UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE INGENIEROS AGRÓNOMOS

DOCTORAL THESIS

**DETERMINATION OF EMERGING CONTAMINANTS IN
ENVIRONMENTAL MATRICES**

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IN ENVIRONMENTAL MATRICES

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HACEN CONSTAR: que el presente trabajo titulado “Determination of emerging contaminants in environmental matrices” ha sido realizado por el Ingeniero Agrónomo Ramón Aznar Roca en el Departamento de Medio Ambiente del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), bajo nuestra dirección, constituyendo la Tesis Doctoral de su autor.

Madrid, 19 de Mayo del 2016

Fdo. Dr. José Luis Tadeo Lluch

Fdo. Dra. Consuelo Sánchez-Brunete Palop

*I was just guessing at numbers and figures
Pulling your puzzles apart
Questions of science; science and progress
Do not speak as loud as my heart*

*Nobody said it was easy
It's such a shame for us to part
Nobody said it was easy
No one ever said it would be this hard
Oh take me back to the start*

Coldplay - The Scientist

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Abbreviations

ACN	→	Acetonitrile
AF4	→	Asymmetric flow field-flow fractionation
BME	→	Bayesian Maximum Entrophy
BSTFA	→	N,O-bis(trimethylsilyl)trifluoroacetamide
CCL	→	Contaminant Candidate List
CCorA	→	Canonical Correlation Analyses
CEs	→	Contaminantes Emergentes
DL	→	Detection Limit
dSPE	→	Dispersive Solid Phase Extraction
ECEW _{max}	→	Maximum Equilibrium Concentration Expected in Water
ECs	→	Emerging Contaminants
EDS	→	Energy Dispersive Spectroscopy
EE2	→	17- α -Ethinylestradiol
EM	→	Electron Multiplier
EPA	→	Environmental Protection Agency
EtAc	→	Ethyl Acetate
E1	→	Estrone
E2	→	17- β -Estradiol
E3	→	Estriol
GC	→	Gas Chromatography
GCB	→	Graphitized Carbon Black
GIS	→	Geographical Information System
ICH	→	International Conference on Harmonization
ICP	→	Inductively Coupled Plasma
K _d	→	Adsorption Coefficient
K _{oc}	→	Organic-Carbon soil partition coefficient
K _{ow}	→	Octanol-Water partition coefficient
LLE	→	Liquid Liquid Extraction
LOD	→	Limit of Detection

LOQ → Limit of Quantification
MAE → Microwave Assisted Extraction
MC → Matrix matched
MDL → Method Detection Limit
MIP → Molecularly Imprinted Polymers
MMI → Multimode Inlet
MRM → Multiple Reaction Monitoring
MS → Mass Spectrometry
MSD → Mass Spectrometric Detector
MSPD → Matrix Solid Phase Dispersion
MS/MS → Tandem Mass Spectrometry
MTBSTFA → N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide
NIST → National Institute of Standards and Technology
NP → Nanoparticle
NSAIDs → Non-Steroidal Anti-Inflammatory Drugs
OC → Organic Carbon
OM → Organic Matter
PAHs → Polycyclic Aromatic Hydrocarbons
PBDEs → Polybrominated Diphenyl Ethers
PCPs → Personal Care Products
pK_a → Acid Dissociation Constant
PLE → Pressurized Liquid Extraction
PSA → Primary Secondary Amine
PSD → Particulate Size Distribution
PVP → Polyvinylpyrrolidone
PYs → Pyrethroids
Q → Qualifier Ion
QA → Quality Assurance
QuEChERS → Quick, Easy, Cheap, Effective, Rugged and Safe
RSD → Relative Standard Deviation
SC → Neat solvent Calibration

SD → Standard Deviation
SFE → Supercritical Fluid Extraction
SI → Supplementary Information
SLE → Supported Liquid Extraction
SP → Single Particle
SPME → Solid Phase Microextraction
T → Target Ion
TBDMCS → Tert-Butyldimethylchlorosilane
*t*BDMS → *t*-Butyldimethylsilyl
TE → Transport Efficiency
TEM → Transmission electron microscopy
TMS → Trimethylsilyl
 t_R → Retention time
UAE → Ultrasound Assisted Extraction
USDA → United States Department of Agriculture
WWTP → Wastewater Treatment Plant

RESUMEN

Los contaminantes emergentes (CEs) son compuestos químicos que pueden poseer el potencial de causar efectos adversos al medio ambiente y/o a la salud humana. También pueden ser considerados emergentes si se ha descubierto una nueva fuente o una nueva vía de introducción al medio ambiente. Hoy en día, las siguientes familias de compuestos pueden ser clasificados como CEs: biocidas, retardantes de llama, hidrocarburos aromáticos policíclicos (HAPs), plastificantes, surfactantes, hormonas, productos de higiene personal, compuestos farmacéuticos y nanopartículas. Debido al elevado uso de estos compuestos y su continua emisión pueden denominarse como pseudo-persistentes, haciendo que sea una prioridad monitorear su presencia en el medio ambiente. Estos CEs son generados principalmente en hospitales, granjas y zonas urbanas e industriales. La principal vía de introducción en el medio ambiente es a través de las aguas residuales. Los CEs no son eliminados completamente en las depuradoras y, consecuentemente, son introducidos en el medio ambiente a través de los efluentes. Además, los lodos de depuradora y los residuos orgánicos de las granjas son usados en la agricultura para aumentar el contenido de materia orgánica y nutrientes en el suelo, siendo otra fuente de introducción de CEs en el medio ambiente.

Sin embargo, aunque las fuentes de contaminación son conocidas, es necesario estudiar la presencia y el comportamiento de los CEs en los diferentes compartimentos del medio ambiente. Para evaluar la presencia de estos compuestos se requieren métodos multirresiduos robustos. Así, en esta tesis, se presentan seis trabajos para evaluar los CEs en varias matrices: fármacos en lodos de depuradora; retardantes de llama, HAPs y biocidas en gallinaza; fármacos y biocidas en el suelo, compuestos representativos de los CEs en plantas acuáticas y nanopartículas de plata en muestras acuosas.

Para la determinación de fármacos en lodos de depuradora se desarrolló un método usando la técnica “supported liquid extraction” y su detección mediante GC-MS/MS. El método se validó y usó para evaluar la presencia de fármacos en lodos recogidos en

España, donde la cafeína, el ibuprofeno, el ácido salicílico y el fenoprofeno fueron detectados en todas las muestras analizadas.

Un método multirresiduos para detectar 41 contaminantes incluyendo retardantes de llama, HAPs y biocidas en gallinaza se desarrolló basado en una modificación del método “QuEChERS” y en su posterior determinación mediante GC-MS. Una vez validado el método se aplicó a muestras recogidas en diferentes granjas de Castilla y León. Se confirmó la presencia de los siguientes compuestos: biocidas, HAPs y 4,4’DDT y sus metabolitos.

Para evaluar la presencia de CE en suelo se desarrollaron dos métodos. Un primer método para la detección de 15 fármacos (ácidos, neutros y básicos) en suelo, basado en la extracción asistida por ultrasonidos seguido de GC-MS se aplicó a suelos agrícolas de diferentes regiones de España (Segovia, Murcia y Valencia). El análisis mostró la presencia ubicua de varios compuestos como ácido salicílico y paracetamol en las tres áreas estudiadas, así como fenoprofeno en Segovia y alopurinol y carbamacepina en Valencia. El segundo método fue desarrollado para detectar 10 piretroides en suelos agrícolas mediante la extracción asistida por ultrasonidos y GC-MS. El método fue aplicado para monitorear la presencia de los compuestos seleccionados en campos de arroz en Valencia, mostrando su ubicua presencia así como la identificación de las aguas de depuradoras como una de las principales fuentes de contaminación usando sistemas de información geográfica.

Otro método multirresiduos fue desarrollado para la determinación de 31 CE en plantas acuáticas por GC-MS, donde la técnica la dispersión de la matriz en fase sólida asistida por ultrasonidos fue utilizada para extraer los compuestos de interés de *Typha angustifolia*, *Arundo donax*, *Oryza sativa* and *Lemna minor*. El método fue validado y aplicado a las plantas recolectadas en tres ríos de tres regiones españolas (Madrid, Andalucía y Valencia). Seis de los treinta y un compuestos estudiados (biocidas y productos de higiene personal) fueron cuantificados.

Para finalizar, se puso a punto una metodología para detectar y caracterizar el tamaño y distribución (PSD) de nanopartículas de plata (Ag-NPs) en productos de consumo y

aguas ambientales usando SP-ICP-MS. El pretratamiento de la muestra (AF4 o filtración por centrifugación) no mejoraron la medida directa del SP-ICP-MS. El método se aplicó a productos de consumo, permitiendo la caracterización en tiritas del PSD de las Ag-NPs. Similarmente, en las aguas filtradas se pudo caracterizar con éxito las nanopartículas en términos de tamaño y número. Sin embargo, las concentraciones encontradas en aguas recolectadas en Italia estuvieron por debajo del límite de detección del ICP-MS.

ABSTRACT

Emerging contaminants (ECs) are chemicals that may have the potential to cause adverse ecological and/or human health effects. They can also be considered emerging contaminants because of the discovery of a new source or a new pathway of introduction to the environment. Nowadays, the following families of compounds have been classified as ECs: biocides, flame retardants, polycyclic aromatic hydrocarbons (PAHs), plasticizers, surfactants, hormones, personal care products, pharmaceuticals and nanoparticles. Due to the high use of these compounds and their continuous release they can be classified as pseudo-persistent, making it a priority to monitor their occurrence. These ECs are generated principally in hospitals, farms and urban and industrial areas. The main pathways of introduction into the environment are the polluted residual waters. Most of these polluted waters will reach a wastewater treatment plant (WWTP), but even in this case, most of the ECs are not completely degraded during the depuration processes and are discharged in the effluent waters, resulting in contamination of the environment. Moreover, sewage sludge from WWTPs and animal litter or manure from farms are used in agriculture to increase the content of organic matter and nutrients in soil, which may be another source of introduction of ECs into the environment.

However, although the input sources are well-known, the fate and behavior of these emerging contaminants in the different environment compartments need further work. Thus, reliable and robust multiresidue methods are needed to assess their presence into different environmental matrices. In this thesis, six works are presented to assess the occurrences of ECs in various matrices: pharmaceutical compounds in sludge; flame retardants, PAHs and biocides in poultry manure; pharmaceuticals and biocides in soil; representative ECs in aquatic plants and silver nanoparticles in aqueous matrices.

A method to determine pharmaceutical compounds in sludge was developed using supported liquid extraction and GS-MS/MS detection. The method was validated and used to assess the occurrence of pharmaceutical compounds in sludge samples collected in Spain, where caffeine, ibuprofen, salicylic acid and fenoprofen were detected in all the samples analyzed.

A multiresidue method to detect 41 selected contaminants including flame retardants, PAHs and biocides in poultry manure was developed, based on a modified QuEChERS method and GC-MS determination. The validated method was applied to poultry manure samples collected from farms located in Castilla-León. The presence of some of the studied contaminants was confirmed, being biocides, PAHs and 4,4' DDT and its metabolites the compounds mainly detected.

To assess the presence of ECs in soil two methods were developed. Firstly, a multiresidue method to detect 15 pharmaceutical compounds (acid, neutral and basic) in soil, based on an ultrasound assisted extraction procedure followed by GC-MS was validated and applied to agricultural soils from several Spanish areas (Segovia, Murcia and Valencia), showing the ubiquitous presence of various pharmaceutical compounds, such as salicylic acid and paracetamol, in the three areas studied, as well as fenopropfen in Segovia and allopurinol and carbamazepine in Valencia. The second method was developed to detect ten pyrethroids in agricultural soils by ultrasound-assisted extraction and GC-MS. The method was validated and applied to monitor their presence in a paddy field area in Valencia, showing the ubiquitous presence of them as well as the identification of WWTPs effluents as one of the main sources of pollution using a GIS program.

A multiresidue method was developed for the determination of 31 ECs in aquatic plants, where ultrasound-assisted MSPD was applied to extract the studied compounds from *Typha angustifolia*, *Arundo donax*, *Oryza sativa* and *Lemna minor* followed by GC-MS determination. The method was validated and applied to aquatic plants collected from three rivers located in different Spanish regions (Madrid, Andalucia and Valencia). Six of the thirty one compounds studied (biocides and personal care products) were found and quantified.

Finally, a methodology to detect and characterize the particle size distribution (PSD) of Ag-NPs in consumer products and environmental waters using SP-ICP-MS was evaluated. The sample pretreatment (AF4 fraction collection or centrifugal filtration) did not improve the analytical performance of direct SP-ICP-MS analysis. The

procedure was applied to consumer products and plasters, allowing the determination of the complete PSD just for plasters. Similarly, the PSDs of Ag-NPs in filtered tap waters were successfully characterized in terms of size and number. However, the concentration levels found in freshwater samples from Italy were below the ICP-MS method dynamic range.

OBJECTIVES

Nowadays almost everything that surrounds us has been in touch or involved in a chemical process. Thus, we cannot consider an intensive agriculture without pesticides, a home without plastics, furniture without flame retardants, health without pharmaceuticals and body beauty without personal care products, showing the dependence of the population on chemical compounds.

Nevertheless, the high production and consumption of these products may have some drawbacks to the environment where they may be toxic to the flora and fauna and to human health if they are introduced into the food chain among others pathways. Today, with the developments in analytical instrumentation, we can detect these substances at low concentrations and assess their occurrence and risk. Therefore, it is necessary to develop multiresidue methods, environmental friendly, fast, robust and cheap, to detect these compounds in the different compartments of the environment (organic residues, soil, plants and water).

In this work, most of the families of compounds that form the group of emerging contaminants (ECs), such as pharmaceuticals, hormones, personal care products and nanoparticles, have been considered. Different studies have been carried out to detect ECs in various environmental matrices of agricultural interest, such as sewage sludge, poultry manure, soil, plants and water. Thus, objectives of the present work are the following:

- Selection of sample preparation techniques to extract ECs from different environmental matrices of agricultural interest.
- Development and in-house validation of new multiresidue methods to separate and determine the target compounds in different matrices, using gas chromatography and inductively coupled plasma as separation techniques coupled with mass spectrometry or tandem mass spectrometry.
- Finally, determination of the studied ECs in sewage sludge, poultry manure, soil, aquatic plants and water in samples collected around Spain and Italy.

CHAPTER ONE – INTRODUCTION

1.1 What is an Emerging Contaminant?

Public and scientific concern regarding the possible drawbacks of the use of some chemicals started to be a reality thanks to Rachel Carson and her well-known book “Silent Spring” published in 1962 (Carson, 1962). She documented the detrimental effects on the environment (particularly on birds) of the indiscriminate use of pesticides such as dichlorodiphenyltrichloroethane (DDT). After that, other scientists started to question the safety of the use of some chemicals, Theo Colborn and coworkers in 1996 published the book entitled “Our Stolen Future” (Colborn et al., 1996) where they indicated that some chemicals could present endocrine disruptor activity (chemicals that may interfere at certain doses with the body’s endocrine system and produce adverse development, reproductive, neurological, and immune effects in both humans and wildlife).

Since the publication of these books, numerous new compounds have been produced by industry to improve the overall quality of life. The use of these compounds has allowed to increase agricultural productivity and to improve animal health to feed the uprising population that also is living longer and they also help to make our life easier.

Environmental scientists focused on industrial and agricultural pollutants (such as polychlorinated biphenyls and DDT) during late 1960s and 1970s, and only a few scientists pointed out a new source of contamination connecting human activities, such as drug consumption, and the subsequent release of these drugs and their metabolites into the environment. The first known report indicating that steroid hormones are not completely eliminated by wastewater treatment was published by Stumm-Zollinger and Fair in 1965. Moreover, clofibric acid was detected in groundwater in 1976 (Garrison et al., 1976) and one year later salicylic acid was reported in wastewater effluents (Hignite and Azarnoff, 1977). Despite these early findings, the issue of steroids and pharmaceuticals in wastewater outfalls did not gain significant attention until the 1990s, when the occurrence of natural and synthetic steroid hormones in wastewater was linked to reproductive impacts in fish living downstream of outfalls (Purdom et al, 1994)

implying possible drawbacks to the environment and human health and driving the scientific community to face a new challenge.

The most recent findings of ECs in the environment can be attributed to improvements in analytical instrumentation with new and improved equipment and techniques such as gas or liquid chromatography coupled with mass spectrometry allowing to detect and quantify these new compounds at trace levels, opening a broad range of previously undetected pollutants present in different matrices including soil, water, food, tissue, blood and breast milk (Bao et al., 2015; Chefetz et al., 2008; Corcellas et al., 2012; Goldstein et al., 2014; Wingfors et al., 2005). Thus, it was how scientists started to be aware of a new family of contaminants that could be defined as follows:

- Emerging contaminants are any synthetic or naturally occurring chemical or any microorganism that is not commonly monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects. In some cases, the release of emerging chemicals or microbial contaminants to the environment has likely occurred for a long time, but may not have been recognized until new detection methods were developed. In other cases, the synthesis of new chemicals or changes in use and disposal of existing chemicals can create new sources of these contaminants (<http://toxics.usgs.gov/regional/emc/>). A contaminant also may be "emerging" because of the discovery of a new source or a new pathway to humans (<http://www.epa.gov/fedfac/emerging-contaminants-and-federal-facility-contaminants-concern>).

It is worth it to say that ECs have been named by different authors as “emerging chemicals of concern”, “contaminants of concern”, “unregulated contaminants”, “contaminants of emerging concern”, among others, but in this work they will be called emerging contaminants.

1.2 Emerging Contaminants

Emerging contaminants can be classified attending to different parameters, but in general they are classified regarding their uses (Barceló, 2003). The list of the groups

of compounds that form the family of emerging contaminants can be seen in Fig. 1.1. This figure has been created summarizing until now the most important groups taken into account by different authors (Albero et al., 2015a; Bao et al., 2015; Eljarrat and Barceló, 2003; Jurado et al., 2012; Tadeo et al., 2012a; Verlicchi et al., 2010). Certainly this figure is not fixed and in next years, as the industry continues to develop new compounds, this list will include new ECs.

Figure 1.1 Emerging Contaminants

It is also important to point out that some ECs can be classified in more than one group. A more detailed description of the different groups of ECs is shown below.

1.2.1 Biocides

A biocide is defined in the European legislation as a chemical substance intended to destroy, deter, render harmless, or exert a controlling effect on any harmful organism by chemical or biological means. They are commonly used in agriculture, urban areas and in various industries. There are diverse types of biocides, such as insecticides, disinfectants, preservatives, avicides, molluscicides, piscicides, algaecides, rodenticides, and acaricides. Focusing on insecticides, after the prohibition of organochlorine and organophosphate compounds, new families were developed (pyrethroids and neonicotinoids) and they are attracting the interest of toxicologists because of their potential toxicity to non-target organisms (Henry et al., 2012; Maund

et al., 2012b). Recently, EPA has added some pyrethroids, such as permethrin (Fig 1.2), in the Contaminant Candidate List (CCL) (<http://www.epa.gov/ccl/chemical-contaminants-ccl-4>) to survey those in drinking water, although they are not currently regulated.

Figure 1.2 Structure of pyrethroids: esfenvalerate and permethrin

1.2.2 Flame retardants

Flame retardants are substances that can delay or prevent combustion. These compounds can be placed in four categories - halogenated organics, phosphorus-based, nitrogen-based, and other inorganic compounds, where the brominated compounds are the most commonly used as they are less expensive and quite effective (Fig. 1.3). Polybrominated diphenyl ethers (PBDEs) are used in the electronic industry mainly in printed circuit boards, connectors, plastic covers and cables, but they are also used in carpets, paints and domestic kitchen appliances. Concerns are being raised because of their persistence, bioaccumulation, and potential toxicity, both in animals and in humans. (Birnbaum and Staskal, 2004)

Figure 1.3 Structure of PBDEs: BDE-47 and BDE-100

1.2.3 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) comprise a large group of compounds, with two or more fused benzene rings (Fig. 1.4), widely distributed in the environment. They usually arise from incomplete combustion of organic materials during natural or anthropogenic processes (Pérez et al., 2014a). PAHs are compounds resistant to degradation and possess toxic properties; some of them have been reported to be mutagenic, carcinogenic and teratogenic and therefore they have been included in the EU priority pollutant list (Guzzella et al., 2011).

Figure 1.4 Structure of PAHs: chrysene and benzo[a]pyrene

1.2.4 Plasticizers

Plastic products have been one of the major concerns in terms of toxicity and persistence in the environment. They contain a myriad of additives including the plasticizers that provide them flexibility and durability to serve multiple purposes, ranging from automotive industry to medical and commodity products (Cadogan and Howick, 2010)

It has been reported that some plasticizers are toxic and can exhibit endocrine disrupting properties (Reemtsma et al., 2008). Nowadays, we can find diverse plasticizers (Fig 1.5), such as phthalate and adipate based, trimellitates and organophosphates, among others, to increase flexibility, transparency, durability, and longevity of plastics.

Figure 1.5 Structure of plasticizers: tris(2-chloroethyl)phosphate and bisphenol A

1.2.5 Surfactants

Surfactants are used to lower the surface tension, generally they are amphiphilic, containing both hydrophobic and hydrophilic groups (Rosen and Kunjappu, 2012) (Fig 1.6). The most common use of surfactant compounds is in soaps, dishwashing liquids, laundry detergents and shampoos. Because the high use of them in industrial and urban areas, the main path to be introduced into the environment is through the discharge of wastewater treatment plants (WWTPs).

Figure 1.6 Structure of octaethylene glycol monododecyl ether and nonylphenol

1.2.6 Hormones

Hormones (Fig. 1.7) are natural and synthetic compounds and may pose a powerful impact on human and animal health. The presence of these chemicals in waters caused fish hermaphroditism due to their endocrine disrupting properties was reported for the first time in 1994 (Purdom et al., 1994). The use of hormone therapy in humans and

livestock farming has increased in recent years. In addition, estrogenic compounds are naturally produced; therefore, the presence of these compounds in wastewaters and in the environment is expected.

Figure 1.7 Structure of hormones: estrone (E1) and hexestrol

1.2.7 Personal care products

Personal care products (PCPs) are compounds that can be found in consumer products marketed for use on the human body. These active ingredients are present in cosmetics, skin care, dental and hair care products, sunscreen agents, soaps, antibacterial products, fragrances and insect repellents (Fig. 1.8). As there are not prescribed as pharmaceuticals, they may be overused and can be directly introduced into the environment through regular use, such as showering, spraying or disposal. Thus, due to the high use and continuous release, they can be classified as pseudo-persistent (Barceló and Petrovic, 2007).

Figure 1.8 Structure of personal care products: triclosan and propyl paraben

1.2.8 Pharmaceuticals

Pharmaceuticals may enter the environment as a result of human or veterinary use, industrial discharges and improper disposal. Pharmaceuticals (see Fig. 1.9) can be classified by their therapeutic use: analgesics, antibiotics or antimicrobials, anticonvulsants, antidiabetics, antihistamines, antidepressants, beta-blockers, cytostatics, lipid-regulators, stimulants and X-ray contrast media. They have been detected in water bodies throughout the world (Díaz-Cruz and Barceló, 2008; Karnjanapiboonwong et al., 2011; Kolpin et al., 2002) and may pose a risk to humans and environment because of their continuous release (Lindberg et al., 2007).

Figure 1.9 Structure of pharmaceutical compounds: ibuprofen and paracetamol

1.2.9 Nanoparticles

Nanotechnology is a new broad interdisciplinary area of research and industrial activity that has been growing rapidly worldwide for the past decade. The proposal of definition for nanoparticle (NP) given by the European Commission states that a nanomaterial is a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm (European Commission, 2011/696/EU).

Nanoparticles are used in areas such as electronic, biomedical, pharmaceutical, cosmetics, energy and in catalytic and material applications (Guzman et al., 2006) (see Fig 1.10). Due to their high use, nanoparticles are expected to pollute the environment (Benn and Westerhoff, 2008).

Figure 1.10 Structure of nanoparticles: Azido-functionalized polypyrrole-magnetite core-shell nanoparticles and fullerene C60

1.3 Emerging contaminants studied in this thesis

The compounds studied in this thesis are 60 and below they are grouped following the classification of Fig. 1.1.

- Biocides (10): resmethrin, bifenthrin, fenpropathrin, λ -cyhalothrin, permethrin, cyfluthrin, cypermethrin, τ -fluvalinate, esfenvalerate and deltamethrin.
- PBDEs (5): BDE-47, BDE-66, BDE-100, BDE-153 and BDE-183.
- PAHs (16): naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-c,d]pyrene, benzo[g,h,i]perylene and dibenzo[a,h]anthracene.
- Plasticizers (3): TCEP, TCPP and BPA
- Surfactan (1): nonylphenol
- Hormones (3): hexestrol, diethylstilbestrol and estrone
- Personal care products (5): methyl paraben, propyl paraben, methyl triclosan, triclosan and BP3.
- Pharmaceutical (16): clofibric acid, ibuprofen, caffeine, salicylic acid, allopurinol, paracetamol, gemfibrozil, fenoprofen, amitriptyline, metoprolol, naproxen, mefenamic acid, ketoprofen, carbamazepine, diclofenac and fenofibrate.
- NP (1): silver nanoparticle (Ag-NP) of different sizes.

Having in mind the variety of compounds that will be studied in this work, it is important to notice that to develop an analytical methodology some factors need to be taken into account:

- Physico-chemical properties of the target analytes.
- Characteristics of the matrix, from where the analytes have to be extracted.

1.4 Physico-chemical properties of emerging contaminants

Although all the compounds mentioned above can be considered ECs, they do not present similar properties and, therefore, they do not have a similar analytical or environmental behavior. The compounds studied have wide differences regarding its physico-chemical properties as can be seen in Table 1.1.

From the point of view of their presence in the environment, water solubility can be considered a key factor, as the main pathway to introduce these compounds is through diverse sources of water (freshwater, reused water and groundwater). However, from an analytical development perspective $\text{Log } K_{ow}$ and pK_a can also be regarded as main parameters.

$\text{Log } K_{ow}$ is the logarithm of a coefficient representing the ratio of the solubility of a compound in octanol (a non-polar solvent) and in water (a polar solvent). The higher K_{ow} is more non-polar is the compound, and it can be used as a relative indicator of the tendency of an organic compound to be adsorbed in a matrix such as soil, sludge or manure. Thus, the high K_{ow} values of compounds such as pyrethroids, BDEs, and PAHs (Table 1.1) will play an important role during the selection of the suitable organic solvent in order to extract them from the different matrices. Another key chemical property is the acid dissociation constant (pK_a). It can be seen in Table 1.1 that ibuprofen and paracetamol have a pK_a of 4.5 and 9.4, respectively, so the pH of the solvent plays a key role in the extraction of these compounds from the matrix.

Table 1.1 Physico-chemical properties of some representative ECs

Compounds	Class	Physico-chemical properties		
		Log K _{ow}	pK _a	Water solubility, mg mL ⁻¹ at 25 °C
Permethrin	Pyrethroid	6.5	-	5.5 x 10 ⁻⁶
Esfenvalerate	Pyrethroid	4	-	2 x 10 ⁻⁷
BDE-47	Flame retardant	8.8	-	1.5 x 10 ⁻⁵
BDE-100	Flame retardant	8.9	-	4 x 10 ⁻⁶
Chrysene	PAH	5.7	>15	1.9 x 10 ⁻⁶
Benzo[a]pyrene	PAH	6.1	>15	1.6 x 10 ⁻⁶
TCPP	Plasticizer	2.6	-	1.6
BPA	Plasticizer	3.4	9	12 x 10 ⁻²
Nonylphenol	Surfactant	3.8	10.7	6 x 10 ⁻³
Estrone	Hormone	3.1	10.3	3 x 10 ⁻²
Hexestrol	Hormone	4.8	9.9	1 x 10 ⁻⁵
Triclosan	PCP	4.8	8	2 x 10 ⁻³
Propyl paraben	PCP	2.7	8.4	3.9 x 10 ⁻⁴
Ibuprofen	Pharmaceutical	4.0	4.5	2.2 x 10 ⁻²
Paracetamol	Pharmaceutical	0.5	9.4	14 x 10 ⁻³

K_{ow}: octanol-water partition coefficient

pK_a: acid dissociation constant

Data compiled from previous studies: (Aznar et al., 2014a; Aznar et al., 2016; Bhandari et al., 2008; Gert-Jan de Maagd et al., 1998)

Nowadays, there is a demand to have multiresidue methods, which include a wide range of compounds pertaining to different families, to be able, in just one analysis, to detect numerous compounds, saving time, money, solvents and amount of sample. But as it has been pointed out above, it is difficult to include diverse families of ECs and try to handle them together in only one analysis with good recoveries and low limits of detection.

1.5 Main pathways of emerging contaminants into the environment

One of the main objectives of this thesis is to detect ECs in different environmental compartments. In Fig. 1.11 the main pathways of ECs into the environment according to EPA are shown.

Figure 1.11 Pathways of emerging contaminants into the environment

- 1) Use of ECs by humans and pets at urban areas and release to sewage systems.
- 2) Release of hospital wastes to sewage systems.
- 3) Leach of septic tanks.
- 4) Irrigation with treated effluent from WWTPs.
- 5) Soil amendment with biosolids from WWTPs.
- 6) Release from agriculture: drift, runoff and leaching.
- 7) Direct release to open waters via washing, bathing and swimming.
- 8) Discharge of industrial waste.
- 9) Disposal to landfills via domestic refuse, medical wastes and other hazardous wastes.

As it can be seen in Fig. 1.11, the best case scenario will be that the contaminated water reaches a wastewater treatment plant (WWTP), but unfortunately, nowadays most of the ECs are not completely degraded during depuration processes (Al Aukidy et al., 2012; Gros et al., 2010; Guerra et al., 2014; Stamatis and Konstantinou, 2013). Consequently, they are discharged in treated effluents resulting in the contamination of freshwater, groundwater and drinking water (Carmona et al., 2014; Dominguez-Morueco et al., 2014; Jurado et al., 2012; Petrie et al., 2015). Moreover, sewage sludge (Albero et al., 2014a; Radjenovic et al., 2009a; Ternes et al., 1999) from WWTPs and animal litter or manure (Hanselman et al., 2003; Kjaer et al., 2007; Topp et al., 2010)

from farms are used in agricultural soils to increase their content in organic matter and nutrients, but ECs may be also introduced into the agricultural soils (Chen et al., 2013; Vazquez-Roig et al., 2012). Finally, inappropriate disposal of these compounds is another source that cannot be neglected (Yu and Wu, 2012). Nonetheless, the input sources are well-known, but the fate and behavior of these emerging contaminants in the environment (Guzman et al., 2006; Kohler and Triebskorn, 2013; Lindberg et al., 2007) and their possible introduction into the food chain (plant uptake) (Goldstein et al., 2014; Wu et al., 2013) need further study.

In spite of the sizeable amount of emerging contaminants released and detected in the environment, environmental risk assessment and regulation are deeply missing. Thus, reliable and robust multiresidue methods are needed to assess their presence into different environmental matrices.

1.6 Environmental matrices studied

The environmental matrices of agricultural interest studied in the present thesis are: sludge, manure, soil, aquatic plants and water.

1.6.1 Sludge

Until now, in the Web of Science, there are 271 published papers regarding the presence of ECs in sludge (Albero et al., 2013; Albero et al., 2015a; Masia et al., 2015).

As shown in Fig. 1.12, sludge has received very little attention in comparison to other matrices. The low number of studies published could be explained due to the complexity of this matrix owing to their heterogeneity and high content of organic matter, containing lipids and other compounds that make difficult to extract the ECs. However, it is necessary to develop new methods to assess the occurrence of ECs in sludge because tones of sludge are produced daily and applied to amend soil. This may have the drawback of spreading some ECs into the environment if there is no control of their occurrence.

Figure 1.12 Evolution of papers published on ECs analysis. Source: WEB OF SCIENCE

The works published until now focus in the determination of few compounds in sludge, but the need to cover the detection of a wider range of ECs makes necessary the development of new multiresidue methods.

1.6.2 Manure

Manure is the matrix less studied, there are only 45 papers for manure (Albero et al., 2014b; Ho et al., 2012; Motoyama et al., 2011) regarding the presence of ECs (Fig. 1.12). Manure presents the same difficulties to work with as sludge. Moreover, it may pose the same potential risk as sludge, as it is used to improve physico-chemical properties of soil, spreading the ECs that may contain. Because it is one of the less studied matrices, compounds such as PAHs are new in this kind of matrix, and they may be considered as ECs, whereas they have been extensively studied in other matrices (e.g. soils).

1.6.3 Soil

Pesticides have been widely studied in soil, but on the contrary, this matrix has received very little attention regarding the presence of ECs, almost the same as sludge as it can

be seen in Fig. 1.12. Although soil is a cleaner matrix compared with manure or sludge, it is a heterogeneous matrix (containing organic matter, sand, silt and clay among others) with different ionic exchange and other physico-chemical properties from one soil to another. The ability that chemicals have to be adsorbed on soil, the use of poor quality waters for irrigation and the application of manure or sludge as organic amendments, make the soil matrix one of the most important reservoirs of contaminants and it needs to receive more attention (Arias-Estevez et al., 2008; Chefetz et al., 2008; Gonçalves and Alpendurada, 2005; Tejada and Gonzalez, 2008).

1.6.4 Plant material

After water, plant material is the second matrix that has received more attention as can be seen in Fig. 1.12. This fact may be explained by the concern of ECs being introduced in the food chain as some authors have highlighted (Cortes et al., 2013; Goldstein et al., 2014; Hurtado et al., 2016; Wu et al., 2015). Preliminary studies are pointing out that some edible plants may accumulate ECs into the roots, leaves or fruits, due to the presence of these pollutants in soil, water and organic amendments as mentioned previously.

It is not an easy matrix to work with being the main problems the high amount of fiber (cellulose), sugars and chlorophyll among others. Scarce information can be found regarding the presence of ECs in aquatic plants (no edible plants). Aquatic plants such as *Typha angustifolia* or *Lemna minor* are typically used in phytoremediation to eliminate organic and inorganic compounds. Recently some experiments in green filters and ponds have given evidence of the ability of aquatic plants for phytoremediation of some ECs (Bouldin et al., 2006; Olette et al., 2008), but it is not clear the exact role that these plants may play as, until now, no methodologies have been developed to assess the presence of ECs in aquatic plants. Once developed, they could be used to give a better understanding of the parameters involved in phytoremediation (including but not limited to microorganisms, degradation, hydrolysis, and photolysis).

1.6.5 Water

Water is the main pathway of ECs into the environment (Fig. 1.11), but it is also one of the cleanest matrices to work with. This makes water an attractive and interesting matrix for researchers to develop new methodologies as they will be simpler (no need for clean-up as the matrix usually is clean) and low limits of detection can be achieved (possibility of using a high amount of sample), detecting ECs in water at trace levels (sometimes levels lower than parts per trillion).

The total number of papers published in Web of Science regarding ECs is currently 5616, as it can be seen in Fig. 1.12. The progression of the total ECs works published is due to the high number of works regarding the occurrence of ECs in water (freshwater, reused water and groundwater) (Aguilar et al., 2014; Carmona et al., 2014; Esteban et al., 2014). However, when silver nanoparticles (Ag-NPs) is included in the search of ECs and water, the result is only 30 papers published.

1.7 Analysis of emerging contaminants in environmental samples

1.7.4 Sample pretreatment

In order to have an accurate determination it is important to have a representative sample. Hence, the pretreatment and the correct conservation of samples until analysis are essential. The main processes used are the following:

Solid matrices:

- Drying
- Lyophilization,
- Grinding
- Homogenization
- Freezing and refrigeration

Liquid matrices:

- Filtration

- pH adjustment
- Microorganism inhibition
- Freezing and refrigeration

1.7.5 Extraction methods

Extraction is defined as a process to separate a target analyte when it is mixed with other compounds. The sample is brought into contact with an acceptor phase (solvent) in which the substance of interest is soluble and the other substances present are insoluble or less soluble. The techniques can be classified regarding if the matrix is a solid or a liquid:

1.7.2.1 Liquid samples

Liquid-Liquid Extraction is one of the simplest technique to extract a compound from a liquid sample. It is based on the distribution of the target analyte in two immiscible liquids (partition coefficient). Various factors involved during the process of extraction being the affinity of the analytes to the extraction solvent one of them main. Fig. 1.13 shows the steps involved during the extraction using Liquid-Liquid Extraction (LLE).

Figure 1.13 Scheme of the steps involved during Liquid-Liquid Extraction

- 1) A solvent with affinity to the target compounds and immiscible with the sample matrix is selected.

- 2) It is shaken/mixed (solvent-sample) to get the target compounds in contact with the solvent and assist the diffusion from the sample to the solvent.
- 3) Due to different density the sample and solvent are separated and the target compounds can be collected.

The LLE is used in extraction methods such as:

- Dispersive Liquid-Liquid Microextraction (DLLME), this method is based on LLE, where an extraction and disperser solvent are rapidly injected into the aqueous sample by syringe as it is shown in Fig. 1.14. The mixture is shaken and a cloudy solution (water/disperser solvent/extraction solvent) is formed in the tube. After centrifugation, the fine particles of extraction solvent are sedimented in the bottom of the conical tube. The resultant sedimented phase is taken with a microsyringe and can be analyzed (Zang et al., 2009).

Figure 1.14 *Dispersive Liquid-Liquid Microextraction*

This technique has been recently used in aqueous samples, such as tap and creek water to determine pharmaceuticals, hormones and PCPs (Yao et al., 2011) and in herbal infusions to analyze pharmaceuticals and hormones (Albero et al., 2015b)

- Supported Liquid Extraction (SLE) is analogous to traditional liquid-liquid extraction (LLE) and employs the same water immiscible solvent systems for analyte extraction. Instead of shaking the two immiscible phases together, the aqueous sample is immobilized on an inert support in SLE (Fig. 1.15), and the organic phase flows through the support, eliminating problems such as emulsion formation and low analyte recoveries.

Figure 1.15 *Supported Liquid Extraction*

The SLE method, although based on LLE, is usually combined with other techniques such as Solid Phase Extraction or Magnetic Solid Phase Extraction. The SLE has been used to detect ECs such as pharmaceutical and personal care products in water (Díaz-Alvarez et al., 2014; Moliner-Martínez et al., 2011) and in this Thesis has been used in Chapter 2.

- Solid Phase Extraction (SPE) the extraction takes place when, after extraction in aqueous phase, this phase passes through a cartridge where the analytes are retained. An elution with a solvent with affinity to the analytes is needed to extract them from the cartridge (Fig. 1.16). Normally four steps are needed: conditioning of the cartridge, loading of the sample, washing of the interferences and finally elution of the target compounds. This technique can

be also used as an extra cleanup for solid samples when the extract is too dirty.

Figure 1.16 Solid Phase Extraction

The SPE method has been extensively used to detect ECs in water, because a high amount of sample can be passed through a cartridge concentrating the analytes and eluting them subsequently with a small solvent volume. This leads to have a high factor of preconcentration, allowing low limits of detection and quantification (around parts per trillion) (Matamoros et al., 2010).

New sorbents, such as Molecularly Imprinted Polymers (MIPs) are tailor-made synthetic materials obtained by copolymerizing a monomer with a cross-linker in the presence of a template molecule. Even so, MIPs methods still need to deal with the drawback of not being able to compete with other techniques to produce the demanded multiresidue methods. However, because of the selectivity and low solvent use (green chemistry), MIPs are already used as routine technique to detect, for example, antibiotics in baby

food and parabens in soil (Díaz-Alvarez et al., 2009, Díaz-Alvarez et al., 2016).

Magnetic Solid Phase Extraction (mSPE) is one of the newest techniques developed. It is based on using magnetic nanoparticles (MNPs) and it has been applied to the separation and pre-concentration of pollutants in environmental matrices, mainly in water samples (Pérez et al., 2014a). MNPs' surface is modified in order to protect and maintain its stability but, in many cases, this modification can be used for further functionalization in order to promote the extraction capacity and specificity of the MNPs. For the extraction of contaminants in liquid matrices, mSPE is an interesting technique because MNPs can be attracted to a magnetic field. Thus, the SPE sorbent is directly added to the solution and, after the adsorption of the analytes onto the magnetic sorbent, the MNPs are isolated by placing a magnet on the wall of the flask and discarding the solution, as can be seen in Fig. 1.17. Finally, the target compounds can be eluted from the sorbent with a low quantity of an adequate solvent to be analyzed (Pérez et al., 2014b).

Figure 1.17 Magnetic Solid Phase Extraction

The mSPE method uses low solvent volumes and MNPs can be also reused reducing analyses cost. The mSPE has been used, for example, to detect hormones in water (Pérez et al., 2014b).

- Solid Phase Microextraction (SPME) is a not exhaustive extraction technique that involves the use of a fiber coated with an extracting phase, which can be a liquid or a solid, to extract the target compounds from different kinds of matrices (liquid or gas) (Arthur and Pawliszyn, 1990). Then analytes are desorbed in a subsequent step into a liquid or gas phase (Fig. 1.18).

Figure 1.18 Solid Phase Microextraction

The SPME method reduces the amount of solvents used and concentrates the analytes in a small volume. The SPME has been used to extract the soil/water suspension by direct immersion of the fiber (DI-SPME) or by headspace extraction (HS-SPME). Generally, HS-SPME reduces the matrix effect because the fiber is not in direct contact with the matrix and only volatiles or semi-volatiles compounds are released into the headspace. It has been used to detect for example flame retardants in sediments and soils (Salgado-Petinal et al., 2006b).

1.7.2.2 Solid samples

Solid Liquid Extraction, is one of the simplest technique to extract a compound from a solid sample. It is based on the contact of certain amount of sample with an appropriate solvent to extract the target compounds from a solid matrix (Turiel and Martín-Esteban, 2008), Figure 1.19 shows the steps involved during Solid Liquid Extraction.

Figure 1.19 Scheme of the steps involved during Solid-Liquid Extraction

- 1) Solvent penetrates inside the pores of the solid matrix.
- 2) Desorption of the analytes bounded to the matrix active sites.
- 3) Analytes diffuse through the matrix.
- 4) Analytes are dissolved in the extracting solvent.
- 5) Analytes leave the pores of the matrix.
- 6) Solvent is collected along with the analytes.

Extraction methods, based on Solid Liquid Extraction are:

- Shaking extraction consists in mixing (manually or automatically) (Fig. 1.20) the sample in presence of an appropriated solvent for a certain period of time, where little energy is provided to the system. Its use is recommended when the compounds that need to be extracted are highly soluble in the acceptor liquid phase.

Figure 1.20 *Shaking by hand*

This method can be used both for solid and liquid samples, and although is mostly used during the cleanup step, it has also been used to determine pharmaceutical compounds in sediments (Minten et al., 2011) and hormones in sludge (Gomes et al., 2004) among others.

- Soxhlet extraction is a traditional technique that consists in an exhaustive extraction of a component from a solid sample with a high volume of solvent (300-500 mL) and the use of high temperature (Fig. 1.21). Thus, it will be used in samples where the target compounds present a medium-low volatilization and are thermally stable.

Figure 1.21 *Traditional Soxhlet extractor*

Despite the use of a high volume of solvents, it is still being used in official methods to extract for example pesticides from soils or fatty acids from meat.

It has been mostly applied all over the years to extract pesticides from different matrices and although has been used to extract ECs, as surfactants from sludge and sediments (Martínez-Carballo et al., 2007) or flame retardants from soil (Hassanin et al., 2004), this technique has been replaced for more ecofriendly techniques that consume less amount of solvents and are faster.

- Pressurized Liquid Extraction (PLE), also called Accelerated Solvent Extraction (ASE), combines elevated temperatures and pressures (Richter et al., 1996) to keep the solvent in a liquid state to extract the target compounds, reducing solvent volume and time required (Fig. 1.22). However, it cannot be applied to thermolabile compounds as they may suffer degradation if elevated temperatures are used. This technique works better if it is applied to samples with small particle size and low content of water (dry solid samples).

Figure 1.22 Pressurized Liquid Extraction

PLE has been recently applied to extract hormones from sludge (Fernández et al., 2009) and pharmaceuticals from soil (Duran-Alvarez et al., 2009) although using a relatively high amount of sample (10 grams).

- Supercritical Fluid Extraction (SFE), initially used to extract scents from plants, i.e. coffee among others. This technique uses supercritical fluids with densities similar to liquids but with lower viscosity which makes the extraction faster than with organic solvents (Ahmed, 2001), being carbon dioxide the fluid most often used (Fig. 1.23). Samples must be dried, as in the case of PLE, and extraction of non-polar target compounds is preferable.

Figure 1.23 *Supercritical Fluid Extraction*

The SFE technique has not been intensively used because the equipment is expensive. Even so, it has been used to determine BDEs and PAHs in biota samples with good results (Fidalgo-Used et al., 2007).

- Ultrasound Assisted Extraction (UAE) uses ultrasonic energy to cause an effect known as cavitation, which generates numerous tiny bubbles in liquid media (Fig 1.24). The mechanical effect of ultrasound induces a greater penetration of solvent into solid matrices and mechanical erosion of solids, including particle rupture, which improves mass transfer, leading to enhanced sample-extraction efficiency (Albero et al., 2015a).

Figure 1.24 *Ultrasound Assisted Extraction*

It is one of the techniques most used to extract ECs from environmental samples (Albero et al., 2015a) as it is cheap and fast. Thus it has been widely applied in complex matrices such as sludge and sediment to extract flame retardants and hormones, respectively (Salgado-Petinal et al., 2006a; Sánchez-Brunete et al., 2011).

- Microwave Assisted Extraction (MAE) uses microwave energy to heat solvents and increase the pressure in contact with a sample in order to partition analytes from the sample matrix into the solvent (Fig. 1.25). The ability to rapidly heat the sample solvent mixture is inherent to MAE and the main advantage of this technique (Eskilsson and Bjorklund, 2000), requiring water in the system, a solvent with dipolar moment or the use of Weflon to transfer the heat.

Figure 1.25 *Microwave Assisted Extraction*

The MAE technique although it is not selective during extraction process and can affect thermolabile compounds, it is fast and with low consumption of solvent. MAE has been used to extract flame retardants and hormones from sediments (Liu et al., 2004; Yusa et al., 2006). Recently, MAE has been used to analyze 90 ECs in sludge and different waters (Petrie et al., 2016).

- Matrix Solid Phase Dispersion (MSPD) involves the direct mechanical blending of the sample with a solid sorbent, such as alkyl-bonded silica, alumina, Florisil and sand among others, in a mortar (Fig. 1.26). The added abrasive sorbent promotes the disruption of the gross architecture of the sample, while sample constituents will disperse into the solid phase, causing a complete disruption of the sample and its dispersion over the surface. When blending or mixing is complete, the homogenized mixture is packed into an empty column or cartridge and eluted with a solvent (Tadeo et al., 2010a).

Figure 1.26 Matrix Solid Phase Dispersion

The MSPD technique is normally used in samples such as sludge, sediments, and soils with a certain degree of humidity. The main drawback could be the human factor during the mechanical blending as it can differ from one operator to another or from one day to another. Nevertheless, it is one of the techniques most used because the enormous possibilities of adaptation (adsorbents and solvents) and because it is fast and does not require expensive instrumentation. For example, it has been used to extract different biocides from sludge and soil (Lozowicka et al., 2012; Sánchez-Brunete et al., 2008), but in this Thesis it has been adapted to be used in aquatic plants, Chapter 5.

- Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS), this methodology consists of two steps, extraction of the analytes from the matrix and dispersive Solid Phase Extraction (dSPE) clean-up (Fig. 1.27). The advantages of the QuEChERS method include rapidity, simplicity and robustness, low solvent consumption, practically no glassware needs and low cost (Albero et al., 2014b). These characteristics and advantages make QuEChERS an interesting procedure to use.

Figure 1.27 *Quick, Easy, Cheap, Effective, Rugged and Safe*

QuEChERS method was developed for the multiresidue determination of pesticides in fruits and vegetables (Anastassiades et al., 2003). Since then, this technique has been applied to many matrices and diverse target compounds. For example Alberro and coworkers (2014) developed a method to detect hormones in sludge and poultry manure. Also QuEChERS has been used to detect ibuprofen and PCP in soil and fish tissue, respectively (Braganca et al., 2012; Norli et al., 2011). In this Thesis QuEChERS method has been used in Chapter 3 to extract ECs from poultry manure.

1.7.3 Clean-up

The clean-up step depends on the characteristics of the matrix. The extraction step is usually a nonselective procedure, which means that other compounds present in the matrix are coextracted with the target compounds and may interfere and hamper the determination and quantification of the target compounds. Thus, some extracts will need a clean-up step before determination.

The SPE technique is often used and diverse sorbents can be employed depending on the structure and polarity of the target compounds, being alumina, activated charcoal, Florisil, C18, C8, PSA, Chlorofiltr and MIPs the solid phases most used.

Particularly, activated charcoal and PSA will not be used in the present work, because planar compounds such as PAHs are retained in the activated charcoal (with low release) and acidic compounds (such as pharmaceuticals) get bounded to the amine group of the PSA making difficult to reach satisfactory recoveries.

1.7.4 Determination of emerging contaminants

After all the above sample preparation steps, target analytes are analyzed mainly using chromatographic techniques, where analytes are first separated on a stationary phase with the aid of a mobile phase, and then they are subsequently detected with a suited detector. Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) are the chromatographic techniques commonly used for the separation of ECs. However, GC or HPLC cannot be used for the analysis of inorganic nanomaterials and, for the detection and quantification of these nanoparticles Inductively Coupled Plasma (ICP) combined with mass spectrometry is becoming the most promising technique.

- High Performance Liquid Chromatography (HPLC) uses pressurized liquid solvents, e.g. water, acetonitrile and/or methanol referred to as a "mobile phase" that transport the sample mixture through a column filled with a solid adsorbent. Each component in the sample interacts with the adsorbent material in a different manner, leading to the separation of the components as they flow out the column. Because the separation in HPLC is mainly

driven by the polarity, compounds such as antibiotics with affinity for water and thermally labile can be just separated with HPLC, but cannot be determined by GC which is the technique used in the present work.

- Gas chromatography (GC) is a technique where the analytes can be vaporized without decomposition at the working temperature (volatile compounds). In GC, the mobile phase is an inert carrier gas (helium or nitrogen) and the stationary phase is a layer of polymer on an inert solid support (capillary column). The volatilized compounds being analyzed interact with the stationary phase and this causes each compound to separate eluting at different time, known as the retention time of the compound.

Most of the compounds studied possess functional groups with active hydrogen atoms (amines, amides, hydroxyl or phenolic groups) and a chemical derivatization of these groups is needed before their analysis by GC to increase their thermal stability and volatility. Silylation with different reagents (BSTFA and MTBSTFA) that lead to the formation of trimethylsilyl (TMS) or t-butyltrimethylsilyl (tBMS) derivatives (Fig 1.28) is an effective approach for their determination.

Figure 1.28 Silylation reactions with BSTFA and MTBSTFA derivatization agents

- Inductively Coupled Plasma (ICP) is a plasma that is energized (ionized) by inductively heating the gas with an electromagnetic coil (Okumura, 2010) and contains a sufficient concentration of ions and electrons to make the gas (argon) electrically conductive. The working temperature of the plasma is very high, of the order of 1000 °C. It is mainly used to elementary analysis in different matrices.

After separation, it is necessary to detect and quantify the analytes. Nowadays, there are several detection techniques available, but for its sensibility, selectivity and reliability mass spectrometry is the technique most often used.

- Mass spectrometry (MS) is an analytical technique that quantifies the content and identifies the type of chemical present in a sample. It works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios.

The ions are subsequently detected and the results are displayed as spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio. The compounds in a sample can be identified by a characteristic fragmentation pattern.

- Tandem Mass Spectrometry (MS/MS) uses the ions separated in the first mass spectrometer to obtain fragments that are detected in a second mass spectrometer. Thus, with MS/MS higher sensitivity can be achieved, due to the decrease of interferences prior to ion measurement, and detection limits can be improved in comparison with MS due to the higher selectivity of MS/MS.

1.7.5 Analytical quality control and validation procedures

Method Validation is defined as: “*establishing documented evidence that provides a high degree of assurance that a specific method, and the ancillary instruments included in the method, will consistently yield results that accurately reflect the quality characteristics of the product tested*” (Ghulam, 2004).

The key criteria defined by the International Conference on Harmonization (ICH) guidelines (ICH, 1997) for evaluating analytical methods are: selectivity/specificity, accuracy, precision, linearity, range, limit of detection, limit of quantitation, ruggedness, and robustness, where these terms are defined as follow (Araujo, 2009):

- Selectivity refers to the extent to which a method can determine a particular analyte in a complex mixture without interference from other components in the mixture, and specificity, which is considered to be the ultimate in selectivity; it means that no interferences are supposed to occur.
- Accuracy is the degree of agreement between the experimental value, obtained by replicate measurements, and the accepted reference value. It has been pointed out that the accuracy is the most crucial aspect that any analytical method should address.
- Precision is defined as the closeness of agreement between quantity values obtained by replicate measurements of a quantity under specified conditions.
- Linearity, in the context of the previously described analysis system consisting of the basic elements input→converter→output, is the assumption that there is a straight line relationship between the input (x) and output (y) variables that can be written mathematically by the expression $y = f(x)$ if the straight line crosses through the origin or by the expression $y = f(x) + b$ if the straight line does not cross through the origin. It is common practice to check the linearity of a calibration curve by inspection of the correlation coefficient (r) . A correlation coefficient close to unity ($r = 1$) is considered sufficient evidence to conclude that the experimenter has a perfect linear calibration.
- Range of an analytical procedure can be defined as the interval between the upper and lower concentration of analyte for which suitable precision, accuracy and linearity have been demonstrated.
- The limit of detection (LOD) is commonly defined as the lowest amount of analyte in a sample that can be reliably detected but not necessarily quantitated by a particular analytical method.

- The limit of quantification (LOQ) is defined as the lowest concentration or amount of analyte that can be determined with an acceptable level of precision and accuracy.
- Ruggedness evaluates the constancy of the results when external factors such as analyst, instruments, laboratories, reagents, days are varied deliberately.
- Robustness evaluates the constancy of the results when internal factors (no external factors as in ruggedness) such as flow rate, column temperature, injection volume, mobile phase composition or any other variable inherent to the method of analysis are varied deliberately.

Moreover, considering how demand for quality assurance (QA) has grown in analytical laboratories (Olivares and Lopes, 2012) the International Standard Organization ISO/IEC 17025 for QA and validation (International Standard Organization, 2005) and the document from the European Commission guidance on analytical QA and validation procedures for pesticides residues (SANCO-12571, 2013) were used to provide a harmonization and ensure:

- Quality assurance and quality control system (sampling, transport, traceability and storage)
- Quality and comparability of the analytical results
- Acceptable accuracy
- False positive and false negative avoidance

1.8 Analytical techniques selected in this thesis

A summary of the different works presented in this thesis is shown in Table 1.2, where it is indicated the matrix, the compounds and the sample treatment that has been selected for each study.

Table 1.2 Summary of the matrices, compounds and sample preparation and determination used in this thesis

	Matrix	Compounds	Pretreatment	Extraction	Clean-up	Determination
Chapter II	Sewage Sludge	Pharmaceuticals	Freezing Grinding	SLE	None	GC-MS/MS
Chapter III	Manure	Pesticides PAHs BDEs	Lyophilization	QuEChERS	MgSO ₄ PSA C18	GC-MS
Chapter IV	Soil	Pharmaceuticals Pesticides	Drying Sieving Freezing	UAE	None	GC-MS
Chapter V	Aquatic plants	Pesticides BDEs Plasticizers Surfactants PCPs Hormones Pharmaceuticals	Grinding Freezing	UA-MSPD	C18	GC-MS
Chapter VI	Water	Silver-NP	None	None	None	ICP-MS

CHAPTER TWO - PHARMACEUTICAL COMPOUNDS IN SLUDGE

PHARMACEUTICAL COMPOUNDS IN SLUDGE

As it has been shown in Fig. 1.2, one of the main paths to introduce ECs into the environment is through WWTPs. There is a growing concern on the increasing amounts of residues produced by WWTPs. It has been estimated that Spain produces 1200000 tons of sludge (dry weigh) per year. Different approaches to dispose this residue are being conducted in Spain:

- Application in agricultural soils (after stabilization of the residue; compost) (76%)
- Landfill disposal (15%)
- Incineration (7%), possible use in cogeneration
- Others (2%)

The high use of sludge in agriculture can be explained because fields are nearby to the WWTPs (less cost of transport) and because Spanish soils, on average, present a low content on organic matter. Moreover, sludge contains nitrogen, phosphorus, potassium, calcium, magnesium and micronutrients necessary for the correct plant development. But on the other hand, pathogens and heavy metals as cadmium, mercury and zinc can also be incorporated. For these reasons, currently there is a legislation to control the application of sludge in agricultural soil limiting the application depending on the compounds above described.

Because of the presence of undesirable compounds, the concern of the safety use of sludge in agricultural soils should be considered. Consequently, it is necessary to develop analytical methods for the determination of ECs in sludge in order to control the amount of these compounds reaching the environment.

DETERMINATION OF SELECTED PHARMACEUTICAL COMPOUNDS IN BIOSOLIDS BY SUPPORTED LIQUID EXTRACTION AND GAS CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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Abstract

In this work, an analytical method was developed for the determination of pharmaceutical drugs in biosolids. Samples were extracted with an acidic mixture of water and acetone (1:2, v/v) and supported liquid extraction was used for the clean-up of extracts, eluting with ethyl acetate:methanol (90:10, v/v). The compounds were determined by gas chromatography–tandem mass spectrometry using matrix-match calibration after silylation to form their *t*-butyldimethylsilyl derivatives. This method presents various advantages, such as a fairly simple operation for the analysis of complex matrices, the use of inexpensive glassware and low solvent volumes. Satisfactory mean recoveries were obtained with the developed method ranging from 70 to 120% with relative standard deviations (RSDs) $\leq 13\%$, and limits of detection between 0.5 and 3.6 ng g⁻¹. The method was then successfully applied to biosolids samples collected in Madrid and Catalonia (Spain). Eleven of the sixteen target compounds were detected in the studied samples, at levels up to 1.1 µg g⁻¹ (salicylic acid). Ibuprofen, caffeine, paracetamol and fenofibrate were detected in all of the samples analyzed.

Keywords: Pharmaceuticals; Sludge; Biosolids; Gas chromatography-tandem mass spectrometry; Supported liquid extraction

2.1 Introduction

Pharmaceutical substances are a source of increasing environmental concern and together with endocrine disrupting chemicals have been classified as the most frequently detected organic contaminants in the environment (Samaras et al., 2011). The determination of pharmaceuticals in the environment has focused on the aquatic compartment because it has been reported that wastewater treatment plants (WWTPs), especially those with conventional technology, cannot accomplish the removal of these compounds before the effluents are discharged to surface waters (Gros et al., 2010). Microbial degradation and the high tendency of some pharmaceuticals to remain adsorbed onto activated sludge are mechanisms that may explain the removal of pharmaceuticals during wastewater treatment (Saleh et al., 2011). Biosolids are the nutrient-rich organic materials resulting from the treatment of sewage sludge. When treated and processed, sewage sludge becomes biosolids, which can be safely recycled and applied as fertilizer to sustainably improve and maintain productive soils and stimulate plant growth. In the European Union, around 4 million metric tons (dry weight) of biosolids are annually applied to agricultural land (Macherius et al., 2012). Therefore, it is important to develop analytical methods for the detection of pharmaceuticals at trace levels to study their occurrence, behavior and fate in this complex matrix. The analysis of pharmaceutical drugs in sludge or biosolids has been focused on a limited set of compounds, mainly antibiotics (Chenxi et al., 2008; Gobel et al., 2005; Nieto et al., 2010) or non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. naproxen, diclofenac, ketoprofen, and ibuprofen) (Dobor et al., 2010; Saleh et al., 2011), however recent studies have included many more compounds belonging to several therapeutic classes that exhibit very different physico-chemical properties (Jelic et al., 2009; Peysson and Vulliet, 2013; Radjenovic et al., 2009a).

However, the determination of these compounds in solid environmental samples is still scarcely documented, due primarily to a lack of appropriate analytical methods. Several methodologies have been developed for determination of pharmaceuticals in sewage sludge using pressurized liquid extraction (Ding et al., 2011; Jelic et al., 2009; Nieto et al., 2007; Radjenovic et al., 2009a; Saleh et al., 2011), ultrasound assisted extraction

(Chenxi et al., 2008; Martín et al., 2010; Samaras et al., 2011), microwave assisted extraction (Dobor et al., 2010) and QuEChERS (Peysson and Vulliet, 2013).

Most published works regarding the analysis of pharmaceutical compounds in the environment were initially performed using mass spectrometry coupled to gas chromatography (GC-MS) (Antonic and Heath, 2007; Bossio et al., 2008; Dobor et al., 2010; Pintado-Herrera et al., 2013; Sagrista et al., 2010) or liquid chromatography (LC-MS) (Minten et al., 2011). Nowadays, the majority of the analytical methods for the separation and detection of pharmaceuticals uses liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Baker and Kasprzyk-Hordern, 2011; Löffler and Ternes, 2003; Radjenovic et al., 2009a; Sabourin et al., 2009; Vazquez-Roig et al., 2011; Vazquez-Roig et al., 2012). Nevertheless, GC-MS/MS is an interesting alternative to LC due to its high resolution, lower operation costs and reduced solvent waste. Moreover, there is a clear impact on electrospray ionization of these compounds when working with complex matrices such as biosolids producing either ion enhancement or suppression that hinders the quantification of target compounds (Samaras et al., 2011). GC-MS/MS was used in the determination of pharmaceuticals in sewage water with very low detection limits (Verenitch et al., 2006), however, its performance has not been evaluated for biosolid samples. Most pharmaceuticals possess functional groups with active hydrogen atoms (amines, amides, and hydroxyl or phenolic groups) and a chemical derivatization of these groups, to reduce their polarity while increasing their thermal stability and volatility, is needed before their analysis by GC. Silylation with different reagents that lead to the formation of trimethylsilyl (TMS) or *t*-butyldimethylsilyl (*t*BDMS) derivatives is an effective approach for their determination.

Supported liquid extraction (SLE) is a relatively new technology that has been developed to replace classical liquid-liquid extraction (LLE). SLE involves the immobilization of aqueous samples over a solid inert phase, generally high purity diatomaceous earth that has a high capacity to retain water. The subsequent extraction is performed with any solvent that is immiscible with water. This technique presents several advantages over LLE, such as the reduced sample and solvent volumes required

and no formation of emulsions (Edel et al., 2013). Since emulsions are not an issue, solvent mixtures can be used regardless of density (Liu et al., 2013). One advantage of SLE over solid-phase extraction (SPE) is that no preconditioning of the column is required; hence, the sample is directly loaded into the solid support, which shortens the whole extraction procedure. Unlike SPE, the whole sample is absorbed onto the solid support and there is no flow-through. This technique has been mainly applied with biological fluids such as plasma (Edel et al., 2013; Wingfors et al., 2005), although it has also been used in the determination of chemical warfare agents in water (Kanaujia et al., 2009), polyphenols in wine (Nave et al., 2007) and pesticides in honey (Pirard et al., 2007).

The aim of this work was to develop a rapid and sensitive multiresidue method based on SLE followed by GC-MS/MS for determination of 16 frequently used pharmaceuticals in biosolid samples. GC-MS/MS was selected to analyze a high number of compounds of different classes in a single run with a high selectivity. The selected pharmaceuticals present wide range of physico-chemical properties and belong to different therapeutic classes: NSAIDs, antiepileptics, antidepressants, lipid regulators, nervous stimulants and β -blockers. The simultaneous determination of pharmaceutical drugs with different physico-chemical characteristics requires a compromise in the selection of experimental conditions for all the compounds studied. A significant advantage of the described procedure is the reduction in the consumption of organic solvents and time versus previously published methodologies. To the best of our knowledge; this is the first time that SLE has been applied in the multiresidue determination of pharmaceutical drugs in biosolids.

2.2 Materials and Methods

2.2.1 Sewage sludge collection

Pelletized biosolids from Madrid (Spain) were used in the development and optimization of the analytical method. Samples were put in amber glass jars (250-500 g) and stored at -20 °C until processing. The developed method was applied to the analysis of biosolids from WWTPs located in Madrid and Catalonia (Spain).

2.2.2 Reagents and standards

Methanol, ethyl acetate, acetone and acetonitrile (ACN), residue analysis grade, were purchased from Scharlab (Barcelona, Spain). A Milli-Q water purification system from Millipore (Bedford, MA, USA) was used to provide ultrapure water in this study. Formic acid was purchased from Sigma-Aldrich (St Louis, MO, USA). ISOLUTE SLE+ 1 mL supported liquid extraction columns were purchased from Biotage (Uppsala, Sweden).

N-*t*-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA, purity > 97%), a mixture of MTBSTFA and *t*-butyldimethylchlorosilane (*t*BDMCS) (99:1, v/v), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, purity > 99%) and the mixture of BSTFA and trimethylchlorosilane (TMCS) (99:1, v/v), used as silylation reagents, were purchased from Aldrich (Steinheim, Germany). Pyridine was purchased from Panreac (Barcelona, Spain).

The standards of clofibric acid, ibuprofen, caffeine, salicylic acid, paracetamol, allopurinol, gemfibrozil, fenoprofen, amitriptyline, metoprolol, naproxen, mefenamic acid, ketoprofen, carbamazepine, diclofenac and fenofibrate were of analytical grade (purity >99%) and purchased from Sigma-Aldrich (St Louis, MO, USA).

Individual stock standard solutions were prepared in methanol at a concentration of 5 $\mu\text{g mL}^{-1}$ and stored in amber vials at -18 °C in the dark. A working mixture solution containing 1000 ng mL^{-1} of all compounds was prepared in ACN weekly by dilution of the stock solution.

2.2.3 Sample preparation

Pelletized biosolids (0.5 g), previously ground, were weighed into a 15 mL screw-cap glass tube and for the recovery assays, aliquots of the working mixture solution were added to reach final concentrations of 100, 50 and 25 ng g^{-1} allowing 24 h before extraction at 4 °C to reach equilibrium. After the addition of 2 mL acetone 1% aqueous formic acid (2:1, v/v), the mixture was stirred intensively by magnetic agitation for 60 min. Then, the extract was centrifuged at 4000 rpm for 4 min. A 500 μL aliquot of the

supernatant was transferred to a glass tube and diluted (1:1, v/v) with 1% aqueous formic acid. The diluted sample was loaded on the SLE column and left 5 min for the sample being completely absorbed. Then, it was placed in a multiport vacuum manifold (SupelcoVisiprep, Madrid, Spain) and the analytes were eluted with 2×5 mL of ethyl acetate:methanol (90:10, v/v), applying vacuum for 5 min to complete elution. The extract was evaporated to dryness using a Genevac EZ-2 evaporator (NET Interlab, S.A.L., Spain) and the analytes reconstituted in 100 μ L acetonitrile and pipetted into a 2 mL vial with a micro insert. The *t*BDMS derivatives were prepared by the addition of 50 μ L of MTBSTFA:*t*BDMCS (99:1, v/v), then the vial was capped and placed in an oven at 70 °C for 1 h. After the derivatization step, the vial was left to cool down before performing the chromatographic analysis.

2.2.4 Gas chromatography-tandem mass spectrometry analysis

GC–MS/MS analysis was performed with an Agilent 7890A gas chromatograph equipped with a multimode inlet (MMI) and coupled to a triple quadrupole mass spectrometer, Model 7000 (Waldbronn, Germany). The MMI was operated in solvent-vent mode with gas liner with deactivated glass wool. During the 2 μ L injection, at a rate of 40 μ L min⁻¹, the split vent was open for 0.18 min with an inlet pressure of 5 psi and a flow rate of 100 mL min⁻¹. Once the entire sample has been injected, the inlet was switched to splitless mode for analyte transfer. After 2.68 min, the purge valve was activated at a 60 mL min⁻¹ flow rate. The MMI program started at 60 °C kept for 0.18 min after injection, then ramped to 325 °C at 600 °C min⁻¹, held 5 min, and finally decreased to the initial temperature cooling with compressed air. The chromatographic analysis was carried out using a fused silica capillary column ZB-5MS, 5% phenyl polysiloxane as nonpolar stationary phase (30 m $\times$ 0.25 mm i.d. and 0.25 μ m film thickness) from Phenomenex (Torrance, CA). Helium (purity 99.995%) was used as carrier gas at a constant flow rate of 1 mL min⁻¹. The oven was programmed to start at 60 °C (held for 3 min) and reach 300 °C at 20 °C min⁻¹ where it was maintained 1.5 min. The total analysis time was 16.5 min and the run was carried out with a solvent delay of 10 min.

Table 2.1 MS/MS parameters for the analyses of target pharmaceuticals in biosolids

Compound	t _R (min)	Q ^a	q ^b
clofibric acid	10.19	143 > 69 (5)	271 > 143 (5)
ibuprofen	10.34	263 > 75 (15)	263 > 161 (20)
caffeine	10.54	194 > 55 (30)	194 > 109 (15)
salicylic acid	10.75	309 > 73 (20)	309 > 195 (15)
allopurinol	11.16	307 > 193 (25)	307 > 166 (35)
paracetamol	11.29	322 > 248 (30)	322 > 150 (35)
gemfibrozil	11.64	243 > 83 (10)	243 > 73 (30)
fenoprofen	11.83	299 > 75 (20)	300 > 75 (20)
amitriptyline	11.91	202 > 200 (40)	202 > 201 (25)
metoprolol	12.06	223 > 72 (10)	324 > 239 (10)
naproxen	12.16	287 > 75 (25)	287 > 185 (25)
mefenamic acid	12.59	298 > 224 (20)	224 > 180 (35)
ketoprofen	12.61	311 > 75 (35)	311 > 295 (10)
carbamazepine	12.84	193 > 165 (30)	193 > 167 (25)
diclofenac	12.97	352 > 75 (25)	214 > 179 (25)
fenofibrate	13.05	139 > 111 (15)	273 > 139 (15)

^aQ: quantifier transition (Collision energy, eV).^bq: qualifier transition (Collision energy, eV).

The mass spectra and the retention time of each of the analytes were acquired operating in full scan mode with a mass range from 50 to 540 m/z, scan time of 300 ms and an ion source and transfer line temperatures of 230 and 280 °C, respectively. The mass spectrometer was operated in electron impact (EI) ionization mode at 70 eV. The electron multiplier was set with a gain factor of 6. Precursor ions for each analyte were selected taking into consideration a high ion m/z-value and abundance. The product ion spectra were obtained by the dissociation of the precursor ions at collision energies ranging from 5 to 50 eV. For quantitative analysis, multiple reaction monitoring (MRM) mode was employed with one quantifier and one qualifier transitions for each target compound. The dwell time in the different time segments was kept between 10 and 30 ms to achieve chromatographic peaks that gave good quantitative data. Analytes were confirmed by their retention time and the identification of target and qualifier transitions. Retention times must be within ± 0.2 min of the expected time and qualifier-to-target ratios within a 20% range for positive confirmation. Table 2.1 summarizes the

retention times and the transitions with their optimal collision energy used for quantification and confirmation of each analyte in MRM mode.

2.3 Results and discussion

2.3.1 Sample preparation

Pharmaceuticals selected in this study present different physicochemical properties but most are acidic compounds, thus the extraction from biosolids (0.5 g) was carried out with 2 mL of acetone- 0.5% aqueous formic acid (2:1, v/v). In a preliminary study, six pharmaceuticals (ibuprofen, salicylic acid, paracetamol, naproxen, ketoprofen and diclofenac) were extracted from samples spiked at 200 ng g⁻¹ with the assistance of sonication (1 h) or magnetic stirring (1 h). Sonication produced lower extraction yields than those obtained with magnetic stirring (e.g. salicylic acid: 55% and 73%, respectively). Therefore, the extraction of pharmaceuticals from biosolids was performed with the assistance of magnetic stirring.

An aliquot of the centrifuged extract (0.5 mL) was diluted (1:1, v/v) with 1% aqueous formic acid before loading the SLE column to promote an even flow of the sample through the column, and analytes were eluted with 2 × 2.5 mL of ethyl acetate. In this first cleanup step, three different extraction mixtures (2 mL) were assayed: a) 1% formic acid, b) acetone- 1% formic acid (1:1, v/v) and c) acetone- 1% formic acid (2:1, v/v). In general, higher extraction yields were obtained when a higher proportion of acetone was used (Fig. 2.1).

Figure 2.1 Effect of the extraction solvent in the recovery of the studied pharmaceuticals.

In order to improve extraction yields, the elution from the SLE columns with 3×2.5 mL and 2×5 mL of ethyl acetate was assayed. An increase in the recovery was observed for salicylic acid (78%), fenoprofen (89%), gemfibrozil (95%) and diclofenac (78%) when higher elution volumes were applied, but the recoveries with ethyl acetate were low for pharmaceuticals such as clofibric acid, mefenamic acid, metoprolol and allopurinol. To improve the recovery of these compounds, the polarity of the extraction solvent was increased and a mixture of ethyl acetate:methanol (90:10, v/v) was tested. The elution with this mixture provided extraction yields $> 70\%$ for all the pharmaceuticals in biosolid samples spiked at 100 ng g^{-1} (Table 2.2).

Derivatization, Silylation, commonly used for the derivatization of pharmaceuticals before their GC analysis, was employed in this study (Verenitch et al., 2006; Xu et al., 2008). Two different silylation reactions were evaluated leading to the formation of TMS and tBDMS derivatives. Hence, BSTFA: pyridine (1:1, v/v), and BSTFA:TMCS (99:1, v/v) were tested to prepare the former and MTBSTFA: pyridine (1:1, v/v) and MTBSTFA: tBDMCS (99:1, v/v) to form the latter.

Table 2.2 Average recovery of pharmaceutical compounds from biosolids spiked at three concentration levels ($n=4$), and limits of quantification (LOQ) and detection (LOD)

Compound	Recovery (%) ^a			LOD (ng g ⁻¹)	LOQ (ng g ⁻¹)
	100 ng g ⁻¹	50 ng g ⁻¹	25 ng g ⁻¹		
clofibric acid	72 ± 4	76 ± 2	115 ± 11	3.1	10.3
ibuprofen	95 ± 4	99 ± 2	102 ± 3	1.0	3.3
caffeine	99 ± 11	103 ± 11	107 ± 3	1.7	5.5
salicylic acid	89 ± 5	87 ± 6	115 ± 12	1.1	3.6
allopurinol	79 ± 8	83 ± 9	100 ± 10	0.7	2.4
paracetamol	92 ± 13	102 ± 10	117 ± 8	2.5	8.3
gemfibrozil	70 ± 2	87 ± 11	120 ± 6	1.5	4.8
fenoprofen	95 ± 8	97 ± 11	80 ± 5	3.1	10.4
amitriptyline	107 ± 4	108 ± 5	98 ± 10	3.6	12.1
metoprolol	84 ± 6	85 ± 7	106 ± 8	0.5	1.8
naproxen	91 ± 6	86 ± 3	107 ± 9	0.8	2.6
mefenamic acid	93 ± 13	73 ± 4	89 ± 3	1.6	5.2
ketoprofen	80 ± 4	95 ± 8	114 ± 4	1.7	5.6
carbamazepine	96 ± 9	109 ± 7	90 ± 5	1.5	5.0
diclofenac	98 ± 7	94 ± 8	108 ± 7	0.7	2.3
fenofibrate	90 ± 8	95 ± 4	96 ± 4	1.2	4.2

^a Results are the mean (%) of four replicates ± RSD (%)

The derivatization conditions used for the silylation were based on those applied in our previous works where other phenolic compounds were derivatized (Albero et al., 2013; Sánchez-Brunete et al., 2009) 50 µL of the reagent were added to 100 µL of 5 µg mL⁻¹ standard in ACN in a reaction vial that was capped and then heated at 70 °C for 30 min. The derivatized pharmaceuticals were then analyzed by GC-MS in the scan mode monitoring m/z from 50 to 540. Fig. 2.2 shows the peak areas obtained with the four different derivatization reagents. It was observed that pyridine was not as effective as the corresponding chlorosilane catalyst and lower peak areas were achieved when the silylating agent was mixed with pyridine.

Figure 2.2 Effect of the derivatization reagent on peak area of the 16 pharmaceuticals studied. Paracetamol I represents mono-*t*BDMS derivative and Paracetamol II is the di-*t*BDMS derivative

The preparation of *t*BDMS derivatives resulted in a significant increase in the response of several compounds, such as salicylic and clofibric acids, carbamazepine and allopurinol. In the case of paracetamol, the di- *t*BDMS derivative obtained presented a considerably higher peak area than the mono- *t*BDMS derivative. A 30 min derivatization step is not enough time for the complete reaction of two reactive hydrogen atoms of paracetamol. On the other hand, when BSTFA is used to derivatize paracetamol only one TMS moiety is attached but its chromatographic response is much lower than that obtained with the other reagent. As shown in Fig 2.2, MTBSTFA-*t*BDMCS (99:1, v/v) was the reagent that produced, in general, the derivatives with higher chromatographic response and was chosen to enhance the volatility of the studied pharmaceuticals. Furthermore, it has been reported that *t*BDMS derivatives are about 1000-10,000 fold more stable to hydrolysis conditions than the TMS derivatives (Poole, 2013), which is an important feature taking into account that the analytes were extracted from biosolids using an aqueous solution.

The effect of derivatization time was studied at 30, 60 min and 24 h. After 30 min, the derivatization of paracetamol was not complete and a 60 min period was necessary to ensure the derivatization of all target compounds and the chromatographic response of the analytes after 24 h did not increase. The experimental conditions for the simultaneous derivatization of these compounds were set as follows: acetonitrile was selected as solvent for the silylation reaction and of 50 μ L of MTBSTFA:*t*BDMCS, were added to a 100 μ L sample and the mixture was kept at 70 °C during 60 min.

2.3.2 Determination by gas chromatography-tandem mass spectrometry

Once the derivatization conditions were optimized and the formation of *t*BDMS derivatives was accomplished, the GC-MS/MS method was optimized. The most abundant ion observed in the mass spectra of the *t*BDMS derivatives was $[M-57]^+$ that results from the loss of the *t*-butyl moiety and was chosen as one of the precursor ions to evaluate possible product ions. Product ions of two different precursor ions were obtained performing the dissociation of the precursor ions at collision energies that ranged from 5 to 50 eV. Three of the studied pharmaceuticals, caffeine, fenofibrate and amitriptyline, do not have active hydrogen atoms so they do not undergo silylation as the rest of the studied pharmaceuticals. The fragmentation of amitriptyline using EI ionization produces a very poor spectrum and the most abundant ion is m/z 58, corresponding to the tertiary ammonium moiety, and ions above m/z 60 are of relatively low abundance. In the GC-MS analysis of amitriptyline, ions m/z 58 and 202 are usually monitored (Ghambarian et al., 2012; Ito et al., 2011) and we selected the latter as precursor ion. Due to the low abundance of this ion, the resulting MRM used for quantification was the lowest of all the pharmaceuticals studied. To reduce the possible memory effects of the column, the inlet was flushed by heating at 300 °C for 30 min before the analysis of samples and procedural blanks were analyzed after every four samples.

The chromatographic response of target analytes may be affected by the presence of matrix components. Matrix effects were evaluated preparing two sets of standard solutions, one set of standards was solvent-based ranging from 10 to 1000 ng mL⁻¹, and

the other was prepared spiking blank biosolid extracts in the same concentration range. For matrix-matched standards, the different concentration levels were prepared using the same volume of extract with the aim of maintaining the same amount of matrix throughout the range of concentrations studied. The slopes obtained by plotting peak area against seven concentration levels, following linear regression analysis, were compared. No significant matrix effects ($< 10\%$) were observed in seven of the analytes studied: clofibric acid, ibuprofen, salicylic acid, fenoprofen, naproxen, carbamazepine and fenofibrate. An enhancement of the chromatographic response due to matrix components was only observed in two compounds, allopurinol (13%) and diclofenac (74%). Although in gas chromatography matrix coextractives, in general, enhance analytes response, signal suppression was observed in the rest of the pharmaceuticals. The derivatization step is carried out with the reagent in excess, nevertheless, matrix components may compete with our analytes and thus a lower chromatographic response is observed in samples prepared spiking biosolid extracts. A decrease of 28-37% in the chromatographic response was observed for gemfibrozil, paracetamol, amitriptyline and metoprolol, whereas for mefenamic acid and ketoprofen, the signal suppression was 76% and 86%, respectively.

2.3.3 Method validation

After optimization, the developed method was evaluated in terms of linearity, precision, accuracy and detection limits before it was used to determine pharmaceutical drug residues in biosolids. The validation procedure was carried out with matrix-matched calibration to counteract the observed matrix effect.

Linearity. Biosolid extracts spiked at seven concentration levels ranging from 5 to 500 ng g^{-1} were used to obtain the calibration curve of all the target analytes. Each calibration solution was injected three times, and a good linearity was obtained in the range assayed with correlation coefficients ≥ 0.990 , except for paracetamol and diclofenac that was 0.982 for both.

Recovery. Recoveries were determined as the average of the analyses of four replicates of samples spiked at three concentration levels (25, 50 and 100 ng g^{-1}) by comparing

these results with those obtained for biosolid extracts that were spiked after the sample preparation and prior to the derivatization step. Good recoveries, ranging from 70 to 120%, were obtained for the three spiking levels assayed (Table 2.2). At the lowest spiking level, the recoveries and standard deviations obtained were somewhat higher. The background levels of some target analytes could explain the higher uncertainty in the recoveries at this concentration level.

Repeatability. The repeatability of the chromatographic method was evaluated by performing the analysis of a derivatized biosolid extract spiked at 50 ng g⁻¹. The sample was injected 10 times and the relative standard deviations (RSD) obtained for peak areas ranged from 1.5 to 12%. The highest RSD value for peak areas was obtained for paracetamol. The reaction rate of amide groups with silyl reagents is lower than that of phenol groups and, thus, after the derivatization step is possible that both mono- and di-*t*BDMS paracetamol derivatives are formed, although during the chromatographic analysis the derivatization reaction is completed. The repeatability of the whole analytical procedure was determined by analyzing seven samples spiked at 50 ng g⁻¹ within a given day and the RSD calculated for the studied compounds ranged from 1.5% to 11.0%.

Limits of detection (LOD) and quantification (LOQ). Ten replicates of biosolid extracts spiked at 10 ng g⁻¹ level were analyzed in order to determine LODs and LOQs of the developed method. The equation to calculate the LODs was the following: $LOD = t_{99} \times S$, where t_{99} is Students' *t* value appropriate for a 99% confidence level and *n*-1 degrees of freedom and *S* is the standard deviation of the replicate analyses. The LOQ was calculated as 10 times the standard deviation of the results of the replicate analysis used to determine LODs. Low limits were obtained due to the high selectivity and sensitivity of GC–MS/MS, allowing the determination of these compounds at the trace levels found in biosolids. As shown in Table 2.2, LODs ranged from 0.5 to 3.6 ng g⁻¹ and LOQs from 1.8 to 12.1 ng g⁻¹, being amitriptyline the compound with the highest limit, since it is the one that presents the quantifier MRM with lowest abundance. The LODs obtained with the proposed method are in the same range or lower than those reported by other authors in the analysis of pharmaceutical compounds in biosolids (Dobor et al., 2010;

Martín et al., 2010; Peysson and Vulliet, 2013; Radjenovic et al., 2009a; Samaras et al., 2011).

2.3.4 Analysis of biosolid samples

The optimized method was applied to the determination of the concentration of pharmaceuticals in ten biosolid samples collected in the areas of Madrid and Catalonia. The analysis of samples was conducted in triplicate and the measured concentration of the pharmaceuticals found and the corresponding standard deviations are summarized in Table 2.3. Eleven of the sixteen studied compounds were found and at least four pharmaceuticals were detected in all the samples analyzed. Fig. 2.3 shows the GC-MS/MS chromatograms of two biosolids analyzed.

Table 2.3 Levels of pharmaceutical found in biosolid samples (ng g^{-1} d.w., $n=3$)

Compound	BS1	BS2	BS3	BS4	BS5	BS6	BS7	BS8	BS9	BS10
ibuprofen	95 ± 4	238 ± 19	21 ± 2	82 ± 4	350 ± 29	322 ± 26	444 ± 12	64 ± 3	344 ± 12	418 ± 14
caffeine	74 ± 5	50 ± 6	153 ± 12	37 ± 2	86 ± 3	104 ± 6	63 ± 7	154 ± 3	132 ± 10	69 ± 4
salicylic acid	825 ± 96	494 ± 55	126 ± 24	210 ± 10	414 ± 87	892 ± 88	651 ± 19	n.d.	751 ± 47	1111 ± 34
paracetamol	192 ± 34	201 ± 27	24 ± 1	37 ± 5	119 ± 2	116 ± 14	161 ± 23	45 ± 7	134 ± 15	290 ± 37
allopurinol	5.3 ± 0.1	6.4 ± 0.7	4.1 ± 0.8	3.6 ± 0.2	5.1 ± 0.5	7.8 ± 2.2	n.d.	n.d.	n.d.	7.5 ± 0.4
gemfibrozil	57 ± 3	67 ± 3	n.d.	37 ± 2	61 ± 4	n.d.	n.d.	n.d.	n.d.	n.d.
fenoprofen	58 ± 8	53 ± 2	n.d.	n.d.	n.d.	66.5 ± 9	50 ± 3	n.d.	70 ± 1	66 ± 12
amitriptyline	177 ± 14	n.d.	n.d.	27 ± 3	n.d.	n.d.	n.d.	156 ± 10	n.d.	n.d.
naproxen	n.d. ^a	n.d.	n.d.	n.d.	n.d.	23 ± 7	n.d.	n.d.	15 ± 2	12 ± 1
diclofenac	278 ± 19	627 ± 69	n.d.	34 ± 4	97 ± 7	514 ± 56	180 ± 14	n.d.	258 ± 28	390 ± 35
fenofibrate	302 ± 18	32 ± 1	13 ± 1	44 ± 4	135 ± 20	48 ± 4	47 ± 2	68 ± 1	101 ± 5	44 ± 3

^a n.d.: not detected (<LOD)

Figure 2.3 GC-MS/MS chromatograms of the pharmaceuticals found in two of the biosolids samples analyzed

In general, the highest concentrations of pharmaceutical residues correspond to NSAIDs, with salicylic acid found at levels up to 1111 ng g⁻¹, which are considerably higher than those reported by (Martín et al., 2010) in digested sludge. Three out of the seven NSAIDs monitored (ibuprofen, salicylic acid, and fenoprofen) were detected in all the samples analyzed. Some NSAIDs, particularly ibuprofen, ketoprofen, naproxen and diclofenac, are common target compounds in the analysis of pharmaceuticals in the environment. In our study, we did not find residues of ketoprofen in any of the samples analyzed and naproxen was only found in three samples at low concentrations, from 12 to 23 ng g⁻¹ (Jelic et al., 2011) performed the monitoring of pharmaceuticals in three conventional WWTPs over a period of two years and no residues of both NSAIDs were detected in any of the samples analyzed concluding that these compounds do not accumulate in sludge. Ketoprofen was not detected or quantified in sewage sludge from WWTPs in Athens (Greece) (Samaras et al., 2011) and Seville (Spain) (Martín et al., 2010), respectively. Saleh et al. analyzed four NSAIDs in sewage sludge from a Swedish WTP, and the highest concentration levels were found for ibuprofen in the range of 304-588 ng g⁻¹ and the lowest concentration levels were for naproxen in the range 7 to 14 ng g⁻¹. It should be noted that these results are similar to those obtained in this work. Sagrista et al. reported data on the occurrence of NSAIDs in sludge from Swedish STPs and they show concentrations about 29, 39, 122 and 138 ng g⁻¹ for ketoprofen, diclofenac, ibuprofen and naproxen, respectively. Differences of these values with those obtained in this work could be attributed to the treatment processes performed in the WWTP or their concentration in inflow waters according to their use by consumers.

The anti-epileptic drug carbamazepine was not found in any of the samples at levels above LOD. Carbamazepine has a very low removal regardless of the treatment applied and does not adsorb onto the sludge (Jelic et al., 2011; Radjenovic et al., 2009b) although it has been found in sludge from different locations (Nieto et al., 2007, 2010a; Radjenovic et al., 2009a).

The presence of caffeine in sludge is not only due to its therapeutic application as nervous stimulant, diuretic or to enhance the effect of analgesics but mainly because it is a constituent of a variety of beverages (coffee, tea, soft drinks) and food products (Buerge et al., 2003). Caffeine was detected in all of the samples analyzed in the present study at levels that ranged from 37 to 154 ng g⁻¹, higher than the concentrations found by Nieto et al. 2007, up to 74 ng g⁻¹, and by Okuda (Okuda et al., 2009), 16 ng g⁻¹. Allopurinol, drug used primarily to treat hyperuricemia, was the pharmaceutical compound found at the lowest levels with average concentrations in a range from 3.6 to 7.8 ng g⁻¹. To the best of our knowledge, this is the first time this compound has been determined in biosolids.

2.4 Conclusions

In this work, a method that combines SLE and GC–MS/MS was developed for the analysis of 16 pharmaceuticals in biosolids. Due to the reduced amount of sorbent used in the SLE procedure, only small quantities of both samples and organic solvents were required that resulted in a considerable saving of analysis time. An important advantage of this method is the ease of use and the wide range of compounds that could be extracted efficiently. Another significant advantage of the described procedure is the reduction in the consumption of organic solvents versus previously published methodologies.

Satisfactory results were obtained for all the compounds studied in terms of reproducibility (RSD ≤ 13%) and sensitivity with LODs ranging from 0.5 to 3.6 ng g⁻¹. The proposed method was applied to biosolids from two Spanish areas, Madrid and Catalonia, and at least 4 of the 16 target pharmaceuticals were detected in all the samples analyzed.

CHAPTER THREE – EMERGING CONTAMINANTS IN POULTRY MANURE

EMERGING CONTAMINANTS IN POULTRY MANURE

As it has been exposed for sewage sludge, the livestock industry generates thousands of tones of residues that need to be handled properly. Manure is a rich source of organic matter with high content of nitrogen, phosphorus and potassium among other nutrients that plants need to develop properly. Because of that, animal litter residues are mainly being incorporated to agricultural fields as organic amendments to improve the physico-chemical properties of soils.

One of the organic amendments more commonly used is poultry manure because of the high demand of chicken that is growing as the population grows. Poultry manure, in comparison with other amendments, presents lower levels of water and higher concentration of nitrogen. In Spain, prior to its application to soil, it is subjected to the law “*Real Decreto- Ley 261/1996*”, which adapts the 91/676/CEE regulation from the European Commission regarding the protection of fresh water from the runoff of nitrogen coming from agriculture and also the “*Real Decreto 824/2005*” about fertilizer products, which legislates primarily the amount of heavy metals that can contain. Until now, no mention at all regarding ECs in organic amendments has been done.

ECs may be toxic to soil and water organisms, therefore, a multiresidue method able to detect and measure these compounds in poultry manure is needed and it may help to adapt in the future the current legislation and take ECs into account.

MULTIRESIDUE ANALYSIS OF INSECTICIDES AND OTHER SELECTED ENVIRONMENTAL CONTAMINANTS IN POULTRY MANURE BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY.

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Abstract

In the present work, an analytical method was developed for the simultaneous determination in poultry manure of 41 organic contaminants belonging to different chemical classes: pesticides, polycyclic aromatic hydrocarbons, polychlorinated biphenyls and polybrominated diphenyl ethers. Poultry manure was extracted with a modified QuEChERS method and the extracts were analyzed by isotope dilution gas chromatography-mass spectrometry. Recovery of these contaminants from samples spiked at levels ranging from 25 to 100 ng g⁻¹ was satisfactory for all of the compounds. The developed procedure provided method detection limits from 0.8 to 9.6 ng g⁻¹. The analysis of poultry manure samples collected in different farms confirmed the presence of some of the studied contaminants. Pyrethroids and polycyclic aromatic hydrocarbons were the main contaminants detected. DDT and its metabolite DDE were also found but at relatively low concentrations.

Keywords: Poultry manure, QuEChERS, Pesticides, Organic pollutants

3.1 Introduction

Spreading poultry manure as fertilizer on agricultural fields has been actively promoted by national authorities as an economic way of recycling. However, poultry manure may contain contaminants and other toxic substances, which could be incorporated into crops or be distributed in the environment (Plaza-Bolanos et al., 2012). It is important to know the levels of contaminants in manure, in order to assess if their presence may pose a risk to the environment or consumers when used as fertilizer in agricultural soil. In addition, the widespread use of pyrethroids in animal husbandry can lead to the transfer of their residues to poultry manure (Niewiadowska et al., 2010). Moreover, persistent organic pollutants (POPs) are a very important group of compounds with a high potential of bioaccumulation and resistance to degradation. POPs are prone to long-range atmospheric transport and deposition and as a result they have been detected in remote areas. In the Stockholm Convention of 2001 (<http://chm.pops.int/Convention/tabid/54/Default.aspx>), polychlorinated biphenyls (PCBs) and organochlorine pesticides, among others, were included as POPs and, in 2009, polybrominated diphenyl ethers (PBDEs) were added to this list (WHO/IPCS, 1994). In addition, other contaminants such as polycyclic aromatic hydrocarbons (PAHs) have been included in the European Union (EU) list of priority pollutants (Guzzella et al., 2011).

Several analytical methodologies are available in the literature for the determination of individual or a combination of several of these compounds in different matrices. Due to the concern regarding the presence and fate of persistent contaminants in the environment as well as the high cost and duration of analysis, there is a need to introduce fast and sensitive multiresidue methods capable of analyzing different classes of contaminants in one analytical procedure. The simultaneous analysis of several groups of compounds with different physical-chemical properties generally requires a compromise in the selection of experimental conditions, but these methods allow a reduction in time and cost of analysis (Tadeo et al., 2012a). Extraction methods such as ultrasonic assisted

extraction (UAE) (Pérez et al., 2013; Sun et al., 2012; Tadeo et al., 2010b) and pressurized liquid extraction (PLE) (Albero et al., 2012a; Qin et al., 2011) have been employed to this aim.

Anastassiades et al. (Anastassiades et al., 2003) developed in 2003 the procedure called QuEChERS (quick, easy, cheap, effective, rugged and safe), originally designed and widely applied to the determination of pesticides in fruit and vegetable matrices with high water content. To a much lesser extent, it has also been used in the extraction of different contaminants from other matrices. There are several published papers on the determination of PAHs, PCBs, PBDEs, organochlorine pesticides and pyrethroids in various matrices, such as fish tissue, soil and water (Kao et al., 2012; Lehotay, 2011; Lozano et al., 2012; Norli et al., 2011; Sapozhnikova and Lehotay, 2013; Tadeo et al., 2012b). In general, the QuEChERS methodology consists of two steps, an extraction/partitioning step followed by a dispersive solid-phase extraction (d-SPE) clean-up step. Moreover, the determination of these compounds in very complex matrices requires a powerful tool such as mass spectrometry (MS) coupled to gas chromatography (GC) or liquid chromatography (LC) to provide high quality results (Tadeo et al., 1996).

The aim of this study was to develop and validate a rapid and simple multiresidue method, based on QuEChERS, for the identification and quantification of 41 organic compounds, belonging to various chemical classes (4 organochlorine pesticides, 8 pyrethroids, 6 PBDEs, 7 PCBs and 16 PAHs) in poultry manure, using isotope dilution GC-MS with selected ion monitoring mode (SIM). To the best of our knowledge, a multiresidue method for the determination in poultry manure of all the compounds listed in this study applying a QuEChERS method is not available in the scientific literature. The linearity, recovery, precision and detection limits were evaluated in order to assess the performance of the proposed method that was then applied to the determination and quantification of these compounds in poultry manure collected from four poultry farms located in Castilla-León, Spain.

3.2 Material and Methods

3.2.1 Reagents and standards

Ethyl acetate (EtAc) and acetonitrile (ACN), residue analysis grade, were purchased from Scharlab (Barcelona, Spain). Bondesil-C18 and primary secondary amine (Bondesil-PSA, 40 μm of diameter) sorbent were from Varian (Palo Alto, CA, USA). A Milli-Q water purification system from Millipore (Bedford, MA, USA) was used to provide ultrapure water in this study. Magnesium sulfate anhydrous (MgSO_4) and anhydrous sodium acetate (NaOAc) were purchased from Merck (Darmstadt, Germany).

A standard solution of the 16 EPA-priority PAHs ($2000\text{ }\mu\text{g mL}^{-1}$ each) was supplied by Sigma-Aldrich (Steinheim, Germany). EPA 525 fortification solution B from Supelco (Bellefonte, PA), containing acenaphthene-d10 (Ace-d10), phenanthrene-d10 (Phen-d10), perylene-d12 (Pery-d12) and chrysene-d12 (Chr-d12) at $500\text{ }\mu\text{g mL}^{-1}$ in acetone, was used as internal standard. A mixture of 7 PCBs (Code BP-D7) at a concentration of $10\text{ }\mu\text{g mL}^{-1}$ in nonane:toluene (96.5:3.5, v/v) (PCB 28, 52, 101, 118, 138, 153 and 180), and a $5\text{ }\mu\text{g mL}^{-1}$ nonane:toluene (93:7, v/v) solution of seven $^{13}\text{C}_{12}$ -labeled PCBs (Code MBP-D7) were purchased from Wellington Laboratories (Guelph, Ontario, Canada). A standard solution of PBDEs (Code BDE-MXD) containing BDE 17, 47, 66, 100, 153 and 183 ($5\text{ }\mu\text{g mL}^{-1}$ each in nonane:toluene (74:26, v/v), and a $5\text{ }\mu\text{g mL}^{-1}$ nonane solution of three $^{13}\text{C}_{12}$ -labeled PBDEs (BDE 47, 99 and 153) (Code MBDE-MXA), were supplied by Wellington Laboratories (Guelph, Ontario, Canada).

Pesticides lindane, α -BHC, 4,4'-DDD, 4,4'-DDE, 2,4'-DDT and 4,4'-DDT, ($100\text{ }\mu\text{g mL}^{-1}$ in nonane purity higher than 98%) were supplied by Supelco (Bellefonte, USA), λ -cyhalothrin, permethrin, cyfluthrin-I, cyfluthrin-II, cyfluthrin-III, cyfluthrin-IV, α -cypermethrin and deltamethrin ($100\text{ }\mu\text{g mL}^{-1}$ in nonane purity 99%) were supplied by Riedel-de Haën (Seelze, Germany), whereas $^{13}\text{C}_6$ -cyfluthrin, mix of stereoisomers, (cyfluthrin-I-II-III-IV, $100\text{ }\mu\text{g mL}^{-1}$ in nonane purity 99%) were supplied by Cambridge Isotope Laboratories (Andover,

MA,USA).The list of investigated compounds, together with their abbreviations, is shown in Table 3.1.

Table 3.1 Retention times (t_R) and selected ions (m/z) of the compounds studied

	Name	IUPAC	t_R	T^a	Q_1^b	Q_2^b	IS^c
1	Naphthalene	Naph	4.093	128	129 (10)	102 (10)	Ace-d10
2	Acenaphthylene	Acyl	6.017	152	151 (20)	153 (10)	Ace-d10
3	Acenaphthene-d10	Ace-d10	6.304	162	164 (95)	160 (45)	-
4	Acenaphthene	Ace	6.341	152	154 (90)	152 (50)	Ace-d10
5	Fluorene	Fl	7.146	166	165 (90)	163 (13)	Ace-d10
6	α -Hexachlorocyclohexane	α -HBC	8.084	284	286 (80)	282 (50)	Lindane
7	Lindane	Lindane	8.523	230	228 (80)	193 (75)	-
8	Phenanthrene-d10	Phen-d10	8.86	188	189 (15)	184 (15)	-
9	Phenanthrene	Phen	8.911	178	176 (20)	179 (15)	Phen-d10
10	Anthracene	Anth	9.021	178	176 (20)	179 (15)	Phen-d10
11	2,4,4'-Trichlorobiphenyl	PCB-28	9.672	258	256 (95)	186 (70)	PCB-28L
12	2,4,4'-Trichlorobiphenyl-L	PCB-28L	9.665	268	266(95)	198 (65)	-
13	2,2',5,5'-Tetrachlorobiphenyl-L	PCB-52L	10.317	304	232 (95)	302 (85)	-
14	2,2',5,5'-Tetrachlorobiphenyl	PCB-52	10.324	292	220 (90)	290 (80)	PCB-52L
15	Fluoranthene	F	11.679	202	203 (20)	201 (10)	Phen-d10
16	2,2',4,5,5'-Pentachlorobiphenyl-L	PCB-101L	12.059	338	336 (75)	340 (70)	-
17	2,2',4,5,5'-Pentachlorobiphenyl	PCB-101	12.074	326	328 (75)	324 (70)	PCB-101L
18	Pyrene	Py	12.242	202	200 (20)	203 (20)	Phen-d10
19	4,4'-DDE	4,4'-DDE	12.696	246	248 (65)	318 (60)	4,4'-DDD
20	2,2',4-Tribromodiphenyl ether	PBDE-17	13.172	248	246 (95)	406 (50)	PBDE-47L
21	2,3',4,4',5-Pentachlorobiphenyl-L	PCB-118L	13.48	338	336 (75)	340 (70)	-
22	2,3',4,4',5-Pentachlorobiphenyl	PCB-118	13.487	326	328 (65)	324 (60)	PCB-118L
23	4,4'-DDD	4,4'-DDD	13.641	243	245 (65)	173 (45)	-
24	2,4'-DDT	2,4'-DDT	13.743	235	237 (65)	165 (55)	4,4'-DDD
25	2,2',4,4',5,5'-Hexachlorobiphenyl-L	PCB-153L	13.978	372	370 (80)	302 (65)	-
26	2,2',4,4',5,5'-Hexachlorobiphenyl	PCB-153	13.992	360	362 (80)	290 (70)	PCB-153L
27	4,4'-DDT	4,4'-DDT	14.644	235	237 (65)	165 (55)	4,4'-DDD
28	2,2,3,4,4',5'-Hexachlorobiphenyl-L	PCB-138L	14.652	372	374 (80)	302 (60)	-
29	2,2,3,4,4',5'-Hexachlorobiphenyl	PCB-138	14.659	360	362 (80)	290 (60)	PCB-138L
30	Benzo[a]anthracene	BaA	16.035	228	226 (25)	229 (20)	Chr-d12
31	Chrysene-d12	Chr-d12	16.057	240	236 (25)	241 (20)	-
32	Chrysene	Chr	16.152	228	226 (30)	229 (20)	Chr-d12
33	2,2',3,4,4',5,5'-Heptachlorobiphenyl-L	PCB-180L	16.431	394	396 (95)	324 (85)	-
34	2,2',3,4,4',5,5'-Heptachlorobiphenyl	PCB-180	16.438	406	408 (95)	394 (90)	PCB-180L
35	2,2',4,4'-Tetrabromodiphenyl ether-L	PBDE-47L	16.643	326	486 (60)	488 (40)	-
36	2,2',4,4'-Tetrabromodiphenyl ether	PBDE-47	16.65	338	498 (60)	496 (45)	PBDE-47L

37	2,3',4,4'-Tetrabromodiphenyl ether	PBDE-66	17.199	326	486 (35)	328 (20)	BDE-99L
38	λ -Cyhalothrin	λ -CYHA	17.536	181	197 (70)	208 (45)	CYFL-I- ¹³ C ₆
39	Permethrin	PERM	18.854	183	163 (20)	165 (10)	CYFL-I- ¹³ C ₆
40	2,2',4,4',6-Pentabromodiphenyl ether	PBDE-100	19.118	406	404 (95)	564 (60)	BDE-99L
41	Cyfluthrin-I- ¹³ C ₆	CYFL-I- ¹³ C ₆	19.796	212	232 (55)	-	-
42	Cyfluthrin-I	CYFL-I	19.824	163	206 (35)	226 (20)	CYFL-I- ¹³ C ₆
43	Benzo[b]fluoranthene	BbF	19.848	252	250 (25)	253 (20)	Pery-d12
44	Benzo[k]fluoranthene	BkF	19.958	252	250 (25)	253 (20)	Pery-d12
45	2,2',4,4',5-Pentabromodiphenyl ether-L	BDE-99L	19.967	416	418 (95)	577 (55)	-
46	Cyfluthrin-II- ¹³ C ₆	CYFL -II- ¹³ C ₆	19.984	212	232 (40)	-	-
47	Cyfluthrin-II	CYFL -II	20.013	163	206 (40)	226 (10)	CYFL -II- ¹³ C ₆
48	Cyfluthrin-III- ¹³ C ₆	CYFL -III- ¹³ C ₆	20.072	212	232 (50)	-	-
49	Cyfluthrin-III	CYFL -III	20.099	163	206 (55)	226 (30)	CYFL -III- ¹³ C ₆
50	Cyfluthrin-IV- ¹³ C ₆	CYFL -IV- ¹³ C ₆	20.161	212	232 (40)	-	-
51	Cyfluthrin-IV	CYFL -IV	20.186	163	206 (50)	226 (10)	CYFL -IV- ¹³ C ₆
52	α -Cypermethrin	α -CYPE	20.641	181	163 (95)	165 (10)	CYFL -IV- ¹³ C ₆
53	Benzo[a]pyrene	BaP	21.021	252	253 (20)	125 (10)	Pery-d12
54	Perylene-d12	Pery-d12	21.241	264	260 (25)	263 (20)	-
55	2,2',4,4',5,5'-Hexabromodiphenyl ether-L	BDE-153L	23.203	496	494 (75)	656 (40)	-
56	2,2',4,4',5,5'-Hexabromodiphenyl ether	PBDE-153	23.218	484	482 (75)	642 (15)	BDE-153L
57	Deltamethrin	DELT	23.335	253	253 (75)	251 (40)	CYFL -IV- ¹³ C ₆
58	Indeno[1,2,3-c,d]pyrene	IcdPy	24.844	276	277 (25)	138 (20)	Pery-d12
59	Dibenzo[a,h]anthracene	DbahA	24.99	278	276 (50)	139 (10)	Pery-d12
60	Benzo[g,h,i]perylene	BghiP	24.605	276	277 (25)	138 (23)	Pery-d12
61	2,2',3,4,4',5',6-Heptabromodiphenyl ether	PBDE-183	26.359	562	462 (90)	724 (20)	BDE-153L

^a T: target ion; ^b Q₁ and Q₂: qualifier ions (their relative abundances in the mass spectra are in brackets); ^c IS: internal standard used in the quantification.

Individual stock solutions of each compound were stored in the dark at 4 °C up to 8 weeks. A working mixture solution at 200 ng mL⁻¹ was prepared weekly by dilution of the stock solution in ethyl acetate.

The compounds: λ -Cyhalothrin, cyfluthrin-I, cyfluthrin-II, cyfluthrin-III, cyfluthrin-IV, α -cypermethrin, deltamethrin, 4,4' DDT, PCB-138 and PBDE-153, due to their low chromatographic response, were made up to 400 ng mL⁻¹. A

standard solution containing all the isotope labeled compounds used as internal standards was prepared in ethyl acetate at the same concentration as the working mixture solution. All standard solutions were stored at 4 °C prior to use.

3.2.2 Sample preparation

Poultry manure samples were collected in glass jars (100-250 g) from 4 farms located in Castilla-León, Spain. In the laboratory, samples were lyophilized and ground to pass through a 2 mm screen. They were stored at -20 °C until processing.

Lyophilized poultry manure (0.5 g) was weighed into a 15 mL Sovirell tube (Pobel, Spain) and spiked with a mixture of compounds to reach final concentrations of 100, 50 and 25 ng g⁻¹, except for those that were prepared at double concentration. The surrogate standards were added at a 100 ng g⁻¹ level and the samples were left 24 h before extraction at 4 °C to reach equilibrium. A short vibration using a Vortex mixer was used to disperse solvent and analytes well throughout the sample. After addition of 3 mL ACN and 2 mL of MilliQ water, the sample was placed in an ultrasonic water bath (Raypa, Barcelona, Spain) for 15 min. After addition of MgSO₄ (0.8 g) and NaOAc (0.3 g), the mixture was then shaken intensively by hand for 1 min, vortexed to induce phase separation and centrifuged at 4500 rpm, 15 min (Model Medifriger from J.P. Selecta, Barcelona, Spain). An aliquot (2.5 mL) of the supernatant (ACN layer) was cleaned-up by d-SPE employing MgSO₄ (0.35 g), PSA (0.1 g) and C18 (0.1 g). The tube was closed, shaken vigorously by hand for 1 min and centrifuged for 15 min at 4500 rpm. The supernatant was transferred to a graduated tube and concentrated to 0.5 mL using an evaporator (Genevac EZ-2 NET Interlab, S.A.L., Spain)

3.2.3 Gas chromatography–mass spectrometry

GC-MS analysis was performed with an Agilent 6890 (Waldbronn, Germany) gas chromatograph equipped with an automatic injector, Model HP 7683 and an inert mass spectrometric detector (MSD), Model HP 5973N, equipped with an inert ion source. A fused silica capillary column ZB-5MS, 5% phenyl polysiloxane as

nonpolar stationary phase (30 m $\times$ 0.25 mm i.d. and 0.25 μ m film thickness), from Phenomenex (Torrance, CA), was used for the analysis. Operating conditions were as follows: injector port temperature 300 °C; helium (purity 99.99%) as carrier gas at a flow-rate of 1.0 mL min⁻¹ and pulsed splitless mode (pulsed pressure 45 psi = 310 kPa for 1.5 min) with the splitless injector purge valve activated 1.5 min after sample injection, in a double-taper glass liner with a nominal volume of 800 μ L. The column temperature was maintained at 80 °C for 0.5 min, then programmed at 20 °C min⁻¹ to 180 °C, then increased to 230 °C at a rate of 8 °C min⁻¹, followed by a final ramp to 300 °C at a rate of 5 °C min⁻¹ and held for 5 min. The total analysis time was 30.75 min and the equilibration time was 3 min.

The mass spectrometric detector (MSD) was operated in electron impact ionization mode with an ionizing energy of 70 eV, scanning from m/z 100 to 800 at 3.62 s per scan, an ion source temperature of 230 °C and a quadrupole temperature of 150 °C. The electron multiplier (EM) voltage was operated with gain of 3 and a solvent delay of 3.5 min. Table 3.1 lists the compounds and the internal standards along with their retention times and selected ions. The SIM program used to determine and confirm the compounds in manure is indicated in Table 3.2. The target and qualifier abundances were determined by injection of standards under the same chromatographic conditions using full-scan with the mass/charge ratio ranging from 100 to 800 m/z . The compounds were confirmed by their retention times, the identification of target and qualifier ions and the determination of qualifier to target ratios.

Table 3.2 SIM program used to analyze organic contaminants in manure

Group	Time	Compounds	m/z	Dwell time (ms)
1	3.70	Naph	102, 127, 128	100
2	6.00	Acyl	150, 151, 152	100
3	6.25	Ace-d10, Ace	152, 153, 154, 160, 162, 164	50
4	7.00	Fl	163, 165, 166	100
5	7.90	α -BHC	282, 284, 286	100

6	8.40	Lindane	193 228, 230	100
7	8.65	Phen-d10, Phen	176, 178, 179, 184, 188, 189	50
8	9.00	Anth	176, 178, 179	100
9	9.50	PCB-28, PCB-28L	186, 198, 256, 258, 268, 270	50
10	10.00	PCB-52L, PCB-52	220, 232, 290, 292, 302, 304	50
11	11.00	F	201, 202, 203	100
12	11.90	PCB-101L, PCB-101	254, 266, 326, 328, 336, 338	50
13	12.20	Py, 4,4'DDE	200, 202, 246, 248, 303, 318	50
14	13.00	PBDE-17, PCB-118L, PCB-118	246, 248, 324, 326, 328, 336, 338, 340, 406	30
15	13.60	4,4'DDD, 2,4 DDT	165, 173, 235, 237, 243, 245	50
16	13.90	PCB-153L, PCB-153	290, 302, 360, 362, 372, 374	50
17	14.20	4,4 DDT, PCB-138L, PCB-138	165, 235, 237, 290, 302, 360, 362, 372, 374	30
18	15.20	BaA, Chr-d12, Chr	226, 228, 229, 236, 240, 241	50
19	16.30	PCB-180L, PCB-180	324, 336, 394, 396, 406, 408	50
20	16.50	PBDE-47L, PBDE-47	326, 338, 486, 488, 496, 498	50
21	17.00	PBDE-66, λ -CYHA	181, 197, 208, 326, 328, 486	50
22	18.00	PERM, PBDE-100	163, 165, 183, 404, 406, 564	50
23	19.50	CYFL-I-II-III-IV ¹³ C ₆ , CYFL-I-II-III-IV, BbF, BKF, BDE-99L	163, 165, 206, 212, 232, 250, 252, 253, 416, 418, 578	25
24	20.40	α -CYPE, BaP, Pery-d12	163, 165, 181, 250, 252, 253, 260, 263, 264	30
25	23.00	BDE-153L, PBDE-153, DELT	181, 251, 253, 482, 484, 494, 496, 642, 656	30
26	24.00	IcdPy, DbahA, BghiP	138, 139, 276, 277, 278	60
27	26.00	PBDE 183	562, 564, 724	100

Retention times must be within ± 0.2 min of the expected time and qualifier-to-target ratios within a 20% range for positive confirmation. The quantification was accomplished by calibration with internal labeled standards. A calibration solution was prepared with the standards containing 200 ng mL⁻¹ of the internal labeled standards. The linear range was established by a five point calibration curve in the range 5-100 ng mL⁻¹.

3.3 Results and discussion

3.3.1 Sample preparation

QuEChERS has been frequently applied to the determination of pesticides and other contaminants in fruit and vegetables, owing to the short extraction and purification time as well as the low solvent consumption. Recently, QuEChERS has been applied to other matrices such as soil and fish (Drozdzyński and Kowalska, 2009; Lehotay et al., 2010; Norli et al., 2011). In our case, with a very

different matrix, the extraction and clean-up conditions have been evaluated to adapt this method.

Two solvents, ACN and EtAc, were compared for extraction efficiency. Although there were only slight differences in recovery with both solvents, the GC-MS chromatograms showed less interferences when ACN was used as the extraction solvent (data not shown). Therefore, ACN was used as extraction solvent. In the QuEChERS method, MgSO_4 was added to induce phase separation and to increase the recovery of polar compounds, allowing the control of the water content in the organic phase. In our case, among the different salt amounts studied, 0.8 g of MgSO_4 and 0.3 g of NaOAc for 0.5 g of manure were enough to completely saturate the sample and appropriate to provide a homogenization of the sample during the vortex step.

Poultry manure is a very complex matrix, therefore, a purification step is necessary to extend column life and reduce the presence a large amount of interferences in the chromatogram. For the clean-up step, a d-SPE may be employed using a combination of PSA to remove polar organic acids, C18 to eliminate some sugars and lipids as well as other components, Graphitized Carbon Black (GCB) to remove pigments and MgSO_4 to reduce the remaining water in the extract. In our case, it has to be taken into account that some planar compounds such as PAHs have a great affinity to GCB, therefore, GCB was not used. Various amounts of salts and adsorbents were assayed, but no significant differences were found. According to these results, 0.35 g of MgSO_4 , 0.1 g of PSA and 0.1 g of C18 were used in the clean-up procedure. Clean chromatograms without interferences from other components were obtained with this modified QuEChERS method.

3.3.2 Determination by gas chromatography-mass spectrometry

The studied compounds were determined by GC-MS-SIM. Three ions were selected, one as quantifier and two as qualifiers. Table 3.1 lists the compounds with their retention times, and their target and qualifier ions. The SIM program

used in the analyses is indicated in Table 3.2 and Fig. 3.1 presents a SIM chromatogram of a standard mixture solution in the conditions indicated above.

Figure 3.1 GC-MS-SIM chromatogram of a standard mixture of contaminants ($25 \mu\text{g L}^{-1}$) with internal standards ($40 \mu\text{g L}^{-1}$). See Table 3.1 for peak identification

The chromatographic method was divided in 27 time segments where the dwell time was adjusted to maintain the cycle time constant. To reduce the possible memory effects of the column, every day, prior to the analysis of samples, the inlet was flushed by heating at 300°C for 30 min and procedural blanks were analyzed after every four samples. Quantification was carried out with internal standard calibration. The isotopically labeled compounds used as internal standards for each analyte are shown in Table 3.1.

3.3.3 Method validation

After optimization, the developed method was evaluated in terms of linearity, accuracy precision and detection limits before it was used to determine the levels of contaminants in poultry manure.

Linearity. The linearity of the method was evaluated injecting five standard solutions prepared at 5 to 100 ng mL⁻¹ levels for all the studied compounds, except for some compounds that were prepared at double-concentration due to their low response. Labeled internal standards were added at a fixed concentration (200 ng mL⁻¹) to all the calibration solutions. Each calibration solution was injected four times. A good linearity was obtained with correlation coefficients equal or higher than 0.990 for all the compounds studied.

Accuracy and precision. The accuracy of the method was evaluated performing the recovery of target analytes from manure samples spiked with standard solutions at three concentration levels 100, 50 and 25 ng mL⁻¹ and adding a mixture of surrogate standards to reach a concentration of 100 ng mL⁻¹. Unspiked samples were also extracted and analyzed to subtract the background levels of the analytes. Good recoveries ranging from 80 to 111% were obtained for the spiking levels assayed (Table 3.3).

The intra-day precision was determined by analyzing five spiked samples on the same day. The inter-day precision was evaluated by determining five replicates on five consecutive days. The intra- and inter-day precisions RSD were lower than 12% and 17%, respectively.

Table 3.3 Recoveries (%) with their relative standard deviations (RSD %, $n=5$) of the studied contaminants.

Compound	Fortification levels (ng g ⁻¹)					
	100		50		25	
	Mean	RSD	Mean	RSD	Mean	RSD
Naph	104.8	3.6	100.8	3.1	102.6	8.6
Acyl	104.1	5.2	101.3	2.7	79.5	3.4
Ace	94.2	7.4	95.0	0.5	99.8	2.6
Fl	102.4	9.5	108.7	3.3	102.8	5.4
HBC	101.5	10.8	97.1	4.6	98.1	4.9
Phen	78.4	3.1	104.7	3.1	104.6	0.9

Anth	106.3	7.0	92.4	1.3	81.5	5.0
F	104.4	3.8	78.4	2.7	103.0	1.4
Py	101.8	3.8	108.3	3.8	100.1	0.9
PCB-28	103.0	4.0	84.3	1.6	91.9	1.2
PCB-52	106.5	5.5	100.7	2.8	105.2	1.0
PCB-101	103.3	3.5	108.4	3.3	110.1	4.1
PBDE-17	109.3	3.5	86.6	1.6	95.0	1.1
PBDE-47	95.6	4.8	101.4	6.6	88.1	3.1
PCB-118	106.0	5.0	101.9	2.8	90.4	1.1
2,4' DDT	91.3	7.6	95.5	2.5	100.7	5.0
4,4' DDT	96.4	7.0	95.8	2.9	104.6	6.5
4,4'DDE	102.9	6.3	102.3	2.7	102.8	9.2
PCB-153	105.4	6.5	109.6	4.5	111.0	1.3
PCB-138	104.4	11.0	98.7	8.7	100.4	3.0
BaA	104.7	3.5	102.3	0.7	100.1	0.8
Chr	93.7	6.1	99.2	1.0	98.6	0.5
PCB-180	107.0	4.8	103.9	4.5	98.2	0.6
λ -CYHA	102.7	6.2	86.9	1.4	88.2	2.9
PERM	101.2	8.3	80.3	2.8	107.1	1.1
PBDE-66	87.7	7.2	106.8	4.1	101.0	1.6
PBDE-100	91.7	5.8	95.4	3.6	98.9	2.8
CYFL-I	83.8	6.8	96.3	2.9	110.7	10.3
BbF	100.3	9.5	99.7	1.6	110.3	7.6
BkF	103.0	5.1	98.2	1.7	111.0	1.2
CYFL-II	99.9	5.0	85.0	8.9	88.4	10.9
CYFL-III	103.1	10.7	92.7	3.6	106.2	8.4
CYFL-IV	95.9	11.9	87.3	3.8	98.9	1.5
α -CYPE	99.0	10.6	80.9	7.7	103.4	10.0
BaP	99.6	1.8	102.3	4.2	110.1	0.5
PBDE-153	103.9	5.1	88.8	7.7	101.1	1.6
DEL	96.0	10.7	87.8	3.2	86.1	10.5
IcdPy	108.7	3.7	92.4	3.5	101.9	2.5
DbahA	83.4	3.0	106.0	3.9	109.7	4.7
BghiP	96.3	10.6	108.2	4.3	106.3	9.7
PBDE-183	90.8	9.7	91.3	1.8	104.2	1.4

The repeatability was evaluated by performing the analysis of a standard solution at 50 ng mL⁻¹. The sample was injected 8 times with an automatic injector and the relative standard deviations (RSD) obtained for peak areas were < 8.7% (Table 3.4). Within-laboratory reproducibility of the chromatographic determination was evaluated at different days during two consecutive weeks and was found to be lower than 11% for all of the compounds, expressed as RSD.

Limits of detection (LOD) and quantification (LOQ). Ten replicates of poultry manure extracts spiked at 5 ng mL⁻¹ level were analyzed in order to determine LOD and LOQ of the developed method. The equation to calculate the LOD is the following: $LOD = t_{99} \times S$, where t_{99} is the Student's t value for a 99% confidence level and n-1 degrees of freedom and S is the standard deviation of the replicate analyses. The LOQ was calculated as 10 times the standard deviation of the results of the replicate analysis used to determine LOD.

Low limits were obtained due to the high selectivity and sensitivity of GC–MS, allowing the determination of these compounds at the trace levels found in manure. As shown in Table 3.4, LODs ranged from 0.8 to 9.3 ng g⁻¹ and LOQs from 1.7 to 21.1 ng g⁻¹, being deltamethrin and PBDE-183 the compounds with the highest limits.

Matrix effect. The chromatographic response of target analytes may be affected by the presence of matrix components that may produce an improvement in analyte transfer from the inlet to the column. The matrix effect occurring in the GC analysis of some organic compounds has a negative impact on the accuracy of the results. Elimination of this effect is essential for quantification of pollutants at trace levels in complex environmental matrices (Kao et al., 2012; Sánchez-Brunete et al., 2005; Sánchez-Brunete et al., 2008).

Table 3.4 Limit of detection (LOD, ng g⁻¹), limit of quantification (LOQ, ng g⁻¹), and repeatability of the studied compounds

Compound	LOD ^a	LOQ ^a	Repeatability (RSD %) ^b	
			t _R	Peak Area
Naph	1.3	2.9	0.009	1.1
Acyl	6.8	15	0.002	0.5
Ace	2.0	5.8	0.002	1.4
Fl	4.5	13.3	0.027	0.4
HBC	2.7	8.5	0.052	2.4
Phen	0.8	1.7	0.014	1.5
Anth	7.9	17.3	0.016	7.5
F	4.0	10.8	0.011	1.0
Py	8.1	17.8	0.012	0.2
PCB-28	7.8	17.1	0.012	3.5

PCB-52	4.2	13.5	0.005	3.1
PCB-101	8.4	18.6	0.004	5.9
PBDE-17	7.4	16.3	0.009	1.8
PBDE-47	3.3	7.2	0.004	6.0
PCB-118	6.5	17.3	0.007	5.1
2,4' DDT	9.5	21.0	0.003	3.2
4,4' DDT	3.7	8.1	0.003	6.0
4,4'DDE	5.7	18.2	0.006	3.6
PCB-153	5.0	15.8	0.002	2.8
PCB-138	6.5	19.4	0.005	3.4
BaA	1	3.2	0.002	4.2
Chr	4.9	13.1	0.002	4.0
PCB-180	1	3.2	0.001	1.1
λ -CYHA	5.8	12.8	0.016	6.3
PERM	7.7	17.0	0.011	8.0
PBDE-66	5.8	17.6	0.011	4.3
PBDE-100	5.4	18.0	0.008	8.7
CYFL-I	2.9	9.7	0.008	4.5
BbF	5.7	15.2	0.010	1.0
BkF	3.6	9.5	0.010	1.4
CYFL-II	4.4	14.6	0.012	1.4
CYFL-III	5.1	15.1	0.017	3.0
CYFL-IV	6.0	16.1	0.007	2.6
α -CYPE	8.3	20.6	0.012	6.8
BaP	5.2	16.7	0.027	2.8
PBDE-153	6.4	17.0	0.025	4.3
DELT	9.6	21.1	0.022	7.0
IcdPy	7.7	17.0	0.022	4.8
DbahA	5.1	11.1	0.025	4.9
BghiP	4.0	8.9	0.054	5.3
PBDE-183	8.6	18..9	0.084	5.1

^a Number of replicates (n=8); ^b RSD: relative standard deviation of retention times and peak areas (n=8)

Matrix effect was evaluated preparing two sets of standard solutions ranging from 5 to 400 ng mL⁻¹, one set of standards was solvent-based and the other was prepared spiking blank poultry manure extracts. The slopes obtained by plotting five concentration levels against peak area, following linear regression analysis, were compared. A significant increase of the chromatographic response was observed for PCBs-28, 118, 138, BDE-100, IcdPy, DBahA, BghiP, and organochlorine pesticides.

There are several approaches to counteract matrix-induced effects, such as exhaustive cleanup-steps to reduce the amount of matrix components extracted, the use of inert surfaces in the GC system, and different calibration methods (Sánchez-Brunete et al., 2008). Among the calibration methods employed to overcome matrix effects, matrix-matched standards are often used in spite of its main drawback, which is the lack of an appropriate blank matrix. On the other hand, some regulatory agencies, like the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) in USA do not permit matrix-matched standardization for enforcement purposes. Another way to overcome matrix effects is the use of analyte protectants (Sánchez-Brunete et al., 2005), these compounds, added to the standards and sample extracts, may protect analytes from degradative interactions, which can take place in the injection port.

Another alternative to matrix-matched calibration is the use of isotopically labelled standards, which avoids the dependence of the results obtained from the sample matrix, although they are usually expensive and sometimes not available. To evaluate if isotope labelled internal standards could overcome matrix effects, calibration curves for standards prepared in neat solvent and in spiked soil blanks from 5 to 100 ng mL⁻¹, which included internal standards at 100 ng mL⁻¹ were obtained. Fig. 3.2 shows the slope ratios between matrix-matched and neat solvent calibration for some compounds with a significant matrix effect, using external and internal standard quantification. Isotope labelled standards, used as internal standards, can overcome the increase in the chromatographic response due to matrix components achieving slope ratios of ca. 1.0 for most of compounds. Therefore, quantification was carried out using labelled standards as internal standards.

Figure 3.2 Slope ratios between matrix-matched and neat solvent-based standards of a selection of target analytes applying external standard (ESTD) or internal standard (ISTD) quantification

3.3.4 Analysis of real samples

Once the method was optimized and validated it was used to monitor the contaminants selected for this study in poultry manure samples from four Spanish farms. As shown in Table 3.5, pyrethroids and PAHs were the compounds found in poultry manure at higher concentration. DDT and its metabolite DDE were also found in samples but at relatively low levels. PCBs were not found in any of the samples analyzed. A representative chromatogram of a poultry manure sample containing cyfluthrin (mix of stereoisomers, cyfluthrin-I 209.7 ng g⁻¹, cyfluthrin-II 254.2 ng g⁻¹, cyfluthrin-III 51.8 ng g⁻¹ and cyfluthrin-IV 108.3 ng g⁻¹) and acenaphthene (36 ng g⁻¹) is depicted in Fig. 3.3. No information on concentrations of these compounds in real samples of poultry manure has been found in the available scientific literature.

Table 3.5 Concentration of the studied compounds found in poultry manure collected in farms located in Castilla-Leon (ng g^{-1} , $n=4$)

Compound	Sample 1		Sample 2		Sample 3		Sample 4	
	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
Naph	22.6	4.1	16.8	3.3	22.4	3	28	6.4
Acyl	30	9.3	26.1	4.9	20.4	2.9	25.8	1.3
Ace	36	1.1	29.6	4.1	26.3	0.8	30.2	3.9
Fl	42.8	9.9	16.8	7.6	14.5	7.2	18.9	1.6
Phen	8.6	3.1	9	0.4	12.1	0.3	9.2	0.5
Anth	4.8	7.4	2	0.2	68.7	2.1	12.6	2.3
F	1.8	0.2	0.5	0.1	0.7	0	0.3	0
Py	4.2	1.7	2.7	0.2	3	0.1	2.6	0.1
4,4' DDT	44.6	2	33.5	0.3	19	1.6	33.3	2.6
4,4'DDE	53.1	2.6	28	1.1	30.9	1.9	38.5	2.2
PERM	125.5	3.5	124.3	2.6	209.7	4.4	149.6	5.4
CYFL-I	125.5	3.5	124.3	2.6	209.7	4.4	189.6	5.4
BbF	3.2	1.6	2	0.1	2.1	0.1	1.9	0.7
BkF	2.6	0.7	1.7	0.4	2.2	0.4	1.9	1
CYFL-II	122.9	1.6	122.6	0.6	254.2	5.1	138.1	4.6
CYFL-III	109.5	6.9	105.9	3.3	51.8	6.4	109.8	3.7
CYFL-IV	111.1	7.4	134.7	1.5	108.3	0.6	121.2	1.4
α -CYPE	109.5	6.9	105.9	3.3	51.8	6.4	109.8	3.7
DEL	111.1	7.4	134.7	1.5	108.3	0.6	121.2	1.4
IcdPy	16.9	0.7	10.5	1.5	10.5	1	17.1	1.1

Figure 3.3 Partial view of a GC-MS-SIM chromatogram of a manure extract containing: (A) cyfluthrin (mix of stereoisomers, cyfluthrin-I 209.7 ng g^{-1} , cyfluthrin-II 254.2 ng g^{-1} , cyfluthrin-III 51.8 ng g^{-1} and cyfluthrin-IV 108.3 ng g^{-1}) and (B) acenaphthene (36 ng g^{-1})

3.4 Conclusions

A multiresidue method for the determination in manure of 41 selected environmental contaminants and pesticides, based on a modified QuEChERS method, has been developed and validated. Isotope labelled internal standards were used to compensate the matrix induced chromatographic response enhancement observed, avoiding the use of matrix-matched calibration. The modified QuEChERS method allowed the extraction of a wide range of compounds with satisfactory recoveries. GC-MS was used for its high selectivity allowing the identification and quantification of the studied contaminants at trace levels. This method was applied to poultry manure samples collected from farms located in Castilla-León, Spain, and the presence of some of the studied contaminants was confirmed, being pyrethroids, PAHs, 4,4' DDT and DDE the compounds mainly detected.

**CHAPTER FOUR – EMERGING CONTAMINANTS
IN SOIL: PHARMACEUTICALS AND
PYRETHROIDS**

EMERGING CONTAMINANTS IN SOIL: PHARMACEUTICALS AND PYRETHROIDS

Emerging contaminants have been extensively studied in fresh water, but not in other matrices of agricultural interest such as soil. Agricultural soil is an environmental compartment where ECs may accumulate because they are in general non polar compounds.

Several pathways can lead these compounds to appear in agricultural soil, such as the use of sludge and manure, incorporating undesirable contaminants. Moreover, the use of effluents from WWTP (water with poor quality), where ECs are not completely degraded to irrigate fields, may incorporate ECs into soil.

The need to have methods to detect ECs in soil to identify areas where edible plants may uptake ECs and introduce them into the food chain is necessary. Moreover, these methods can be used to control techniques to remediate the pollution of ECs in soil.

Thus, in this Chapter IV two methods are presented:

- The first one to detect basic, neutral and acid pharmaceutical compounds in soil that was applied to samples taken from several agricultural areas of Spain.
- The second work is a method to detect a wide range of pyrethroid biocides in soil. As samples collected in Valencia showed the highest levels of pollution of selected pharmaceutical compounds, this work focus in an extensive monitoring of PYs in the area of the Natural Park of Albufera, Valencia.

OCCURRENCE AND ANALYSIS OF SELECTED PHARMACEUTICAL COMPOUNDS IN SOIL FROM SPANISH AGRICULTURAL FIELDS

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Abstract

This work describes the analysis of 15 pharmaceutical compounds, belonging to different therapeutic classes (anti-inflammatory/analgesics, lipid regulators, anti-epileptics, β -blockers and antidepressants) and with diverse physical-chemical properties, in Spanish soils with different farmland uses. The studied compounds were extracted from soil by ultrasound assisted extraction (UAE) and determined, after derivatization, by gas chromatography with mass spectrometric detection (GC-MS). The detection limits (LODs) ranged from 0.14 ng g⁻¹ (naproxen) to 0.65 ng g⁻¹ (amitriptyline). At least two compounds were detected in all samples, being ibuprofen, salicylic acid and paracetamol, the most frequently detected compounds. The highest levels found in soil were 47 ng g⁻¹ for allopurinol and 37 ng g⁻¹ for salicylic acid. The influence of the type of crop and the sampling area on the levels of pharmaceuticals in soil, as well as their relationship with soil physical-chemical properties, was studied. The frequent and widespread detection of some of these compounds in agricultural soils show a diffuse contamination, although the low levels found do not pose a risk to the environment or the human health.

Keywords: Pharmaceuticals, UAE, Soil, Agricultural fields, Environment levels, GC-MS, Spain.

4.1 Introduction

At present, approximately 3000 different pharmaceutical ingredients are used in the European Union (Termes and Joss, 2006). Pharmaceutical compounds are administered in large quantities to humans and animals and have been detected in diverse environmental matrices (Antonic and Heath, 2007; Pintado-Herrera et al., 2013; Quintana et al., 2007). After consumption, these drugs are released into the environment mainly through discharge of wastewater treatment plants (WWTPs) or from biosolids applied to land (Al Aukidy et al., 2012). Septic system effluents discharge and disposal of sewage sludge are other potential sources that can introduce pharmaceuticals into the environment (Kinney et al., 2006). The presence of pharmaceutical chemicals in the environment has long been recognized as a concern because it is likely that some of these compounds may pose a risk to flora and fauna (Oetken et al., 2005; Pomati et al., 2004; Pounds et al., 2008; Stuart et al., 2012). Moreover, residual pharmaceuticals have the potential to be taken up by edible plants and then enter the food chain (Shenker et al., 2011; Winker et al., 2010). Most of the studies carried out for the determination of pharmaceuticals in the environment have focused on the aquatic compartment, as a consequence of the polar nature of the majority of these compounds and since the main route to enter the environment is through the discharge of wastewater effluents into surface water. Although most pharmaceutical drugs are designed to be hydrosoluble and biodegradable, many compounds have high log K_{ow} (Table 4.1), and therefore, present a high affinity to sludge or soil. The presence of pharmaceuticals in soil may be explained by the spreading of sludge or manure to fertilize agricultural soils together with the use of contaminated water for irrigation. Residues of pharmaceutical compounds, usually found at low levels, may cause negative effects due to their continuous introduction into the environment (Xu et al., 2009b).

Among the pharmaceutical compounds, anti-inflammatory/analgesics, lipid regulators, anti-epileptics, β -blockers and anti-depressants are often consumed

(Fent et al., 2006). Analgesics are consumed in large quantities and have been frequently found in water (Kosjek et al., 2005; Vazquez-Roig et al., 2011) and sediments (Antonic and Heath, 2007). Other pharmaceuticals, such as lipid regulators and β -blockers, have been also found in sediments due to its elevated lipophilicity with log K_{ow} in the range 3-5 (Pérez-Carrera et al., 2010). A number of researchers have detected pharmaceutical compounds in environmental samples throughout North America and Europe (Al Aukidy et al., 2012; Antonic and Heath, 2007; Metcalfe et al., 2003; Verenitch et al., 2006), generally originated from discharge of treated wastewater effluents, use of reclaimed water for irrigation or soil application of biosolids. Although they report levels of pharmaceuticals in soils located in different environmental areas, information regarding the concentration of these compounds in agricultural soils growing different crops is scarce in the available scientific literature, and only residues of a small number of pharmaceuticals have been reported (Braganca et al., 2012; Walker et al., 2012).

Table 4.1 *Physico-chemical properties of the target compounds*

Compound	Therapeutic class	Log K_{ow}	pK _a	Water solubility, mg mL ⁻¹ at 25 °C
Clofibric acid	lipid regulator	2.6	3.35	0.583
Ibuprofen	NSAID	3.97	4.47	0.022
Salicylic acid	NSAID	1.2	3.5	2
Allopurinol	anti-gout	2.9	9.3	0.48
Paracetamol	NSAID	0.5	9.4	0.014
Gemfibrozil	lipid regulator	4.7	4.4	0.01
Fenoprofen	NSAID	3.9	4.21	15
Amitriptyline	antidepressant	4.92	9.76	0.01
Metoprolol	β -blocker	1.9	9.5	157
Naproxen	NSAID	3.2	4.8	0.016
Mefenamic acid	NSAID	5.1	4.2	0.02
Ketoprofen	NSAID	3.1	4.45	0.05
Carbamazepine	anti-epileptic	2.45	13.9	0.017
Diclofenac	NSAID	4.5	4.1	0.02
Fenofibrate	lipid regulator	4.8	-4.9	0.25

NSAID (Nonsteroidal anti-inflammatory drugs)

The analysis of trace quantities of pharmaceuticals requires powerful and selective techniques, such as, high performance liquid chromatography (Vazquez-Roig et al., 2010) or gas chromatography (GC) (Bisceglia et al., 2010; Weigel et al., 2004) coupled with mass spectrometry (MS) or tandem mass spectrometry (MS/MS). The presence of polar functional groups with active hydrogens require the use of a derivatization procedure to reduce their polarity and enhance their volatility before GC analysis. Ultrasound assisted extraction (UAE) is a technique frequently applied to the extraction of various contaminants from soil (Sánchez-Brunete et al., 2010). Nevertheless, no method has been found on its application to the simultaneous analysis of acid, neutral and basic pharmaceuticals in soil. The short extraction time and low solvent consumption of UAE, besides its robustness and easy handling, are some of the advantages of this extraction technique.

The aim of this study was to develop a rapid and robust analytical method, based on UAE and GC-MS, for the simultaneous determination in soil of 15 widely used pharmaceutical compounds, belonging to different therapeutic classes with a wide range of physical-chemical properties, and to evaluate their presence in soil from agricultural fields located in three Spanish regions (Valencia, Murcia and Segovia). In addition, the influence of the type of crop and the sampling area on the levels of pharmaceuticals in soil, as well as their relationship with soil physical-chemical properties, was also studied.

4.2 Materials and methods

4.2.1 Soil Sampling and Area description

A number of 31 samples were collected from several Spanish areas: 8 samples from rice fields, 6 from citrus orchards, 5 from horticultural fields (potatoes and cabbage) and 12 from cereal farmland (wheat and barley). Samples were taken from surface soil (0-10 cm), where the contamination is generally higher (Chen et al., 2011), using a manual sampling drill, collected in glass jars (100-250 g) and stored in a portable refrigerator during transport to the laboratory. Samples were

air dried over night at room temperature, ground to pass through a 2 mm screen and stored frozen -20 °C until analyzed.

For recovery assays, an alfisol soil (Soil Survey Staff, 2009) was collected from the plough layer (0-10 cm) of an experimental plot located in the region of Madrid (Spain), sieved (2 mm) and stored frozen (-20 °C) until being used. The characteristics of this control soil sample were: pH 7.69, total organic matter content (OM) 0.97%, sand 44.34%, silt 37.44% and clay 18.22%.

Valencia is a known area of rice and citrus production in Spain. All the rice field samples were taken from the Natural Park of “L’Albufera” (Valencia) at two sampling areas: one with irrigation water from either Júcar River or the Albufera Lake and the other with water coming from Pinedo WWTP. Citrus and vegetables fields in this area, irrigated with water from Turia River, were also sampled. In Murcia, soil samples were taken from citrus, vegetables and cereal (barley and wheat). Fields were irrigated with groundwater from the aquifer “Campo de Cartagena” that is around 150 m deep and impermeable. Soil from barley and wheat fields in Segovia was sampled as it is a representative area of dry-land crops that is irrigated using groundwater from a more superficial and permeable aquifer (“Los Arenales”).

4.2.2 Soil physical-chemical properties

Soil samples were air-dried over night at room temperature and standard soil analyses were carried out. Three soil granulometric fractions (sand, silt, clay) were determined for each sample following the pipette method. Soil pH was measured in a 1:5 (soil/distilled water) extract, shaken for 5 min and left standing for 2 h. OM was analyzed by the Walkley–Black method. USDA soil taxonomy classification was used (Soil Survey Staff, 2006). All the physical-chemical properties of the soils sampled and the soil taxonomy classification of Valencia, Murcia and Segovia are described in Table 4.2.

Table 4.2 Soil sample properties and USDA classification

Area	Crop	USDA	n ^a	pH (1:5)	% Sand	% Clay	% OM
Segovia	cereal	alfisol	1 - 4, 9	6.2 - 9.1	21 - 97	1 - 55	0.2 - 4.3
	cereal	inceptisol	5, 7	5.5 - 7.8	55 - 72	16 - 31	0.7 - 1.2
	cereal	psamments	6, 8	7.6 - 8.2	53 - 63	11 - 18	1.1 - 2.6
Murcia	citric	xerosols	10 - 12	7.8 - 8.3	23 - 57	21 - 30	1.6 - 2.1
	vegetable	xerosols	13 - 14	8.2 - 8.3	34 - 56	20 - 21	1.5 - 1.6
	cereal	xerosols	15 - 17	8.1 - 8.2	34 - 43	11 - 38	0.9 - 1.5
Valencia	vegetable	fluvents	18 - 20	8.2 - 9	30 - 40	32 - 45	3.2 - 3.9
	citric	fluvents	21 - 23	7.9 - 8.4	36 - 39	37 - 39	2.9 - 3.4
	rice	fluvaquents	24 - 31	8 - 8.4	17 - 36	37 - 50	3.3 - 4.7

^a n: sample number, OM: Organic Matter

4.2.3 Reagents

Acetonitrile (ACN) and ethyl acetate (EtAc), residue analysis grade, and ammonium hydroxide (NH_4OH) $\geq 32\%$ were purchased from Scharlab (Barcelona, Spain). Anhydrous sodium sulfate was obtained from Aldrich (Steinheim, Germany), heated for 24 h at 180 °C and, after cooling, stored in a dark vessel in a desiccator before use. All the pharmaceutical chemicals (purity $\geq 98.8\%$), the derivatization agent N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA, purity $\geq 95\%$) with 1% tert-butyldimethylchlorosilane (TBDMCS) and 96% formic acid were purchased from Aldrich (Steinheim, Germany).

Separate stock solutions of individual compounds made up at 50 $\mu\text{g mL}^{-1}$ were prepared in ACN and stored at -18 °C. A working mixture solution containing each compound at 1 $\mu\text{g mL}^{-1}$ was prepared weekly by dilution of the stock solution in ACN. Table 4.1 reports the pharmaceuticals selected in this study, showing their therapeutic class, and their physical-chemical properties.

4.2.4 Instruments

For extraction process, an ultrasonic water bath (Raypa, Barcelona, Spain) was used in the extraction step. The generator of this ultrasonic water bath has an output of 150 W and a frequency of 35 kHz. A vacuum manifold (Supelco, Visiprep,

Madrid, Spain) was employed for collecting the extracts that were then evaporated to dryness using an evaporator (Genevac EZ-2 NET Interlab, S.A.L., Spain).

All measurements were performed by GC-MS with an Agilent 6890 gas chromatograph (Waldbronn, Germany) equipped with an automatic injector, Model HP 7683, and an inert mass spectrometric detector (MSD), Model HP 5973N, equipped with an inert ion source. A fused silica capillary column ZB-5MS, 5% phenyl polysiloxane as nonpolar stationary phase (30 m $\times$ 0.25 mm i.d. and 0.25 μ m film thickness), from Phenomenex (Torrance, CA), was used for the analysis. Operating conditions were as follows: injector port temperature 285 $^{\circ}$ C; helium (purity 99.995%) as carrier gas at a flow-rate of 1.2 mL min $^{-1}$ and pulsed splitless mode (pulsed pressure 45 psi = 310 kPa for 1.5 min) with the splitless injector purge valve activated 1.5 min after injection of 2 μ L volume into a liner with deactivated glass wool. The column temperature was maintained at 80 $^{\circ}$ C for 0.5 min, then programmed at 20 $^{\circ}$ C min $^{-1}$ to 285 $^{\circ}$ C and held for 2 min. The total analysis time was 12.75 min and the equilibration time was 3 min.

MSD was operated in electron impact ionization mode with an ionizing energy of 70 eV, an ion source temperature of 230 $^{\circ}$ C and a quadrupole temperature of 150 $^{\circ}$ C. The electron multiplier (EM) voltage was operated with a gain of 3 and a solvent delay of 7.5 min. Table 4.3 lists the compounds along with their retention times and selected ions. The target and qualifier abundances were determined by injection of standards under the same chromatographic conditions using full-scan with the mass/charge ratio ranging from 45 to 450 m/z. The compounds were confirmed by their retention times and the identification of target and qualifier ions. Retention times must be within $\pm$ 0.2 min of the expected time and qualifier-to-target ratios within a 20% range for positive confirmation.

Table 4.3 Retention times (t_R , min) and selected ions (m/z) of the compounds studied

Name	t_R	T ^a	Q1 ^b	Q2 ^b
Clofibric acid-tbdms	7.93	143	271	185
Ibuprofen-tbdms	8.13	263	264	117

Salicylic acid-tbdms	8.66	309	310	195
Allopurinol-ditbdms	9.16	307	308	193
Paracetamol-ditbdms	9.33	322	248	323
Gemfibrozil-tbdms	9.76	243	179	307
Fenoprofen-tbdms	9.98	299	197	206
Amitriptyline	10.07	58	202	277
Metoprolol-tbdms	10.30	72	324	223
Naproxen-tbdms	10.40	287	185	288
Mefenamic acid-tbdms	10.96	298	224	355
Ketoprofen-tbdms	10.98	311	295	105
Carbamazepine-tbdms	11.26	193	194	293
Diclofenac-tbdms	11.47	352	354	214
Fenofibrate	11.57	121	273	139

^a T: target ion; ^b Q1 and Q2: qualifier ions

4.2.5 Sample preparation

Two Whatman No. 1 paper circles of 2 cm diameter (Whatman, Maidstone, UK) were placed at the end of a 20 mL glass column (Normax, Portugal) and anhydrous sodium sulfate (3 g) was added as a layer over the paper filter then, sieved soil (5 ± 0.001 g) was placed in the column. For the recovery studies, soil samples were previously fortified with a mixture of the different pharmaceuticals to reach final concentrations of 20-10-5 ng g⁻¹. Samples were left 60 min before extraction to reach equilibrium. ACN containing 2% of NH₄OH (8 mL) was added to the column that was placed for 15 min in an ultrasonic water bath at room temperature. The water level in the bath was adjusted to equal the extraction solvent level inside the columns, which were supported upright in a tube rack and closed with one-way stopcocks. After extraction, the columns were placed on the multiport vacuum manifold where the solvent was collected in graduated tubes, samples were washed with 1 mL of additional basic solvent (ACN containing 2% of NH₄OH), and extracts were evaporated to dryness. The soil samples were extracted a second time in the ultrasound bath (15 min) with 8 mL of ACN containing 2% of formic acid and samples were then washed with 1 mL of acidic solvent. The extracts were collected in the graduated tubes used in the first extraction step, evaporated to dryness and reconstituted to 1 mL with ACN. An aliquot (100 µL) of the extract

was transferred into a 2 mL reaction vial, followed by the addition of 50 μL of MTBSTFA:TBDMCS (99:1, v/v). The vials were closed and the mixture left to react for 1 h at 70 $^{\circ}\text{C}$ before the GC-MS analysis. Chromatographic standards were prepared spiking blank soil extracts.

4.2.6 Gas chromatography-mass spectrometry analysis

The quality assurance and quality control criteria used for this method included analyses of laboratory blanks (solvent blanks) and laboratory control samples. One laboratory blank was run with each set of samples to check for contamination from the preparative steps and to demonstrate laboratory background levels. Using the proposed procedure, background levels of laboratory blanks were below the detection limits. Control soil samples were used in the recovery assay. A recovery in the range of expected levels was carried out in each set of samples to confirm that results were in the accepted recovery range. Solvent was injected every 4 samples and after each sample containing potentially high levels of the target contaminants to avoid possible cross-contamination. To overcome memory effects, the inlet was flushed every day, prior to the analysis of samples, by heating at 300 $^{\circ}\text{C}$ for 30 min. Repeatability and reproducibility were determined at two concentrations, namely 5 and 10 ng g^{-1} . Repeatability was evaluated by performing extraction and injection of both solutions 10 times on the same day. To determine reproducibility, extraction and injection were performed three times per day on six different days for each level.

A standard statistical analysis (mean, median, and standard deviation) was carried out to determine the levels of pharmaceutical compounds in soil. Significant differences between means (crops and agricultural areas) were assessed through non-parametric ANOVA and Kruskal-Wallis tests ($\alpha = 0.01$). To study the relationship between soil properties and levels of pharmaceutical compounds found in soil, canonical correlation analyses (CCorA) were used, where the aim was to maximize the covariance between two sets of variables and to minimize their respective variance (Jobson, 1992). All the statistical analyses were carried out using the XLSTAT (Addinsoft Version 2012.2.02).

4.3 Results and discussion

4.3.1 *Sample preparation*

ACN and EtAc were evaluated as extraction solvents in the optimization of the extraction of the studied pharmaceutical compounds from soil. Although there were only slight differences in recovery yields with both solvents, the GC-MS chromatograms showed less interferences when ACN was used as the extraction solvent (data not shown). Moreover, ACN is the solvent recommended to carry out the derivatization process; hence, it was selected as extraction solvent. Nevertheless, when UAE was initially performed using ACN, extraction yields > 70% were only achieved for three compounds (paracetamol, carbamazepine and fenofibrate (Fig. 4.1).

An increase of the polarity of the solvent was considered, and ACN with 10% of methanol was assayed, but no significant improvement was observed with this modification. In order to enhance the recovery of acidic compounds, such as clofibric, salicylic and mefenamic acids, ACN containing 2% of formic acid was tested. These acidic compounds showed a clear improvement (Fig. 4.1), but the recoveries were still very low for compounds like allopurinol, amitriptyline and metoprolol. For this reason, another extraction step with ACN containing 2% NH_4OH (Li et al., 2013) was implemented in the UAE procedure before the extraction with acidic ACN. As shown in Fig. 4.1, recoveries with this two-step UAE were close to 100% for all compounds except for allopurinol, which presented recoveries around 50%, but with good reproducibility. A purification step of sample extracts was not necessary, and clean chromatograms without interferences from other components were obtained with this UAE method.

Figure 4.1 Comparison of the extraction efficiency of different solvents in samples spiked at 20 ng g⁻¹, n=4

4.3.2 Determination by gas chromatography-mass spectrometry

Table 4.3 lists the compounds with their retention times, and their target and qualifier ions. The GC method was optimized, seeking to reduce time and increase the sensibility in the determination of our target analytes. Different inlet temperatures, 325, 300, 275 and 250 °C, were evaluated and 275 °C was selected because it provided the best results in terms of abundance and peak shape. The oven was programmed to start at 60, 70, 80 or 90 °C and reach 285 °C at 20 °C min⁻¹ and an initial oven temperature of 80 °C was selected because it provided high peak abundances while reducing the analysis time from 17.5 min to 12.25 min.

Figure 4.2 A GC-MS chromatogram of a blank soil extract spiked at 10 ng g^{-1}

Prior to the GC-MS determination, the studied analytes need to be derivatized. The derivation agent MTBSTFA with TBDMCS as catalyst was selected, as it was used in various works where pharmaceutical compounds are derivatized before the GC determination (Rice and Mitra, 2007). After testing different oven temperatures and reaction times, the derivatization at 70°C during 1 h provided the highest reaction yields. Fig. 4.2 presents a chromatogram of a blank soil extract spiked at 10 ng g^{-1} obtained in the conditions indicated above.

4.3.3 Method validation

After optimization, the developed method was evaluated in terms of linearity, accuracy, precision, reproducibility, repeatability and detection limits before it was used to determine pharmaceutical residues in soil.

The linearity of the method was evaluated injecting seven blank soil extracts spiked at concentration ranging from 0.2 to 20 ng g^{-1} for all the studied compounds. Each calibration solution was injected four times. A good linearity was obtained with correlation coefficients equal or higher than 0.999 for all the compounds studied.

The accuracy of the method was evaluated performing the recovery of target analytes from soil samples spiked with standard solutions at three concentration levels 20, 10 and 5 ng g⁻¹. Good recoveries, ranging from 80 to 115%, were obtained for all the compounds except for allopurinol, which were near 50% with good reproducibility (Table 4.4). In this work, higher extraction yields were achieved for fenofibrate and diclofenac in comparison to those obtained in soil using pressurized liquid extraction (Vazquez-Roig et al. 2011). Our results are similar to those reported by other authors that have applied microwave assisted extraction for the determination of pharmaceuticals in soil (Azzouz and Ballesteros, 2012).

The repeatability and reproducibility were determined at two concentrations, 5 and 10 ng g⁻¹. Repeatability values $\leq 8.0\%$, expressed as relative standard deviation (RSD), were obtained for most of the compounds, indicating good precision. The highest reproducibility RSD value was 12%, also indicating the robustness of the method.

Limits of detection (LOD) and quantification (LOQ) of the developed method were determined using ten replicates of soil extracts, spiked at 0.2 ng g⁻¹. The equation to calculate the LOD was the following: $LOD = t_{99} \times S$, where t_{99} is the Student's value for a 99% confidence level and $n-1$ degrees of freedom and S is the standard deviation of the replicate analyses. The LOQ was calculated as 10 times the standard deviation of the results of the replicate analysis used to determine LOD. Low limits were obtained due to the high selectivity and sensitivity of GC-MS, allowing the determination of these compounds at the trace levels found in soil. As shown in Table 4.4, LODs ranged from 0.04 to 0.24 ng g⁻¹ and LOQs from 0.14 to 0.65 ng g⁻¹, being amitriptyline, metoprolol and fenofibrate the compounds with the highest limits. The LODs and LOQs determined in this work are lower than those reported by other authors (Vazquez-Roig et al., 2011).

Table 4.4 Recoveries (%) with their relative standard deviations (RSD %, $n=8$) and limit of detection and quantification (LOD, LOQ, ng g^{-1} , $n=10$) of the studied contaminants

Compound	Recovery $\pm$ RSD (%)			LOD	LOQ
	20 ng g^{-1}	10 ng g^{-1}	5 ng g^{-1}		
Clofibric acid	106.6 $\pm$ 3.7	112.8 $\pm$ 1.7	104.5 $\pm$ 6.4	0.14	0.38
Ibuprofen	104.4 $\pm$ 3.4	102.8 $\pm$ 3.6	97.8 $\pm$ 3.4	0.07	0.21
Salicylic acid	103 $\pm$ 4.2	93.8 $\pm$ 4.1	80.3 $\pm$ 3.3	0.14	0.38
Allopurinol	53.0 $\pm$ 1.7	40.8 $\pm$ 2.2	50.0 $\pm$ 5.8	0.07	0.21
Paracetamol	86.2 $\pm$ 4.7	97.4 $\pm$ 7.2	92.0 $\pm$ 4.4	0.07	0.24
Gemfibrozil	84.2 $\pm$ 4.6	108.5 $\pm$ 1.6	92.8 $\pm$ 2.9	0.04	0.16
Fenoprofen	109.0 $\pm$ 4.0	102.6 $\pm$ 3.0	100.8 $\pm$ 4.7	0.09	0.26
Amitriptyline	98.0 $\pm$ 2.9	105.1 $\pm$ 2.1	101.8 $\pm$ 3.1	0.24	0.65
Metoprolol	102.5 $\pm$ 1.8	87.3 $\pm$ 2.5	96.3 $\pm$ 2.4	0.18	0.55
Naproxen	111.6 $\pm$ 3.2	101.5 $\pm$ 1.7	98.5 $\pm$ 4.9	0.04	0.14
Mefenamic acid	115.1 $\pm$ 4.8	107.5 $\pm$ 3.3	94.3 $\pm$ 3.6	0.16	0.42
Ketoprofen	105.4 $\pm$ 4.7	99.2 $\pm$ 1.6	95.6 $\pm$ 4.5	0.14	0.38
Carbamazepine	107.8 $\pm$ 3.7	92.8 $\pm$ 3.9	99.7 $\pm$ 4.7	0.16	0.44
Diclofenac	104.4 $\pm$ 3.3	97.5 $\pm$ 3.1	106.6 $\pm$ 3.2	0.16	0.48
Fenofibrate	108.1 $\pm$ 4.6	94.3 $\pm$ 4.5	105.8 $\pm$ 3.9	0.17	0.53

The matrix effect occurring in the GC analysis of some organic compounds has a negative impact on the accuracy of the results. There are several approaches to counteract matrix-induced effects, such as exhaustive cleanup-steps, the use of inert surfaces in the GC system, and different calibration methods (Sánchez-Brunete et al., 2008). Matrix effect was evaluated preparing two sets of calibration solutions, one set was solvent-based ranging from 1 to 100 ng mL^{-1} , and the other was prepared spiking blank soil extracts at the same concentrations. In both cases, a good linearity of the calibrations curves was obtained in the studied range. The slopes obtained by plotting seven concentration levels against peak area, following linear regression analysis, were compared. A significant increase of the chromatographic response was observed for ketoprofen, carbamazepine, diclofenac and fenofibrate. Plots of calibration curves for metoprolol (without matrix effect) and fenofibrate (with a significant matrix effect) in neat solvent (SC) and matrix-matched (MC) soil extracts are shown in Fig. 4.3. Analysis of pharmaceuticals in neat solvent produced for most compounds calibration curves

with lower slopes when compared to matrix matched standards thus, quantification was carried out using matrix-matched standards.

Figure 4.3 Comparison of calibration curves of metoprolol and fenofibrate obtained by injection of standards in neat solvent (SC, ◆) and spiked soil extracts (MC, ■)

4.3.4 Analysis of real samples

The proposed method was applied to analyze pharmaceutical drug residues in soil samples collected from agricultural fields. Table 4.5 summarizes the range of concentrations found together with the detection frequencies for each compound. Three out of the fifteen pharmaceuticals studied (amitriptyline, ketoprofen and diclofenac) were not detected in any of the samples and one compound

(metoprolol) was detected but not quantified. This fact could be explained by their low consumption and also because some of them have a low soil adsorption (Lin and Gan, 2011; Xu et al., 2009a; Xu et al., 2009b). The rest of pharmaceuticals studied were found at concentration levels up to 47 ng g⁻¹. Fig. 4.4 shows a representative GC-MS chromatogram of a soil sample containing ibuprofen (0.7 ng g⁻¹) and salicylic acid (6.8 ng g⁻¹).

Table 4.5 Concentration of studied pharmaceutical compounds in soil

Compound	Range (ng g ⁻¹)	Mean $\pm$ SD (ng g ⁻¹)	Detection rate (%)
Clofibric acid	n.d - 0.7	0.7 $\pm$ 0.0	3
Ibuprofen	n.d - 1.5	0.5 $\pm$ 0.4	81
Salicylic acid	1.4 - 37.1	4.4 $\pm$ 6.2	100
Allopurinol	n.d - 47	1.3 $\pm$ 16.2	45
Paracetamol	n.d - 0.5	0.4 $\pm$ 0.1	71
Gemfibrozil	n.d - 5.4	3.6 $\pm$ 2.1	6
Fenoprofen	n.d - 3.2	0.8 $\pm$ 1.1	42
Amitriptyline	n.d	n.d	n.d
Metoprolol	n.d - n.q	n.q	3
Naproxen	n.d - 5.9	0.7 $\pm$ 2.2	19
Mefenamic acid	n.d - 2.7	1.5 $\pm$ 0.8	32
Ketoprofen	n.d	n.d	n.d
Carbamazepine	n.d - 2.8	1.2 $\pm$ 1.0	45
Diclofenac	n.d	n.d	n.d
Fenofibrate	n.d - 7.8	7.0 $\pm$ 1.0	6

^a n = 31; n.d, not detected; n.q, not quantified

As shown in Table 4.5, salicylic acid was found in all samples, followed by ibuprofen (81%) and paracetamol (71%), which can be explained by the high consumption of these compounds by the population. The concentration of ibuprofen in all samples is relatively small (< 1.5 ng g⁻¹), due to its low soil adsorption and high biodegradability (Lin and Gan, 2011; Winker et al., 2010). In comparison with other studies on pharmaceuticals in soil, the levels found for the majority of compounds were similar to those reported in our study. In soil samples from a Spanish marsh area, (Vazquez-Roig et al., 2011) found some of the compounds studied in our work but with a low detection frequency and

concentration of fenofibrate and ibuprofen. In soil samples from USA (Xu et al., 2009b), clofibric acid, ibuprofen and naproxen were detected at slightly higher levels. Salicylic acid was reported at higher concentrations in samples from China (Chen et al., 2011) and in UK similar levels of carbamazepine were detected in soil (Walker et al., 2012).

Figure 4.4 Ion chromatograms of a rice soil extract containing ibuprofen (0.7 ng g^{-1}) and salicylic acid (6.8 ng g^{-1}) with the main ions of their mass spectra

To study the relation between soil properties (Table 4.2) and the concentration of the pharmaceutical compounds CCorA were applied. The CCorA results were plotted in Fig. 4.5 and proved to be highly significant. The first two factors (F1 and F2) in the multivariate analysis represented an 83% of total variance and showed, as expected, that the concentration of pharmaceutical compounds are negatively affected by the sand percentage in soil. On the other hand, OM, pH and clay content are positively related with carbamazepine, allopurinol and paracetamol. Regarding carbamazepine and allopurinol, they tend to group with a higher OM and clay content, which may favor the retention of these compounds in the soil matrix.

Figure 4.5 Ordination diagram based on the CCorA of the soil parameters (% OM, pH, Sand and % Clay) and levels of pharmaceutical compounds (ibuprofen, salicylic acid, allopurinol, paracetamol, fenoprofen, mefenamic acid and carbamazepine)

Table 4.6 shows the obtained data grouped into area of sampling and type of crop. Soil samples from rice fields showed the highest levels of ibuprofen, allopurinol, mefenamic acid and carbamazepine ($p < 0.01$). Gemfibrozil and fenoprofen were detected in half of the rice fields, which were irrigated with wastewater treatment plant (see Supplementary Information SI-Table 1).

Table 4.6 Statistical summary (mean $\pm$ standard deviation) of the levels of pharmaceutical compounds

	Crop			
	Cereal	Vegetables	Citrus	Rice
Ibuprofen	0.0 $\pm$ 0.1 (a)	0.0 $\pm$ 0.1 (a)	0.1 $\pm$ 0.2 (a)	0.4 $\pm$ 0.4 (b)
Salicylic acid	3.2 $\pm$ 2.2 (a)	2.8 $\pm$ 1.8 (a)	3.8 $\pm$ 11.4 (a)	4.8 $\pm$ 2.5 (a)
Allopurinol	0.0 $\pm$ 0.0 (a)	0.0 $\pm$ 3.5 (a)	0.0 $\pm$ 15.5 (a)	0.1 $\pm$ 0.7 (b)
Paracetamol	0.1 $\pm$ 0.1 (a)	0.1 $\pm$ 0.1 (a)	0.1 $\pm$ 0.2 (a)	0.1 $\pm$ 0.1 (a)
Fenoprofen	0.0 $\pm$ 0.7 (a)	0.0 $\pm$ 0.0 (a)	0.0 $\pm$ 1.0 (b)	0.0 $\pm$ 0.4 (a)
Mefenamic acid	0.0 $\pm$ 0.5 (b)	0.0 $\pm$ 0.0 (a)	0.0 $\pm$ 0.0 (a)	0.0 $\pm$ 0.4 (b)
Carbamazepine	0.0 $\pm$ 0.0 (a)	0.0 $\pm$ 0.3 (b)	0.0 $\pm$ 0.3 (b)	1.4 $\pm$ 1.0 (c)

	Agricultural Areas		
	Murcia	Segovia	Valencia
Ibuprofen	0.1 $\pm$ 0.1 (a)	0.1 $\pm$ 0.1 (a)	0.1 $\pm$ 0.3 (a)
Salicylic acid	3.5 $\pm$ 2.0 (a)	3.8 $\pm$ 2.3 (a)	3.9 $\pm$ 7.2 (a)
Allopurinol	0.0 $\pm$ 0.1 (a)	0.0 $\pm$ 0.1 (a)	0.1 $\pm$ 9.7 (b)
Paracetamol	0.0 $\pm$ 0.0 (a)	0.0 $\pm$ 0.1 (a)	0.1 $\pm$ 0.2 (b)
Fenoprofen	0.0 $\pm$ 0.0 (a)	0.3 $\pm$ 0.8 (b)	0.0 $\pm$ 0.7 (a)
Mefenamic acid	0.0 $\pm$ 0.0 (a)	0.1 $\pm$ 0.6 (b)	0.0 $\pm$ 0.3 (a)
Carbamazepine	0.0 $\pm$ 0.0 (a)	0.0 $\pm$ 0.0 (a)	0.5 $\pm$ 1.0 (b)

a, b and c: Significant difference ($p < 0.01$) between types of crop (Cereal, Vegetables, Citrus and Rice) and Agricultural areas (Murcia, Segovia and Valencia).

Regarding the area of sampling, Valencia showed the highest concentration of three compounds, paracetamol (although at low levels), allopurinol found (in 85.7% of the Valencia's samples), and carbamazepine (a pharmaceutical persistent in the environment (Williams et al., 2006)), present in all the samples. Moreover, fenoprofen (42% of the total samples) and mefenamic (32% of the total samples) were found predominantly in Segovia, in cereal crops. This fact could be explained by the type of irrigation (Shenker et al., 2011), with surface water in the area of Valencia, water from a shallow aquifer in Segovia and from a deeper and impermeable aquifer in Murcia.

Although the levels found of the compounds studied are relative low, the continuous release and chronic exposure to these substances make necessary monitoring their environmental levels to avoid adverse effects to aquatic life and the potential risk to human health (Stuart et al., 2012).

4.4 Conclusions

An analytical method has been developed for the simultaneous extraction and determination of 15 pharmaceutical compounds with diverse physical-chemical properties in different farmland soils. The developed method, based on an ultrasound assisted extraction procedure followed by GC-MS determination, is rapid, reliable and accurate, allowing the determination of these compounds in soil at the low levels expected.

The application of this method to the analysis of soil samples from agricultural fields in several Spanish areas showed the presence of various pharmaceutical compounds although at low concentrations, highlighting the ubiquitous presence of the widely used anti-inflammatory drugs ibuprofen, salicylic acid and paracetamol in the three areas studied, as well as fenoprofen in Segovia and allopurinol and carbamazepine in Valencia.

SPATIO-TEMPORAL DISTRIBUTION OF PYRETHROIDS IN SOIL IN MEDITERRANEAN PADDY FIELDS

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Abstract

Purpose The demand of rice by the increase in population in many countries has intensified the application of pesticides and the use of poor quality water to irrigate fields. The terrestrial environment is one compartment affected by these situations, where soil is working as a reservoir, retaining organic pollutants. Therefore, it is necessary to develop methods to determine insecticides in soil and monitor susceptible areas to be contaminated, applying adequate techniques to remediate them.

Materials and methods This study investigates the occurrence of 10 pyrethroid insecticides (PYs) and its spatio-temporal variance in soil at two different depths collected in two periods (before plow and during rice production), in a paddy field area located in the Mediterranean coast. Pyrethroids were quantified using gas chromatography–mass spectrometry (GC-MS) after ultrasound-assisted extraction with ethyl acetate. The results obtained were assessed statistically using non-parametric methods and significant statistical differences ($p < 0.05$) in pyrethroids content with soil depth and proximity to wastewater treatment plants were evaluated. Moreover, a geographic information system (GIS) was used to monitor the occurrence of PYs in paddy fields and detect risk areas.

Results and discussion Pyrethroids were detected at concentrations $\leq 57.0 \text{ ng g}^{-1}$ before plow and $\leq 62.3 \text{ ng g}^{-1}$ during rice production, being resmethrin and cyfluthrin the compounds found at higher concentrations in soil. Pyrethroids were detected mainly at the top soil and a GIS program was used to depict the obtained results, showing that effluents from wastewater treatment plants (WWTPs) were the main sources of soil contamination. No toxic effects were expected to soil organisms, but it is of concern that PYs may affect aquatic organisms, which represents the worst case scenario.

Conclusions A methodology to determine pyrethroids in soil was developed to monitor a paddy field area. The use of water from WWTPs to irrigate rice fields is one of the main pollution sources of pyrethroids. It is a matter of concern that PYs may present toxic effects on aquatic organisms, as they can be desorbed from soil. Phytoremediation may play an important role in this area, reducing the possible risk associated to PYs levels in soil.

Keywords: Soil, Wetlands, Geographical information system, Insecticides, Pyrethroids, Gas chromatography-mass spectrometry

4.5 Introduction

Rice (*Oryza sativa* L.) is the cereal grain most widely consumed and it represents the third highest worldwide production (<http://faostat.fao.org/site/339/default.aspx>). Its cultivation under hydric conditions is a very complex system due to water-soil interactions and anthropic interventions (Nawaz et al., 2013). Moreover, the high demand of water to keep the fields continuously flooded and the low rainfall in the Mediterranean areas make necessary the use of poor quality water such as regained water from wastewater treatment plants (WWTPs). Unfortunately, as some authors have pointed out, the treatment of this water will not satisfactorily remove all contaminants (Alonso et al., 2012; Campo et al., 2013; Feo et al., 2010; Weston et al., 2013). Hence the spreading of contaminants, such as insecticides and biocides, through agricultural soils may take place (Arias-Estevez et al., 2008), where they can be considered pseudo-persistent due to their daily release into the environment.

Table 4.7 Properties of the target compounds and abbreviations

Chemical	Abbreviation	Log K _{ow}	Log K _{oc}	Solubility (mg L ⁻¹)	Soil aerobic half-life (days)	Soil anaerobic half-life (days)
Resmethrin	RESM	5.4 ^a	5 ^e	<1 ^c	-	-
Bifenthrin	BIFE	6.0 ^a	5.4 ^b	0.1 ^a	96.3 ^b	425 ^b
Fenpropathrin	FENP	6.0 ^a	5 ^e	0.014 ^a	22 ^d	276 ^d
λ-Cyhalothrin	CYHA	6.9 ^a	5.5 ^b	0.003 ^a	42.6 ^b	-
Permethrin	PERM	6.5 ^a	5.4 ^b	-	39.5 ^b	197 ^b
Cyfluthrin	CYFL	5.9 ^a	5.1 ^b	0.002 ^a	11.5 ^b	33.6 ^b
α-Cypermethrin	CYPE	6.6 ^a	5.5 ^b	0.004 ^a	27.6 ^b	55 ^b
τ-Fluvalinate	FLUV	4.3 ^a	-	0.002 ^a	-	-
Esfenvalerate	ESFE	4.0 ^a	5.4 ^b	0.0002 ^a	38.6 ^b	90.4 ^b
Deltamethrin	DELT	6.1 ^a	-	<0.002 ^a	24 ^d	29 ^d

K_{ow}: octanol-water partition coefficient, K_{oc}: organic carbon-soil partition coefficient

^a: (Oros and Werner 2005)

^b: (Laskowski 2002)

^c: http://www.inchem.org/documents/pds/pds/pest83_e.htm#1.3.2

^d: http://www.cdpr.ca.gov/docs/registration/reevaluation/chemicals/environmental_fate.pdf

^e: http://www.pesticideinfo.org/Detail_Chemical.jsp?Rec_Id=PC34303

Pyrethroid insecticides (PYs) were derived from chrysanthemic acid to obtain more stable compounds in the environment. They have been intensively used in agricultural, industrial and urban areas (Amweg et al., 2005; Aznar et al., 2014b; Song et al., 2015), since they are a replacement of other banned pesticides, such as organochlorine and organophosphate pesticides. The occurrence of PYs is of concern because although they are retained in soil due to their hydrophobicity and low water solubility (see Table 4.7), PYs can be toxic to the aquatic life (Amweg et al., 2005; Song et al., 2015; Weston et al., 2005).

However, in contrast to the data of PYs levels documented in aquatic ecosystems, information on the levels of these insecticides in soil ecosystems is scarce. Given the universal dependence on hydric soils for rice production and their high ecological value, their maintenance in good environmental conditions is crucial. Hence, it is necessary to monitor the presence of PYs regularly and evaluate their potential risk to the environment (Huang et al., 2015).

The aim of this work was to monitor and assess the occurrence and distribution of PYs in soil samples collected from paddy fields in a Mediterranean region at different depths (0-40 and 40-60 cm) and during two campaigns (plow and rice production periods). To determine PYs in soil, a method based on ultrasound assisted extraction and gas chromatography-mass spectrometry (GC-MS) was developed. In addition, a geographical information system (GIS) was used to assess the main sources of pollution as well as to identify and indicate areas where PYs may be toxic and phytoremediation may be a good management practice to mitigate contamination (Mahabali and Spanoghe, 2014; Moore et al., 2009). To the best of our knowledge, this is the first time that these insecticides are studied and monitored in soil at different depths in paddy fields.

4.6 Materials and methods

4.6.1 Site description

The study was carried out in Albufera of Valencia, a Natural Park located in the Spanish eastern coast (Fig. 4.6). This area is a wetland composed of three distinct environments: the lake, the marsh area where rice is cultivated and the sand barrier. The area was formed due to sedimentary contributions of the Turia and Júcar Rivers closing a gulf in the Mediterranean Sea. In the 18th century the lake had an area of 300 km², but nowadays the lake's area is 23 km², being currently the largest freshwater lake in Spain. The lake's area reduction was caused by two main processes: the natural process of silting (sediments from both rivers over the years) and anthropogenic processes to gain land to produce rice over the last century (Pascual-Aguilar et al., 2015). This area is usually flooded due to rice production management and the presence of the water table near the soil surface. Following the Soil Taxonomy classification (Soil Survey Staff 2014), soils are defined as Entisols and Aridisols (Moreno-Ramón et al., 2015). These soils are carbonated, saline and show a moderate surface organic carbon content due to the rice management (incorporation of post-harvest residues).

Figure 4.6 Map of the sites sampled in the rice fields at the Natural Park in Valencia, Spain

The area studied is ruled by the rice production cycle (Fig. 4.7). It starts with a period of fallow when the lake reaches its maximum level, flooding part of the rice fields (November-January). In January, the gates connecting with the Mediterranean Sea are opened and the fields are drained reaching the lake its normal water level. From the end of February till May, paddy fields are dried, so they can be plowed and prepared prior to sowing. In May, the rice growing season starts and water flows around the whole park and the paddy fields are flooded again. In September, the period of harvest starts and paddy fields are drained to allow harvest by the heavy machinery, and the rice cultivation cycle will start again. Water inputs come from the Júcar and Turia Rivers that run south and north in the area of study, respectively. Due to the shortage of fresh water during summer, water from two WWTPs located 6-8 km from the lake (Fig. 4.6) is used to irrigate rice fields.

Figure 4.7 Hydrological cycle of rice production and the two sampling periods

4.6.2 Determination by gas chromatography-mass spectrometry

Standards and reagents, ethyl acetate (EtAc) and Florisil (magnesium silicate adsorbent, 150-250 μm , 60-100 mesh for chromatography) were purchased from Scharlab (Barcelona, Spain). Sodium sulfate (purity $\geq 99\%$) was obtained from Aldrich (Steinheim, Germany). Insecticides resmethrin (RESM), bifenthrin (BIFE), fenpropathrin (FENP), λ -cyhalothrin (CYHA), permethrin (PERM),

cyfluthrin (CYFL), α -cypermethrin (CYPE), τ -fluvalinate (FLUV), esfenvalerate (ESFE) and deltamethrin (DELT) (purity 99%) were supplied by Riedel-de Haën (Seelze, Germany), whereas the surrogate standard trans-permethrin-D6 (purity > 99%) was supplied by Symta (Madrid, Spain). The list of investigated compounds is shown in Table 4.7 along with their physicochemical properties. Individual stock solutions of each compound at 500 $\mu\text{g mL}^{-1}$ were prepared in EtAc and stored in the darkness at 4 °C up to 8 weeks. A mixed stock solution of 1000 ng mL^{-1} containing all analytes was prepared by dilution with EtAc of the individual stock solutions. A working mixture solution at 200 ng mL^{-1} was prepared weekly by dilution with EtAc of the mixed stock solution. A solution containing the surrogate standard was prepared in EtAc at the same concentration as the working mixture solution.

Apparatus, glass columns (20 mL) of 10 cm x 20 mm i.d., Afora, Spain, and Whatman No.1 filter paper circles of 2 cm diameter (Whatman, Maidstone, UK) were used. An ultrasonic water bath (Raypa, Barcelona, Spain) was used in the extraction step. A vacuum manifold (Supelco, Visiprep, Madrid) was employed to collect the extracts. Gas chromatography-mass spectrometry (GC-MS) analysis was performed with an Agilent 6890 (Waldbronn, Germany) gas chromatograph equipped with a mass spectrometric detector, Model HP 5977A. The operating conditions are summarized in SI- Table 2. The target and qualifier abundances were determined by injection of standards under the same chromatographic conditions using full-scan with the mass/charge ratio ranging from 50 to 400 m/z . The compounds were confirmed by their retention times, the identification of target and qualifier ions and the determination of qualifier to target ratios. Retention times must be within ± 0.1 min of the expected time and qualifier-to-target ratios within a 20% range for positive confirmation. The quantification was accomplished by calibration with the surrogate standard at 10 ng g^{-1} . To reduce possible memory effects of the column, prior to the analysis of samples, the inlet was flushed by heating at 300 °C for 30 min and procedural blanks were analyzed after every four samples.

4.6.3 Samples

Sample collections, soils from thirty-three sites were sampled in rice fields at two different depths (0-40 and 40-60 cm). Sampling points were located with a virtual reference station (Leica GPS 1200) that supplied the UTM (universal transverse mercator) coordinates for the geostatistical treatment (SI-Tables 3-4). First layer (0-40 cm) of soil is the plow surface in which rice crop residues are incorporated. On the other hand, the deeper layer of soil (40-60 cm) remains unchanged and is usually saturated by the presence of a saline water table. A stainless steel Eijkelkamp auger was used for soil sampling according to a stratified sampling design. After soils were sampled, they were transported to the laboratory, where they were air dried at room temperature (21 °C) in darkness to avoid PYs photodegradation (Katagi, 2004), sieved through a 2 mm mesh, thoroughly mixed and kept frozen (-18 °C) in glass containers until analysis. Two sampling campaigns were carried out. The first campaign at the end of February, before plow period, when fields are dried to prepare them to produce rice and the second sampling was in July when the fields are flooded (Fig. 4.7).

Physical-chemical properties of soil samples, soil properties may affect insecticides behavior (transport, persistence, leaching, etc.) and, therefore, they were determined. Granulometric fractions of soil (sand, silt, clay) were determined for each sample following the Bouyoucos method. Soil pH was measured in a 1:2.5 (soil/distilled water) extract shaken for 15 min and measured after 2 h. Soil organic carbon was analyzed by the ignition method and carbonate content by Bernard calcimeter method. Finally, soil salinity was measured by the electrical conductivity (EC) 1:5 (soil/distilled water) (SI-Tables 3-4). All the methodologies described in this paper have been carried out according to Soil Survey Staff (2009).

Sample preparation, extraction of PYs from soil was carried out by ultrasound assisted extraction as one of the most favorable techniques to extract the target compounds (Albaseer et al., 2010). Briefly, 1 g of sieved soil was placed in a glass column containing 1 g sodium sulfate and 1.5 g of Florisil over a paper filter and

a frit. Soil samples were extracted twice for 15 min in an ultrasonic water bath with 5 mL EtAc and an additional 1 mL was used to wash the glass material. The combined extracts were collected in 10 mL graduated tubes using a multiport vacuum manifold, concentrated to 0.1 mL using a gentle stream of air and analyzed by GC-MS. To counteract matrix effects a surrogate standard was used.

4.6.4 Method validation and quality control

In order to evaluate the method developed for the detection of insecticides in soil, different quality parameters were studied: recoveries, reproducibility, linearity and sensitivity.

Table 4.8 Mean recoveries (%) with their relative standard deviation (RSD, %), limit of detection (LOD, ng g⁻¹) and limit of quantification (LOQ, ng g⁻¹) of the studied insecticides

Compounds	Fortification levels (ng g ⁻¹) ^a				LOD ^b	LOQ ^b
	10		2			
	Mean	RSD	Mean	RSD		
RESM	104	3	75	1	0.4	1.2
BIFE	103	2	100	8	0.1	0.3
FENP	107	3	107	3	0.2	0.7
CYHA	95	9	96	9	0.1	0.4
PERM	94	5	98	11	0.1	0.5
CYFL	97	4	102	4	0.3	1.1
CYPE	96	7	97	10	0.2	0.8
FLUV	92	8	107	4	0.3	1.0
ESFE	101	8	106	7	0.3	1.0
DELT	99	7	75	2	0.3	0.9

^a: (n=8); ^b: (n=10)

For the recovery studies, samples were previously fortified with a mixture of the different analytes to reach final concentrations of 10 and 2 ng g⁻¹ and the labeled surrogate standard at 10 ng g⁻¹. They were kept at room temperature overnight to allow solvent evaporation. The recoveries obtained for all the studied compounds were satisfactory, ranging from 75 to 107% (Table 4.8). The precision of the analytical procedure, expressed as relative standard deviations (RSD, %) of the analysis of four replicates, ranged between 1 and 11% (Table 4.8).

Limits of detection (LODs) and quantification (LOQs) of the developed method were determined using ten replicates of soil extracts, spiked at 1 ng g⁻¹. The equation to calculate the LOD was the following: $LOD = t_{99} \times S$, where t_{99} is the Student's value for a 99% confidence level and $n-1$ degrees of freedom and S is the standard deviation of the replicate analyses. The LOQ was calculated as 10 times the standard deviation of the results of the replicate analysis used to determine LOD. Low limits were obtained due to the high selectivity and sensitivity of GC-MS. As shown in Table 4.8, LODs ranged from 0.1 to 0.4 ng g⁻¹ and LOQs from 0.3 to 1.2 ng g⁻¹ allowing the detection of insecticides at trace levels in soil samples.

A multipoint calibration curve with five standard solutions at different concentration levels (from 1 to 100 ng g⁻¹), appropriate to the levels found in soil samples, was used. The surrogate standard was added at the concentration of 10 ng g⁻¹ for all levels.

4.6.5 Risk assessment

In order to carry out the risk assessment, the concentrations of PYs in soil as well as in water were considered, because PYs can be desorbed into fresh water due to the hydric conditions of the area. Therefore, the maximum equilibrium concentration expected in water ($ECEW_{max}$) of PYs was calculated. The maximum concentration of each PY found in the soils studied and the adsorption coefficient (K_d) of each compound were used as shown in the following equation:

$$ECEW_{max} = \text{Concentration in soil}_{max} / K_d$$

The K_d of each PY was calculated using the organic carbon-soil partition coefficient (K_{oc}) values from Table 4.7 and the corresponding value of the organic carbon (OC) from the top layer of soil (Table 4.9).

$$K_d = K_{oc} \times OC$$

Moreover, with the aim of evaluating the possible toxic effects of PYs in the studied area, the potential toxicity of PYs was assessed for soil and aquatic organisms. It was accomplished by comparing the ecotoxicological index of lethal dose (LC_{50}) of terrestrial organisms, reported in previous publications (Commission, 2002, 2004, 2005), with the levels of PYs found in soil in the present study. In the case of aquatic organisms, the calculated $ECEW_{max}$ was compared with the median effective concentration (EC_{50}) or the no observed effect concentration (NOEC) values for three aquatic species obtained from previously published studies (Fojut et al., 2012; Hill, 1985; Maund et al., 2012a).

4.6.6 Software

Standard statistical analyses were carried out with SPSS statistical program (Mann-Whitney and Spearman correlation test) to determine the levels of insecticides in soil. The use of non-parametric methods was confirmed by the outcome of Shapiro-Wilk test, which did not show a normal distribution. The compounds included in the statistical analysis were those with detection rates higher than 70%. To create the matrix, a pretreatment of the data was necessary. Values below quantification limit were converted in numerical results, by adding a value of half their limit of quantification.

Cartography was performed by the Bayesian maximum entropy method (BME) which allowed a complete stochastic description of those non-sampling areas (Money et al., 2009). The maps showed gentle transitions between the different mapping units which reflected the normal behavior of continuous variables like water contaminants. The software used was ARCGIS 9.3 with a BMEGUI module.

4.7 Results and discussion

4.7.1 Spatial and temporal distribution of pyrethroids in soil

In general, soils sampled had an electrical conductivity of 0.72-0.95 dS m⁻¹ and many of them were calcareous. The maximum values registered in the $EC_{1/5}$ (2.89 dS m⁻¹) revealed that there was soil salinization in the area (Table 4.9). Regarding

particle size, 39% of the samples were classified as silty clay, followed by 30% of the samples classified as clay loam according to USDA textural classes.

Table 4.9 *Soil characteristics*

	0-40 cm			40-60 cm		
	Min.	Max.	Mean $\pm$ RSD	Min.	Max.	Mean $\pm$ RSD
EC (dS m ⁻¹)	0.4	2.0	0.6 $\pm$ 0.4	0.4	2.8	0.7 $\pm$ 0.5
Ph	7.2	8.1	7.6 $\pm$ 0.2	0.5	8.4	7.6 $\pm$ 1.3
Carbonate (g kg ⁻¹)	278	502	358 $\pm$ 38	260	530	357 $\pm$ 55
OC (g kg ⁻¹)	18.6	104.9	31.2 $\pm$ 18.5	4.3	60.1	23.4 $\pm$ 11.3
SOC (g kg ⁻¹)	0.0	1.1	0.4 $\pm$ 0.3	0.0	1.2	0.4 $\pm$ 0.3

EC: electrical conductivity; OC: organic carbon; SOC: soluble organic carbon

The developed method was applied to the analysis of PYs in soils from paddy fields collected in two periods, before plow and during rice production. The overall results obtained are summarized in Table 4.10, showing the range of concentrations found and the detection frequencies for each compound. The complete set of concentration values are shown in SI-Tables 5-8.

Before plow period (March), when there is no water flowing through the rice fields, six out of the ten PYs studied were detected (Table 4.10). The compounds most often detected were RESM, CYFL, CYPE and ESFE up to 70% of the analyzed samples, with levels up to 57 ng g⁻¹ in the case of ESFE near to an area of discharge of the North WWTP (Fig. 4.8). Four PYs studied were not detected in any of the samples (BIFE, PERM, FLUV and DELT) and CYHA was quantified only in one sample. However, during rice production (July), when freshwater flows through the fields, the soil sampled presented a higher detection rate (almost 100%) of RESM, BIFE, FENP, CYFL, CYPE and ESFE, being seven PYs detected, up to 62.3 ng g⁻¹ for RESM nearby the area close to the North WWTP (see SI-Table 7).

Table 4.10 Levels (ng g⁻¹) and detection rate (%) of PYs during plow and rice production period from 33 soil sampling points at different depths

Plow period									
	0-40 cm					40-60 cm			
	Min.	Max.	Mean	% det.		Min.	Max.	Mean	% det.
RESM	0.0	52.0	19.5	97.0	RESM	1.6	53.4	23.9	100.0
BIFE	nd	nd	nd	Nd	BIFE	nd	nd	nd	nd
FENP	nd	44.9	6.2	24.2	FENP	13.7	29.8	8.1	42.4
CYHA	nd	nd	nd	Nd	CYHA	nd	1.5	0.0	3.0
PERM	nd	nd	nd	Nd	PERM	nd	nd	nd	nd
CYFL	nd	54.2	20.3	90.9	CYFL	nd	27.3	11.0	81.8
CYPE	nd	17.9	5.0	69.7	CYPE	nd	11.1	1.7	42.4
FLUV	nd	nd	nd	Nd	FLUV	nd	nd	nd	nd
ESFE	nd	57.0	19.4	84.8	ESFE	nd	46.3	19.7	90.9
DELT	nd	nd	nd	Nd	DELT	nd	nd	nd	nd
Rice production period									
	0-40 cm					40-60 cm			
	Min.	Max.	Mean	% det.		Min.	Max.	Mean	% det.
RESM	2.0	62.3	23.2	100.0	RESM	4.5	57.9	28.2	100.0
BIFE	nq	32.2	4.2	100.0	BIFE	nq	13.5	3.0	100.0
FENP	nd	47.5	13.9	97.0	FENP	nq	40.2	13.3	100.0
CYHA	nd	20.7	3.0	93.9	CYHA	nd	41.1	7.7	97.0
PERM	nd	nd	nd	Nd	PERM	nd	nd	nd	nd
CYFL	nq	39.0	15.7	100.0	CYFL	nq	54.9	22.0	100.0
CYPE	nd	26.2	3.9	84.8	CYPE	nd	31.9	4.1	97.0
FLUV	nd	nd	nd	Nd	FLUV	nd	nd	nd	nd
ESFE	nd	57.1	23.4	87.9	ESFE	nd	48.8	20.3	97.0
DELT	nd	nd	nd	Nd	DELT	nd	nd	nd	nd

nd: not detected; nq: not quantified

Figure 4.8 Spatial representation of CYFL and ESFE, A) First sampling of top soil (0-40 cm depth), B) First sampling of deep soil (40-60 cm depth)

The presence of PYs could be explained by their application to local crops as well as their non-efficient removal during WWTPs processes (Campo et al., 2013). During rice production period, when there is not enough freshwater to keep fields flooded to produce rice properly, the use of regained water from WWTPs is required. However, effluents from WWTPs are an important source of PYs release, as reported by other authors (Weston et al., 2013), and as a result these compounds are introduced into the environment increasing the contamination of soil, which is an important reservoir. The outcome of non-parametric statistical analyses (Table 4.11) showed that water source had a clear influence over PYs levels in the area, particularly for CYFL and ESFE.

Table 4.11 Statistical analysis of PYs levels in relation with soil depth and origin of irrigation water (Mann-Whitney and Spearman tests)

Factor	Subfactor	Average rank (Mann-Whitney)						
		RESM	CYFL	CYPE	ESFE	BIFE	FENP	CYHA
Water origin	WWTPs	70.3a ¹	79.6a	76.9a	85.2a	31.9a	29.2a	39.2a
	Rivers	64.6a	59.9b	61.3b	57.1b	34.3a	35.7a	30.6a
Soil depth	0-40	58.5a	69.4a	77.0a	67.2a	33.7a	33.2a	30.9a
	40-60	74.5b	63.6a	56.0b	65.8a	33.3a	33.8a	36.1a
		Spearman coefficients						
		RESM	CYFL	CYPE	ESFE	BIFE	FENP	CYHA
Distance to WWTP		0.216*	0.418**	0.254**	0.288**	0.046	0.006	0.3*

¹ Different letter means $p < 0.005$ (inside the same factor); * ($p < 0.05$); ** ($p < 0.001$)

The distribution in the area of these compounds shows that the main sources were the WWTPs (Fig. 4.8). In the case of BIFE, the increase of contamination along the area studied (Fig. 4.9) could be explained by its enrichment during transport by runoff (Gan et al., 2005), resulting in progressively higher pesticide levels in the soil downstream from the source. However, the contamination of CYFL and ESFE decreased along the park indicating that the marsh area may act as a buffer, retaining the contamination before reaching the lake. Therefore, phytoremediation may be a good management practice to mitigate contamination as it has been proven to work in wetlands (Mahabali and Spanoghe, 2014; Moore et al., 2009). Nevertheless, further work needs to be done to assess the main paths of pollutant

dissipation in the marsh area. *Arundo donax*. and *Typha angustifolia*, among the typical plants used in phytoremediation and rice may play an important role reducing the concentration of contaminants and improving the environmental conditions of the area studied.

Figure 4.9 Spatial representation of BIFE showing the areas marked in red, where BIFE levels (> 10.1 ng g⁻¹) may present a negative effect to aquatic invertebrates

4.7.2 Distribution of pyrethroids with soil depth

Soil organic carbon showed a decrease in depth due to the rice management in the area, because straw is incorporated in soil after the harvest at first 40 cm increasing its content in the top soil. The average content was around 31 g kg⁻¹ of soil. On the contrary, the soluble organic carbon showed an increase in depth, and this trend can be explained due to the hydric characteristics of soils. Soluble compounds were accumulated in depth because at 40-60 cm there was a permanent water table. In the upper parts, the water table can be intermittent depending on the crop management period.

In general, the target compounds tend to be found in the first 40 cm of soil, where higher content of organic matter is present. However, PYs concentrations against depth showed that CYFL, BIFE, FENP, CYHA and ESFE (Table 4.11) did not present that trend whereas RESM and CYPE show significant statistical differences (p value < 0.001). The compound RESM, which presents the highest water solubility of the studied family of insecticides (Table 4.7), may be translocated deeply under hydric conditions and accumulated at the second layer studied (40-60 cm). On the other hand, the low solubility in water of CYPE, and their application during rice production to eradicate common armyworm, may explain the accumulation of this insecticide in the top layer.

Comparing the maps generated by GIS depicted in Figure 4.8, it can be observed that CYFL and ESFE contamination on top soil matched the highest points of pollution at deep soil, which are nearby WWTPs discharge (Fig. 4.6). On the other hand, the adsorption of PYs is higher in organic matter and mineral particles with a large surface area (Zhou et al., 1995). Moreover, wetland soils due to natural conditions and paddy soils due to the rice management tend to accumulate organic matter in the surface layers. Thus, the presence of PYs in the area studied may be explained by the content of organic matter in soils, where PYs can be bounded making more unlikely their degradation.

The correlation between distance to the WWTP and insecticides levels showed a significant statistical relationship. It should be noted that RESM, CYFL, CYPE and ESFE showed higher concentration in the locations near North WWTP discharge. The Spearman coefficient between CYFL and distance showed a high-moderate correlation ($r = 0.42$ $p < 0.001$), whereas the rest of the data set showed a low-moderate correlation grade (Table 4.11).

4.7 3 *Ecotoxicological assessment*

The purpose of this study was to identify the areas that may be of special concern with respect to the present status of contamination. The toxicity of PYs to soil organisms, earthworms and other non-target soil organisms, is very low, with $LC_{50} > 1,000,000$ ng g⁻¹ for *Eisenia fetida* (Commission, 2002, 2004, 2005). Thus, the concentrations found in this field-based study indicate negligible toxic effects for terrestrial organisms. However, invertebrates have been found to be the species most sensitive to PYs, presenting low LC_{50} (Amweg et al., 2005). These invertebrates are present in aquatic and semiaquatic habitats and are an important food supply for fish and insectivorous birds, and the alteration of invertebrates' population could break the ecological equilibrium of the area. Thus, due to the hydromorphic conditions of the soils studied (Fig. 4.7), PYs in soil can be desorbed and aquatic organisms should be also taken into account in this study.

The maximum equilibrium concentration expected in water, calculated as indicated in materials and methods section, and the toxic effects of PYs in three aquatic trophic levels are summarized in Table 4.12. In algae, the EC_{50} is high for all PYs (*Scenedesmus subspicatus* EC_{50} (72 h) $> 1 \times 10^7$ ng L⁻¹ for CYFL) (Maund et al., 2002) and no toxic effect is expected. Moreover, as shown in Table 4.12, the NOEC data of PYs for fish is higher than the calculated equilibrium concentration in water in the studied area (no toxic effects expected). On the other hand, the NOEC data for invertebrates is closer to those equilibrium concentrations in water as aquatic invertebrates are the most sensitive organisms to PYs (Fojut et al., 2012; Hill, 1985). Particularly, the calculated equilibrium values of BIFE in water are

higher than the NOEC, which means that some toxic effects may be produced to the invertebrate community in the area studied.

Table 4.12 *Ecotoxicological assessment in the studied area on aquatic organisms*

Compound	K_d (L kg ⁻¹)	ECEW _{max} (ng L ⁻¹) in the studied area	EC ₅₀ (ng L ⁻¹)	NOEC (ng L ⁻¹)	
			Algae	Invertebrates	Fish
				<i>Daphnia magna</i>	<i>Pimephales promelas</i>
BIFE	7787	4	-	1.3 ^a	40 ^a
CYHA	9803	2	> 1000000 ^c	3.8 ^a	31 ^a
CYFL	7787	7	> 991000 ^c	20 ^a	140 ^a
CYPE	9803	3	> 1300000 ^c	20 ^b	77 ^a

K_d : adsorption coefficient, ECEW_{max}: maximum equilibrium concentration in water, EC₅₀: median effective concentration, NOEC: no observed effect concentration

^a: Fojut et al. (2012)

^b: Hill (1985)

^c: Maund et al. (2012)

The areas in which the concentration of BIFE in soil may present harmful effects for aquatic invertebrates are those with levels higher than 10.1 ng g⁻¹, which corresponds to an equilibrium concentration in water of the NOEC value (Table 4.12). The GIS program reported above was used to identify these areas where mitigation measures should be applied (Fig. 4.9).

4.8 Conclusions

Pyrethroid insecticides were monitored at two depths in soils collected during two seasons in a paddy field area within the Natural Park of Albufera to assess their occurrence in the environment. During the period before plow, RESM, CYFL, CYPE and ESFE were the compounds detected more often, up to 70% of detection rate, but at lower concentrations than during the second sampling period (rice production), when soils sampled presented a higher rate of detection (almost 100%) of RESM, BIFE, FENP, CYFL, CYPE and ESFE. The results provided in this field-based study combined with GIS showed that water from WWTPs and field application are the main sources of soil contamination by these insecticides. It was a matter of concern that the levels of BIFE may cause harmful effects on the

aquatic invertebrates within the area monitored, and an area where BIFE levels may present a risk was highlighted. Phytoremediation can be applied to reduce this risk but further work needs to be done to assess how phytoremediation should be performed to be effective in situ.

CHAPTER FIVE – EMERGING CONTAMINANTS IN AQUATIC PLANTS

EMERGING CONTAMINANTS IN AQUATIC PLANTS

Contaminants present in soil may be transported by runoff to surface waters or by lixiviation to groundwater being a source of pollution for fresh water. Aquatic plants have been used extensively in phytodepuration and phytoremediation and have proved their efficiency on the removal of several undesirable chemical compounds such as heavy metals. Several factors can be involved during the processes of phytodepuration and phytoremediation, such as hydrolysis, photolysis and biodegradation among others. Consequently, in this chapter an analytical methodology is presented to detect ECs in several well-known aquatic plants, commonly used in phytodepuration and phytoremediation to help to understand the role that these plants may play. Also it will help to select the best plants to monitor or eliminate the contaminants present in the environment.

In addition, *Oryza sativa* (rice) needs high amounts of water to be grown properly. In Mediterranean areas, the shortage of freshwater from rivers, and the scarcity of rainfall during rice production time have lead the farmers to use wastewater effluents to irrigate agricultural fields to overcome the drought. Consequently, undesirable compounds such as ECs (e.g. pharmaceuticals, personal care products, and hormones) may be introduced into the environment or the food chain, with the possible drawbacks to the environment and human health. Thus, this work also presents a screening of ECs in rice plants due to the agricultural interest.

SIMULTANEOUS DETERMINATION OF MULTICLASS EMERGING CONTAMINANTS IN AQUATIC PLANTS BY ULTRASOUND ASSISTED MATRIX SOLID PHASE DISPERSION AND GC-MS

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Abstract

A multiresidue method was developed for the simultaneous determination of 31 emerging contaminants (pharmaceutical compounds, hormones, personal care products, biocides and flame retardants) in aquatic plants. Analytes were extracted by ultrasound assisted-matrix solid phase dispersion (UA-MSPD) and determined by gas chromatography-mass spectrometry after silylation. The method was validated for different aquatic plants (*Typha angustifolia*, *Arundo donax* and *Lemna minor*) and a semiaquatic cultivated plant (*Oryza sativa*) with good recoveries at concentrations of 100 and 25 ng g⁻¹ wet weight, ranging from 70 to 120%, and low method detection limits (0.3 to 2.2 ng g⁻¹ wet weight). A significant difference of the chromatographic response was observed for some compounds in neat solvent versus matrix extracts and therefore quantification was carried out using matrix-matched standards in order to overcome this matrix effect. Aquatic plants taken from rivers located at three Spanish regions were analyzed and the compounds detected were parabens, bisphenol A, benzophenone-3, cyfluthrin and cypermethrin. The levels found ranged from 6 to 25 ng g⁻¹ wet weight except for cypermethrin that was detected at 235 ng g⁻¹ wet weight in *Oryza sativa* samples.

Keywords Aquatic plants, Ultrasound assisted-matrix solid phase dispersion, GC-MS, Emerging contaminants, *Typha angustifolia*, *Oryza sativa*, Pharmaceuticals, Biocides

5.1 Introduction

Emerging contaminants (ECs) have raised concern among the scientific community because of their ubiquitous presence in different environmental compartments (Aznar et al., 2014a; Bell et al., 2013; Wang et al., 2012; Wu et al., 2011) and the risk they may pose to the environment and human health, taking also into account their potential bioaccumulation in animals (Corcellas et al., 2015; Ortiz de Garcia et al., 2013) and uptake by plants (Tanoue et al., 2012; Wu et al., 2011), which are routes for entering the food chain (Calderon-Preciado et al., 2011; Goldstein et al., 2014; Rodil et al., 2012).

ECs (pharmaceuticals, personal care products, flame retardants and biocides) are widely used and can reach the environment through effluent waters and the use of biosolids (Al Aukidy et al., 2012; Albero et al., 2012b) or manure (Aznar et al., 2014b) applied to improve agricultural soils. Nevertheless, their continuous release through septic system effluents (Kinney et al., 2006) and discharge of wastewater treatment plants (WWTPs), after being inefficiently removed by conventional methods, are the main routes to reach different environmental compartments (Harada et al., 2008; Thomas and Foster, 2005). Thus, ECs can be considered pseudo persistent, reaching the environment uninterruptedly and making them a priority to the scientific community.

Among biocide substances, synthetic pyrethroids (PYs), derived from chrysanthemic acid, are widely used indoors to control household insects, such as mosquitoes, termites and other harmful insects, in place of more toxic organophosphorus and organochlorine insecticides. Indeed, they are much more effective against a wide spectrum of pests than organochlorines and organophosphates and, consequently, they are used in many applications. However, several pyrethroids are known to affect the central nervous system of humans and to have endocrine disrupting effects (Kohler and Triebskorn, 2013). Moreover, PYs may have a negative impact on the environment, primarily on water bodies, due to their toxicity to arthropods (Song et al., 2015; Wang et al.,

2012; Weston et al., 2005) and their bioaccumulation potential in fish (Corcellas et al., 2015). There are several processes involved in the contamination mitigation, such as hydrolysis, photolysis, adsorption, microbial degradation and plant uptake. Several authors have addressed the ability of plants to uptake and translocate organic contaminants (Calderon-Preciado et al., 2011; Herklotz et al., 2010; Tanoue et al., 2012; Wu et al., 2013). Thus, aquatic plants, which are continuously exposed to organic contaminants transported by water, may be used as bio-indicators of the occurrence of these contaminants in freshwater as well as for phytoremediation. Dordio et al. (2011a, 2011b) showed that constructed wetland treatment of wastewaters showed removal efficiencies of 96%, 97% and 75% for ibuprofen, carbamazepine and clofibric acid, respectively (Dordio et al., 2011a; Dordio et al., 2011b). Reinhold et al. (2010) reported on constructed treatment wetlands and demonstrated that they have the potential to reclaim wastewaters through the removal of trace concentrations of emerging organic pollutants, including personal care products, pharmaceuticals and pesticides (Reinhold et al., 2010). These studies demonstrated that aquatic plants contributed both directly and indirectly to the aqueous depletion of emerging organic pollutants through both active and passive processes.

Multiresidue analyses of the organic pollutants in plants are limited by the complexity of the matrix and the properties of the analytes, requiring powerful and selective techniques such as gas chromatography (GC) coupled to mass spectrometry (MS) (Bisceglia et al., 2010; Niewiadowska et al., 2010) or liquid chromatography (Matamoros et al., 2012). GC-MS is a robust and less expensive technique generally available in most analytical laboratories, but the presence of polar functional groups with active hydrogens in some of the compounds studied requires the use of a derivatization procedure to reduce their polarity and enhance their volatility before GC analysis. Several authors have pointed out a gap of multiresidue methods for ECs in plants (Matamoros et al., 2012) and no multiresidue method has been found for the simultaneous analysis of multiclass ECs commonly found in freshwater, including pharmaceuticals, personal care products, flame retardants and pyrethroids in aquatic plants.

Therefore, the aim of this study was to develop an analytical method, based on ultrasound assisted-matrix solid phase dispersion (UA-MSPD) and gas chromatography-mass spectrometry (GC-MS), for the simultaneous determination of 31 organic pollutants (pharmaceuticals, personal care products, hormones, flame retardants, and biocides) in aquatic plants (*Typha angustifolia*, *Arundo donax*, *Oryza sativa* and *Lemna minor*) and evaluate their presence in plants taken from three rivers in different Spanish regions.

5.2 Material and methods

5.2.1 Standards and reagents

Standards of bifenthrin, fenprothrin, λ -cyhalothrin, permethrin, cyfluthrin, α -cypermethrin, τ -fluvalinate, esfenvalerate, deltamethrin, triclosan and methyl triclosan (purity > 99%) were supplied by Riedel-de Haën (Seelze, Germany). Standards of estrone, hexestrol, diethylstilbestrol, ibuprofen, gemfibrozil, fenoprofen, naproxen, mefenamic acid, ketoprofen, carbamazepine, fenofibrate, nonylphenol, bisphenol A (BPA), benzophenone-3 (BP3), 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), 2,2',4,4',6-penta-bromodiphenyl ether (BDE-100) methyl paraben and propyl paraben (purity > 97%), were purchased from Sigma-Aldrich (St Louis, MO, USA). Tris(2-carboxyethyl) phosphine (TCEP) and tris(2-chloroisopropyl) phosphate (TCPP) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). All target compounds are shown in Table 5.1.

Ethyl acetate (EtAc), acetonitrile (ACN), residue analysis grade, ammonium hydroxide (NH_4OH) $\geq 32\%$, Silica Bondesil-C18, particle diameter of 40 μm , and primary secondary amine (PSA) (Bondesil-PSA, 40 μm) were purchased from Varian (Palo Alto, CA, USA). Environ-Clean Bulk Chlorofiltr was acquired from Carlo Erba (Madrid, Spain). Graphitized carbon black (GCB) (Supelclean ENVI-Carb 120/400) was purchased from Supelco (Madrid, Spain). The derivatization agent N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA, purity $\geq 95\%$) with 1% tert-butyldimethylchlorosilane (TBDMCS) and formic acid were obtained from Sigma-Aldrich (St Louis, MO, USA). Florisil, 150-250 μm

(60-100 mesh), was supplied by Aldrich (Steinheim, Germany). Magnesium sulfate anhydrous (MgSO_4) was purchased from Merck (Darmstadt, Germany).

Table 5.1 Physicochemical properties, retention times (t_R , min) and selected ions (m/z) of the compounds studied (T : target ion, Q_1 and Q_2 : qualifier ions)

Name	Physicochemical properties*		SIM parameters			
	Log K_{ow}	pK_a	t_R	T	Q_1	Q_2
Methyl Paraben	1.7	8.2	11.27	209	210	266
TCEP	1.6	-	11.38	249	250	63
TCP	2.6	-	11.57	125	99	277
Ibuprofen	4	4.5	11.85	263	264	117
Propyl Paraben	2.7	8.4	12.15	237	238	294
Methyl Triclosan	5.2	-	13.38	302	304	252
Nonylphenol	3.8	10.7	13.38	334	277	278
Gemfibrozil	4.7	4.4	13.45	243	179	307
Fenoprofen	3.9	4.2	13.68	299	197	206
BP3	3.7	7.6	14.11	285	242	286
Naproxen	3.2	4.8	14.13	287	185	288
Triclosan	4.8	8	14.34	347	345	200
Mefenamic acid	5.1	4.2	14.64	298	224	355
Ketoprofen	3.1	4.5	14.65	311	295	105
Bifenthrin	6	-	14.8	181	165	166
Fenpropathrin	6	-	14.87	125	181	265
Carbamazepine	2.5	13.9	14.95	193	194	293
BDE-47	8.8	-	15.16	486	326	488
Fenofibrate	4.8	-4.9	15.19	121	273	139
λ -Cyhalothrin	6.9	-	15.24	197	181	208
Permethrin	6.5	-	15.74	183	163	165
BPA	3.4	9	15.7	441	442	456
BDE-100	8.9	-	15.92	404	406	566
Cyfluthrin	5.9	-	16.06	163	206	226
Hexestrol	4.8	9.9	16.35	249	250	337
α -Cypermethrin	6.6	-	16.41	163	165	181
Diethylstilbestrol	5.1	-	16.5	496	497	498
τ -Fluvalinate	4.3	-	17.09	250	252	181
Estrone	3.1	10.3	17.1	327	384	328
Esfenvalerate	4	-	17.2	125	167	181
Deltamethrin	6.1	-	17.74	181	253	251

*: Data compiled from previous studies: (Aznar et al., 2014a; Bhandari et al., 2009; Oros and Werner, 2005)
 K_{ow} : octanol water partition coefficient; pK_a : acid dissociation constant

Separate stock solutions of individual compounds were made up at $50 \mu\text{g mL}^{-1}$ in ACN and stored at -18°C . A mixed stock solution of $1 \mu\text{g mL}^{-1}$ containing all analytes was prepared by dilution with ACN of the individual stock solutions. A working mixture solution at 500 ng mL^{-1} was prepared weekly by dilution with

ACN of the mixed stock solution. All solutions were stored in the darkness at 4 °C up to 8 weeks.

5.2.2 Plant material

Samples of *Typha angustifolia*, *Arundo donax*, and *Lemna minor* were provided by the School of Agricultural Engineering, Polytechnic University of Madrid, Spain. *Oryza sativa* was taken from the natural park of Albufera (Valencia, Spain) within an area irrigated with pure artesian water. Plants were maintained in darkness at 4 °C and transported to the laboratory where representative portions of the aerial part of the selected plants were ground using a food processor and kept at – 20 °C until analyses. These samples, after preliminary screenings, did not show any of the target compounds included in this work and they were used as blanks. *Typha angustifolia* was employed in the optimization of the method because it was the most complex matrix.

The validated method was applied to plants taken from rivers located at different Spanish regions (Valencia, Madrid and Andalucía) during a sampling campaign carried out in the summer 2015 to assess the presence of pollutants.

5.2.3 Sample preparation

Ultrasound Assisted-Matrix solid phase dispersion (UA-MSPD). UA-MSPD was performed mixing 1 g of aquatic plant with 4 g of Florisil and 2 g of MgSO₄ in a glass mortar. Then the mixture was blended with a glass pestle for 5 min to yield a homogeneous material and placed in a 20 mL glass column (10 cm x 20 mm I.D., from Becton-Dickinson, Madrid, Spain) over 2 paper filters (Whatman No. 1 paper circles of 2 cm diameter, Maidstone, UK) at the end with 2 g of MgSO₄. EtAc with 3% NH₄OH (8 mL) was added to each column and 2 mL were used to wash the mortar and pestle. Columns were sonicated for 15 min in an ultrasonic water bath (Raypa, Barcelona, Spain) at room temperature. The water level in the bath was adjusted to equal the extraction solvent level inside the columns, which were supported upright in a tube rack and closed with one-way stopcocks. Then extracts

were collected in tubes using a multiport vacuum manifold (Supelco, Visiprep, Madrid) and evaporated near dryness. The extraction was repeated twice with 5 mL ACN containing 4% formic acid to ensure the complete extraction of the acidic target compounds. The extract was evaporated to 1 mL using a Genevac EZ-2 evaporator (purchased from NET Interlab, Spain) before the cleanup step.

Cleanup. Aquatic plant extracts (1 mL) were cleaned through a 5 mL glass column (Normax, Lisbon, Portugal) with 2 paper filter (Whatman No. 1, Maidstone, UK) containing 1 g of MgSO₄ and 1 g of C18. Analytes were eluted with 5 mL of ACN and extracts were collected in tubes using a multiport vacuum manifold, evaporated to dryness and reconstituted to 0.5 mL with ACN before their derivatization.

Derivatization. Prior to the GC-MS determination, some of the studied analytes need to be derivatized to increase their volatility. The derivation agent MTBSTFA: TBDMCS (99:1, v/v) was selected, as it presents the best performance for pharmaceutical and personal care products in comparison with other derivatization agents (Schummer et al., 2009). Thus, the *t*-butyldimethylsilyl derivatives were prepared by the addition of 50 µL of MTBSTFA: TBDMCS (99:1, v/v) to an aliquot (100 µL) of the plant extract and transferred into a 2 mL reaction vial with a micro insert. Vials were closed and the mixture left to react for 1 h at 70 °C before the GC-MS analysis.

5.2.4 Gas chromatography-mass spectrometry analysis

Gas Chromatography-mass spectrometry (GC-MS) analysis was performed with an Agilent 6890 (Waldbronn, Germany) gas chromatograph equipped with an automatic injector and a mass spectrometric detector, model HP 5977A. A fused silica capillary column ZB-5MS, 5% phenyl polysiloxane as nonpolar stationary phase (30 m × 0.25 mm i.d. and 0.25 µm film thickness), from Phenomenex (Torrance, CA), was used for the analysis.

Operating conditions were in solvent-vent mode as follows: 2 μL of plant extracts were injected in a simple-taper glass liner with a nominal volume of 800 μL with glass-wool. The split vent was open for 0.1 min with an inlet pressure of 5 psi and a flow rate of 100 mL min^{-1} . Once the sample introduction was completed, the inlet was switched to splitless mode for analyte transfer. After 2.6 min, the purge valve was activated at a 60 mL min^{-1} flow rate. Helium (purity 99.995%) was used as carrier gas at a constant flow rate of 1.2 mL min^{-1} . The temperature program of the injector started at 50 $^{\circ}\text{C}$, kept for 0.1 min, then ramped to 300 $^{\circ}\text{C}$ at 600 $^{\circ}\text{C min}^{-1}$, held 5 min, and finally decreased to the initial temperature cooling with compressed air. The column temperature was maintained at 50 $^{\circ}\text{C}$ for 2.6 min, then programmed at 20 $^{\circ}\text{C min}^{-1}$ to 300 $^{\circ}\text{C}$ and held for 5 min. The total analysis time was 20.1 min and the equilibration time was 4 min.

The mass spectrometric detector was operated in electron impact ionization mode with an ionizing energy of 70 eV, an ion source temperature of 230 $^{\circ}\text{C}$ and a quadrupole temperature of 150 $^{\circ}\text{C}$. The electron multiplier voltage was set with a gain factor of 4 and a solvent delay of 10.5 min was used. Table 5.1 lists the compounds with their retention times and selected ions to be used in SIM mode. The target and qualifier abundances were determined by injection of standards under the same chromatographic conditions using full-scan with the mass/charge ratio ranging from 50 to 550 m/z . The compounds were confirmed by their retention times, the identification of target and qualifier ions and the determination of qualifier to target ratios. Retention times must be within ± 0.1 min of the expected time and qualifier-to-target ratios within a 20% range for positive confirmation. The quantification was accomplished by matrix-matched calibration.

In order to evaluate the method developed for the detection of ECs in aquatic plants, different quality parameters were studied: recoveries, precision, linearity and sensitivity.

Plants were spiked with the analytes at two levels (25 and 100 ng g⁻¹) with four sample replicates, to study the recoveries and the accuracy of the method. The precision of the method was evaluated in terms of repeatability (intra-day precision) and reproducibility (inter-day precision) at 100 and 25 ng g⁻¹. The repeatability was assessed by the application of the whole procedure on the same day and the reproducibility was evaluated performing the complete procedure in different days. The results were expressed as % RSD (six replicates).

To evaluate the sensitivity, method detection limit (MDL) and limit of quantification (LOQs) of the developed method were determined using ten replicates of plant extracts, spiked at 2.5 ng g⁻¹. The equation to calculate the MDL was the following: $MDL = t_{99} \times S$, where t_{99} is the Student's value for a 99% confidence level and $n-1$ degrees of freedom and S is the standard deviation of the replicate analyses. The LOQ was calculated as 10 times the standard deviation of the results of the replicate analysis used to determine MDL.

Finally, to evaluate linearity and matrix effect two sets of calibration solutions were prepared in the range from 1 to 400 ng g⁻¹, one set was solvent-based and the other was prepared spiking blank plant extracts at the same concentrations. There are several approaches to counteract matrix-induced effects but due to the high price and the nonexistence of internal standards for some of the 31 organic pollutants studied, matrix-matched calibration was selected (Aznar et al., 2014a).

In order to reduce possible memory effects of the column, prior to the analysis of samples, the inlet was flushed by heating at 300 °C for 30 min and one laboratory blank was run with each set of samples to check for memory effects and demonstrate laboratory background levels.

Standard statistical analyses were carried out to study the significant differences of the method using STATGRAPHIC CENTURION. One way ANOVA was applied to determine significant differences at a $p \leq 0.01$ level.

5.3 Results and discussion

5.3.1 Sample preparation

In a first assay, 1 g of *Typha angustifolia* spiked at 100 ng g⁻¹ was placed in a mortar and blended with 4 g of Florisil and 2 g of MgSO₄. Different organic solvents and combination of them changing the pH were tested to evaluate extraction yields. Figure 5.1 shows the recoveries obtained with different extraction solvents for a representative selection of the studied compounds. Firstly, two extractions with ACN did not present good recoveries, particularly for the acidic compounds (i.e. mefenamic acid, etc., data not shown). Thus, the pH of the second extraction was changed (adding 3% of formic acid), and the recoveries of those compounds clearly improved. In order to improve the extraction yields of the target analytes, particularly the more lipophilic compounds, such as pyrethroids and the brominated flame retardants (BDE-47 and BDE-100), a less polar solvent was selected to perform the first step of the procedure, therefore, the extraction with EtAc and ACN containing 3% formic acid was carried out, but yields of basic compounds (i.e. carbamazepine) (Table 5.1) were low and an extraction with basic solvent was required. The best performance was reached using EtAc with 3% NH₄OH and two extractions with ACN containing 4% formic acid as can be seen in Figure 5.1. The addition of formic acid resulted in recoveries around 100% for the acid compounds, and the use of NH₄OH during the first extraction improved the recovery of the basic compounds, particularly for carbamazepine. Moreover, significant differences ($p \leq 0.01$) were found in the statistical analysis of the selection of extraction solvent and the best recoveries were obtained with EtAc 3% NH₄OH + 2 x ACN 4% formic acid, as shown in Figure 5.1.

Figure 5.1 Solvent selection using *Typha angustifolia* spiked at 100 ng g^{-1} . Different letters indicate significant differences ($p \leq 0.01$)

Although target analytes can be determined in the conditions indicated above, plant extracts showed high content of chlorophyll and some interference appeared during MS quantification; for these reasons a cleanup step was necessary. Dispersive solid-phase extraction (dSPE) was carried out with 1 mL blank extracts spiked at 100 ng g^{-1} and 0.1 g of sorbent. Four sorbents were tested: GCB, PSA, Chlorofiltr and C18. GCB showed the best performance removing chlorophylls completely, but it showed the lowest recoveries because planar compounds are retained in its surface (data not shown). PSA removes pigments and sugars, but showed the poorest removal of green pigments, and all the acidic compounds studied were not recovered at all because of the amine group of the PSA, as described by other authors (Paya et al., 2007). Recoveries using Chlorofiltr were much higher, but the extracts remained slightly green, although its main purpose is to eliminate chlorophyll. C18 showed the best performance eliminating most of the chlorophyll and other interferences with the best recoveries as shown in Figure 5.2. Thus, C18 was selected as sorbent for the purification of the extracts using 5

mL glass columns. The influence of the amount of C18 (0.5, 1 or 2 g) was also studied and 0.5 g were not enough to remove interferences but with 1 g and 2 g similar recoveries and removal of interferences were obtained. Therefore, the cleanup was performed with 1 g of C18.

Figure 5.2 Clean-up sorbent selection using *Typha angustifolia* spiked at 100 ng g⁻¹. Different letters indicate significant differences ($p \leq 0.01$)

5.3.2 Method validation

In order to determine the accuracy of the method, recoveries were carried out spiking four different aquatic plants at two levels, 100 and 25 ng g⁻¹ (Table 5.2). Satisfactory recoveries were obtained with the four plants for most of the compounds. In comparison to other studies in plants (Winker et al., 2010; Wu et al., 2012), where less compounds were studied, our work showed in general better recoveries. Repeatability was evaluated by analyzing six replicates within a given day and reproducibility by determining the recoveries of six replicates within different days and RSD lower than 6% and 10%, respectively, were obtained.

Table 5.2 Recoveries (%) and relative standard deviations (RSD ($n=4$), % in parenthesis) obtained for target compounds in different aquatic plants

	<i>Typha angustifolia</i> *		<i>Arundo donax</i> *		<i>Lemna minor</i> *		<i>Oryza sativa</i> *	
	100	25	100	25	100	25	100	25
Methyl paraben	70 (8)	82(8)	76 (5)	72 (9)	83 (5)	88 (8)	75 (5)	81 (6)
TCEP	84 (4)	80 (9)	74 (3)	94 (6)	94 (7)	85 (6)	90 (3)	73 (3)
TCP	94 (9)	81 (8)	100 (3)	96 (9)	83 (8)	91 (5)	99 (3)	74 (6)
Ibuprofen	100 (3)	71 (3)	70 (6)	72 (5)	75 (2)	73 (3)	120 (7)	112 (9)
Propyl paraben	89 (3)	80 (6)	86 (2)	80 (5)	88 (2)	90 (4)	78 (6)	74 (10)
Methyl triclosan	88 (3)	74 (7)	87 (4)	83 (8)	111 (3)	79 (4)	86 (4)	73 (5)
Gemfibrozil	86 (6)	81 (5)	77 (7)	79 (10)	77 (3)	77 (8)	81 (9)	71 (4)
Nonylphenol	87 (2)	83 (8)	70 (2)	77 (5)	88 (3)	79 (4)	98 (2)	80 (3)
Fenoprofen	78 (9)	71 (3)	72 (8)	78 (2)	78 (3)	81 (3)	87 (5)	74 (6)
BP3	97 (4)	98 (6)	78 (7)	80 (4)	79 (3)	76 (4)	120 (5)	71 (6)
Naproxen	82 (8)	98 (2)	86 (3)	79 (5)	81 (4)	82 (9)	99 (3)	75 (2)
Triclosan	89 (4)	84 (2)	94 (11)	80 (7)	84 (2)	85 (5)	99 (2)	78 (3)
Mefenamic acid	91 (8)	94 (8)	70 (9)	107 (3)	74 (8)	87 (4)	80 (8)	79 (3)
Ketoprofen	73 (2)	120 (2)	75 (10)	98 (8)	72 (4)	100 (3)	98 (9)	73 (2)
Bifenthrin	86 (3)	89 (7)	94 (5)	82 (5)	91 (4)	93 (3)	88 (6)	75 (4)
Fenpropathrin	84 (3)	89 (7)	90 (6)	73 (5)	97 (9)	95 (9)	95 (4)	71 (2)
Carbamazepine	90 (2)	83 (2)	88 (3)	75 (9)	98 (2)	96 (4)	85 (5)	79 (3)
BDE-47	76 (8)	84 (9)	89 (3)	82 (6)	86 (5)	88 (6)	90 (4)	74 (3)
Fenofibrate	83 (3)	82 (8)	89 (5)	83 (5)	87 (5)	91 (6)	94 (3)	80 (4)
λ -Cyhalothrin	83 (11)	80 (5)	85 (6)	90 (11)	93 (6)	82 (7)	91 (3)	101 (8)
Permethrin	83 (2)	83 (8)	91 (5)	79 (6)	89 (6)	83 (3)	92 (5)	86 (5)
BPA	77 (7)	77 (4)	107 (10)	105 (3)	80 (10)	80 (4)	97 (5)	70 (5)
BDE-100	86 (2)	80 (9)	88 (4)	79 (9)	86 (8)	87 (4)	91 (4)	86 (4)
Cyfluthrin	80 (4)	84 (7)	87 (5)	82 (8)	82 (9)	88 (7)	90 (7)	96 (6)
Hexestrol	90 (0)	80 (7)	85 (3)	78 (6)	92 (2)	100 (2)	99 (2)	77 (4)
α -Cypermethrin	81 (3)	79 (8)	83 (4)	87 (10)	87 (8)	89 (7)	91 (3)	95 (8)
Diethylstilbestrol	97 (6)	101 (2)	111 (4)	86 (6)	84 (3)	72 (3)	77 (2)	72 (3)
τ -Fluvalinate	74 (3)	85 (8)	82 (6)	86 (6)	76 (7)	87 (8)	86 (5)	92 (9)
Estrone	103 (11)	97 (9)	95 (5)	74 (10)	98 (3)	81 (3)	107 (9)	89 (7)
Esfenvalerate	82 (11)	74 (6)	95 (6)	89 (2)	80 (5)	83 (6)	95 (7)	92 (8)
Deltamethrin	80 (5)	73 (11)	83 (7)	95 (7)	83 (6)	90 (7)	85 (8)	89 (5)

*: ng g⁻¹

MDLs and LOQs obtained for the different aquatic plants are shown in Table 5.3. MDLs ranged from 0.3 to 2.2 ng g⁻¹. The differences reported were related to the background noise and the recoveries obtained in the four matrices. Low limits were achieved for all the aquatic plants, being similar to those reported by Wu et al. (2012) in vegetables and better than the ones published by other authors (Calderon-Preciado et al., 2009; Winker et al., 2010).

Table 5.3 Method Detection Limits (MDL, ng g⁻¹) and limits of quantification (LOQ, ng g⁻¹) obtained for the analytes in the different aquatic plants studied

	<i>Typha angustifolia</i>		<i>Arundo donax</i>		<i>Lemna minor</i>		<i>Oryza sativa</i>	
	MDL	LOQ	MDL	LOQ	MDL	LOQ	MDL	LOQ
Methyl Paraben	0.4	1.2	0.5	1.7	0.4	1.5	0.8	3.0
TCEP	1.5	3.2	0.5	1.9	0.6	2.1	1.2	4.2
TCPP	0.8	2.4	0.3	1.0	0.9	2.9	0.8	2.8
Ibuprofen	0.8	2.7	0.7	2.6	0.8	2.7	0.8	2.8
Propyl Paraben	0.3	1.0	0.3	1.0	0.3	1.2	1.1	3.8
Methyl Triclosan	1.3	4.0	0.3	1.0	0.5	1.6	1.5	5.3
Gemfibrozil	0.5	1.5	1.1	3.2	0.3	1.1	0.8	2.7
Nonylphenol	0.4	1.5	1.2	3.8	1.4	3.8	0.7	2.5
Fenoprofen	0.9	2.6	1.3	3.7	0.6	2.0	1.0	2.9
BP3	0.8	2.5	1.0	3.2	0.8	2.7	0.4	1.4
Naproxen	1.0	3.1	1.0	3.1	0.7	2.4	1.0	3.3
Triclosan	0.3	1.0	1.0	3.5	0.6	1.9	0.6	1.9
Mefenamic acid	0.4	1.4	0.9	2.7	0.8	2.5	0.9	3.1
Ketoprofen	0.3	1.0	1.5	4.8	1.1	3.2	2.1	6.3
Bifenthrin	0.4	1.3	0.3	1.0	0.5	1.9	0.7	2.5
Fenpropathrin	1.2	3.6	0.3	1.0	0.5	1.6	0.6	2.1
Carbamazepine	0.5	1.5	0.7	2.4	0.3	1.1	0.3	1.0
BDE-47	0.7	2.5	1.1	3.8	0.8	2.6	0.3	1.0
Fenofibrate	1.0	2.7	0.7	2.3	0.7	2.0	0.5	1.6
λ-Cyhalothrin	0.9	3.0	0.8	2.8	0.9	3.1	0.8	2.7
Permethrin	1.1	3.6	0.3	1.0	0.5	1.8	0.7	2.3
BPA	1.5	3.2	1.1	3.4	0.6	1.9	0.9	3.0
BDE-100	1.1	3.6	0.4	1.3	1.0	3.5	0.3	1.0
Cyfluthrin	1.0	3.1	0.6	2.1	0.8	2.6	0.3	1.0
Hexestrol	0.5	1.8	0.4	1.3	0.4	1.3	0.9	3.1
α-Cypermethrin	1.1	3.5	0.3	1.0	0.8	2.7	0.9	3.1
Diethylstilbestrol	0.3	1.0	0.9	3.0	0.7	2.4	2.2	6.7
τ-Fluvalinate	0.9	2.8	0.6	2.0	0.6	2.0	0.4	1.2
Estrone	0.5	1.8	0.9	3.1	0.7	2.5	0.4	1.2
Esfenvalerate	0.6	2.0	0.8	2.8	1.1	3.7	1.4	4.2
Deltamethrin	1.3	3.3	0.6	2.2	2.2	4.8	1.2	4.0

The chromatographic response of analytes may be affected by the presence of matrix components. Therefore, matrix effects were evaluated preparing two multipoint calibration curves using a set prepared with solvent-based standards and the other with matrix-matched standards. A good linearity was obtained in the range of 1 to 400 ng g⁻¹, with correlation coefficients ≥ 0.994 for all the compounds studied. The slopes obtained by plotting the seven concentration levels against response, following linear regression analysis, were compared (Figure 5.3). A significant difference of the chromatographic response was observed for bifenthrin

(response increase) and for carbamazepine (response suppression). On the other hand, compounds such as diethylstilbestrol were not affected by matrix components. The analysis of pharmaceuticals in neat solvent produced, in general, calibration curves with higher slopes when compared to matrix matched standards. Hormones did not show differences and the analysis of pyrethroids in neat solvent produced calibration curves with lower slopes when compared to matrix matched standards for most compounds. In general, an enhancement of analyte response is observed in gas chromatography due to the presence of co-extractives that may improve the transfer of some compounds, i.e. pyrethroids, by blocking active sites in the chromatographic system. On the other hand, although the derivatization step is carried out with an excess of reagent, matrix components may compete with target analytes and thus a lower chromatographic response is observed, as it occurs in general with pharmaceuticals. Thus, quantification was carried out using matrix-matched standards in order to overcome the matrix effects observed and have more accuracy in the quantification of samples.

Figure 5.3 Comparison of calibration curves of bifenthrin, carbamazepine and diethylstilbestrol, obtained by injection of standards in neat solvent (◆) and spiked plant extracts (■)

5.3.3 Analysis of real samples

The developed analytical method was applied to different aquatic plants collected from rivers located in three Spanish regions (Valencia, Madrid and Andalucía) in

order to show the feasibility of the analytical technique to detect environmental levels of the selected contaminants in common aquatic plants of the studied area.

Table 5.4 shows the compounds found in the different aquatic plants analyzed. Methyl and propyl paraben, BPA, BP3, cyfluthrin and cypermethrin were found at levels ranging from 6 to 25 ng g⁻¹ wet weight, except for cypermethrin that was detected at 235 ng g⁻¹ wet weight in *Oryza sativa* samples.

The concentration and fate of emerging contaminants in water, where several processes like adsorption, transport and degradation are involved, affect their bioavailability to plants. In the studied area of Turia and Manzanares Rivers (Valencia and Madrid, respectively), methyl and propyl paraben, BPA, triclosan and nonylphenol were often detected in water (Carmona et al., 2014; Esteban et al., 2014). On the contrary, no residues were reported in the area of Guadalfeo River (Andalucia), which is located in a sparsely populated area with scarce industrial activity and, consequently, no residues were found in the aquatic plants from this river. Among the compounds most frequently detected in water, BPA and methyl and propyl paraben, were also detected in aquatic plants, whereas triclosan and nonylphenol were not found, although they have been reported at higher levels in water from the studied area (Carmona et al., 2014; Esteban et al., 2014). A greater translocation has been reported for compounds with log K_{ow} between 1 and 3.5, whereas compounds with higher log K_{ow} tend to be not taken up (Eggen et al., 2013; García-Valcárcel et al., 2016). This may explain why triclosan and nonylphenol were not detected in the aquatic plants studied (see Table 5.4).

Cypermethrin was detected in *Oryza sativa* collected from Albufera (Valencia) that is fed by Turia River. In this river, cypermethrin was found in water and fish due to the intense agricultural use of the surroundings (Ccanccapa et al., 2016; Corcellas, 2015).

Table 5.4 Analytes detected in aquatic plants. Data are expressed as ng g^{-1} wet weight, mean $\pm$ SD for $n=3$

	Turia River (Valencia)			Albufera Lake (Valencia)	Manzanares River (Madrid)		Guadalejo River (Andalucía)
	<i>Typha angustifolia</i>	<i>Arundo donax</i>	<i>Lemna minor</i>	<i>Oryza sativa</i>	<i>Typha angustifolia</i>	<i>Arundo donax</i>	<i>Arundo donax</i>
Methyl Paraben	n.d.	n.d.	n.d.	n.d.	12 ± 1	n.d.	n.d.
Propyl Paraben	7 ± 1	n.d.	n.d.	14 ± 1	n.d.	n.d.	n.d.
BP3	8 ± 1	n.d.	n.d.	n.d.	17 ± 6	20 ± 2	n.d.
BPA	15 ± 1	18 ± 3	18 ± 2	25 ± 6	n.d.	n.d.	n.d.
Cyfluthrin	6 ± 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cypermethrin	n.d.	n.d.	n.d.	235 ± 19	n.d.	n.d.	n.d.

n.d.: not detected

5.4 Conclusions

A multiresidue method was developed and validated for the determination of 31 organic pollutants in aquatic plants. UA-MSPD was applied to extract target compounds from *Typha angustifolia*, *Arundo donax*, *Oryza sativa* and *Lemna minor* at two levels (100 and 25 ng g⁻¹ wet weight). These contaminants were determined by gas chromatography-mass spectrometry after reaction with MTBSTFA:TBDMCS to derivatize amine and hydroxyl polar groups of the target analytes. Good recoveries were obtained for most of the compounds in the four aquatic plants studied, with low limits of detection and quantification. The developed method was applied to aquatic plants collected from three rivers located in different Spanish regions to demonstrate the feasibility of the analytical technique to detect the selected contaminants in common aquatic plants at environmental levels. Six of the compounds studied were detected at concentrations ranging from 6 to 25 ng g⁻¹ wet weight, except cypermethrin that was detected at 235 ng g⁻¹ wet weight in *Oryza sativa* samples. Given the diverse physical-chemical properties of the compounds considered in the present study, this method can be used for monitoring other organic contaminants in aquatic plants. Nevertheless, further research needs to be done on the use of the developed analytical technique to assess the contamination of rivers and to better understand the role that aquatic plants may play regarding pollution mitigation in aquatic environments such as wetlands and rivers.

CHAPTER SIX – SILVER NANOPARTICLES IN AQUEOUS SAMPLES

SILVER NANOPARTICLES IN AQUEOUS SAMPLES

Nanotechnology is an uprising and promising industry which is constantly developing new technology based on nanomaterials. As the development of these compounds in comparison with the other ECs is quite new, the knowledge about their presence in the environment and the potential drawbacks that may have is scarce.

In contrast to all the other ECs, the nature of the nanoparticles that are gathering more attention are inorganic (i.e. silver nanoparticles, Ag-NPs).

For this reason, to fulfill this thesis, the research was performed in partnership with the European Commission at the Joint Research Center (JRC) in Ispra, Italy, where the instruments to detect nanoparticles of silver were available, as a visiting scientist during three months as a part of the international Ph.D. program.

The need to have a methodology to detect and quantify Ag-NPs to legislate properly their use and presence is nowadays a must for the European Commission. For this reason, a methodology has been implemented to study silver nanoparticles in aqueous samples of diverse interest, such as fresh waters, consumer products, medical devices and food contact materials.

QUANTIFICATION AND SIZE CHARACTERISATION OF SILVER NANOPARTICLES IN CONSUMER PRODUCTS AND AQUEOUS SAMPLES BY SINGLE PARTICLE ICP-MS: EVALUATION AND PRACTICAL CONSIDERATIONS

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Submitted

Abstract

Single particle-ICP-MS (SP-ICP-MS) is a promising technique for the implementation of the EC recommendation on the definition of nanomaterial (2011/696/EU) as the technique is able to generate the number based-particle size distribution (PSD) of nanoparticles (NPs) in aqueous suspensions. However, SP-ICP-MS analysis is not consolidated as routine-technique yet and is not typically applied to real test samples with unknown composition. This work presents a methodology to detect, quantify and characterize the number-based PSD of Ag-NPs in different consumer products and real environmental aqueous samples using SP-ICP-MS. The procedure is built from a pragmatic view and involves the analysis of serial dilutions of the original sample until no variation in the measured size values is observed while keeping particle counts proportional to the dilution applied. After evaluation of the analytical figures of merit, the SP-ICP-MS method exhibited excellent linearity ($r^2 > 0.999$) in the range $(1 - 25) \times 10^4$ particles mL^{-1} for 30, 50 and 80 nm nominal size Ag-NPs standards. The repeatability was studied according to the RSDs of the measured size and particle number concentration values and a t-test ($p=95\%$) at the two intermediate concentration levels was applied to determine the trueness of SP-ICP-MS size values. The method showed good repeatability and an overall acceptable trueness in the studied concentration range. The experimental minimum detectable size for Ag-NPs ranged between 12-15 nm. Additionally, results derived from direct SP-ICP-MS analysis were

compared to the results conducted for fractions collected by asymmetric flow-field flow fractionation (AF4) and supernatant fractions after centrifugal filtration. The method has been successfully applied to determine the presence of Ag-NPs in seven different liquid silver-based consumer products; migration solutions (pure water and sweat simulant) from plasters; lake water; tap water; and tap water filtered by a filter jar. Results obtained by SP-ICP-MS were confirmed by transmission electron microscopy (TEM) and energy dispersive spectroscopy (EDS) characterization, suggesting that the proposed methodology can be applied as a positive screening test in the simultaneous quantification and size characterization of Ag-NPs in samples of environmental interest.

Keywords: SP-ICP-MS, Single particle, Nanoparticles, Silver, Consumer products, Environmental waters

6.1 Introduction

During the last two decades the emergence of nanoparticles (NPs) technology has driven the industry to a new revolution due to its polyvalence. Nanoparticles technology is applied to a wide range of products in the fields of cosmetics, biomedicine, food and food packaging, bioremediation, coatings, electronics, catalysis and material sciences, among many others (Nel et al., 2006) (Christensen et al., 2010) (Nowack et al., 2011) (2016). Particularly, silver nanoparticles (Ag-NPs) are used in consumer products due to a proved antimicrobial properties attributable to the release of Ag^+ (Zhou et al., 2014) (Xiu et al., 2012). Because of the massive and uprising tendency to use NPs in general and Ag-NPs in particular (Nowack et al., 2011), the release of nano-residues into different compartments in the environment should be taken into account (López-Serrano et al., 2014) (Schaumann et al., 2015), being water bodies particularly at risk, as they act as a sink for most environmental contaminants (Farré et al., 2011). Moreover, regarding human health, insufficient knowledge in relation with their safety has led to concerns (Kawata et al., 2009).

Facing the new trend of goods produced based on nanotechnology, the European Commission published a recommendation on the definition of nanomaterial which establishes a threshold number of particles (50% of the total number of particles) with at least one dimension in the size range 1-100 nm (2011). As a consequence, analytical scientists are dedicating efforts to develop selective, robust and reliable fit-for-purpose methods that are able to separate, isolate and measure NPs to characterise the number based-particle size distribution (PSD). To date, Asymmetric Flow Field Flow Fractionation (AF4) (Poda et al., 2011) (Bolea et al., 2011) (Loeschner et al., 2013) (Mitrano et al., 2012a), chromatographic techniques (Zhou et al., 2014) and filtration (Mitrano et al., 2015) are among the most frequently used techniques to separate NPs, often coupled to light scattering, UV or Inductively Couple Plasma Mass Spectrometry (ICP-MS) detectors (Barahona et al., 2016) (Cascio et al., 2014). Each measuring technique and approach exhibits its own advantages and limitations and a combined use of all of them following a

multimethod approach has been sometimes proposed since the information from each individual method is usually complementary (Bouwmeester et al., 2014) (Hagendorfer et al., 2012).

In this context, in recent years single particle-ICP-MS (SP-ICP-MS) has arisen as a promising alternative technique for the implementation of the EC recommendation on the definition of nanomaterial (Laborda et al., 2013) primarily because SP-ICP-MS allows simultaneous counting and sizing of NPs present in diluted aqueous samples. The potential of SP-ICP-MS analysis to characterise suspensions of NPs is well accepted and fundamental principles of SP-ICP-MS have been already well explained in other works (Laborda et al., 2014). The positive aspects of SP-ICP-MS include elemental selectivity, low limits of detection comparable with typical concentrations occurring in the environment and almost null existence of matrix effect due to dilution. However, there are still important knowledge gaps hampering its consolidation as routine-technique and, with meritorious exceptions, SP-ICP-MS is still limited to proof-of-concept research. Among the limiting factors, current detection limits (DLs) in SP-ICP-MS strongly depend on the nature of the element and they could lead to miss part of the whole PSD (Lee et al., 2014). In addition to this, SP-ICP-MS cannot distinguish between primary NPs and aggregated NPs, so that the protocol according to which the sample is prepared becomes critical. In this regard, although far from being an universal technique and despite showing size DLs that also depend on the target species, the information derived from Transmission Electron Microscopy (TEM) analysis is still usually considered as confirmatory technique (Verleyesen et al., 2015; Ramos et al., 2016).

In order to move from exploratory towards a routine technique, the evaluation of the analytical parameters of quality in SP-ICP-MS methodologies should be addressed like in any other measuring technique. Such an issue has been recently approached by some researchers, like Peters and co-workers, who in-house validated a method to analyse Ag-NPs in chicken meat spiked with standards (Peters et al., 2014). Linsinger and co-workers also reported on an inter-laboratory

study evaluating the SP-ICP-MS measurements of Au-NPs standards (Linsinger et al., 2014). Regarding to the analysis of real samples, published applications include the characterisation of Ag-NPs released from plastic food containers (Ramos et al., 2016), analysis of Ag-NPs in environmental waters (Tuoriniemi et al., 2012) and wash waters (Mitrano et al., 2015) among some others (Laborda et al., 2016), but the applicability to real unknown and polydispersed samples still needs further research. Moreover, the development of systems for data analysis should be directed to fast, pragmatic and user-friendly tools, as contributions from researchers (Peters et al., 2015; Tuoriniemi et al., 2014) and manufacturers (Hineman and Stephan, 2014; Dan et al., 2015) already suggested.

This work presents a methodology to detect, quantify and characterise the number-based PSD of Ag-NPs in different consumer products and real environmental aqueous samples using SP-ICP-MS by analysing consecutive dilutions of pristine samples. As it is advisable for any other analytical procedure, the evaluation of the analytical performance of the technique has been systematically addressed and the outcome is reported here. Parameters such as limits of detection, linearity, repeatability and trueness were studied using Ag-NPs standards. Additionally, centrifuge filtration and AF4 were evaluated as preparative techniques prior to SP-ICP-MS. The developed methodology has been applied to the analysis of real consumer products, aqueous samples derived from migration tests as well as environmental water samples. Transversally, aiming to contribute to the still limited knowledge on SP-ICP-MS experimental handling, this article reports the results obtained using different calibration strategies and different calibrant NPs standards, and discusses some practical considerations as well as main advantages and limitations.

6.2 Materials and methods

6.2.1 Reagents and materials

Reference material of citrate gold NPs 60 nm nominal diameter (5 mL ampoules, 51.86 $\mu\text{g g}^{-1}$) (RM 8013) used for SP-ICP-MS calibration was supplied by the

National Institute of Standards and Technology (NIST, U.S. Department of Commerce, Gaithersburg). Stock suspension was stored in dark at room temperature following the supplier recommendations. Polyvinylpyrrolidone (PVP) capped Ag-NPs 75 nm-PVP (1 mL, 1 mg L⁻¹), used for SP-ICP-MS calibration and PVP-capped Ag-NPs standards with nominal diameters of 10, 20, 30, 40, 50, 60, 70, 80 and 100 nm, respectively, and nominal concentration of 0.02 mg L⁻¹, were purchased from nanoComposix (nanoComposix Inc., San Diego, CA, US). Ag-NPs 75 nm-PVP supplied by nanoComposix has been used by NIST for recent certification of reference material RM 8017. Dissolved ionic silver (1 mg L⁻¹, 25 mL) in 2% HNO₃/water was acquired from Analytical Technology (Milan, Italy). For external calibrations, working solutions of Ag⁺ were prepared in ultrapure water or 1% HNO₃ from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, USA) at pertinent concentrations. Stock suspensions of ionic Ag were stored in dark and at 4 °C following the supplier recommendations. Dilutions and working suspensions from the stock material were freshly prepared using 15 mL polypropylene tubes from PerkinElmer (USA). L-Hystidine, NaCl, Na₂HPO₄ and NaOH (0.1 M) were all purchased from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, USA). Sodium hydroxide (Acros Organics, Geel, Belgium) was used to adjust the pH of ultrapure water to pH 9.5 for the AF4 carrier.

6.2.2 Samples

Consumer products. Seven different liquid silver-based consumer products were acquired either on the internet or in pharmacies (products 1-7 in SI-Table 9). The information provided on the product labels was scarce and even sometimes contradictory about the silver concentrations. Some of them claimed to contain Ag-NPs.

Release of Ag-NPs from plasters. The release from plasters claiming silver content was studied as well. The plasters were purchased in a local supermarket in Italy. Two procedures were conducted to provoke the release of silver: A) 4 cm² of the plaster was placed into a polypropylene tube and 4 mL of pure water was added

prior to 5 min of ultrasonic treatment in an ultrasound bath; B) 4 cm² of the plaster was placed into a polypropylene flask covered by stainless steel spheres (0.5 cm diameter) and 4 mL of sweat simulant were added, prior to 18 h under orbital shaking (100 rpm) to simulate friction. Artificial sweat was produced using L-Hystidine (0.5 g L⁻¹), NaCl (5 g L⁻¹), Na₂HPO₄ (2.5 g L⁻¹) and NaOH (0.1 M) to bring the pH to 8. Such a formulation was taken from an existing ISO standard (2013) method for textiles testing.

Water samples. They were taken from different sources. Fresh water from Lake Maggiore (Italy), tap water and filtered tap water using a filter jar purchased in a local supermarket in Italy were sampled in falcon tubes of 15 mL. All tubes were rinsed with the sample three times in advanced and stored at 4° C in darkness. Lake waters were also used as matrix to be spiked with suspensions of Ag-NPs in order to evaluate the performance of the methodology.

6.2.3 Equipment

ICP-MS. A PerkinElmer NexIon 300D quadrupole ICP-MS, equipped with a SC Fast peristaltic pump, a Meinhard concentric nebulizer, a glass cyclonic spray chamber and a standard quartz torch (2.5 mm i.d). The system operated in standard mode, dwell time of 50 ms and integration time of 1 s. Operating conditions were optimized to produce maximum sensitivity tuning the nebuliser gas flow, positions of the deflector and the torch.

External calibration was performed by analysis of a blank and five solutions of dissolved Ag in 1% HNO₃ ranging from 0 to 100 µg L⁻¹. The 107 Ag intensity for each solution was then averaged from the entire length of the analysis. Reported values were the average result of 5 measurements. No internal standard was employed, as only 107 Ag was quantified during the run. To ensure the absence of significant instrumental drift over time, a single 100 ng L⁻¹ Ag dissolved calibration check standard was run for every ten samples analysed. Moreover, as zirconium and yttrium could cause interferences during total Ag quantification, zirconium (90 Zr, 91 Zr and 92 Zr) and yttrium (89 Y) were checked during the

analyses of real test samples to ensure that the quantification of total Ag content was correct.

Single Particle-ICP-MS. A PerkinElmer NexIon 300D ICP-MS was also used with identical configuration to the previous section for SP analysis, using the Nano Application Module, present in software Syngistix™, PerkinElmer (Waltham, MA, US). The length of dwell time was set at 50 μ s. The data acquisition time was 60 seconds. The 107 Ag intensity was monitored. The flow rate was measured daily and values ranged from 0.310 to 0.335 mL min⁻¹. The transport efficiency (TE) was calculated every day following different approaches that will be discussed later. Working suspensions of Ag-NPs were made diluting the stock solutions with Milli-Q® ultrapure grade water. Solutions of dissolved Ag⁺ for ionic calibration were also prepared by dilution in pure water.

Separation by centrifugation. In order to separate Ag-NP from dissolved Ag in consumer products 1-7, 4 mL aliquots of the samples were placed in Centrifugal filters Amicon® Ultra-15 (membrana PLBC Ultracel-PL, 3 kDa) supplied by Sigma-Aldrich (Cork, Ireland) and subjected to centrifugation at 4000 rpm during 40 min. To ensure that all the dissolved Ag passed through the filters, a standard solution of 50 ppb was also subjected to the same process. More than 98% of the element was found in the filtrate. The supernatant was reconstituted to 4 mL in water and sonicated for 12 minutes before SP-ICP-MS measurements.

AF4-UV separation and fraction collection. The AF4 instrument comprised an Eclipse Dualtec Separation System from Wyatt Technology Europe and an Agilent 1260 Infinity high performance liquid chromatograph (HPLC) equipped with a degasser (G1322A); an isocratic pump (G1310B); an autosampler (G1329B); a multi wavelength detector (G1365C), from Agilent Technologies. A pre-cut 10 kDa regenerated cellulose membrane and a 350 μ m height spacer were assembled inside the Eclipse SC channel 153 mm length, which was located inside a Thermos[PRO] thermostatic unit, all from Wyatt Technologies. The temperature was set at 25 °C. The eluent was ultrapure water with pH adjusted to 9.5 with

NaOH (0.1 M) which was freshly prepared every day. Processing of AF4-UV data was performed using ASTRA[®] 6.1 software (Wyatt Technology). The fractionation method applied has been described somewhere else (Geiss et al., 2013). Fraction collection was conducted by switching the position of a Rheodyne valve located after the waste outlet of the detector during the time (t) corresponding to the peak elution. Dilution factor was calculated considering the injection volume (50 μL), detector flow rate (500 $\mu\text{L min}^{-1}$) and peak width (min).

Characterisation by Transmission Electron Microscopy (TEM) (JEM 2100, JEOL-Italy) at an accelerating voltage of 200 kV was used to demonstrate the presence of Ag-NPs. Samples (3 μL) were spotted on ultrathin copper-Formvar carbon coated (200 mesh) grids (Tedpella Inc.) and dried overnight. Energy dispersive spectroscopy (EDS) analysis was also performed in order to identify/confirm the presence of Ag.

6.3 Results and discussion

6.3.1 Single particle ICP-MS method

Selection of Transport Efficiency Calibration (TE). To obtain accurate results the selection of an appropriate calibration strategy is crucial. As Pace and co-workers introduced (Pace et al., 2011), the calibration for SP-ICP-MS involves the calculation of the sample introduction system flow-rate and the TE of NPs into the plasma. For this purpose, the most frequently used NP standard is RM 8013 reference material from NIST, which contains Au-NPs with reference nominal size value of 60 nm and information value for concentration. One approach consists in building a calibration curve with ionic dissolved element followed by the measurement of a suspension of spherical NP standards with precisely known concentration (calibration by NP-particle concentration). To analyse a different target analyte, such as Ag-NP, a complementary calibration curve with ionic Ag is needed, assuming equal nebulisation and ionisation behaviours as Au. Related to this, in order to suppress any matrix effect in SP analyses it is advisable to prepare the solutions of ionic element in pure water instead of typical acidic water, as water

is typically the matrix of the samples and the intensity signal is often enhanced by the presence of acid. Regarding the analysis of Ag-NPs, calibration with recently released RM 8017 (75 nm PVP-Ag-NPs) also allows the measurement of NPs of the same nature as the NPs standards applying calibration by NP-particle concentration. (In this work, 75 nm PVP-Ag-NPs were purchased directly from the producer, nanoComposix Inc. and not from NIST). An alternative calibration strategy involves the use of a series of NP standards of different well known sizes to correlate a resulting increasing signal with increasing particle size, and therefore mass (calibration by NP-particle size). In contrast to the described procedures that require the use of NPs as calibrants, the gravimetric analysis of the aspirated solution has been also proposed to calculate the TE. Table 6.1 includes the values of the TE obtained by the different calibration strategies on four different days, together with their respective interday relative standard deviation (RSD). Values for TE differed significantly for gravimetric, particle size and particle concentration approaches. The greater interday RSD for particle size calibration could be attributed to the use of more than one NP standard, which involves the preparation of a greater number of suspensions and consequently greater number of possible associated errors due to the operator or to the condition of the standard materials. It is noteworthy that the TE obtained applying calibration by NP-particle concentration was different when Au-NPs were used instead of Ag-NPs. As it is expected, different TEs lead to different measured size values, as Table 6.1 illustrates for the case of 60 nm Ag-NPs. In this case, results applying calibration with Ag-NPs standards were more accurate than those obtained with Au-NPs. Taking into account a better interday repeatability, TE calibration by NP-particle concentration using 75 nm Ag-NPs was selected as the best performing approach to characterise Ag-NPs in test samples.

Evaluation of analytical performance and analytical figures of merit. As it was introduced before, the evaluation of the analytical performance of a method is also advised for SP-ICP-MS methods. Of particular importance is to characterise well the repeatability, accuracy, limits of detection and SP-linearity range, where the likelihood of particle counting artefacts is low.

Table 6.1 Evaluation of transport efficiency for single particle calibration

Transport Efficiency (%); n=4 (interday)							
		Ag				Au	
Gravimetric		Particle Size ^a		Particle Concentration ^b		Particle Concentration ^c	
Average	RSD (%)	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)
13.8	1.4	11.0	6.6	8.9	2.9	7.1	1.8

60 nm Ag-NPs standard (60.8 ± 6.6 nm) TEM diameter ; n=3 (intraday)							
		Ag				Au	
Gravimetric		Particle Size		Particle Concentration		Particle Concentration	
Mode	RSD (%)	Mode	RSD (%)	Mode	RSD (%)	Mode	RSD (%)
62	0	59.3	1.0	55.3	1.1	49.7	1.2
Average		Average		Average		Average	
64.3	0.9	61.7	0.9	57.3	1.0	51.7	1.1

^a using 30, 50 and 80 nm Ag-NPs standards^b using 75 nm Ag-NPs standard^c using 60 nm Au-NPs standard

Linearity, repeatability and trueness. The linearity range of the present SP-ICP-MS method has been evaluated in terms of particle size and particle number concentration. As SP-ICP-MS is a technique that counts the number of particles, dimensional units for number based concentration are used for analyses in SP mode. Suspensions of standards with 30, 50 and 80 nm nominal size were used to prepare dilutions containing (1, 5, 25, 50) $\times 10^4$ particles mL^{-1} , respectively, assuming perfect spheres and negligible ionic contribution. Figure 6.1A shows the correlation between theoretical number of particles (prepared) and the actual number of particles measured by SP-ICP-MS. The three classes of standards showed acceptable linearity in the whole range under study ($r^2 > 0.99$) and excellent linearity in the range (1 - 25) $\times 10^4$ particles mL^{-1} ($r^2 > 0.999$). The evaluation of the linearity range suggests that the quantification within such a range is possible for spherical monodispersed standards. The repeatability was also studied according to the RSDs of the particle number concentration values found experimentally at four concentration levels. RSDs for three replicates were lower than 4.6% in every case, except 21.1% found for 30 nm at the lowest concentration level (104 particles mL^{-1}), hence proving overall precise quantification in the concentration range studied.

Related to the particle size, Figure 6.1B compares the measured average particle size values for standards 30, 50 and 80 nm with their respective reference values. In general, SP-ICP-MS average values are in acceptable agreement with the expected ones. The measured size values were accompanied by RSDs lower than 3.1% in every case, with the exception of 13.3% found for 30 nm at the lowest concentration level. In addition, trueness was determined applying the t-test ($p=95\%$) at the two intermediate concentration levels. SP-ICP-MS size values for Ag-NPs of each size were compared with the theoretical reference size value. The following equation allowed the calculation of the t-values:

$$t = \frac{|\bar{x} - \mu|}{s} \sqrt{n}$$

Where $\bar{X}$ is the average size measured for the six replicates, μ is the theoretical "should"-value, s is the standard deviation of the 6 replicates, n is the number of replicates for measurement (6 replicates). The resulting t-values were compared with theoretical value for a confidence interval of 95% and $n-1$ degrees of freedom ($t=2.57$). Despite of the fact that the reference size values correspond to TEM measurements, the calculated t values were within the range of acceptance in 3 of 6 measurements. In the sight of these results, the present SP-ICP-MS method exhibits good repeatability an overall acceptable trueness.

Figure 6.1 (A) Evaluation of the performance of the method: correlation between theoretical number of particles (prepared) and the actual number of particles measured by SP-ICP-MS; (B) comparison of the measured average particle size values for standards 30, 50 and 80 nm ($n=3$) with their respective reference values

Limits of detection. The minimum detectable size or detection limit (size DL) has been defined as the smallest ion burst that is distinguished as a particle event (Tuoriniemi et al., 2014), and it is often calculated as a function of the standard deviation (σ) of a data set acquisition (Tuoriniemi et al., 2012; Mitrano et al., 2012b). For instance, Laborda and co-workers applied a criterion based on three times the standard deviation of the continuous background ($\mu+3\sigma$) for discrimination of NP events from the background (Laborda et al., 2013). In practical terms, generally after signals acquisition the raw data files (intensity pulses and time) corresponding to each measurement are exported to a calculation data sheet, the threshold criteria discerning NP signals is set and then applied iteratively until no variation is observed.

As a positive feature of the ICP-MS instrument used for this work, the software automatically establishes the threshold criteria ($\mu+3\sigma$) above which intensity counts are assigned to size values, based on a previously existing calibration. This operation allows monitoring the number PSD on real time (while it is measured). In the present study, calibrations were performed every day and during the period of the experiments, the minimum detectable size observed in the frequency histograms ranged between 12-15 nm. Size DLs improved only slightly ($\sim 1, 2$ nm) after thorough clean-up of the torch and the cones. Experimental values are in agreement with the calculated size DL provided by Lee and co-workers for Ag (13 nm) (Lee et al., 2014). In practice, the minimum complete PSD that can be detected depends also on the polydispersity (width) of the sample. In the current case, it was possible to detect the complete PSD of Ag-NPs standards with 30 nm nominal size, in accordance with previous studies (Mitrano et al., 2012a; Laborda et al., 2011). The average size value of Ag-NPs standards with 20 nm nominal size was accurately measured despite of the fact that the lower end of the PSD fell below the size DL, as it is shown in SI-Figure 1.

6.3.2 Sample preparation prior to SP-ICP-MS.

The direct measurement of the total amount of silver does not provide any information to discern between ionic or particulate. Therefore, to determine the fraction of NP and dissolved material in the samples a separation step such as centrifugal filtration or AF4 is sometimes included before the detector. Analysis by SP-ICP-MS has the potential to overcome this issue. In this regard, monitoring the evolution of the PSD on real time allows the evaluation of the results in-situ. Thus, if eventual dilutions are needed to avoid artefacts, these can be performed subsequently without further data treatment. The last is particularly useful for the analysis of real samples of unknown composition or polydispersed samples. The methodology proposed here involves consecutive analyses of an unknown aqueous sample with different dilution factors until no variation in the particle size is observed, while keeping particle counts proportional to the dilution applied. Table 6.2 illustrates the results obtained after the sequential analysis of a real sample with unknown composition. The results for samples with little dilution (set of results A) led to greater particle size values and no proportional particle counting. Set of results B shows minor changes in the measured particle size and proportional peak counting, suggesting that measurements were conducted under SP premises. The last is supported by complementary NP characterisation by TEM (shown in Figure 6.2). Set of results C provides similar values for particle size, but the number of peaks is too low and lacks of proportionality. Interestingly, although the number of particles measured in set A of results fell within the linearity range studied with PVP-Ag-NPs standards, the measured sizes of the particles released into the sweat simulant revealed partial aggregation. This phenomenon can be attributed to the higher ionic content of suspensions with low dilution factors that would cause the aggregation of uncoated Ag-NPs (El Badawy et al., 2010; Hedberg et al., 2014).

Table 6.2 Direct analysis of a sweat simulant after migration from plaster with Ag (friction) by SP-ICP-MS (consecutive dilutions)

Dilution of original	Most frequent size (nm)	Average size (nm)	Number of measured peaks	Measured Particle number concentration ^o (N mL ⁻¹) x 10 ⁻³	Measured Particle mass concentration ^{a, o} (ng L ⁻¹)	Evaluation of results	
1/2	64	73	941	35.7	51.4	A	Over-estimated size Non-proportional counting
1/10	46	60	952	36.1	19.3		
1/20	36	50	779	29.5	7.6		
1/30	32	45	1000	37.9	6.8	B	Single Particle range
1/50	29	42	576	21.8	3.3		
1/200	29	39	217	8.2	1.1	C	Too few peaks Statistically poor
1/400	31	42	101	3.8	0.6		

^a : estimated assuming spherical shape, bulk density of 10.49 kg m⁻³ and modal value (most frequent value) of particle size as equivalent spherical diameter.

^o : dilution factor correction not applied

Figure 6.2 Results of the analysis of real samples: (A) SP-ICP-MS histogram of Ag-NP released by plaster to sweat simulant under friction during 18 hours; (B) TEM image of Ag-NP aggregate present in the sweat simulant migration solution; (C) SP-ICP-MS histogram of Ag-NP released by a well-used water filter into filtered tap water; (D) TEM image of Ag-NPs present in the filtered water

In order to evaluate the suitability of the direct analysis of diluted monodispersed standards and real test samples by SP-ICP-MS, SP-ICP-MS measurements were also conducted for fractions collected by AF4 and supernatant fractions after centrifugal filtration. Table 6.3 summarises illustrative results, including standard ICP-MS measurements of the elemental content of the different fractions. In general, the average size values obtained did not differ. An increased modal value was observed after filtration and fractionation for 20 nm particles and consumer product 6, suggesting that at least part of the smaller particles were lost during the process. On the other hand, both average and modal particle size values for 60 nm particles were unaltered

regardless the type of treatment, which was expected as 60 nm is not a border-line case in terms of size. Regarding to the mass balance of silver after centrifugal filtration indicates losses of material during the filtration. The same phenomenon was observed for AF4. Moreover, although SP-ICP-MS requires diluted samples, in some cases the sensibility of AF4-UV was the limiting factor, hampering proper fractionation, as it was the case of a migration solution from plaster to water. Worth mentioning that the direct on-line coupling of AF4 (as well as any other flow-analysis technique) to SP-ICP-MS is not straightforward, as a concentration gradient would entail likely artifacts in SP-ICP-MS results. Taking all this into account, direct analyses of diluted samples was preferred.

Table 6.3 Standard ICP-MS and SP-ICP-MS measurements of 20 and 60 nm Ag-NP standards and consumer product 6, following different sample pre-treatment

Product	Direct dilution			Filtration and resuspension of NP content							AF4 Fraction				
	Total Ag content [°]	Size (nm)*		NP Concentration [°]	NP Recovery [°]		Filtrate Concentration [°]	Size (nm)*			NP Concentration [°]	NP Recovery [°]		Size (nm)*	
	(mg L ⁻¹)	Mode	Average	(mg L ⁻¹)	RSD (%)	(%)	(mg L ⁻¹)	RSD (%)	Mode	Average	(mg L ⁻¹)	RSD (%)	(%)	Mode	Average
20 nm Ag-NP	21.6	13	20	16.3	1.5	75.4	0.1	15.3	16	22	11.2	0.5	51.9	16	22
60 nm Ag-NP	23.1	58	61	7.8	6.3	33.8	1.4	12.0	58	61	14.8	6.5	64.1	59	61
Product 6	24.8	13	20	6.5	18.5	26.2	10.8	4.1	20	26	12.8	2.7	51.6	13	14

*: by SP-ICP-MS

°: by ICP-MS using external ionic calibration

6.3.3 Analysis of real samples by SP-ICP-MS.

Description of samples and total Ag content. The list of consumer products studied, together with a brief description of them with the information offered by the producers is included in Table S1. In general, the description of the products does not reflect if they contain only ionic silver, particulate silver or a mixture of them, being the ambiguous term "ionic colloidal silver" often used. The total Ag elemental content of all the consumer products was measured by ICP-MS in standard mode applying six point external calibrations with ionic silver standards. Interestingly, the total concentration of silver differed from the concentrations declared by the producers. In the case of product 1, the measured concentration was less than 10% the declared one. The same type of analysis was performed for tap water before and after filtration with a filter jar for hard waters; lake water and migration solutions from the plaster after ultrasound treatment and friction test (water and sweat simulant, respectively). All the results are summarised in Table 6.4. With the exception of tap water samples, silver was found at $\mu\text{g L}^{-1}$ level in all the migration solutions from plasters and water filters. To discern between dissolved and particulate silver, SP-ICP-MS characterisation was carried out.

Size characterisation and quantification. Direct SP-ICP-MS analyses of the selected samples appropriately diluted were performed applying the methodology described above and the results are included in Table 6.4. SI-Figures 2 A-G show the obtained PSDs of Ag-NPs contained in consumer products 1-7. Quantification of particles number was not possible because the profiles measured were generally incomplete, with the lower ends always falling below the size DL. However, the average sizes of the detectable particles were into agreement with size values derived from complementary techniques. Related to this, all fractograms obtained after AF4 analyses of products 1-7, which are included in SI-Figure 3, showed one peak of variable peak area eluting before the 20 nm Ag-NPs standards. Results obtained by TEM characterisation (SI-Figures 4 A-G) also support the results by SP-ICP-MS, suggesting that a reference nominal value can be correctly assigned to the consumer products under study.

Table 6.4 Ag content and analysis of Ag NP in consumer products and aqueous samples

Consumer products	Total Ag content ^o (mg L ⁻¹)	Ag-NP by SP-ICP-MS			
		Size (nm)		Measured Particle number concentration a (N mL ⁻¹) x10 ⁻³	Measured Particle mass concentration ^{a, b} (ng L ⁻¹)
		Mode	Average		
Product 1*	1.6	13	20	n.a. *	n.a. *
Product 2*	9.5	13	18	n.a. *	n.a. *
Product 3*	8.3	20	27	n.a. *	n.a. *
Product 4*	7.5	16	24	n.a. *	n.a. *
Product 5*	30.3	23	27	n.a. *	n.a. *
Product 6*	24.8	12	20	n.a. *	n.a. *
Product 7*	10.2	12	19	n.a. *	n.a. *
Spikes (50-80 nm) on Product 6	n.a.	48-74	50-76	46.2-77.0	28.0-172.3
Aqueous sampes	Total Ag content ^o (µg L ⁻¹)	Ag-NP by SP-ICP-MS			
		Size (nm)		Measured Particle number concentration a (N mL ⁻¹) x10 ⁻³	Measured Particle mass concentration ^{a, b} (ng L ⁻¹)
		Mode	Average		
Migration from plaster to water (sonication)	224.5	32	47	1147.5	206.5
Migration from plaster to sweat (friction)	38.9	45	54	1090	146
Tap Water	n.d.	n.d.	n.d.	n.d.	n.a.
Tap water filtrated (used filter)	9.3	38	43	93.5	28.2
Tap water filtrated (new filter)	97.1	77	120	1219.3	3057.4
Lake water	19.7	n.d.	n.d.	n.d.	n.d.
Spikes (50-80 nm) on lake water	n.a.	48-74	49-76	47.9-82.6	29.1-183.9

^o: by ICP-MS using external ionic calibration.

^a: by SP-ICP-MS. Final dilution factor correction applied.

^b: estimated assuming spherical shape, bulk density of 10.49 kg m⁻³ and modal value (most frequent value) of particle size as equivalent spherical diameter. Factor dilution factor correction applied.

*: Partial PSD, near to minimum detectable size.

n.d.: Not detected (too few peaks, statistically poor data) ; n.a.: not applicable.

Additionally, 4 cm² pieces of plaster claiming silver were subjected to a migration test by friction into sweat simulant as described in the experimental section. The migration solution was duly diluted and analysed by SP-ICP-MS. Results summarised in Table 6.4 show the presence of Ag-NPs in the migration solution, although these represent approximately less than 0.4% of the total amount of silver in the migration solution. According to the generated PSDs practically all the Ag-NPs measured were below 100 nm as Figure 6.2A displays, with average and modal size values into good agreement with the information derived from TEM images (Figure 6.2B). As it was expected, the release of silver after ultrasound treatment was much higher, as the provided energy was greater. TEM images in SI-Figures 5A-B show the presence of big aggregates in the migration solution that explains such a greater concentration value. Interestingly, the average and modal size values of migrated Ag-NPs were smaller than the values found after the friction test. This can be attributed to a partial dissolution of the NPs promoted by the energy supplied and/or more likely to partial disaggregation of the released aggregates. It is noteworthy that the observed micron-sized aggregates did not affect the measurements under SP mode as the probability of measuring them after dilution is low and the interactive integration window for the histogram curve allows excluding eventual big artefacts in case they appear.

Tap water samples were also measured, before and after filter-jar filtration. Two identical filters were utilised: one was brand new (never used before) and the other one was well-used (after filtration of c.a. 100 L of water. Silver was not found in the unfiltered sample. However, silver could be measured after filtration with both the well-used and the new filter, in approximate ratio of 1/10. SP-ICP-MS analysis revealed the presence of Ag-NPs in both samples. The amounts of particulate silver corresponded to 0.3% and 3.2% of Ag-NPs over the total Ag content for the well-used and new filter, respectively. After examination of the generated PSDs, the size of Ag-NPs was smaller when the well-used filter was employed, being all the NPs below 100 nm, whereas in the case of the new filter only 63.7% of the measured particles were below 100 nm. The differences observed suggest that SP-ICP-MS is well suited to monitor the fate of NPs in

environmental samples and samples of relevance for exposure assessment, as it has been also proposed in previous studies (Mitrano et al., 2014).

To extend the evaluation of the applicability of the present SP-ICP-MS methodology, consumer product 6 and a lake waters sample were also spiked with a bimodal mixture of 50 and 80 nm Ag-NPs, at final concentration of 50×10^3 particles mL^{-1} , each. The resulting histograms are displayed in Figure 6.3 together with their respective frequency curves. For the spiked consumer product, SP-measurements led to average size values of 50 and 76 nm with recoveries of 80.7 and 127.6%, respectively. In the case of spiked lake waters, the measured values were 49 and 76 nm, with recoveries of 83.8 and 135.0% of the original number of particles, respectively. Despite of the presence of a background concentration of dissolved silver and in the case of product 6, also a population of Ag-NPs near the size DL, size measurements of the spiked standards were accurate. On the other hand, although particle number concentrations for 80 nm were overestimated, concentrations measured for 50 nm standard were close to the real value.

Figure 6.3 Histogram of Ag-NP measured by SP-ICP-MS of spikes of 50 and 80 nm Ag-NPs standards on: (A) product 6; (B) lake water

6.4 Conclusions

This work has proposed a methodology to detect and characterise the PSD of Ag-NPs in consumer products and environmental waters using SP-ICP-MS. The method was developed and evaluated using Ag-NPs standards. The analytical figures of merit showed excellent linearity, good repeatability and an overall acceptable trueness in the studied concentration range. The size DLs ranged between 12-15 nm. Nanoparticle sizing by SP-ICP-MS after AF4 fraction collection and centrifugal filtration did not improve the analytical performance of direct SP-ICP-MS analysis.

The procedure has been applied to characterise the PSDs of Ag-NPs in seven different liquid silver-based consumer products. Although the existence of size DLs hampered

the determination of the lower part of the PSD by SP-ICP-MS, it was possible to assign a reference nominal size value to the detectable particles. Such SP-ICP-MS nominal size values were into agreement with size values derived from complementary AF4-UV and TEM characterisations. Moreover, the quantification and size characterisation of the Ag-NPs migrated under specific conditions from plasters claiming silver into water and sweat simulant was investigated by the proposed methodology, allowing the determination of the complete PSD, which fell below 100 nm. Similarly, the PSDs of Ag-NPs in filtered tap water were successfully characterised in terms of size and number. Concentration levels found in unfiltered water and lake water were below the SP-ICP-MS method dynamic range. In general, AF4 and TEM results supported the SP-ICP-MS size characterisation.

Due to the existence of technical DLs, which depend on the nature of each analyte, the results presented in this paper indicate that SP-ICP-MS is well suited to be used as a positive screening test in the simultaneous quantification and size characterisation of Ag-NPs in consumer products and samples of environmental interest. Similarly, SP-ICP-MS can be used to monitor the fate of NPs in samples of relevance for exposure assessment.

CHAPTER SEVEN – CONCLUSIONS

CONCLUSIONS

The results of the investigation obtained during the present work have allowed to fulfil the main objectives of this thesis: the development and validation of analytical methods to detect emerging contaminants in different environmental samples of agricultural interest. Below, are summarized the most relevant conclusions of the works presented.

CHAPTER TWO – PHARMACEUTICALS IN SLUDGE

1. A multiresidue method that combines SLE and GC–MS/MS was satisfactorily developed for the analysis of 16 pharmaceuticals (acid, neutral and basic) in biosolids.
2. The proposed method was applied to biosolids from two Spanish areas (Madrid and Catalonia), where caffeine, ibuprofen, salicylic acid and fenopfen were detected in all the samples analyzed.

CHAPTER THREE – EMERGING CONTAMINANTS IN POULTRY MANURE

1. A multiresidue method for the determination in manure of 41 selected environmental contaminants by GC-MS, based on a modified QuEChERS method has been developed and validated.
2. This method was applied to poultry manure samples collected from farms located in Castilla-León, and the presence of some of the studied contaminants was confirmed, being pyrethroids biocides, PAHs, 4,4' DDT and metabolites the compounds mainly detected.

CHAPTER FOUR – EMERGING CONTAMINANTS IN SOIL: PHARMACEUTICALS AND PYRETHROIDS

Pharmaceuticals:

1. A multiresidue method to detect 15 pharmaceutical compounds (acid, neutral and basic) in soil, based on an ultrasound assisted extraction procedure followed by GC-MS determination was satisfactorily developed.
2. The method was applied to agricultural fields in several Spanish areas (Segovia, Murcia and Valencia), showing the ubiquitous presence of various pharmaceutical compounds, such as salicylic acid and paracetamol, in the three areas studied, as well as fenoprofen in Segovia and allopurinol and carbamazepine in Valencia.

Pyrethroids:

3. A method to detect ten pyrethroids in agricultural soils by GC-MS, after ultrasounds assisted extraction, was satisfactorily developed. It was applied to monitor the presence of PYs in a paddy field area in Valencia, showing their ubiquitous presence as well as the identification of WWTPs effluents as one of the main sources of pollution using a GIS program.
4. A section with levels of bifenthrin that may cause harmful effects on the aquatic invertebrates was identified within the monitored area, where phytoremediation may be applied to reduce this risk.

CHAPTER FIVE – EMERGING CONTAMINANTS IN AQUATIC PLANTS

1. A multiresidue method was developed and validated for the determination of 31 ECs in aquatic plants by GC-MS, based on UA-MSPD to extract the studied compounds from *Typha angustifolia*, *Arundo donax*, *Oryza sativa* and *Lemna minor*.
2. The developed method was applied to aquatic plants collected from three rivers located in different Spanish regions (Madrid, Andalucia and Valencia). Six of the thirty one compounds studied (pyrethroid biocides and personal care products) were quantified.

CHAPTER SIX – SILVER NANOPARTICLES IN AQUEOUS SAMPLES

1. A methodology to detect and characterize the particle size distribution (PSD) of Ag-NPs in consumer products and environmental waters using SP-ICP-MS was evaluated. The sample pretreatment (AF4 fraction collection or centrifugal filtration) did not improve the analytical performance of direct SP-ICP-MS analysis, therefore direct analysis was used.
2. The procedure was applied to seven different liquid silver-based consumer products and plasters, allowing the determination of the complete PSD just for plasters. Similarly, the PSDs of Ag-NPs in filtered tap waters were successfully characterized in terms of size and number. However, the concentration levels found in fresh water samples from Italy were below the SP-ICP-MS method dynamic range.

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SUPPLEMENTARY INFORMATION

SI-Table 1 Results of analyses of soils from different agricultural areas in Spain

	1	2	3	4	5	6	7	8	9
Clofibric acid	0.7 ± 0.1	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Ibuprofen	0.3 ± 0.0	0.2 ± 0.0	n.q	n.q	n.q	n.q	n.q	0.3 ± 0.0	0.2 ± 0.0
Salicylic acid	3.1 ± 0.3	3.6 ± 0.4	1.4 ± 0.2	5.4 ± 0.1	7.0 ± 0.1	4.0 ± 0.4	4.9 ± 0.1	5.6 ± 0.6	11.0 ± 0.5
Allopurinol	n.q	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.5 ± 0.0
Paracetamol	n.d	n.d	n.d	n.q	n.q	n.d	n.q	n.q	0.4 ± 0.0
Gemfibrozil	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Fenoprofen	0.3 ± 0.0	0.4 ± 0.0	n.d	n.d	0.4 ± 0.0	1.4 ± 0.0	0.5 ± 0.1	3.0 ± 0.3	2.8 ± 0.2
Amitriptyline	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Metoprolol	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Naproxen	0.3 ± 0.0	n.d	0.3 ± 0.0	n.d	n.d	1.9 ± 0.8	n.d	n.d	5.9 ± 0.4
Mefenamic acid	n.q	n.d	n.d	n.d	n.q	1.7 ± 0.4	0.6 ± 0.0	n.d	2.7 ± 0.1
Ketoprofen	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Carbamazepine	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Diclofenac	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Fenofibrate	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

n.d, not detected; n.q, not quantified

SI-Table 1 (continued)

	10	11	12	13	14	15	16	17	18
Clofibric acid	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Ibuprofen	1.5 ± 0.1	n.q	n.q	0.2 ± 0.1	n.q	n.d	n.d	n.d	n.q
Salicylic acid	9.7 ± 0.5	6.1 ± 0.2	37.1 ± 0.8	5.1 ± 0.7	3.2 ± 0.4	1.7 ± 0.3	3.2 ± 0.5	1.6 ± 0.1	1.8 ± 0.9
Allopurinol	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.q
Paracetamol	n.d	n.q	n.q	n.d	n.d	n.q	0.1 ± 0.0	n.q	0.4 ± 0.1
Gemfibrozil	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Fenoprofen	n.d	3.2 ± 0.1	0.9 ± 0.1	n.d	n.d	n.d	n.d	n.d	n.d
Amitriptyline	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Metoprolol	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.q	n.d
Naproxen	n.d	n.d	n.d	n.d	n.d	n.d	0.3 ± 0.1	n.d	n.d
Mefenamic acid	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Ketoprofen	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Carbamazepine	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.q
Diclofenac	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Fenofibrate	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

n.d, not detected; n.q, not quantified

SI-Table 1 (continued)

	19	20	21	22	23	24	25	26	27
Clofibric acid	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Ibuprofen	n.d	n.d	n.q	n.d	n.q	0.7 ± 0.0	n.q	0.5 ± 0.1	0.7 ± 0.0
Salicylic acid	2.7 ± 0.2	4.9 ± 0.2	4.0 ± 0.2	3.4 ± 0.1	3.8 ± 0.2	5.3 ± 0.1	4.6 ± 0.2	7.6 ± 0.9	5.4 ± 0.1
Allopurinol	0.3 ± 0.0	10.0 ± 0.2	47.0 ± 1.0	0.9 ± 0.1	n.d	0.3 ± 0.0	n.q	2.6 ± 0.7	n.q
Paracetamol	n.q	0.2 ± 0.0	0.5 ± 0.1	n.q	0.4 ± 0.1	n.q	0.4 ± 0.0	0.2 ± 0.1	n.d
Gemfibrozil	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Fenoprofen	n.d	n.d	0.4 ± 0.0	n.d	n.d	n.d	n.d	n.d	n.d
Amitriptyline	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Metoprolol	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Naproxen	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Mefenamic acid	n.d	n.d	n.d	n.d	n.d	n.d	n.d	1.3 ± 0.1	n.d
Ketoprofen	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Carbamazepine	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	0.5 ± 0.0	n.q	1.0 ± 0.0	2.1 ± 0.2	1.2 ± 0.1	2.8 ± 0.1
Diclofenac	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Fenofibrate	n.d	n.d	n.d	n.d	n.d	n.d	7.8 ± 0.3	n.d	6.3 ± 1.5

n.d, not detected; n.q, not quantified

SI-Table 1 (continued)

	28	29	30	31
Clofibric acid	n.d	n.d	n.d	n.d
Ibuprofen	0.7 ± 0.1	1.2 ± 0.2	0.7 ± 0.0	0.2 ± 0.1
Salicylic acid	6.8 ± 0.5	5.4 ± 0.3	3.9 ± 0.1	3.1 ± 0.3
Allopurinol	n.q	0.2 ± 0.0	n.q	n.d
Paracetamol	n.q	n.d	n.q	n.q
Gemfibrozil	2.5 ± 0.1	5.4 ± 0.2	n.d	n.d
Fenoprofen	1.1 ± 0.3	n.d	0.5 ± 0.1	0.6 ± 0.0
Amitriptyline	n.d	n.d	n.d	n.d
Metoprolol	n.d	n.d	n.d	n.d
Naproxen	0.6 ± 0.1	n.d	n.d	n.d
Mefenamic acid	n.q	n.q	n.q	n.d
Ketoprofen	n.d	n.d	n.d	n.d
Carbamazepine	3.5 ± 0.2	1.3 ± 0.1	1.5 ± 0.1	2.1 ± 0.2
Diclofenac	n.d	n.d	n.d	n.d
Fenofibrate	n.d	n.d	n.d	n.d

n.d, not detected; n.q, not quantified

SI-Table 2 Operational conditions of GC-MS

GC conditions:

Carrier gas	Helium at 1.2 mL min ⁻¹
Injection	Solvent-vent, 5 µL, 50 °C (0.1 min) → 600 °C min ⁻¹ → 300 °C (5 min)
Column	ZB-5MS (5% phenyl polysiloxane), 0.25 mm i.d. × 30 m Film thickness: 0.25 µm
Column temperature	60 °C (2.6 min) → 20 °C min ⁻¹ → 300 °C (5 min)

MS conditions:

Ionization mode	electron impact ionization (70 eV)
Ion source temperature	230 °C
Quadrupole temperature	150 °C
Solvent delay	14 min
Detection mode	Selected ion monitoring (SIM)

Compound	Retention time (min)	Target ion (m/z)	Qualifier ion 1 (m/z)	Qualifier ion 2 (m/z)
PERM-D6	15.91	171	169	184
RESM	14.63	123	171	128
BIFE	14.90	181	165	166
FENP	14.98	181	125	265
CYHA	15.40	181	197	208
PERM	15.86	183	163	165
CYFL	16.36	163	206	226
CYPE	16.59	181	163	165
FLUV	17.38	250	252	181
ESFE	17.43	125	167	181
DELT	17.96	253	251	181

SI-Table 3 Physico-chemical properties of soil (0-40 cm), 1st sampling (plow period)

Sample	x	y	Sand (g kg ⁻¹)	Silt (g kg ⁻¹)	Clay (g kg ⁻¹)	Texture	EC (dS m ⁻¹)	pH	Carbonate (g kg ⁻¹)	SOC (g kg ⁻¹)	OC (g kg ⁻¹)
1	730030	4353641	210	395	395	Clay	0.8	8.1	387	0.3	36.4
2	729132	4353014	230	380	390	Clay	0.5	7.9	435	0.0	21.2
3	727292	4362814	155	450	395	Silty Clay	0.4	7.6	403	0.0	19.6
4	727465	4362358	200	430	370	Silty Clay	0.5	7.8	376	0.0	23.0
5	728019	4363534	180	415	405	Silty Clay	0.6	7.9	391	0.0	28.8
6	727503	4364751	210	450	340	Clay Loam	0.5	7.8	322	0.0	35.2
7	728104	4365204	175	465	360	Silty Clay	0.4	7.7	356	0.5	29.9
8	728115	4365798	210	390	400	Clay	0.6	7.7	362	1.1	104.9
9	725228	4360223	295	475	230	Clay Loam	0.9	7.3	375	0.6	21.9
10	724983	4358520	210	440	350	Clay Loam	0.6	7.5	343	0.8	100.3
11	724644	4358625	230	430	340	Clay Loam	1.0	7.6	359	0.7	37.0
12	729260	4355993	130	435	435	Silty Clay	0.6	7.6	368	0.9	35.8
13	731793	4356293	678	238	125	Sandy Loam	0.7	7.6	325	0.3	18.6
14	730890	4355300	315	400	285	Clay Loam	0.5	7.5	362	0.0	21.4
15	731194	4353858	285	385	330	Clay Loam	0.8	7.4	363	0.7	39.8
16	724412	4355421	390	345	265	Clay Loam	0.5	7.6	326	0.5	22.8
17	725451	4354162	190	505	305	Silty Clay Loam	0.6	7.7	346	0.5	31.2
18	729096	4365217	155	410	435	Silty Clay	0.6	7.7	345	0.5	27.4
19	728660	4365858	240	370	390	Clay	0.6	7.6	336	0.1	32.4
20	729909	4361649	415	330	255	Clay Loam	0.7	7.3	278	0.2	29.7
21	725487	4362542	290	445	265	Loam	0.9	7.2	347	0.1	35.0
22	726249	4362060	145	440	415	Silty Clay	0.5	7.6	358	0.1	37.4
23	723835	4357354	400	345	255	Loam	0.5	7.7	299	0.2	22.5
24	723892	4356141	320	340	340	Clay Loam	0.6	7.6	330	0.4	24.4
25	724131	4356182	220	405	375	Clay Loam	0.7	7.6	370	0.4	31.2
26	729183	4354909	195	505	300	Silty Clay Loam	0.7	7.5	333	0.5	31.7
27	729122	4355343	130	390	480	Clay	0.9	7.6	342	0.5	31.4
28	726605	4353810	210	395	395	Clay	0.9	7.7	352	0.6	27.7
29	726200	4353352	335	315	350	Clay	0.5	8.0	502	0.5	24.1
30	725435	4352931	200	415	385	Silty Clay	0.5	7.9	381	0.5	24.6
31	728062	4353713	200	430	370	Silty Clay	1.3	7.3	356	0.2	34.2
32	732071	4353674	190	440	370	Silty Clay	2.0	7.5	360	0.3	40.9
33	731910	4354076	165	415	420	Silty Clay	1.8	7.6	360	0.2	36.9

x & y: UTM (meters) 30S, EC: electrical conductivity, OC: organic carbon; SOC: soluble organic carbon

SI-Table 4 Physico-chemical properties of soil (40-60 cm). 1st sampling (plow period)

Sample	x	y	Sand (g kg ⁻¹)	Silt (g kg ⁻¹)	Clay (g kg ⁻¹)	Texture	EC (dS m ⁻¹)	pH	Carbonate (g kg ⁻¹)	SOC (g kg ⁻¹)	OC (g kg ⁻¹)
1	730030	4353641	180	390	430	Clay	0.9	7.8	392	0.3	26.3
2	729132	4353014	150	330	520	Clay	0.4	7.6	450	0.0	15.0
3	727292	4362814	110	440	450	Silty Clay	0.5	7.7	409	0.0	14.9
4	727465	4362358	210	360	430	Clay	0.8	8.3	357	0.0	16.5
5	728019	4363534	130	430	440	Silty Clay	0.5	8.4	343	1.1	19.4
6	727503	4364751	410	320	270	Clay Loam	0.5	7.8	364	1.2	30.6
7	728104	4365204	80	400	520	Silty Clay	0.5	7.7	322	0.3	23.5
8	728115	4365798	160	460	380	Silty Clay Loam	0.5	7.7	372	0.6	21.7
9	725228	4360223	300	420	280	Clay Loam	0.5	7.5	365	0.3	15.4
10	724983	4358520	210	480	310	Clay Loam	1.2	7.4	421	0.9	31.4
11	724644	4358625	260	390	350	Clay Loam	0.7	7.6	364	0.9	16.2
12	729260	4355993	130	450	420	Silty Clay	0.7	7.7	303	0.9	26.2
13	731793	4356293	925	25	50	Sand	0.4	7.8	369	0.0	4.3
14	730890	4355300	280	460	260	Loam	0.7	7.4	364	0.0	21.1
15	731194	4353858	410	350	240	Loam	0.8	7.3	405	0.7	33.9
16	724412	4355421	510	210	280	Sandy Clay Loam	0.4	7.6	375	0.5	15.3
17	725451	4354162	150	450	400	Silty Clay	0.7	7.8	299	0.7	28.2
18	729096	4365217	150	420	430	Silty Clay	0.4	7.8	260	0.5	12.5
19	728660	4365858	100	410	490	Silty Clay	0.5	7.6	320	0.3	34.8
20	729909	4361649	750	110	140	Sandy Loam	0.9	0.5	268	0.2	8.4
21	725487	4362542	180	500	320	Silty Clay Loam	0.7	7.4	347	0.3	41.7
22	726249	4362060	180	420	400	Silty Clay	0.5	7.5	341	0.3	30.6
23	723835	4357354	540	250	210	Sandy Clay Loam	0.4	8.0	283	0.0	8.4
24	723892	4356141	460	280	260	Loam	0.5	7.8	318	0.0	9.7
25	724131	4356182	370	360	270	Clay Loam	0.5	7.6	343	0.4	22.6
26	729183	4354909	180	530	290	Silty Clay Loam	1.1	7.4	347	0.6	24.9
27	729122	4355343	190	420	390	Silty Clay Loam	1.3	7.4	326	0.6	38.3
28	726605	4353810	230	450	320	Loam	0.8	7.8	351	0.5	26.6
29	726200	4353352	310	430	260	Loam	0.8	7.7	437	0.4	33.0
30	725435	4352931	220	400	380	Clay Loam	0.4	8.0	421	0.4	17.0
31	728062	4353713	60	500	440	Silty Clay	1.3	7.5	409	0.2	23.3
32	732071	4353674	180	460	360	Silty Clay Loam	2.8	7.5	335	0.4	60.1
33	731910	4354076	230	410	360	Clay Loam	2.1	7.5	530	0.2	31.1

x & y: UTM (meters) 30S, EC: electrical conductivity, OC: organic carbon; SOC: soluble organic carbon

SI-Table 5 Concentration of the studied compounds (ng g⁻¹) found in superficial soil (S; 0-40 cm) from the Natural Park of Albufera in Valencia, Spain, during the first sampling (1; plow period)

	RESM	BIFE	FENP	CYHA	PERM	CYFL	CYPE	FLUV	ESFE	DELT
1S1	24.5	nd	nd	nd	nd	23.5	10.4	nd	26.1	nd
2S1	27.3	nd	nd	nd	nd	11.7	nd	nd	20.9	nd
3S1	8.2	nd	nd	nd	nd	14.9	10.4	nd	22.0	nd
4S1	10.6	nd	nd	nd	nd	12.6	3.3	nd	13.2	nd
5S1	9.3	nd	nd	nd	nd	26.8	6.3	nd	22.6	nd
6S1	11.1	nd	nd	nd	nd	24.2	17.1	nd	23.9	nd
7S1	19.8	nd	nd	nd	nd	54.2	5.9	nd	53.2	nd
8S1	13.5	nd	nd	nd	nd	47.3	2.6	nd	56.9	nd
9S1	25.9	nd	nd	nd	nd	13.4	2.4	nd	31.0	nd
10S1	35.4	nd	9.0	nd	nd	nd	5.2	nd	8.3	nd
11S1	4.0	nd	nq	nd	nd	nd	nd	nd	nd	nd
12S1	0.0	nd	nd	nd	nd	4.3	3.7	nd	nd	nd
13S1	23.6	nd	nd	nd	nd	nd	nd	nd	nd	nd
14S1	16.1	nd	nd	nd	nd	23.3	3.0	nd	6.5	nd
15S1	21.2	nd	nd	nd	nd	10.0	1.8	nd	21.1	nd
16S1	13.4	nd	nd	nd	nd	4.7	nd	nd	4.8	nd
17S1	52.0	nd	44.9	nd	nd	24.2	nd	nd	17.5	nd
18S1	46.0	nd	nd	nd	nd	20.3	16.2	nd	nd	nd
19S1	13.7	nd	nd	nd	nd	45.4	17.9	nd	57.0	nd
20S1	22.2	nd	nd	nd	nd	47.2	nd	nd	55.3	nd
21S1	4.3	nd	37.5	nd	nd	20.8	nd	nd	35.6	nd
22S1	36.3	nd	21.7	nd	nd	16.8	5.1	nd	11.4	nd
23S1	15.9	nd	22.1	nd	nd	24.7	2.9	nd	6.9	nd
24S1	24.8	nd	nd	nd	nd	nq	6.1	nd	6.4	nd
25S1	8.2	nd	nd	nd	nd	15.6	9.9	nd	9.1	nd
26S1	43.9	nd	nd	nd	nd	9.5	nd	nd	nd	nd
27S1	19.3	nd	43.2	nd	nd	10.0	5.0	nd	7.6	nd
28S1	18.2	nd	nd	nd	nd	30.3	8.4	nd	31.1	nd
29S1	16.5	nd	25.7	nd	nd	39.0	5.9	nd	38.1	nd
30S1	17.3	nd	nd	nd	nd	39.5	12.2	nd	23.8	nd
31S1	12.9	nd	nd	nd	nd	20.7	nd	nd	13.5	nd
32S1	25.7	nd	nd	nd	nd	20.8	2.7	nd	2.5	nd
33S1	3.7	nd	nd	nd	nd	13.8	nd	nd	14.8	nd

nd: not detected; nq: not quantified

SI-Table 6 Concentration of the studied compounds (ng g⁻¹) found in deep soils (D; 40-60 cm) from the Natural Park of Albufera in Valencia, Spain, during the first sampling (I; plow period)

	RESM	BIFE	FENP	CYHA	PERM	CYFL	CYPE	FLUV	ESFE	DELT
1D1	30.4	nd	nd	nd	nd	4.1	nd	nd	44.4	nd
2D1	31.2	nd	nd	nd	nd	10.6	nd	nd	20.2	nd
3D1	25.2	nd	nd	nd	nd	nd	nd	nd	8.5	nd
4D1	25.8	nd	13.7	nd	nd	17.4	nd	nd	22.8	nd
5D1	13.8	nd	nd	nd	nd	12.1	1.2	nd	31.2	nd
6D1	13.8	nd	15.0	nd	nd	11.6	1.8	nd	28.8	nd
7D1	19.1	nd	nd	nd	nd	14.1	6.3	nd	28.3	nd
8D1	14.0	nd	nd	nd	nd	20.6	nd	nd	22.0	nd
9D1	13.8	nd	16.5	nd	nd	5.1	nd	nd	21.0	nd
10D1	17.0	nd	19.2	nd	nd	nd	nd	nd	7.9	nd
11D1	4.6	nd	18.9	nd	nd	nd	nd	nd	nd	nd
12D1	1.6	nd	nd	nd	nd	5.7	3.5	nd	nd	nd
13D1	43.2	nd	19.5	nd	nd	nd	nd	nd	10.2	nd
14D1	30.7	nd	13.7	nd	nd	2.3	nd	nd	17.2	nd
15D1	49.5	nd	21.1	nd	nd	2.2	nd	nd	17.3	nd
16D1	25.8	nd	29.8	nd	nd	nd	nd	nd	6.8	nd
17D1	53.4	nd	20.2	nd	nd	27.3	nd	nd	35.9	nd
18D1	44.7	nd	nd	nd	nd	12.4	10.3	nd	13.6	nd
19D1	24.3	nd	nd	nd	nd	20.8	11.1	nd	37.7	nd
20D1	24.4	nd	nd	nd	nd	22.8	3.0	nd	34.4	nd
21D1	15.2	nd	20.4	nd	nd	15.0	nd	nd	46.3	nd
22D1	14.3	nd	21.4	nd	nd	14.2	nd	nd	16.0	nd
23D1	16.2	nd	nd	nd	nd	19.6	nq	nd	5.3	nd
24D1	21.5	nd	nd	nd	nd	13.0	0.8	nd	12.3	nd
25D1	11.0	nd	nd	nd	nd	nd	6.2	nd	22.6	nd
26D1	22.3	nd	nd	nd	nd	9.2	nd	nd	nd	nd
27D1	23.2	nd	nd	nd	nd	11.1	5.0	nd	9.4	nd
28D1	32.2	nd	nd	nd	nd	20.7	nd	nd	36.4	nd
29D1	28.4	nd	nd	1.5	nd	26.7	3.6	nd	29.1	nd
30D1	28.3	nd	nd	nd	nd	21.5	3.0	nd	28.8	nd
31D1	38.4	nd	19.7	nd	nd	12.3	nd	nd	13.8	nd
32D1	26.3	nd	17.5	nd	nd	2.1	1.7	nd	12.3	nd
33D1	5.9	nd	nd	nd	nd	8.1	nd	nd	9.8	nd

nd: not detected; nq: not quantified

SI-Table 7 Concentration of the studied compounds (ng g⁻¹) found in superficial soil (S; 0-40 cm) from the Natural Park of Albufera in Valencia, Spain, during the second sampling (2; rice production period)

	RESM	BIFE	FENP	CYHA	PERM	CYFL	CYPE	FLUV	ESFE	DELT
1S2	27.0	nq	3.6	1.0	nd	16.1	2.1	nd	16.4	nd
2S2	20.5	nq	nd	nd	nd	nq	nd	nd	20.3	nd
3S2	3.1	nq	nq	1.0	nd	5.3	nq	nd	15.0	nd
4S2	2.0	32.2	31.0	nq	nd	4.8	1.3	nd	nd	nd
5S2	26.2	2.5	11.0	9.9	nd	13.3	6.0	nd	35.4	nd
6S2	23.3	0.1	nq	nd	nd	11.5	nd	nd	27.2	nd
7S2	26.6	0.5	1.1	2.3	nd	29.2	7.6	nd	37.7	nd
8S2	42.3	3.4	15.5	0.5	nd	27.9	4.0	nd	36.8	nd
9S2	10.0	6.1	9.4	nq	nd	17.3	26.2	nd	19.4	nd
10S2	12.4	13.8	8.9	12.4	nd	14.8	13.2	nd	17.3	nd
11S2	11.8	0.6	16.7	0.7	nd	nq	1.2	nd	35.3	nd
12S2	13.8	0.4	23.1	4.3	nd	3.9	5.4	nd	34.5	nd
13S2	10.6	2.3	4.1	8.1	nd	22.3	2.3	nd	20.3	nd
14S2	10.6	0.7	2.7	1.1	nd	8.2	nd	nd	41.7	nd
15S2	18.4	nq	16.5	0.8	nd	4.9	2.0	nd	22.5	nd
16S2	5.5	nq	10.1	0.7	nd	2.4	6.9	nd	26.7	nd
17S2	9.7	8.0	47.5	nq	nd	24.2	nd	nd	nd	nd
18S21	10.0	0.4	2.9	1.3	nd	5.8	10.6	nd	16.7	nd
19S2	52.1	4.2	18.1	20.7	nd	38.8	3.4	nd	57.1	nd
20S2	58.5	1.3	1.5	3.0	nd	30.5	2.5	nd	44.2	nd
21S2	58.9	12.1	16.2	16.1	nd	28.9	7.2	nd	45.9	nd
22S2	32.4	2.6	5.2	2.0	nd	4.0	2.1	nd	23.6	nd
23S2	10.2	4.5	26.9	0.5	nd	16.5	1.9	nd	nd	nd
24S2	18.6	2.3	nq	nq	nd	3.1	4.5	nd	9.7	nd
25S2	13.2	3.2	14.3	nq	nd	9.2	2.4	nd	18.8	nd
26S2	15.8	1.9	6.8	2.0	nd	21.5	2.1	nd	19.3	nd
27S2	9.8	1.1	3.0	nq	nd	12.1	3.5	nd	7.2	nd
28S2	38.3	11.2	40.6	1.6	nd	21.6	nd	nd	30.2	nd
29S2	31.9	1.7	29.1	0.5	nd	28.9	1.8	nd	25.9	nd
30S2	62.3	6.1	33.9	4.9	nd	22.6	6.6	nd	24.8	nd
31S2	30.1	4.7	16.7	0.8	nd	39.0	1.0	nd	22.7	nd
32S2	27.8	5.4	33.1	0.8	nd	18.4	1.7	nd	21.2	nd
33S2	22.3	6.4	10.4	0.7	nd	11.9	0.5	nd	nd	nd

nd: not detected; nq: not quantified

SI-Table 8 Concentration of the studied compounds (ng g⁻¹) found in deep soil (D; 40-60 cm) from the Natural Park of Albufera in Valencia, Spain, during the second sampling (2; rice production period)

	RESM	BIFE	FENP	CYHA	PERM	CYFL	CYPE	FLUV	ESFE	DELT
1D2	33.8	1.2	6.9	0.6	nd	20.2	nq	nd	20.0	nd
2D2	40.3	0.3	1.1	nq	nd	10.1	1.0	nd	29.3	nd
3D2	4.5	0.8	17.7	1.1	nd	1.6	1.2	nd	13.9	nd
4D2	29.0	1.4	27.8	nq	nd	4.0	15.8	nd	15.5	nd
5D2	30.1	2.1	4.2	nq	nd	21.6	nq	nd	19.1	nd
6D2	44.7	1.3	6.2	2.5	nd	24.3	nq	nd	17.0	nd
7D2	57.9	1.3	27.9	41.1	nd	35.9	1.3	nd	40.2	nd
8D2	31.5	3.1	2.4	2.0	nd	33.6	1.8	nd	32.6	nd
9D2	18.6	4.7	37.2	nq	nd	19.5	6.6	nd	28.3	nd
10D2	17.4	11.5	13.6	23.6	nd	24.5	27.8	nd	21.0	nd
11D2	25.0	3.6	18.9	5.1	nd	nq	2.9	nd	11.0	nd
12D2	9.6	0.4	24.1	11.1	nd	20.0	2.8	nd	48.8	nd
13D2	28.0	2.5	7.9	1.4	nd	21.3	0.9	nd	12.7	nd
14D2	4.9	0.5	4.3	nq	nd	27.9	7.8	nd	10.8	nd
15D2	18.9	0.4	0.9	1.9	nd	4.6	nq	nd	6.8	nd
16D2	13.3	nq	nq	nq	nd	3.3	4.2	nd	32.1	nd
17D2	41.5	0.4	4.1	9.3	nd	4.2	1.1	nd	nd	nd
18D2	13.4	1.5	3.8	0.9	nd	10.1	1.2	nd	5.4	nd
19D2	40.0	1.6	1.6	25.8	nd	54.9	nq	nd	40.8	nd
20D2	52.4	1.4	7.8	23.3	nd	47.4	4.8	nd	27.8	nd
21D2	43.8	13.5	15.3	24.6	nd	42.7	31.9	nd	32.6	nd
22D2	22.8	3.7	17.8	1.5	nd	11.7	6.6	nd	19.7	nd
23D2	26.1	4.0	18.8	nq	nd	19.2	nq	nd	nq	nd
24D2	6.9	2.9	5.0	nd	nd	20.5	2.0	nd	24.8	nd
25D2	27.8	4.5	35.3	2.5	nd	16.1	2.0	nd	10.9	nd
26D2	27.8	6.9	5.4	0.8	nd	19.1	2.5	nd	15.6	nd
27D2	9.3	1.1	4.2	3.8	nd	9.5	1.6	nd	15.4	nd
28D2	50.2	0.9	7.2	20.7	nd	33.5	nq	nd	24.1	nd
29D2	53.2	5.2	30.6	25.1	nd	54.5	2.7	nd	24.6	nd
30D2	35.8	3.9	21.3	23.4	nd	36.1	nd	nd	31.8	nd
31D2	39.1	6.3	40.2	0.6	nd	27.4	1.4	nd	23.1	nd
32D2	15.8	4.3	18.1	0.8	nd	21.5	1.3	nd	12.9	nd
33D2	18.4	3.2	2.4	nq	nd	24.2	2.9	nd	nq	nd

nd: not detected; nq: not quantified

SI-Table 9 *Consumer products information*

Sample					
Number	Code	Use	Acquired	Declared silver concentration (mg L ⁻¹)	Declared silver form
1	Consumer Product 1	Personal care	Online	20	Ag pure suspension
2	Consumer Product 2	Multipurpose antimicrobial	Online	10	Ag ionic colloidal
3	Consumer Product 3	Multipurpose antimicrobial	Online	10	Ag ionic - Nano cristal Ag
4	Consumer Product 4	Multipurpose antimicrobial	Online	15	Ag colloidal
5	Consumer Product 5	Medical device incorporating medicinal product (fluidiser)	Pharmacy	not declared	Ag colloidal
6	Consumer Product 6	Multipurpose antimicrobial	Online	20	NP Ag 0.65 nm
7	Consumer Product 7	Multipurpose antimicrobial	Online	10 /20-30 °	NP Ag 0.63 nm
8	Plaster claiming silver	Medical device for dermal protection	Supermarket	not declared	Silver gauze
9	Domestic filters for tap water (aged and new)	Water filter	Supermarket	not declared	not declared

°: inconsistent information in label

SI-Table 10 Determination of particle size and concentration of Ag-NPs standards measured at 150×10^3 particles mL^{-1} concentration level, $n=3$

Ag NP Standard	Ag-NP					
	Concentration		Size (nm)			
	N Particles mL^{-1}	RSD (%)	Mode	RSD (%)	Average	RSD (%)
AgNP-PVP 10 nm	226536.7	80.2	14.3	4.2	15.7	13.4
AgNP-PVP 20 nm	57949.0	27.9	11.7	58.1	20.0	8.5
AgNP-PVP 30 nm	118508.0	3.7	20.3	7.4	29.3	2.0
AgNP-PVP 40 nm	58309.7	5.9	20.0	17.5	32.3	1.9
AgNP-PVP 50 nm	129682.7	5.1	50.0	2.0	51.7	1.2
AgNP-PVP 60 nm	157005.7	2.3	60.0	1.7	62.3	1.0
AgNP-PVP 70 nm	132067.7	3.1	73.0	1.4	74.3	1.6
AgNP-PVP 75 nm	138849.0	2.4	75.0	1.3	77.3	1.6
AgNP-PVP 80 nm	123899.7	4.8	77.0	1.3	78.7	1.5
AgNP-PVP 100 nm	103244.3	1.2	105.3	1.4	107.7	1.4

SI-Figure 1 SP-ICP-MS Particle Size Distributions of Ag-NPs standards measured at 150×10^3 particles mL^{-1} concentration level: (A) 10 nm Ag-NPs; (B) 20 nm Ag-NPs; (C) 30 nm Ag-NPs ;(D) 50 nm Ag-NPs

SI-Figure 2 SP-ICP-MS Particle Size Distributions of consumer products 1-7 (A-G, respectively)

SI-Figure 3 Fractograms obtained after the AF4-UV analysis of products 1-7. Ag-NP standards of 20 nm, 75 nm and 100 nm are included for reference: (A) intensity axe in normalised scale; (B) absolute scale

A

TEM image showing ZnO nanoparticles after 10 min of synthesis. The particles are small and spherical. Labeled diameters include 12.59 nm, 12.77 nm, 9.21 nm, 15.93 nm, 11.93 nm, 12.55 nm, 15.12 nm, 22.58 nm, and 15.87 nm. A scale bar indicates 200 nm.

B

TEM image showing ZnO nanoparticles after 20 min of synthesis. The particles are small and spherical. Labeled diameters include 11.00 nm, 16.20 nm, 16.22 nm, 11.67 nm, 11.89 nm, 16.78 nm, and 11.75 nm. A scale bar indicates 200 nm.

C

TEM image showing ZnO nanoparticles after 30 min of synthesis. The particles are small and spherical. Labeled diameters include 16.9 nm, 22.76 nm, 13.40 nm, 13.10 nm, 16.97 nm, 16.85 nm, 16.90 nm, 16.52 nm, and 16.9 nm. A scale bar indicates 200 nm.

D

TEM image showing ZnO nanoparticles after 40 min of synthesis. The particles are small and spherical. Labeled diameters include 12.01 nm, 12.64 nm, 12.66 nm, 19.70 nm, 19.41 nm, 19.00 nm, 16.14 nm, 16.91 nm, 16.41 nm, 16.92 nm, 2.78 nm, and 16.00 nm. A scale bar indicates 200 nm.

E

TEM image showing ZnO nanoparticles after 50 min of synthesis. The particles are small and spherical. Labeled diameters include 11.19 nm, 17.33 nm, 16.63 nm, 16.61 nm, and 6.75 nm. A scale bar indicates 200 nm.

F

TEM image showing ZnO nanoparticles after 60 min of synthesis. The particles are small and spherical. Labeled diameters include 15.33 nm, 15.20 nm, 15.04 nm, 7.64 nm, 15.64 nm, 15.99 nm, 15.32 nm, 15.01 nm, 15.01 nm, 15.25 nm, 12.81 nm, 15.25 nm, and 11.31 nm. A scale bar indicates 200 nm.

G

TEM image showing ZnO nanoparticles after 70 min of synthesis. The particles are small and spherical. Labeled diameters include 6.81 nm, 16.50 nm, 16.40 nm, 16.40 nm, 11.10 nm, 16.83 nm, 16.84 nm, 13.36 nm, 13.72 nm, 11.18 nm, 12.75 nm, 16.19 nm, 16.37 nm, and 16.37 nm. A scale bar indicates 200 nm.

SI-Figure 5 TEM images of: (A) Ag-NPs released by a plaster after sonication; (B) detail; (C) TEM image of Ag-NPs present in filtered water

SCIENTIFIC PRODUCTION DURING Ph.D. PERIOD

INTERNATIONAL STAY

Ispira, 27 November 2015

TO WHOM IT MAY CONCERN

Mr. Ramon Aznar Roca has been working in the Joint Research Centre, Institute for Health and Consumer Protection, Chemical Assessment and Testing Unit under my direct supervision for a period of three months (from 1st September till 30th November 2015). He has been working as unpaid visiting scientist in the Product Safety and Innovation competence group which gives scientific and technical support to European Commission services in the field of health and consumers policies. In particular the work of the visiting scientist has been embedded in a project aiming at providing harmonized approaches for testing the release of emerging pollutants, including nanoparticles, from consumer products. During this time he has gained scientific and technical skills and worked with autonomy and commitment in order to deliver high quality results.

In particular, the activities performed by Mr. Aznar Roca have included:

- the revision and analysis of scientific literature and documentation on analysis of silver nanoparticles,
- the execution of research activities for the development/optimization/characterization of a single particle ICP-MS method for determination of number size distribution of silver nanoparticles,
- the application of the developed method to selected environmental samples and selected product categories, and
- the reporting of the outcomes of the research activities undertaken.

Josefa Barrero Moreno

Leader of the Product Safety&Innovation competence group

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PARTCIPATION IN CONGRESSES

Aznar, R.

Poster: Multiresidue analysis of selected pharmaceutical compounds in poultry manure by gas chromatography – mass spectrometry

Euro Chemistry 2016. Roma, Italy 2016

Aznar, R. and Tadeo, J.L

Poster: Ocurrence of emerging contaminants in rice plants from Valencia, Spain

VIII Congress of Students, Science, technology and Agricultural Engineering. Madrid, Spain 2016

Aznar, R. and Tadeo, J.L

Poster: Analysis of emerging contaminants in *Oryza sativa* by ultrasound assisted-matrix solid phase dispersion and GC-MS

VIII Congress of Students, Science, technology and Agricultural Engineering. Madrid, Spain 2016

Aznar, R. Sánchez-Brunete, C. Albero, B. Miguel, E and Tadeo, J.L

Oral communication: Determination of selected pharmaceutical compounds in *Typha angustifolia* and *Oryza sativa* by GC-MS

1st International Caparica Christmas Conference on Translational Chemistry. Caparica, Portugal 2015

Aznar, R. Albero, B. Sánchez-Brunete, C and Tadeo, J.L

Poster: Analysis of pyrethroids and organochlorine insecticides in soil

8th European Congress on Regional Geoscientific Cartography and Information Systems, Barcelona, Spain 2015

Aznar, R. Albero, B. Sánchez-Brunete, C and Tadeo, J.L

Oral communication: Occurrence of pharmaceutical compounds in soil from Spanish agricultural fields

VII Congress of Students, Science, technology and Agricultural Engineering. Madrid, Spain 2015

Aznar, R. Sánchez-Brunete, C. Albero, B and Tadeo, J.L

Poster: Analysis of pyrethroids and organochlorine pesticides in poultry manure and soil by isotope dilution GC-MS

20th International Symposium on Separation Sciences. ISSS Pragma, Czech Republic. 2014

Aznar, R. Sánchez-Brunete, C. Albero, B. Rodriguez, J.A and Tadeo, J.L

Oral communication: Determination of selected pharmaceutical compounds in soil from Spanish agricultural fields

International Congress on Phytoremediation of Polluted Soils. PPS, Vigo, Spain 2014

Albero, B. Aznar, R. Miguel, E Sánchez-Brunete, C and Tadeo, J.L

Poster: Determination of selected pharmaceutical compounds in biosolids by gas chromatography–tandem mass spectrometry

XIII Reunión Científica de la Sociedad Española de Cromatografía y Técnicas Afines. SEyTA, Tenerife, Spain. 2013

Aznar, R. Albero, B. Sánchez-Brunete, C. Miguel, E and Tadeo, J.L

Poster: Multiresidue analysis of insecticides and selected environmental contaminants in poultry manure by gas chromatography-mass spectrometry.

XIII Reunión Científica de la Sociedad Española de Cromatografía y Técnicas Afines. SEyTA, Tenerife, Spain. 2013

COURSES

- Jul 2015, How to write great papers: from title to references, from submission to publication, Elsevier (1 day course). Universidad Politécnica de Madrid
- Jan 2015, Risk prevention, use of chemical products (1 day course). CUALITIS, INIA
- Jan 2015, Risk prevention, laboratory and the use of personal protection (1 day course). CUALITIS, INIA
- Nov 2014, Writing in the Science. University of Stanford (8 weeks). Online course
- 2013 – 2014 Master Agro-Environmental Technology for Sustainable Agriculture. Polytechnic University of Madrid, Spain (9.8 / 10)

CODE	COURSE TITLE	CREDITS	DURATION	TYPE	YEAR	SESSION	GRADE	MARKS
23000252	EXPERIMENTAL DESIGN AND STATISTICAL DATA ANALYSIS	4,00	S	B	2013-14	JUN	A EXCELLENT	9,00
23000253	AGRICULTURAL CHEMICAL ANALYSIS	4,00	S	O	2013-14	FEB	A EXCELLENT	10,00
23000254	GIS APPLIED TO SOIL EVALUATING AND SOIL RESOURCE INVENTORIES	3,00	S	O	2013-14	FEB	A EXCELLENT	9,00
23000258	DIFFUSE AGRICULTURAL POLLUTION	4,00	S	O	2013-14	FEB	A EXCELLENT	10,00
23000259	PROCESSES OF SOIL DEGRADATION	3,00	S	O	2013-14	FEB	A EXCELLENT	10,00
23000263	BIOENERGY: PLANTS FOR A SUSTAINABLE DEVELOPMENT	4,00	S	O	2013-14	JUN	A EXCELLENT	9,00
23000265	AGRICULTURAL REUSE OF ORGANIC RESIDUALS	4,00	S	O	2013-14	JUN	A EXCELLENT	9,50
23000266	ADVANCED SEMINARS	2,00	S	B	2013-14	FEB	A EXCELLENT	9,30
23000267	MASTER THESIS	15,00	I	P	2013-14	JUL	A EXCELLENT	10,00
23000306	SEMINAR MODULE II	3,00	S	B	2013-14	JUL	A EXCELLENT	9,00
23000307	SEMINAR MODULE III	3,00	S	B	2013-14	JUL	A EXCELLENT	9,00
23000308	CONFERENCE	1,00	S	B	2013-14	FEB	A EXCELLENT	10,00
23000352	PRACTICE	10,00	S	O	2013-14	FEB	A EXCELLENT	10,00

Disertation: “Analysis of pyrethroid and organochlorine pesticides in poultry manure and soil by isotope dilution gas chromatography – mass spectrometry and environmental assessment” (10/10)

OTHER CONTRIBUTIONS

- Feb 2015 Master class at the Universidad Politecnica de Madrid. Entitled: “Determination of DDT and its metabolites and pyrethroids pesticides in agricultural soil and water”
- Feb 2015 Technical Discussion Meeting on Emerging contaminants in Environmental samples. Geological Survey of Ireland

GRANTS & ACHIEVEMENTS

Jan 2013 - Jan 2017 Predoctoral fellowship INIA

May 2016 Award best written proceeding. CATEDRA FERTIBERIA.

During the VIII Congress of Agricultural Student Scientist hold in Madrid the Catedra Fertiberia decided to award my work entitled: Occurrence of emerging contaminants in rice plants from Valencia, Spain.

Dec 2015 Ex-aequo excellent shotgun oral presentation. PROTEOMASS Scientific Society

During the congress 1st International Caparica Christmas Conference on Translational Chemistry, I was awarded by PROTEOMASS Scientific Society for the oral presentation entitled: Determination of selected pharmaceutical compounds in *Typha angustifolia* and *Oryza sativa* by GC-MS.

Sep 2015 Award best Master Thesis by CATEDRA FERTIBERIA

For the work “Analysis of pyrethroid and organochlorine pesticides in poultry manure and soil by isotope dilution gas chromatography - mass spectrometry and environmental assessment”

Jun 2015 Award best written proceeding for its future applicability. CATEDRA FUNDACIÓN INGENIO UPM

During the VII Congress of Agricultural Student Scientist hold in Madrid the Catedra Fundación Ingenio UPM decided to award me with the highest price for my work entitled: Occurrence of pharmaceutical compounds in soil from Spanish agricultural fields, because its future applications.

Jun 2015 Award best oral presentation. Escuela técnica superior de ingenieros agrónomos (UPM)

During the VII Congress of Agricultural Student Scientist hold in Madrid the University UPM awarded my oral presentation entitled: Occurrence of pharmaceutical compounds in soil from Spanish agricultural fields. It was decided by the votes of the participants of the Congress.

**I don't know where
I'm going from here,
but I promise it
won't be boring**

David Bowie