micotoxinas presentan ademais unha alta estabilidade química, co que poden sobrevivir ao procesado dos alimentos e chegar ao consumidor final.

Ademais das perdas en canto a produtividade que pode ocasionar a presenza destas toxinas na actividade gandeira, tamén poden presentar efectos adversos para a saúde tanto animal como humana. As micotoxinas poden chegar a xerar problemas inmunolóxicos, hepatotóxicos, disrupcións na síntese do ADN e do ARN, ou presentar efectos carcinoxénicos, entre outros. É por isto que existe unha regulación en canto aos niveis máximos permitidos das micotoxinas máis frecuentemente atopadas en diversas matrices alimentarias.

Xa que o escenario máis habitual é que se dea a co-ocorrencia de dúas ou máis micotoxinas neste tipo de matrices, faise

practicamente imprescindible o desenvolvemento de metodoloxías de análise multi-analito, que poidan ofrecer unha identificación e cuantificación inequívoca destes compostos. Ademais, como xeralmente as matrices nas que se atopan as micotoxinas son complexas, requírense métodos de preparación de mostra rápidos e eficaces.

Para levar a cabo a análise destas familias de compostos, as metodoloxías empregadas baseáronse na cromatografía, tanto líquida (LC) coma de gases (GC). A cromatografía é a técnica máis eficiente para conseguir unha separación axeitada dos analitos nunha mostra. A técnica de detección acoplada aos sistemas cromatográficos empregados foi en tódolos casos a espectrometría de masas (MS), tanto na configuración de espectrometría de masas en tándem (MS/MS) coma de alta resolución (HRMS). A espectrometría de masas en tándem, tanto GC-MS/MS como LC-MS/MS, ofrece unha elevada selectividade e sensibilidade para a identificación e cuantificacións dos compostos de interese. A espectrometría de masas de alta resolución, ofrece tamén algún parámetro adicional de confirmación, como é a masa exacta ou o perfil isotópico, ademais de ser un elemento esencial para os estudos de fotodegradación. A HRMS permite a detección de compostos descoñecidos nunha mostra, co que é a técnica que se empregou para a identificación de posibles produtos de degradación tras as fotólises de funxicidas e filtros UV.

En canto á estrutura da presente tese doutoral, atópase dividida en seis seccións principais.

Na **Sección I** inclúese unha xustificación onde se pon en contexto a relevancia das familias de compostos seleccionados para a realización dos distintos traballos que se inclúen na tese, en canto ao potencial perigo que pode supoñer a súa presenza nas matrices de interese. De

acordo con esta xustificación, expóñense de forma estendida os obxectivos que se pretenden acadar.

A **Sección II** consiste nunha ampla introdución onde se presentan as diferentes familias de compostos analizados nos distintos traballos, atendendo á súa clasificación, as propiedades físico-químicas que presentan, a súa potencial toxicidade e o marco lexislativo no que se atopan no ámbito de interese. Introdúcense tamén nesta sección os procesos de fotodegradación en auga que empregaron, incluíndo os procesos de oxidación avanzada (AOPs) e os tipos de catálise.

Tamén hai nesta sección un epígrafe onde se expoñen as técnicas de preparación de mostra empregadas durante o transcurso da tese, explicando o procedemento xeral de cada unha delas, os factores a ter en conta para optimizar o protocolo co fin de conseguir unha mellor eficacia de extracción e, finalmente, algunhas das aplicacións que teñen en distintos ámbitos descritas na bibliografía. Inclúense nesta sección a extracción en fase sólida (SPE), a extracción en fase sólida dispersiva (DSPE) e a extracción QuEChERS (*Quick, Easy, Cheap, Effective, Rugged, and Safe*). Descríbese tamén a técnica miniaturizada de micro-extracción en fase sólida (SPME), empregada fundamentalmente para as análises levadas a cabo en matrices acuosas.

As técnicas de análise están recollidas igualmente na Sección II, centrándose únicamente nas técnicas de cromatografía líquida e de gases, que foron as empregadas en tódolos traballos presentados nesta tese. Así mesmo, aparecen incluídas as técnicas de detección baseadas na espectrometría de masas, particularmente a espectrometría de masas en tándem e a de alta resolución. Algunhas das aplicacións das configuracións LC-MS/MS, GC-MS/MS e LC-HRMS en diversos ámbitos aparecen comentadas.

Na **Sección III** faise unha breve descrición dos procedementos metodolóxicos empregados nos traballos presentados nesta tese.

Na **Sección IV** -resultados e discusión- exponse e discútese o traballo experimental realizado, así como os resultados obtidos durante a realización da tese doutoral. Os diferentes estudios realizados deron lugar a catro publicacións que se compilan en tres capítulos diferentes atendendo á temática e á familia de compostos estudiada.

Capítulo 1. Determinación e transformación de funxicidas en auga

Neste capítulo inclúense dúas publicacións, a primeira delas baseada na determinación de funxicidas a niveis traza en augas naturais, e a segunda máis centrada na fotodegradación dos funxicidas no medio acuático.

1. Determinación simultánea de niveis traza de funxicidas en augas naturais mediante micro-extracción en fase sólida – cromatografía de gases acoplada a espectrometría de masas en tándem.

Para este traballo seleccionáronse once funxicidas de uso amplamente estendido no ámbito da viticultura: benalaxyl, cyprodinyl, dimethomoph, fenhexamid, iprodione, iprovalicarb, kresoxim-methyl, metalaxyl, myclobutanil, procymidone e tebuconazole.

Describe a optimización dun método simple e rápido baseado na SPME, tendo en conta factores que afectan á eficacia da extracción, coma a temperatura, o tipo de fibra que se empregou e o modo de extracción (inmersión directa ou de espazo de cabeza).

Os extractos obtidos tras o protocolo de SPME foron analizados empregando cromatografía de gases acoplada a un detector de triplo cuadrupolo MS/MS. A metodoloxía proposta foi validada en termos de repetibilidade e de recuperación a tres niveis de concentración distintos e en distintas matrices. Así mesmo, este método foi probado de forma satisfactoria para a detección de funxicidas distintas mostras de auga a niveis de concentración tan baixos coma de ng L^{-1}.

Analizouse tamén a eficacia desta metodoloxía para o estudo de fotodegradación dos funxicidas seleccionados a baixas concentracións, posto que a maioría dos estudos publicados traballan con concentracións máis altas das encontradas en mostras reais. Atopouse que o método SPME-GC-MS/MS optimizado é unha ferramenta perfectamente válida para determinar as cinéticas de degradación destes compostos tras recibir irradiación UVC, incluso a baixas concentracións.

2. Fotodegradación de funxicidas no medio acuático e posterior determinación mediante cromatografía líquida acoplada a espectrometría de masas de alta resolución.

Este segundo traballo publicado céntrase no comportamento dunha selección de 9 funxicidas (benalaxyl, cyprodinyl, dimethomoph, fenhexamid, iprovalicarb, kresoxim-methyl, metalaxyl, myclobutanil e tebuconazole) de distintas clases tras os procesos de fotodegradación en diferentes mostras de auga.

As metodoloxías empregadas para a fotodegradación dos compostos baseáronse primeiro na avaliación da fotólise directa baixo radiación UVA e UVC. A radiación UVC demostrou unha maior eficacia para a eliminación dos compostos fronte á UVA, posto que tras 30 minutos de exposición, acadouse unha eliminación media do 60%. Posteriormente, plantéxase o uso de procesos de oxidación avanzada coma o sistema UVC/H_2O_2 coa intención de obter unha maior porcentaxe de eliminación dos funxicidas nun período inferior de tempo.

Os estudos de fotodegradación leváronse a cabo en catro tipos distintos de augas (augas residuais, auga de consumo, auga de piscina e auga de río) co obxectivo de avaliar a influenza do tipo de matriz no proceso de degradación. Tamén se realizaron a distintos niveis de concentración para constatar se existía relación entre a concentración inicial dos compostos e a efectividade da degradación.

Para a avaliación das cinéticas de degradación dos compostos, empregouse unha metodoloxía baseada na cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS). Este método foi satisfactoriamente validado, obtendo uns límites de detección instrumental do nivel de ng L^{-1}.

Finalmente empregouse un acoplamento de cromatografía líquida cun espectrómetro de masas de alta resolución cunha configuración de cuadrupolo-cuadrupolo cun detector de tempo de voo (QqTOF). Esta conformación permitiu, ademais de realizar o seguimento da degradación dos funxicidas seleccionados, a busca de posibles produtos de degradación que se puideran ter formado tras a exposición fronte á radiación UVC e demais procesos.

Capítulo 2. Fotodegradación de filtros UV no medio acuático

Neste capítulo aparece recollida unha publicación baseada en diferentes estratexias para a fotodegradación simultánea de filtros UV orgánicos de distintas clases no medio acuático, seguida da identificación de posibles fotoprodutos empregando cromatografía líquida acoplada a espectrometría de masas de alta resolución.

Para este estudo fíxose unha ampla selección de 21 filtros UV orgánicos pertencentes a distintas clases (benzofenonas, cinamatos, salicilatos e derivados de canfor entre outras). As probas de fotodegradación realizáronse en primeira instancia empregando fotólise directa con radiación UVA e UVC. Sen embargo, debido á estabilidade de algúns dos compostos fronte a este tipo de fotólise, foi necesaria a utilización de procesos avanzados de oxidación (AOPs) para conseguir a completa eliminación dos filtros UV máis recalcitrantes. Os AOPs empregados baseáronse no sistema de oxidación fotoquímico UVC/H_2O_2 e na fotocatálise heteroxénea. Para este último, utilizáronse nanopartículas de TiO_2 inmobilizadas nunhas estruturas inertes co fin de evitar a liberación das nanopartículas e a súa posterior eliminación da

matriz. Este sistema de fotocatálise ten a vantaxe de ser ademais un proceso respectuoso co medio ambiente.

A evolución dos perfís cinéticos foi elaborada empregando dúas metodoloxías distintas; por un lado, mediante inxección directa-cromatografía líquida acoplada a espectrometría de masas en tándem; e por outro, mediante cromatografía de gases acoplada tamén a MS/MS. Previo á análise mediante GC-MS/MS, realizouse unha extracción e pre-concentración *in situ* con un método SPME, o que permitiu realizar a avaliación da fotodegradación dos filtros UV en niveis traza de poucos ng L^{-1}.

Os experimentos de degradación en matrices de augas recreativas, coma o caso de auga de piscina, leváronse a cabo empregando o sistema UVC/H_2O_2, posto que presentou unha maior efectividade na eliminación dos compostos máis persistentes nun tempo menor.

Coma no caso dos funxicidas no capítulo anterior, para a busca de posibles fotoprodutos empregouse un acoplamento de cromatografía líquida cun espectrómetro de masas de alta resolución cunha configuración de cuadrupolo-cuadrupolo cun detector de tempo de voo (QqTOF). Deste xeito, ademais de identificar os filtros UV de interese, tamén se puideron atopar case vinte posibles produtos de degradación tras a realización dunha busca *non-targeted* e baseándose na bibliografía dispoñible.

Capítulo 3. Determinación de micotoxinas en mostras de alimentación animal

Este último capítulo da Sección IV, recolle unha publicación baseada na optimización dunha metodoloxía de preparación de mostra para a detección simultánea de 26 micotoxinas de diferentes clases (fumonisinas, nivalenol e derivados, zearalenona e derivados, e aflatoxinas entre outras) en mostras complexas de alimentación animal mediante HPLC-HRMS.

A etapa de preparación de mostra proposta consiste nun protocolo QuEChERS (rápido, fácil, barato, eficaz, robusto e seguro) modificado seguido dunha etapa de *clean-up* SPE-DSPE.

A metodoloxía analítica baseada na cromatografía líquida optimizouse en canto á composición da fase móbil empregada, debido á variedade de características físico-químicas do amplo grupo de micotoxinas seleccionado. Para a identificación e cuantificación dos compostos estudiados, LC foi acoplada á espectrometría de masas de alta resolución cunha configuración QqTOF (ou TripleTOF$^{(R)}$). A metodoloxía proposta foi validada en termos de linearidade, precisión, repetibilidade e recuperación.

Debido á complexidade das mostras analizadas, ademais das etapas de limpeza no protocolo de preparación de mostra, o efecto matriz -xeralmente supresor para a maioría das micotoxinas- corrixiuse mediante unha calibración *matrix-matched*.

O modo de adquisición SWATH^{TM} (*sequential window acquisition of all theoretical fragment ion spectra*) empregouse para a inequívoca identificación e cuantificación das micotoxinas estudiadas, debido á posibilidade de comparación da masa exacta, o perfil isotópico do composto e do espectro de MS/MS con bibliotecas de espectros. Do mesmo xeito, SWATH resultou unha ferramenta moi útil para a posterior busca *non-targeted* de posibles formas modificadas das micotoxinas precursoras.

Tras a análise de case 100 mostras, corroborouse a co-ocorrencia de dúas ou máis micotoxinas en todas elas, sendo a combinación máis habitual zearalenona-fumonisina B_1. É por isto que a metodoloxía que se optimizou recolle a determinación simultánea de 26 micotoxinas das máis frecuentemente atopadas.

A **Sección V** inclúe unha discusión xeral que trata de poñer nun contexto conxunto todos os traballos presentados na anterior sección.

Finalmente, na **Sección VI** aparecen descritas as conclusións xerais extraídas dos traballos presentados ao longo desta tese doutoral.

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2. M. Celeiro, R. Facorro, T. Dagnac, M. Llompart, Simultaneous determination of trace levels of multiclass fungicides in natural waters by solid-phase microextraction – gas chromatography – tandem mass spectrometry, Anal. Chim. Acta *1020* (2018) 51-61.

Simultaneous determination of trace levels of multiclass fungicides in natural waters by solid - phase microextraction - gas chromatography-tandem mass spectrometry

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Photodegradation behaviour of multiclass ultraviolet filters in the aquatic environment: Removal strategies and photoproduct identification by liquid chromatography–high resolution mass spectrometry

Author: Maria Celeiro,Rocio Facorro,Thierry Dagnac,Vitor J.P. Vilar,Maria Llompart

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4. R. Facorro, T. Dagnac, M. Llompart, Combined (d)SPE-QuEChERS extraction of mycotoxins in mixed feed rations and analysis by high performance liquid chromatography - high-resolution mass spectrometry, Toxins *12* (2020) 206-226. *(Open Access)*

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