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Development of analytical methodology for determination of emerging contaminants in environment and foods

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ABSTRACT

In recent years, fate, occurrence and potential adverse effect of emerging contaminants (ECs) in the environment have received an increased attention by scientific community. The ECs are a broad category of chemicals, mainly organic compounds, that are not currently covered by existing regulations but they may be candidates for future regulation, as they may be potential threats to human health and environmental safety. The ECs are mainly substances of anthropogenic origin, introduced continuously into the environment in large quantities and distributed ubiquitously in the ecosystem, due to their wide consumption. Recent studies have indicated that most of them are environmentally persistent, bioactive, and certain have a high potential for bioaccumulation. In literature data are still too few regarding their toxicity, distribution and fate and consequently, it is not still possible to assess their real impact on the environment and on human health. For these reasons it is important to design analytical procedures for monitoring specific environmental compartments and to provide the basis for drawing conclusions about the occurrence, the persistence and hazard of ECs in the environment. Currently, the main objectives of the research and monitoring of ECs are the development of accurate and sensitive analytical methods able to simultaneously analyze multiple chemical classes of ECs in different environmental compartments with different complexity. In line with these requirements, in this PhD project, three multi-residue methods were developed for the determination of three different classes of ECs in different and complex environmental matrices.

The pharmaceutical and personal care products (PPCPs) were the first studied class of ECs and a multi-residue method for their determination in different environmental matrices has been developed. The main challenge of this work was to determine simultaneously twenty-two selected PPCPs belonging to different families. The proposed analytical procedure combines solid phase

extraction (SPE) and dispersive liquid-liquid microextraction techniques (DLLME) to perform the extraction (water)/purification (solid matrices) and the ultra-concentration of target PPCPs. An UHPLC-MS/MS multi-residue method was developed for the sensitive and selective quantification and confirmatory analysis of the target analytes with different chemical characteristics. Finally, the proposed methodologies were validated for different aqueous matrices (tap water, sea water, river water and wastewater).

Subsequently, a novel and advantageous analytical procedure, suitable to investigate the presence of eight Organophosphate esters (OPEs) in sludge samples, was developed. Matrix solid-phase dispersion (MSPD) was selected as an extraction technique considering its low cost, reasonable selectivity and previous successful applications dealing with emerging compounds extraction from sludge. OPEs were determined by LC using, for the first time, a hybrid quadrupole time-of-flight MS system, as an alternative to triploquadrupole instruments. Furthermore, the information contained in accurate, scan MS spectra were used to screen the presence of additional OPEs, which had not been included in the quantitative method, in sludge samples.

Finally, a multi-residue method was developed for environmental monitoring of 18 analytes, corresponding to a wide range of drugs of abuse (DAs) and some of their major urinary metabolites, in wastewater samples. The proposed analytical methodology combines the use of mixed-mode solid phase extraction (Oasis MCX) with fractioned elution strategy, to improve the sensitivity of the overall procedure. A selective UHPLC-MS/MS method was developed for a quantitative and confirmatory analysis and the stable isotope dilution assay (SIDA) was used to compensate the matrix effects and losses of DAs during the sample preparation, ensuring a high accuracy and precision to the method. Furthermore, this method was applied to wastewater samples and it was used as tool to estimate the consumption of DAs in Avellino province by sewage epidemiology approach.

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Introduction

The industrialization of society and the growth of the human population, however, have caused an exponential growth in the production of goods and services, and consequently the waste by-products have increased. The indiscriminate discharge of domestic wastes and untreated industrial into aquatic compartment, the diffusion of thousands of tons of particulates and airborne gases into the atmosphere, the "throwaway" attitude toward solid wastes, and the indiscriminate use of newly developed chemicals without considering potential consequences have resulted in major environmental disasters (Munoz, 2009).

For this reason, in recent decades, a constant increase in public awareness of the environmental protection issues has been observed both globally and locally. The international scientific community has focused its attention on the chemical, analytical and toxicological studies of emerging pollutants (ECs) (Kot-Wasik, 2007).

The ECs are substances of anthropogenic origin introduced continuously into the environment in large quantities and distributed ubiquitously in the ecosystem due to their wide consumption. Several studies suggest that this class of contaminants could alter the normal balance of the ecosystem, both directly acting on the normal physiological functions of living beings, and indirectly through contamination of food and water. This type of contaminants are not included in national and international programs of environmental and alimentary regolamentation, therefore, it is of urgent interest to know their distribution in different environmental compartments and their potential toxicity (Daughton & Ternes, 1999; Ferrari, 2003; Jjemba, 2003). They include a diverse group of compounds, pharmaceuticals, personal care products, estrogens, surfactants, perfluorinated compounds, industrial additives, flame retardants, gasoline additives, and transformation products of

regulated/no regulated pollutants. In addition, among other compounds, new classes have been added to the list of emerging organic contaminants in the last few years, such as nanomaterials, swimming pool disinfection by-products and 1,4-dioxane and many others (Richardson, 2012).

In the environment, the ECs are substances released from industrial, domestic, and agricultural sources (Yan, 2010). The environmental compartment in which they are detected more frequently is aquatic environment; in fact, they have been detected in wastewaters, surface waters, ground waters, and in some cases in the drinking water (Bolong, 2009; Pojana, 2011). The ECs have been detected in aquatic environment at levels up to μg L^{-1} (Kasprzyk-Horder, 2007). Despite the few data reported in the literature, regarding their distribution in biota and food, it was found that these compounds are able to bioaccumulate in aquatic organisms and they can reach to human being both directly ingesting contaminated waters and indirectly by feed (Kot-Wasik, 2007). Toxicity studies showed a chronic toxicity caused by their continuous input and persistence in the environment (Braush & Rand, 2011).

Scope of my PhD thesis

The current trend in the study of ECs is based on an interdisciplinary approach that consists in the identification of new pollutants, in the development of highly sensitive analytical methods for the determination of these unwanted substances in various matrices and in the toxicity assessment and persistence in the ecosystem. One of the reasons for the lack of information about the environmental fate, the toxicity on aquatic organisms and the human health of ECs has been the shortage of analytical methods suitable to detection of these compounds at very low concentrations (ppb or ppt).

In line with these requests, the goal of this PhD project is the development of analytical methods which allow the assessment of the distribution and fate of

ECs in the environment and in food. The approach is based on the development of efficient, reliable multi-residue methods for rapid, sensitive and selective determination of a broad range of compounds in complex matrices. Multi-residue analytical methodologies are preferred over single-group analysis, since they reduce overall analysis time and costs.

This thesis focuses on the determination of three groups of emerging organic contaminants: Pharmaceutical and personal care products, Organophosphate esters and illicit drugs. These classes of contaminants were selected based on their origin, environmental persistence, human toxicity and ecotoxicity.

The research plan has been divided in these main aims:

- ***Sensitive determination of selected Pharmaceutical and Personal Care Products (PPCPs) in different environmental matrices by Solid-Phase Extraction combined with Dispersive Liquid-Liquid Microextraction (SPE-DLLME) prior to UHPLC-MS/MS analysis***

The aim of this research has been the development of a new multi residue method for simultaneously determination of twenty-two PPCPs in different environmental matrices. PPCPs are one of the most important classes of emerging contaminants analyzed the last decade. These potentially hazardous contaminants include numerous classes of chemicals with distinctive physical chemical properties and biological activities (Daughton and Ternes, 1999).

Currently, most of the methods for the determination of PPCPs are directed to a single class of PPCPs, because it is very difficult to analyze simultaneously a broad range of compounds with different physical-chemical properties. The main challenge of this work was to determine simultaneously PPCPs belonging to different families.

The method has been developed using solid phase extraction (SPE) followed by dispersive liquid–liquid microextraction (DLLME), to perform the extraction (water)/purification (solid matrices) and ultra concentration of

analytes with a moderate use of solvents. A selective and sensitive UHPLC-MS/MS method was developed to quantitative and confirmatory analysis of the PPCPs. Finally, the developed procedure was validated for different waters (wastewater, tap water, river water and seawater) and it was applied to real samples.

- ***Liquid chromatography quadrupole time-of-flight mass spectrometry quantification and screening of organophosphate compounds in sludge***

For the first time, we assess the performance of liquid chromatography (LC) quadrupole time-of-flight (QTOF) mass spectrometry (MS) for the selective quantification of eight organophosphate compounds (OPEs), used as plasticizers and flame retardants additives, in sludge from urban sewage treatment plants. Some OPEs (e.g. trichloroisopropyl phosphate, TCPP) display limited biodegradation at sewage treatment plants (STPs) and can bioccumulated in sludge (Olofsson *et al.,* 2013). Assuming that around 50% of the sludge generated at STPs is disposed as a fertilizer in agriculture fields (Macherius *et al.,* 2012), evaluation of OPEs discharges in the environment requires not only determining their dissolved concentrations, at the outlet stream of STPs, but also addressing their levels in sludge. This latter issue becomes particularly concerning after having reported i) significant uptakes of polar OPEs by vegetable roots and ii) their capability to migrate from roots to leaves (Eggen *et al.,* 2013); thus, the risk of OPEs introduction in the human food web through livestock animals and vegetables is not negligible.

As regarding the sample preparation, the Matrix solid-phase dispersion (MSPD) was used. Moreover, the developed method was applied to real samples and the usefulness of accurate, full scan MS and MS/MS spectra to screen and to confirm the presence of additional OPEs, without using reference standards, in sludge samples is discussed.

- ***Determination and measurement of illicit drugs in urban wastewater***

A sensitive method has been successfully developed and validated for the simultaneous determination and quantification of 11 abuse drugs and some of their metabolites in wastewater. DAs enter in wastewater as unalterated drug and/or their active metabolites by human excretion after illegal consumption or by accidental or deliberate disposal from clandestine drug laboratories. They are released into surface waters because their removal during sewage treatments is often incomplete (Pedrouzo *et al.,* 2011b; Postigo *et al.,* 2010) and consequently they can even reach drinking water sources (Boleda *et al.,* 2011; Boleda et al., 2009).

The selective extraction and concentration of the basic drugs was obtained by mixed-mode solid phase extraction (Oasis MCX) with fractioned elution strategy. UHPLC-MS/MS with selective reaction monitoring (SRM) was used for quantification. Subsequently, this method has been used to estimate and monitor drug consumption in the population of an Italian province, helping social scientists and authorities to combat drug abuse. Hence, monitoring of DAs in environmental water bodies is very useful from two perspectives: i) epidemiologists can assess the nature and magnitude of drug abuse (Rieckermann and Christakos, 2008) and information on changes in drug abuse trend (Terzic *et al.,* 2010); and ii) environmental scientists and policy makers can implement control strategies to protect the environment from biologically active substances

Chapter 1

Emerging Contaminants

1.1. Emerging Contaminants (ECs)

Over the past years, the attention to environmental pollution by new substances has significantly increased. Nowadays, the awareness of the presence of new and unknown contaminants in the foods and environment has grown significatively, in addition to well-known heavy metals, pesticides and organic halogen compounds (Munoz *et al.,* 2009).

The ECs are a broad category of chemicals, mainly organic compounds, that are not currently covered by existing regulations but they may be candidates for future regulation, as they may be potential threats to environmental ecosystems and human health. Many ECs are environmental persistent, bioactive and as potentially bioaccumulative compounds (Peck, 2006; Mackay and Barnthouse, 2010). Owing to their characteristics of persistence and bioaccumulation, the ECs can move easily in the chain food. Thus, they may pose a human health risk and the major concern related to the presence of ECs in foods is their potential endocrine disruption, carcinogenic effect and other chronic effects (Dirtu, 2012).

Over the last few decades, the adjective "emerging" has been applied to pollutants with increasing frequency, because they are mainly anthropogenic compounds introduced continuously into the environment in large quantities and distributed ubiquitously in the ecosystem (Daughton, 2004). Furthermore, new substances of natural or synthetic origin, and the compounds derived by the transformation/degradation processes are classified as ECs.

Actually, the contaminant's "emerging" status is typically determined by whether the contaminant is persistent or has potentially harmful human or ecological effects. It is often the case that ECs have actually been present in the environment for some time, but they are discovered through a wider search of potential contaminants or through the use of new technologies (LC/MS) that have enabled their discovery and measurement in the environment for the first time (pharmaceuticals). The term "emerging contaminants" is not exclusively referred to

newly introduced substances, but can also indicate natural compounds with previously unrecognized adverse effects on ecosystems. In fact, hormones and algal toxins fall into this category of being naturally occurring, yet can have adverse ecological impacts (Petrovic and Barcelo, 2006)

The ECs are compounds with very different physical-chemical properties, and usually they are classified according to the following characteristics: chemical class, use, toxic effects and mechanism of action (Daughton and Ternes, 1999; Brausch and Rand, 2011; Kantian *et al.,* 2010; Richardson, 2011).

The most studied families of ECs are:

- Pharmaceuticals: analgesics, anti-inflammatories, antibiotics, β-blockers, antidislipidemic, antidepressants, anti-ulcer, hormones, veterinary and illicit drugs;
- Personal Care Products: fragrances, sunscreens, disinfectants, preservatives and insect repellent.
- Endocrine distrupting compounds;
- Industrial Products: perfluorinated compounds, flame retardants, benzotriazoles, nanomaterials, organotin biocides.
- Drinking water and swimming pool disinfection byproducts (DBPs): brominated, iodinated and nitrogen-containing DBPs
- Natural Toxins: marine and plant toxins;
- Metabolites and degradation/transformation products of regulated/non regulated pollutants.
- Food emerging contaminants: sucralose and other artificial sweeteners, Maillard reaction products (acrilamide), compounds formed by the reaction of ethanol with urea or substances containing the cyanide group (ethyl carbamate) (Dorne *et al.,* 2009) and the substances used in the alimentary counterfeit (melamine in milk) (Suna *et al.,* 2010).

1.1.1. Occurrence and toxicity

Despite, in recent years the interest for these compounds has increased and several studies have been conducted, there are still few data on their toxicity, distribution, persistence and bioaccumulation.

Available studies have been shown their occurrence into aquatic, terrestrial and atmospheric environment as a result of antropogenic activities (Daughton and Ternes, 1999). The environmental compartment in which they are detected more frequently is aquatic environment, in fact, they have been detected in wastewaters, surface waters, ground waters, and in some cases in the drinking water (Bolong, 2009; Pojana, 2011; Baker and Kasprzyk-Hordern, 2011). The ECs have been detected in aquatic environment at levels up to μg L^{-1}(Kasprzyk-Horder, 2007). In the aquatic environment, the ECs are mainly released by household, industrial and hospital wastewater and several studies have clearly demonstrated the ineffectiveness of the treatment wastewater plant, in fact only 40-60% of ECs is removed from wastewater (Carballa *et al.,* 2004).

ECs have been detected also in biota, animal and vegetal foodstuffs as result of the bioaccumulation and soil migration phenomena. Occurrence of ECs in foods is related also to contamination by contact with packaging and manufacturing processes.

The toxicity studies conducted on aquatic organism show an irrelevant acute toxicity, because the concentrations in the environment are relatively low. Despite the found low concentrations, the ECs have a high toxic potential due to primarily their continued release in environment, which consequently leads to a prolonged exposure to them, causing chronic toxicity and irreversible damage at vital cycle of organisms (Daughton and Ternes, 1999; Ferrari *et al.,* 2003; Jjemba and Robertson, 2003). It has been observed, in fact, that the presence of ECs in the aquatic environment may induce immunosuppression in invertebrates and vertebrates (Khan and Thulin, 1991; Galloway and Depledge, 2001). Others studies have shown that the innate and acquired immune function (Bols *et al.,* 2001) in fish can

be damaged by classical xenobiotics (Dunier and Siwicki, 1994). Certain ECs can interfere in the normal functioning of the endocrine system and alter the normal growth, development, reproduction and behavior of organism (Damstra *et al.*, 2002). One of the best documented endocrine-distrupting effects is the feminization of fish following exposure to estrogenic compounds (Colborn *et al.,* 1998).

Another important factor to consider is that the compounds in the environment are not present singly but as a mixture of them. For this reason the relative toxicity to a mixture of compounds is different from that referred to the single compound. Cleuvers et al. have shown that the toxicity of a mixture of anti-inflammatory non-steroidal towards Daphnia was considerably higher than the toxicity of each single compound with the same concentration of the mixture (Cleuvers, 2008).

1.1.2. Case studies

The development of sensitive and specific analytical techniques and new equipment, in the field of analysis and environmental monitoring, has made the study of pollutants present in the environment in very low concentrations. This has allowed the identification of wide spectrum of contaminants present in different environmental compartments (Kot-Wasik*et al.,* 2007).

The brominated flame retardants (BFRs) are an example of substances previously believed harmless and subsequently identified as harmful compounds to the environment and to human health. However, the Europe Union has adopted legislation to reduce or end the sale and use of certain BFRs in order to protect human health and the environment. The BFRs are mixtures of man-made chemicals that are added to a wide variety of products, including for industrial use, to make them less flammable. They are used commonly in plastics, textiles and electrical/electronic equipment. The most common are brominated diphenyl ethers (PBDE), polybrominated biphenyls (PPB), hexabromocyclododecane (HBCDD) and tetrabromobisphenol A (TBBPA) (Richardson, 2007). In the last years, the scientific interest about these compounds has increased a lot because several

studies have shown the presence of these pollutants in almost all the environmental compartments (Richardson, 2008). These compounds are persistent environmental lipophilic and bioaccumulate in animals and humans. The increasing risk awareness to human health due to exposure to these chemicals derives from some toxicological studies that have demonstrated neurotoxicity in rats and the possibility of hormonal alterations and, in some cases, cancer (Richardson, 2008). For this reasons, since July 2006, in accordance with Directive 2002/95/EC, all electrical and electronic equipment could no longer contain PBB and PBDE, in any concentration. In July 2008, a third mixture PBDEs, decaBDE, which had originally been exempted from the restrictions, was banned by the European Court of Justice (2002/95/EC).

The same way, the bisphenol A has been used in the production of plastic and epoxy resin without problems since the 1950s, and only in the last twenty years it has been indicated as potentially toxic substance. Generally, the bisphenol A is used in plastic and metal food and beverage containers, thence it can come into contact with food and consequently with the humans. Several studies have shown that this compound can interfere in the normal functioning of the endocrine system, even at low concentrations (Howdeshell *et al.,* 1999). For these reason, since 2006 the EFSA has set for this substance a tolerable daily intake of 0.05 milligrams/kilogram body weight/day (EFSA, 2007). In January 2011 the European Commission adopted Directive 2011/8/EU that prohibits the use of BPA in the production of baby bottles in polycarbonate (2011/8/EU).

The ECs can be also the natural compounds as algal toxin: microcystins, anatoxins, nodularins, cylindrospermopsin, and saxitoxins. Microcystins and nodularins are hepatotoxic with high molecular weight. Anatoxins, cylindrospermopsin, and saxitoxinsare heterocyclic alkaloids; anatoxins and saxitoxins areneurotoxic, and cylindrospermopsin is hepatotoxic (Richardson, 2012). They can accumulate in various marine species such as fish, crabs or shellfish (oysters, mussels, scallops and clams). Nevertheless, when considerable amounts of contaminated foods are

consumed by humans this may cause severe intoxication. Around 60,000 human intoxications yearly with overall mortality of about 1.5% are related to toxins produced by algae (Kantiani *et al.,* 2010).

In recent years, a new class of ECs, the degradation/transformation products, has drawn the attention of the environmental chemistry. In fact, in the environment, the regulated/not regulated pollutants can be subject to degradation reaction, forming transformation products. These by-products can be generated by the normal processes of purification of drinking water and wastewater, such as chlorination, ozonolysis and advanced oxidation processes (TiO_2 photocatalytic oxidation, Fenton reaction) (Ikehata *et al.,* 2006) and by hydrolytic and photochemical processes that commonly occur in nature. Hydrolysis reactions, metabolic transformations and photochemical reactions can in some cases lead to the formation of compounds with chemical-physical and toxicological characteristics different from the parent compound.

These by-products can be a problem both from a toxicological point of view that analytical. In fact the available data demonstrate that in most cases the transformation product can be more toxic than parent compound (Grasso *et al.,* 2002, Sinclair and Boxall, 2003). For example, the Triclosan, a antimicrobial agent used in many product for personal and domestic hygiene, can be converted by sunlight-irradiated, in dioxin (2,7/2,8 -dibenzodicloro-p-dioxin) in aqueous solution (Kanetoshi *et al.,* 1992). As known dioxins are extremely toxic for humans and animals, reaching levels of toxicity evaluable in ng kg^{-1}(EFSA, 2010). This process, according to the researchers of the University of Minnesota, could be responsible for the presence of a part of dioxins that is present in the environment. Although the dioxin thus produced is relatively less toxic, scientists have shown that treatment of sewage with chlorine could lead to the production of a species of dioxin much more toxic (Latch *et al.,* 2003; Lores *et al.,* 2005;).

Other examples are the pesticide transformation products. Pesticides are synthetic chemical or natural substances, used as plant protection products. They have

different properties and potential impact on the environment (Stuart, 2012). Recent studies have focused more on their transformation products because their hydrolysis, oxidation, biodegradation, or photolysis transformation products can be present at greater levels in the environment than the parent compound and can be as toxic or more toxic (Richardson, 2012).

Chapter 2

Analytical methodologies in the analysis of trace contaminants in environment and foods

2.1. Introduction

Nowadays, due to increasing concern for the presence, fate and effects on the environment and humans of ECs, there is an obvious need for fast and sensitive multi-residue methods for the determination of low levels of ECs in the environmental and food matrices (Kasprzyk-Hordern *et al.,* 2008). Typically, the determination of ECs in environmental and food matrices involves a number of steps: sampling, sample preparation, separation and detection, identification and quantification of the target compound.

The conventional sample preparation steps include sampling/homogenisation, extraction, clean-up and concentration. For the determination of trace organic contaminants, the final analysis is achieved using a powerful separation technique, generally chromatographic, combined with an suitable detector (Dorne *et al.,* 2009). However, despite the advances in instrumental techniques and detection systems, the complexity of matrixes requires, in most cases, an extensive sample-preparation step, which is often still the bottleneck of the whole analytical procedure (Ridgway *et al.,* 2007). The objective of the sample preparation is to extract the target analytes from the matrix and clean up the extract to remove possible interfering compounds that might hinder the final instrumental determination.

The choice of the sample preparation methods depends on the analyte and the matrix. Nowadays, sample preparation methods tend to move towards more environmental friendly approaches (less consumption of organic solvents), miniaturization, automation and on-line coupling with the final instrumental determination. These latter strategie allow to extracts the target analytes with less manipulation by the analyst, so decreasing the possibility of experimental errors (Fidalgo-Udes *et al.,* 2007).

Detection systems play a key role in the determination and quantification of analytes, it should be sensitive and selective enough for the unequivocal

determination of the target analytes. In particular, mass spectrometry (MS), coupled to LC and GC, has become an essential tool to provide valuable structural information for identification of the compounds. The use of tandem MS/MS is becoming frequent with the introduction of different mass analyzers that enhance MS^2 capabilities, as improved designs of the triple-quadrupole (QqQ) and hybrid systems as the quadrupole-linear ion trap (Qq-LIT) and quadrupole time-of-flight (Qq-TOF). These systems allow not only a high sensitivity, but also provide further confirmation of the identity of the target analytes (Schuhmacher *et al.,* 2008; Lehotay *et al.,* 2008). While GC-MS is used as a routine technique in many laboratories for the analysis of non-polar, semi-polar, volatile and semi-volatile contaminants, LC-MS or LC-$MS^/$MS has become a powerful tool for qualitative and quantitative analysis of polar and non-volatile contaminants in recent years, with an increased number of applications both in environmental and fields food-safety (Picò and Barrcelo, 2008).

Despite the wide utility and diffusion of the atmospheric pressure ionization in mass spectrometry, it is subjected to relevant drawbacks called matrix effects. These effects result from co-eluting residual matrix components affecting the ionization efficiency of target analytes that could lead to no correct quantitative results. The most important method parameters as well as linearity, precision, and accuracy could be altered due to interfering compounds present in the matrix. The validation of an analytical method can not be accepted without an accurate evaluation of the matrix effects and possible strategies to minimize or to correct their, should be used. There are several strategies to overcome these drawbacks, it is possible to use a suitable sample preparation technique to eliminate or reduce co-extracted constituents matrix or to use a isotopically labeled standards as internal standard. The matrix effects are also minimized in quantitative analysis, by standard addition or matrix-matched calibration approaches (Kaplan, 2013).

2.2. Sample preparation techniques

The determination of trace residues of contaminants in complex matrices, such as environment and food, often requires laborious sample preparation procedures prior to instrumental analysis. Sample preparation is often the bottleneck in analysis and the reduction of the number of steps to decrease error sources and analysis time, is necessary. Nowadays, the use of environmentally friendly techniques (use less solvent and smaller sample sizes) is increasingly required. Optimal sample preparation can decrease error sources, analysis time, improve sensitivity and allow unequivocal identification, determination and quantification (Ridgway *et al.,* 2007).

The selective extraction of the analytes is based on their different chemical and physical properties, including molecular weight, charge, solubility, polarity, and volatility. Numerous techniques exist for extracting the sample, each one suitable for a given type of analyte or matrix (Mitra, 2003).

The extraction of organic compounds from environmental and food samples have normally been done using organic solvents with or without the use of heat (Buldini *et al.,* 2002; Ridgway *et al.,* 2007).

Generally, the used procedures for the separation of analytes from the matrix or from other coexisting components involve a two-phase system where the analyte and interferences are distributed between the two phases.

Classical sample pre-treatment techniques used for solid samples are: *Liquid extraction* and *extraction by Soxleth.*

In the *liquid extraction*, the target analytes are extracted from solid matrices by solvent. The efficiency of extraction is influenced by solubility, penetration of the sample by the solvent (mass transfer) and matrix effects. Solvent with different polarity, from methanol to hexane, also including acetone and ethyl acetate, are used in this technique. Successively, the obtained extract solutions can be treated as a liquid sample and often additional concentration or clean-

up steps, are required. This extraction technique has many drawbacks, it is laborious and time-consuming, requires the evaporation of large volumes of solvents and expensive, furthermore, a relatively large amount of matrix is required (Ridgway *et al.,* 2007).

A *Soxhlet extractor* has been invented in 1879 by Franzvon Soxhleth. It was originally designed for the extraction of lipid from a solid material. Nevertheless, a *Soxhlet* extractor cannot be used only for the extraction of lipids. Normally, a *Soxhlet* extraction is used when the target analyte has a limited solubility in a solvent and the impurities are insoluble in that solvent. While, if the target compound has a significant solubility in a solvent, it can be saperate from the insoluble substance by simple filtration (Soxhlet, 1879). Despite, the *Soxhlet* extraction is exhaustive, this tecnique does not allow a selective extraction and often a further clean-up step is necessary. Furthermore, the high used temperatures can degrade thermolabile compounds, and the large amounts of solvent requires, which must be evaporated before strumental analisys, makes the technique no friendly environment and lengthens the analysis time (6-24 h) (Luque de Castro and Garcìa-Ayuso, 1998).

In routine analysis of liquid samples, *liquid–liquid extraction (LLE*) has been used for many years as the basic, powerful methods for extraction of analytes from organic extracts and aqueous matrices. The *LLE* is based on the principle that a analyte can distribute itself in a certain ratio between two immiscible solvents (generally water and organic solvent). The use of large amounts of sample volumes and harmful organic solvents make this procedure, expensive, time consuming, environmentally unfriendly, laborious and potentially subject to sample contamination, making the analysis unreliable (Anthemidis and Miro, 2009; Pena-Pereira *et al.,* 2009).

Usually, after the extraction process, the sample is subjected to various processes of purification and concentration, often expensive, time-consuming

and environmentally unfriendly. For these reasons it is necessary to use alternative analytical techniques rapid, selective, economic and environmental sustainable.

To this aim, several alternative techniques for the extraction and sample preparation have been developed to overcome the limitations of conventional methods. The most suitable are: *solid phase extraction* (SPE) (Hennion, 1999) *solid-phase microextraction* (SPME) (Prosen and Zupani-Kralj, 1999), *molecular imprinting technique* (MIT) (Vlatakis *et al.,* 1993), *single-drop microextraction* (SDME) (Jeannot and Cantwell, 1996), *hollow fibber-based liquid-phase microextraction* (HF-LPME) [18], *pressurized liquid extraction* (PLE) (Esrafili *et al.,* 2007), *matrix solid phase dispersion* (MSPD) (Barker, 2000) and *dispersive liquid-liquid microextraction* (DLLME)(Rezaee *et al.,* 2006). Due to the large number of techniques for the quantitative determination of trace contaminants, their exhaustive examination goes beyond the scope of my thesis and, therefore, only the used technique applied in this thesis will be discuss.

2.2.1. Solid Phase Extraction (SPE)

Disposable cartridges for SPE have been introduced for more than 30 years (cartridges in 1978, syringe-format types in 1979, pre-columns for the on-line coupling with liquid chromatography in the 1980s) and their development has been slow for many years. In the last decades, the great success of the SPE was due to improvements in formats, automation and introduction of new phases (Hennion, 1999). SPE has gradually displaced the classical LLE and become the routine sample preparation technique in environmental chemistry. The SPE has replaced LLE for its numerous advantages: uses less solvent, improves sample throughput, a suitable choice of stationary phase improves the selectivity of the overall procedure, can be easily automated and avoids the

formation of emulsions. In many cases, SPE provides cleaner extracts and provides higher and more reproducible recoveries (Fontanals *et al.,* 2005).

In SPE, the analytes are partitioned between a solid phase and a liquid phase, and they must have greater affinity for the solid phase than for the sample matrix. SPE is mostly used to analyze aqueous samples (to extract semi-volatile or non-volatile analytes), or to purify organic extracts from solid matrices (Mutavdzic Pavlovic *et al.,* 2007). SPE is used for sample clean-up and analyte concentration before of the chromatographic separation.

2.2.1.1. Sorbent SPE

The choice of sorbent is very important in SPE procedure, because it influences parameters such as, affinity, selectivity and capacity. This choice depends on the target analytes and the interactions of the sorbent with the functional groups of the analytes. Nevertheless, it also depends on the sample matrix and its interactions with the sorbent and the analytes (Fontanals *et al.,* 2005). A wide variety of SPE sorbents are commercially available and the most common are silica-based sorbents and polymeric sorbents.

The silica-based sorbents are constituted by silica particles with diameter between 40 and 60 μm, with a rough surface and pores between 65 and 75 Amstrong and they offer a surface area of 300-500 $m^2 \cdot g^{-1}$. These types of adsorbents are very common and they can be functionalized with the addition of polar or apolar groups to silanol groups ≈Si—OH (Savelli and Bruno 2005).

In the polymeric sorbents instead the support is a polymer based on polystyrene divinyl benzene, very crosslinked. The particles have a surface area ranging between 700 and 1000 $m^2\ g^{-1}$ and a pore size comprised between 80 and 100 Amstrong. Compared to adsorbent siliceous, the particles have a sphericity more regular and a surface more homogeneous (Savelli and Bruno 2005). In addition, the polymeric adsorbents have a greater surface area,

allowing to load higher sample amount. This type of adsorbents can be used at pH range comprised between 2 and 12.

The stationary phases commonly used in SPE cartridges are reversed-phase (C8, C18), ion-exchange (strong anion and strong cation exchange), or normal-phase (silica, cyano, amino) packings. In the last decades, new sorbents, to improve the selectivity and the poor retention of polar compounds, have been developed. A first approach has used antibodies (immunosorbents, ISs), which have allowed a high degree of molecular selectivity. Instead, a second approach has used molecularly imprinted polymers (MIPs), which has allowed to overcome the inherent instability of biological materials (Hennion, 1999).

Reversed-phase

Reversed phase separation uses an apolar stationary phase, able to retain the apolar analytes, and a polar or moderately polar sample matrix (aqueous sample). The most used SPE sorbents in reversed phase are alkyl- or aryl-bonded silicas. The hydrophilic silanol groups are chemically modified with hydrophobic alkyl or aryl functional groups by reaction with the corresponding silanes (C-18 and C-8). The silica based bonded phases show some percentage of residual unreacted silanols that can act as secondary interaction sites. These secondary interactions may be useful in the extraction or retention of highly polar analytes or contaminants, but may also irreversibly bind target analytes.

Moreover, polymeric sorbents based on polystyrene divinyl benzene belong to the reversed-phase sorbent group. These sorbents were developed for the extraction of a wide range of, basic, acidic and neutral compounds from various matrices using a simple, generic protocol. Usually, this type of stationary phase primarily adsorbs the analytes by π-π interactions, which are established between the aromatic rings of the resin and the aromatic groups of

the analytes. Moreover, it is possible to add on the benzene rings the polar or ionic groups (Hannion, 1999).

Ion-exchange

Ion exchange sorbents are used to analyze charged analytes in solution (generally aqueous solution and sometimes organic solution). The analytes are retained on the stationary phase by electrostatic interactions between functional charged groups of analytes and stationary phase. In ion exchange SPE the pH is very important, because the stationary phase and analytes must be at a pH where both are charged.

The positively charged compounds (basic compounds) are retained by cation exchange sorbents containing aliphatic sulfonic acid groups that are negatively charged in aqueous solution (pH 5). While negatively charged compounds (acid compounds) are retained by anion exchange sorbents containing quaternary ammonium groups that have a permanent positive charge in aqueous solutions (pH 9). The adsorbed analytes on the stationary phase are eluted by a solution at a pH able to neutralize the electrostatic interactions between analytes and stationary phase (Zwir-Ferec and Biziuk, 2006).

Normal-phase

Normal phase SPE procedures generally use apolar matrices and a polar stationary phase. Typically, the polar-functionalized bonded silicas are used in normal phase SPE. Retention of an analyte in normal phase SPE is mainly due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface. These include π-π interactions, hydrogen bonding, dipole-dipole interactions, and dipole-induced dipole interactions. The elution of adsorbed analytes is obtained by a solvent able to disrupt the binding mechanism between the analytes and sorbent. Generally, the used solvent is more polar than matrix (Hannion, 1999).

Immunoaffinity solid-phase extraction

Immunoaffinity solid-phase extraction sorbents (immunosorbents, ISs), are based on an antibody-analyte specific binding technology. The use of the antibody-analyte interaction allows a high selectivity and an efficient recovery of the analytes from complex matrices, as environmental and food matrices. The elution of the analytes from the sorbent is obtained by solvent able to denature the protein, so the antibody-analyte interaction is broken. Moreover, an antibody can bind one or more analytes having structure similar to the one used for its preparation, for this the ISs can be used for the analysis of a single analyte and its metabolites. Their main disadvantage is the high cost (Delanauy *et al.,* 2000).

Molecularly imprinted solid-phase extraction

The molecularly imprinted polymers (MIPs) are an optimal solution to the preparation of selective materials for solid phase extraction. The MIPs are composed by highly ramified synthetic resins with internal selective sites for molecular recognition. These materials are prepared by polymerisation of suitable functional monomers in presence of a molecule capable of acting as 'stamp' (template molecule). The monomers are chosen considering their ability to interact with the functional groups of the template molecule.

After removing the template molecule, the obtained sites are able to bind to target molecules with shape, size and functionalities complementary to template molecule. The resulting imprinted polymers are robust, stable and resistant to a wide range of pH, solvents and temperature. Despite, this kind of adsorbent is an alternative to expensive immunoaffinity sorbents, its spread is limited by the difficulty of synthesis of the polymer (Turiel and Martín-Esteban, 2010).

2.2.1.2. Procedure and parameters affecting the SPE process

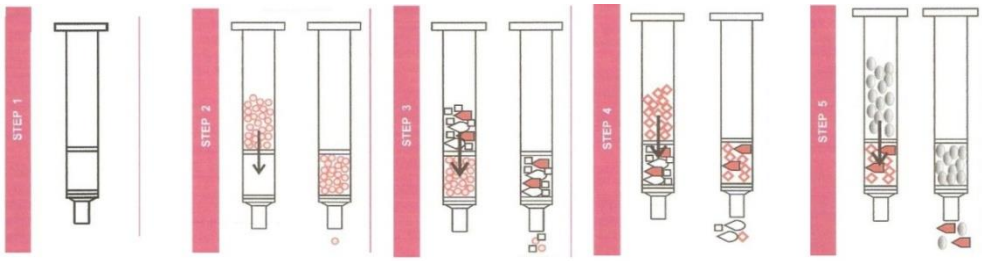
The SPE process provides samples that are in solution, free of interfering matrix components, and concentrated enough for detection.

The extraction is performed in five steps: (Figure 2.1):

- Step 1 - Choice sorbent: the choice of the sorbent is dependent on the matrix, target analytes and interferents;
- Step 2 - Precondition: the functional groups of the sorbent are solvated in order to make them to interact with the analytes;
- Step 3 - Load sample: the analytes are retained on the sorbent phase and the weakly retained matrix compounds are eluted;
- Step 4 - Wash: undesired species are removed;
- Step 5 - Elute: the analytes are desorbed and collected for analysis in a small volume.

Sometimes, the eluent is blown down by evaporation to further concentrate the analyte or to allow redissolution of the analyte in a solvent more compatible with the subsequent chromatographic technique.

Figura 2.1. *SPE procedure*



In SPE process suitable solvent are used in the three main steps: loading, wash and elution. The loading solvent must solubilize the whole sample and, like the washing solvent, it doesn't have to eluate the analytes of the stationary phase. Moreover, the washing solvent must be able to remove the interfering substances and create the suitable environment for the following elution phase of the analytes. Finally, the elution solvent must be able to completely elute the analytes from the sorbent breaking the bonds analyte-sorbent.

The main features of the solvents used for the SPE are represented in table 2.1.

Table 2.1. *Characteristics of Solvents Commonly Used in SPE*

Polarity			Solvent	Miscible in Water?
No polar	Strong Reversed Phase	Weak Normal Phase		
			Hexane	No
			Isooctane	No
			Carbon tetrachloride	No
			Chloroform	No
			Dichloromethane	No
			Tetrahydrofuran	Yes
			Diethyl ether	No
			Ethyl acetate	Poorly
			Acetone	Yes
			Acetonitrile	Yes
			Isopropanol	Yes
			Methanol	Yes
	Weak Reversed Phase	Strong Normal Phase	Water	Yes
Polar			Acetic acid	Yes

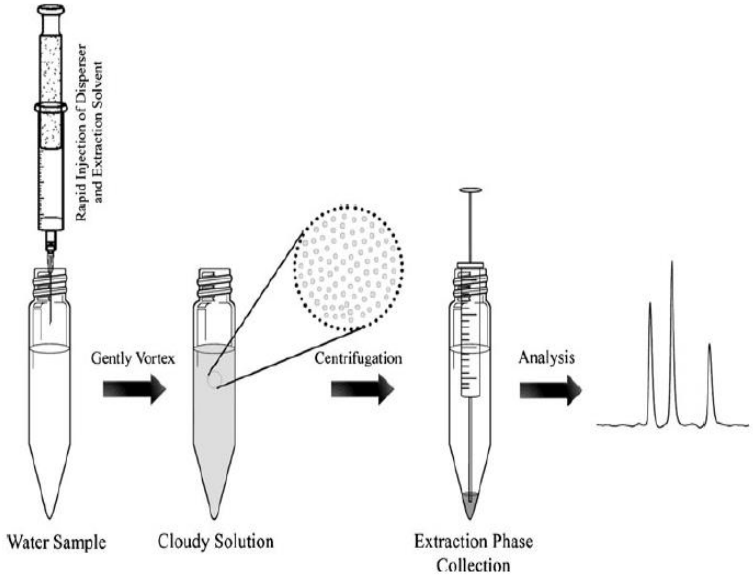
Taking into account all these factors and choosing the right combination, it is possible to achieve a good selectivity and exhaustive recovery of the analyte.
Another important parameter to consider is the volume of sample that can be loaded on the cartridge. There is a threshold value beyond which it is no can get a 100% recovery. This value isn't fixed, but it depends on the type of absorbent, matrix and analytes, for this reason it must be determined experimentally.
In the determination of ionizable analytes the pH value is very important in the SPE process, above all in the ion exchange SPE procedures.

2.2.2. Dispersive Liquid-Liquid MicroExtraction (DLLME)

In recent years, much attention has been paid to the development of environmentally friendly activities such as miniaturizing, replacing toxic reagents and automating extraction techniques. For this reason, microextraction techniques were developed to replace the conventional extraction method (Saraji and Boroujeni, 2013).
DLLME is a recent miniaturized version of conventional LLE technique introduced in 2006, which requires only microliter volumes of solvents (Rezaee *et al.,* 2006). The high enrichment factor and the use of low volumes of extraction solvent and sample together with the simplicity and rapidity of the operation and low cost are the main advantages of this analytical technique (Andruch *et al.,* 2012; Reezae *et al.,* 2006).
This new microextraction technique has been successfully used to determine a wide variety of organic and inorganic compounds in different matrices, including, food, environmental, clinical and forensic samples, as an effective alternative to traditional sample treatments (Reezae *et al.,* 2006).
DLLME is a powerful extraction and/or preconcentration technique based on a ternary solvent system in which a few microliters of extractant (a water-immiscible solvent) are dispersed into an aqueous sample with the help of a

disperser (solvent with high miscibility in both extractant and water) to form a stable cloudy solution containing fine droplets of the extractant. In this state, the infinitely large surface area between extractant and water allows a quick transfer of the target analytes from the aqueous sample to the extractant, reducing the extraction time. The extraction phase is then separated by centrifugation, and the enriched analytes in the settled phase (with or without further treatment) are determined by analytical techniques (Figure 2.2) (Saraji and Boroujeni, 2013; Razaee *et al,* 2010).

Figure 2.2. *DLLME procedure (Saraji and Boroujeni, 2013)*



The performance of DLLME is assessed in terms of two different parameters, namely, the enrichment factor (EF) and the extraction recovery (ER). EF is defined as the ratio of the analyte concentration in the sedimented phase (C_{sed}) to its initial concentration in the aqueous sample (C_0) (Rezaee *et al.,* 2006):

$$\mathbf{EF = C_{sed}/C_0}$$

ER is defined as the proportion of total analyte (n_0) that is extracted into the sediment phase (n_{sed}) (Rezaee *et al.,* 2006):

$$\mathbf{ER = n_{sed}/n_0 = (C_{sed} \cdot V_{sed})/(C_0 \cdot V_{aq})\ 100}$$

where, V_{sed} and V_{aq} are the volumes of sedimented phase and initial sample solution, respectively.

2.2.2.1. Parameters affecting the DLLME process

In order to obtain high extraction efficiency in the DLLME process, it is necessary to study the effect of all experimental parameters that can probably influence the DLLME performance. The extraction efficiency of DLLME is directly influenced by the type of disperser and extractant solvents and by their volumes. Also, ionic strength and pH of aqueous solution play a crucial role in DLLME efficiency (Saraji and Boroujeni, 2013; Reezae *et al.,* 2006; Reezae *et al.,* 2010).

The extraction time (time interval between injection of the mixture of disperser and extraction solvents and centrifugation) does not have a significant effect on the extraction efficiency in DLLME, because the finely dispersed drops of the extraction solvent provide a large surface area between the extraction solvent and the aqueous sample. For this, the transfer of the analyte from the aqueous phase to the extraction phase is very fast, and the equilibrium state is achieved very quickly (Saraji and Boroujeni, 2013; Reezae *et al.,* 2010).

DLLME relies on three essential components the aqueous solution and the extractant disperser solvents, which play complementary roles in the process and must meet some basic requirements.

Extraction solvent

Selecting an appropriate extraction solvent plays an essential role in DLLME procedure. The extraction solvent should be immiscible with the water, should possess high extraction capabilities for the analytes and be heavier than the aqueous phase in order to facilitate phase separation, usually by centrifugation, after extraction. Various organic solvents (mainly halogenated hydrocarbons) have been used as extraction in this context; however, solvents lighter than water have lately gained acceptance for this purpose (Saraji and Boroujeni, 2013; Reezae *et al.,* 2010; Farajzadeh *et al.,* 2010a; Kamankesh *et al.,* 2013).

Disperser solvent

The choice of the disperser solvent is based on its miscibility in both the aqueous phase and the extraction solvent, it directly affects the formation of the cloudy solution, the dispersion of the extraction solvent in the aqueous phase and, consequently, the extraction. Therefore, the type of disperser solvent to be used, and its volume, should be carefully selected in order to ensure adequate efficiency. Acetone, acetonitrile, and methanol are usually used for this purpose. Also, some DLLME modes minimize or avoid the use of a disperser solvent to increase the extraction efficiency. To this end, it is replaced with an auxiliary form of energy such as ultrasound or controlled heating (Saraji and Boroujeni, 2013; Reezae *et al.,* 2010).

Volume of the extraction and disperser solvents

The volume of the extraction solvent and the volume of the disperser solvent have an important effect on the extraction efficiency.

Specifically, the extractant volume directly affects EF: lower volumes of the extraction solvent enhance the EF owing to reduction of the volume of the sedimented phase, but also decrease the ER. Therefore, the optimum volume of the extractant is generally a compromise between ER and EF. Furthermore,

the volume of the extractant should be kept as low as possible, due to toxicity of most of the extraction solvents (Saraji and Boroujeni, 2013; Reezae *et al.,* 2010).

The disperser solvent volume directly influences the ease of formation of the cloudy solution and hence the extraction efficiency. The ratio between the extraction solvent volume and the disperser solvent volume is also important and it should be adjusted to obtain a cloudy solution (Saraji and Boroujeni, 2013; Reezae *et al.,* 2010).

Ionic strength and pH

Addition of salt can improve the extraction yield in DLLME due to the increase of the solubility of the analyte in the extractant. In addition, higher ionic strength of aqueous solution improves the phases separation. However, owing to decreasing solubility of the extraction solvent in water, the sedimented phase volume is increased, thereby decreasing the EF (Saraji and Boroujeni, 2013; Reezae *et al.,* 2006).

Aqueous solution pH is very important in the case of ionizable analytes because it influences the dissociation equilibrium from ionized to the un-ionized form and consequently the solubility of the analyte in water and organic phases (Reezae *et al.,* 2010).

2.2.2.2. DLLME application

DLLME has been successfully applied to extraction and concentration of wide variety of organic compounds and metal ions, mainly from water sample.

The first application of DLLME was performed by Rezaee *et al*. for extraction and preconcentration of polycyclic aromatic hydrocarbons (PAHs) in water samples (river water, surface water and well water) and their determination by gas chromatography (GC)–flame ionization detection (FID) (Rezaee *et al.,* 2006). This method had a good linear range (approximately 104) and high EFs

(603–1113) and low detection limits (0.007–0.030 μg L^{-1}) for determination of PAHs. Furthermore, the results showed that the method can be safely applied for the determination of organic compounds in real water samples (Rezaee *et al.,* 2006).

DLLME has been mainly used for the analysis of pesticides. Pesticides are a widely studied class of analytes and a large number of studies have been conducted to determine them, using DLLME as extraction/preconcentration technique. The great interest in this group of analytes is due their connection with environmental and food pollution. The pesticides were determinated mainly in the water matrices (Berijani *et al.,* 2006; Nagaraju and Huang, 2007; Wei *et al.,* 2007; Melwanky and Fuh, 2008; Farhadi *et al.,* 2009; Farajzadeh *et al.;* 2010a; He *et al.;* 2010; Wang *et al.,* 2011). Moreover, DLLME was used for preconcentration of many other organic compounds. Several studies were devoted to analysis of phenols and pharmaceuticals. Two groups of phenol were extracted from water samples: endocrine distruptors phenols and clorophenols (Fattahi *et al.,* 2007a; Moradi *et al.,* 2010). The pharmaceutical were analyzed mainly in water samples (tap water, river water, well water, sea water, and lake water) (Negreira *et al.,* 2010; Martín *et al.,* 2013; Saraji and Marzban, 2010) and biological samples (Zeeb *et al.,* 2010; Saraji *et al.,* 2011).

DLLME was also used for determination of personal care products from environmental matrices, such as polycyclic musks (Panagiotou *et al.,* 2009), UV filters (Negreira *et al.,* 2010), antimicrobial agent (Guo *et al.,* 2009) and parabens (Farajdaze *et al.,* 2010b; Hou *et al.,* 2013).

Other groups of compounds as PAHs, polybrominated diphenyl ethers (PBDEs) and polychlotinated biphenyls (PCBs) were analyzed by DLLME. These compounds are persistent environmental and toxic contaminants. PCBs were extracted from water (Dai *et al.,* 2010), soil and fish (Hu *et al.,* 2009a) PBDEs were analyzed in water (Li *et al.,* 2008a), plants and animal tissue (Liu *et al.,* 2009a). PAHs were extracted from water (Rezaee *et al.,* 2006; Xu *et al.,*

2009; Pena *et al.,* 2009; Shi and Lee, 2010) and marine sediments (Rezaee *et al.,* 2010). Obviously, solid samples have to be subjected to extraction with appropriate solvent that could be used in the second extraction step as a constituent of a ternary solvent mixture in a DLLME procedure.

Usually, DLLME is chosen for the analysis of samples with a simple matrix. The technique has sample clean-up efficiency and low selectivity, so the matrix most commonly studied are different types of water samples (Rezaee *et al.,* 2006; Martín *et al.,* 2013). Nevertheless, in recent years, few applications of DLLME have been conducted on more complex matrices such as food matrices: wine (Fariña *et al.,* 2007; Campone *et al.,* 2010; Arroyo-Manzanares *et al.,* 2012), fruit and juice (Ling Yan *et al.,* 2009; Viñas *et al.,* 2013) honey (Chen *et al.,* 2009a; Zacharis *et al.,* 2012), milk (Campillo, 2013, Campone *et al.,* 2013) and cereals (Campone *et al.,* 2011; Campone *et al.,* 2012).

Table 2.2. *DLLME applications in the analysis of contaminant compounds in different matrices (Zang et al., 2009)*

Analytes	Matrix	Method	Extraction solvent	Disperser solvent	EF	LOD (μgL^{-1})
PAHs	Water	GC-FID	Tetrachloroethylene	Acetone	603-1113	0.007-0.030
Organophosphorus pesticides	Water	GC-FPD	Chlorobenzene	Acetone	789-1070	3-20 ngL^{-1}
Trihalomethanes	Drinking water	GC-ECD	Carbon disulfide	Acetone	116-355	0.005-0.040
Chlorobenzenes	Water	GC-ECD	Chlorobenzene	Acetone	711-813	0.0005-0.05
PCBs	Water	GC-ECD	Chlorobenzene	Acetone	383-540	0.001-0.002
Pyrethroid pesticide	Water	GC-ECD	Chlorobenzene	Acetone	708-1087	0.10-0.04
Triazine herbicide	Water	GC-MS	Chlorobenzene	Acetone	151-722	0.021-0.12
Fragrances, phthalate	Water	GC-MS	Chloroform		100	6.0-133 ngL^{-1}
PAHs	Water	GC-FID	Chloroform	Methanol	225-257	0.02-0.18
Parabens	Water	HPLC-MS	Chloroform	Acetone	287-906	0.010-2.0
Butyl and phenyltin	Water	GC-FPD	Carbon tetrachloride	Ethanol	825-1036	0.2-1 ng^{-1}
Anilines	Waste water	GC-MS	Chlorobenzene	Acetone	212-645	0.04-0.09
Organophosphorus pesticides	Watermelon, cucumber	GC-FPD	Chlorobenzene	Acetonitrile	41-50	0.010-0.190
Volatile phenols	Red wines	GC-MS	Carbon tetrachloride	Acetone		28-44
Captan, Folpet and Captafol	Grape	GC-ECD	Chlorobenzene	Acetone	788-876	6.0-8.0 $\mu g\ Kg^{-1}$
Triclosan	Water	HPLC-TUV	tetrachloroethane	Methanol	215	0.3
Phthalate esters	Water	HPLC-VWD	Carbon tetrachloride	Acetonitrile	44-196	0.64-8
Polybrominateddiphenyl ethers	Water	HPLC-VWD	Tetrachloroethane	Acetonitrile	268-305	12.4-55.6 ng L^{-1}

2.2.3. Matrix Solid Phase Dispersion (MSPD)

The extraction of target analytes from different matrices (environmental, food or biological) is always a fundamental step in the development of an analytical procedure, and sometimes a previous disruption of the general sample architecture is needed (Barker *et al.,* 1993).

MSPD is an analytical technique first introduced in 1989 by Barker et al. (Barker *et al.,* 1989), for the extraction of compounds of interest from solid samples. In MSPD process, the extraction of target analytes is obtained by dispersing tissues onto a solid support, so the mainly encountered difficulties in the classical SPE process (sample homogenization and incomplete cell disruption), are avoided. The mechanical disruption of the matrix structure is carried out by blending of sample with abrasive solid support, and this was confirmed by scanning electron microscopy observations (Barker, 2000).

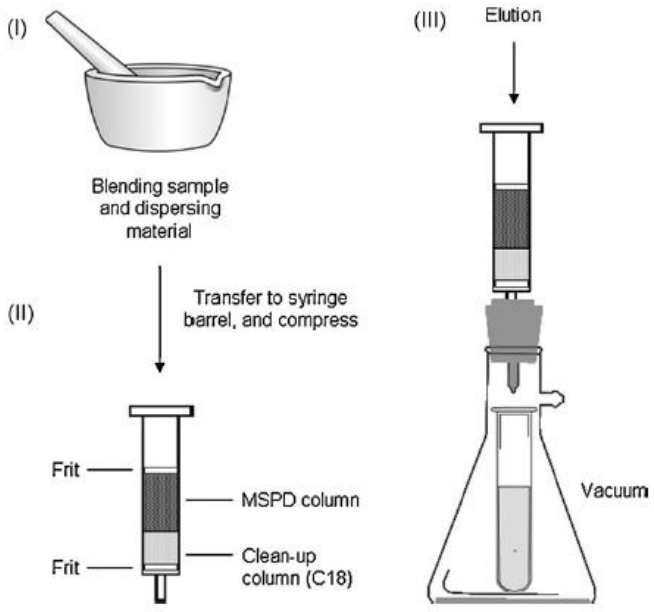
Since its introduction, MSPD has been applied, with some modifications, to the extraction of a large number of exogenous and endogenous organic compounds (contaminants, drugs, pesticides, food and bacteria components) from solid, semi-solid, and viscous matrices (animal tissues, blood, milk, bacteria, fruits, vegetables, etc.) (Barker *et al.,* 1989; Baker, 2000; Garcìa-Lopez *et al.,* 2008).

The great success of MSPD is due to its numerous advantages, it is a very quick, easy and environmentally friendly technique and thanks to its simplicity and flexibility it has replaced the classical sample preparation techniques, in many applications (Barker, 2007, Kristenson *et al.,* 2006). Moreover, MSPD process requires mild extraction condition (atmospheric pressure and room temperature), respect to others analytical techniques as soxhlet, microwave-assisted extraction, pressurized liquid extraction and supercritical-fluid extraction and, furthermore, this technique provides accetable yield and good selectivity (Garcìa-Lopez *et al.,* 2008).

After extraction, according to compounds of interest and instrumentation employed for their detection, further sample clean-up is required (Barker *et al.,* 1989). Usually, after MSPD, gas-chromatography (GC) or a liquid-chromatography (LC) separation is followed by mass spectrometric determination (MS); less frequently, LC is coupled to UV or fluorescence detection (FLD), and GC to electron capture detection (ECD).

The main steps of the MSPD process are three (figure 2.3). In the first step, the sample is blended with the dispersant material in a mortar with a pestle; in the second step the homogenized powder is transferred in a solid-phase extraction cartridge, and compressed; finally, in third step the analytes are eluted with a suitable solvent or solvent mixture, this operation is performed by the aid of a vacuum pump (Capriotti *et al.*, 2010).

Figure 2.3. *MSPD procedure (Capriotti et al., 2010)*



2.2.3.1. Parameters affecting the MSPD process

According to Baker, MSPD process is based on retention properties that seem a mix of adsorption, partition and paired ion/paired chromatography. The distribution of compounds in the material depends on the interaction between the analytes and the solid support and, further by the molecular size. (Barker *et al.,* 1993). In MSPD process the selectivity and the extraction efficiency strictly depend on both the type of the sorbent materials and the elution solvent used (Kristenson *et al.,* 2006).

Eluent solvent

The selection of the eluent solvent strictly depends on both the target analytes and the nature of solid material. Normally, organic solvent mixtures are employed, but in some applications (mainly belonging to PLE procedures), hot water gave satisfactory results (Bogialli and Di Corcia, 2007). Apolar solvents, as hexane, dichloromethane, or mixture of both, are used to elute apolar substances. Instead, acetonitrile, acetone, ethyl acetate or mixtures of water with methanol or ethanol are employed to elute substances of medium or high polarity (Garcìa-Lopez *et al.,* 2008).

Sorbents

The sorbent dispersant should be selected considering physical and chemical properties of the target analytes and the composition of the matrix. The parameters to consider are: the particle size, the consistency of resultant sorbent-sample mixture and the best ratio between the amount of sample material and dispersant (Garcìa-Lopez *et al.,* 2008, Kristenson *et al.,* 2006). In MSPD can be used three types of sorbents: reverse phase material (C18 and C8 bonded silica), normal phase inorganic materials (alumina and florisil) and non-retentive supporting materials (sand and diatomaceous).

Reversed phase materials such as C18 and C8 are mainly employed for the extraction of anthropogenic and natural contaminants from environmental and food matrices, in addition, these lipophilic sorbents show good extraction efficiency also in matrices with high lipid content (Capriotti *et al.,* 2010).

Normal phase inorganic materials such as floris, alluminia and silica are employed as dispersant in many MSPD applications, particularly; they are employed for analysis of environmental pollutants, but in recent years these sorbent were employed also for analysis of animal tissue and plants. Probably, the mechanical disruption of the cells sufficiently permits the analyte migration to the sorbent surface (Capriotti *et al.,* 2010).

The use of inert materials for MSPD leads to cost-effective methods at expense of selectivity, which is just regulated by the molecule solubility (Capriotti *et al.,* 2010).

Another important parameter, that influences MSPD process, is the ratio between the sample and the dispersing material. Usually, about 0.5 g of sample are disprsed with sorbent, with ratios ranging from 1:1 to 1:4, with some exceptions. The ratio 1:4 is the most common employed, nevertheless this ratio has to be optimized in function of both sample complexity and physical–chemical features of the material (Abhilash *et al.,* 2007).

2.2.3.2. On-line clean-up of MSPD extracts

Sometimes the accurate selection of the elution solvent and dispersant sorbent in MSPD procedure does not generate a ready-to-analyse extract. In these cases, an on-line clean-up step can be added to the sample-preparation process. One possible strategy is to add a layer of co-sorbent in MSPD cartridge to purify the raw extract. The choice of co-sorbent depends on both the characteristics of target analytes and interfering matrix. The polar interferences can be eliminated using normal-phase materials as co-sorbents and C18 or other reversed-phase functionalised silica sorbents as disperdsent

(Garcia-Lopez *et al.,* 2008; Kristenson *et al.,* 2006). For example, silica has been used as co-sorbent in the extraction of pesticides, fungicides aflatoxin and insecticides from vegetable and fruit samples (Fernandez *et al.,* 2000; Blesa *et al.,* 2003) Carbon is also useful for removing interfering pigments co-extracted from chilli powder and vegetables (Hu *et al.,* 2006).

Other studies have used C18 as dispersant and Florisil as co-sorbent (Pensado *et al.,* 2005; Canosa *et al.*, 2007). Sometimes, a layer of acidified silica was placed in MSPD cartridge, for analysis of compounds with high chemical stability, as PBDEs, PCBs, and PBBs (Carro *et al.,* 2005). This strategy leads to clean extracts, and a further solvent and consuming time off-line clean-up step, is avoided.

Furthermore, the selectivity of MSPD extractions can be increased by rinsing the sample before analyte extraction. For example, the sugars and polar compounds can be removed, from vegetables and fruit disperded on C18, by washing with water before elution of analytes (Garcia-Lopez *et al.,* 2008; Kristenson *et al.,* 2006). Instead, hexane often is employed to remove apolar interferences (fatty acid and lipids), before elution of analytes of interest with polar solvent mixtures (Garcia-Lopez *et al.,* 2008; Kristenson *et al.,* 2006).

In leterature both clean-up strategy were considered to increase selectivity in the extraction of medium-polar analytes from complex samples (Garcia-Lopez *et al.,* 2008; Kristenson *et al.,* 2006).

2.2.3.3. MSPD application

MSPD can be considered as a good alternative to the classical sample preparation techniques, eparticularly for semisolid samples, because it allows the extraction and cleanup in a single step using solid sorbents with significant reduction in both the sample amount and the solvent consumption (Smith, 2002). MSPD was applied to the analysis of several analytes in different matrices (Barker, 2000).

In the field of food safety, MSPD has been used to extract the four main aflatoxins from olive oil and a reversed phase materials (C18) has been used. The methods has shown good recoveries (92-107%) and low quantification limits, ranged between 0.04 and 0.12 μg kg^{-1} (Cavaliere *et al.*, 2007).

Regarding to drug residue analysis, Sergi et al. have developed a MSPD protocol for determination of sulphonamides in raw meat and meat-based baby food. The sample was dispersed with C18 and successively, the target analytes were eluting with methanol at 0°C in order to reduce its eluotropic power. This strategy has resulted a high selectivity and quantitative recovery and the co-elution of fat or proteic substances from the matrix was reduced (Sergi *et al.*, 2007):

Several authors have used MSPD for determination of pesticides and environmental contaminants in food. Cunha et al. (Cunha, 2007) have developed a MSPD protocol for analysis of phosmet and its metabolites in olives and olive oil. The proposed method has used C18 and $MgSO_4$ as dispersant sorbent and No further clean-up was required prior to GC–MS analysis. Maldaner et al. have developed a method for the determination of six pesticides (imazaquin, imazethapyr, carboxin, metsulfuron–Me, chlorimuron–Et, and tebuconazole) in soybeans, using MSPD with silica and a clean-up step with C8 as co-sorbent. The additional clean-up step before HPLC–DAD determination was added to remove the large quantities of co-extracted fat and proteins. The developed method has shown acceptable recoveries (60-120%) and very low quantification limits (Maldaner, 2008).

Recently, a multiresidue method was proposed for determination of 13 emerging and priority contaminants in lettuce (PAHs, pesticides, pharmaceuticals, personal care products, and phenolic estrogens). In this method was combined the MSPD with pressurized fluid extraction (PFE) followed by GC–MS/MS (Caldero-Preciado, 2011).

Table 2.3. *MSPD applications (Garcia-Lopez et al., 2008)*

Analyte	Matrix	Sample preparation	LOQ	Detection
MCs and nodularin	Fish muscle	1 g sample + 5 g sand. Extraction with 4 mL H_2O (80 °C, pH=2) pH adjustment and filtration	1.6–4 ng g^{-1}	LC–MS
PCBs	Biota	2 g sample + 4 g Florisil; 6 g Florisil as co-sorbent. Washing with 20 mL DCM–pentane (15:85). Extraction with 16 mL pentane–acetone (1:1).	/	GC–ECD, GC–MS
Chloramphenicol	Muscle tissue	2 g sample + 3 g C_{18}; 0.5 g C_{18} co-column. Washing with 10 mL hexane and 12 mL ACN–H_2O (5:95). Elution with 10 mL ACN–H_2O (1:1). LLE clean-up with 2×5 mL H_2O saturated with EtOAc	4 ng g^{-1}	GC–ECD
Organophosphate ester	Dust	0.5 g dust + 0.5 g Na_2SO_4anh. + 0.5 g Florisil; 0.5 g Al_2O_3 cosorbent. Washing with 2 mL hexane; elution with 3 mL acetone, addition 1 mL EtOAc and concentration. Solution filtration	40–50 ng g^{-1}	GC–NPD
Phenolic compounds	Leaves	0.5 g plant + 2 g C_{18}. Elution with 20 mL MeOH–H_2O (7:3, v/v). Dryness and reconstitution with 5 mL MeOH–H_2O (7:3, v/v)	/	LC–UV
Isoflavonoids	Medicinal herb	0.5 g sample + 0.5 mL H2O + 1 g C_{18}; 1 g C_{18} as co-sorbent. Washing 10 mL H_2O, elution with MeOH–H_2O (90:10, v/v). Dryness and reconstitution with 1 mL MeOH	/	LC–UV, LC–MS
Fungicides	Fruits and vegetables	0.5 g sample + 0.5 g C_{18}. Elution with 10 mL EtOAc	4–100 ng g^{-1}	LC–MS
Pesticides	Fruit, vegetables and cereal	5 g sample + 10 g silica gel. Elution with hexane–diethyl ether or MeOH–DCM. Extract to dryness and reconstitution	0.02–0.25 µg mL^{-1}	LC–UV
Aflatoxins	Chilli powder, green bean, black sesame	1 g sample + 2 g Al_2O_3; 70 mg graphitic carbon black as cosorbent. Elution with 5 mL ACN and concentration	0.1–0.25 ng g^{-1}	LC–FLD
Pesticides	Fruit	0.5 g sample + 0.5 g C_{18}. Elution with 100 mL DCM–MeOH (1:1) and concentration	0.05–2 µg g^{-1}	LC–MS
Benzoic acid derivatives	Medicinal plant	g sample + 2 g C_{18} + 1 mL hexane. Washing with 10 mL hexane and 10 mL DCM. Extraction with 10 mL MeOH–HCOOH (8:2)	/	LC–UV
Parabens and triclosan	Dust	0.5 g dust + 0.5 g Na_2SO_4anh. + 1.25 g C_{18}; 2 g Florisil co-sorbent. Washing with 10 mL DCM; elution with 10 mL acetonitrile. Concentration to 1 mL. Solution filtration	0.6–2.6 ng g^{-1}	GC–MS

2.3. Analytical Techniques

New methodological procedures and novel equipment in the field of analysis and environmental monitoring have made it possible to evaluate the level of environmental contamination, to identify the pollutants and to measure their concentration in different compartments. New analytical methods have better reproducibility and repeatability, and lower detection limits (Kot-Wasik *et al.,* 2007).

The analytical methods for detecting and quantifying emerging contaminants in waters are generally based on gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS) or tandem MS (MS^2). The choice between GC and LC is normally based on the physic-chemical properties of the target analytes. LC-MS, particularly LC-MS^2, is the main choice to determine ultra-trace concentrations of polar and less volatile compounds, while GC-MS is used to identify and to quantify less polar and volatile or volatilizable compounds (fragrances, UV filters, flame retardants and antioxidants). Combining LC and GC is a very powerful approach to develop multi-residue analytical methods for wide range screening of contaminants (Pietrogrande and Basaglia, 2007).

LC-MS/MS methods continue to dominate new methods developed for emerging contaminants, and the use of multiple reaction monitoring (MRM) with MS/MS has become commonplace for quantitative environmental analysis (Richardson, 2007).

The mass spectrometry is a powerful analytical technique used to identify unknowns, for quantitative determinations of known compounds and to clarify the structural and chemical properties of the molecules. Unlike spectroscopic techniques, the MS is not based on the interaction between radiation and matter, it is a destructive technique and characterized by high sensitivity. The analysis of MS can be performed with extremely limited amount of sample (in

some cases less than one pg) at very low concentrations in complex mixtures (up to one ppt).

An approach for increasing selectivity and avoiding false-positive findings is use of high-resolution MS, such as time-of-flight-MS (TOF-MS), quadrupole-TOF (Q-TOF) or quadrupole linear ion trap (QqLIT). Ionic trap-MS has proved to be very useful analytical tool, due to its ability to determine multi-stage fragmentation pathways of molecules through MS^n experimentes. The major advantages of the liner ion trap over ion trap are larger ion-storage capacity and higher trapping efficiency. The QqLIT offers an additional possibility to operate the third quadrupole as a normal quadrupole or in the LIT mode. TOF-MS represent an indispensable analytical tool for the non-target screening and structural elucidation of metabolites and transformation products, due to its full-scann sensitivity, high selectivity (lack interference) and specificity (correct empirical formula assigned), given by the high mass resolution and mass accuracy of TOF analyzers (Radjenovic *et al.,* 2009). In the last years, these techniques are rapidly becoming important analytical tools and particularly they are used to indentify emerging contaminants and their degradation products in wastewaters and environmental waters (Gros, 2006; Bueno, 2007).

One of the limitations of LC-MS is the susceptibility of atmospheric pressure ionization interfaces to co-extracted matrix component. The matrix effect typically causes the suppression or the enhancement of the analyte signal. In general, the matrix effect can be reduced by taking into account the variability of matrix within the set of samples to be analyzed (river water, sewage treatment plant influent and effluent, and sediment extracts). Moreover, an appropriate internal standard (structurally similar unlabeled compound or isotopically-labeled standard) can be used to compensate both matrix effect and the losses of analytes in sample preparation step. However, the matrix effect can strongly depend upon the chromatographic retention time, and more

than one internal standard may be needed, although finding a suitable internal standard for each analyte can be difficult (Kot-Wasik *et al.,* 2007).

Nowadays, the environmental chemistry has directed its attention more towards the detection of parent compounds, while the analysis of metabolites and transformation products is still scarce. Elimination of pharmaceuticals, especially polar ones, during wastewater and drinking water treatment, is not satisfactory, thence more research is needed to determine the breakdown pathways and to evaluate the fate of transformation products. Moreover, disinfection processes applied in waterworks (either chlorination or ozonation) potentially shift the assessment of the risk of human consumption from the parent compound to its degradation products. For these reasons, the development of analytical protocols that allow the simultaneous determination of parent compounds and their metabolites is required. Additionally, time-of-flight (ToF)-MS and quadrupole (Q)-ToF-MS instruments, with the capacity to achieve accurate mass determination at sensitivities comparable to those of a triple-quadrupole (QqQ) instrument operating in selected reaction monitoring (SRM) mode, are expected to be applied increasingly to screening and identification of unknown metabolites (Kot-Wasik *et al.,* 2007).

Chapter 3

Sensitive determination of selected Pharmaceutical and Personal Care products (PPCPs) in different environmental matrices by Solid-Phase Extraction combined with Dispersive Liquid-Liquid Microextraction (SPE-DLLME) prior to UHPLC-MS/MS analysis

3.1. Introduction

Pharmaceutical and personal care products (PPCPs) are one of the most important classes of emerging contaminants analyzed the last decade. These potentially hazardous contaminants include numerous classes of chemicals with distinctive physical chemical properties and biological activities (Daughton and Ternes, 1999).

PPCPs constitute a group of a wide number of compounds largely consumed in modern societies, including drugs (tranquillizers, antibiotics, drugs of abuse, anti-epileptics, etc.), X-ray contrast media, hormones (natural and synthetic), additives, musk fragrances, etc., which, until recently, have not been of major concern with regard to their environmental effects.

PPCPs are introduced to the environment via a number of pathways, most notably wastewater treatment plant (WWTP) effluent and runoff from sources such as animal feeding operations (Daughton, 2001). These contaminants are found in wastewater at levels of up to a few μg L^{-1}, and they have been detected in surface waters and drinking water sources as a result of their persistence through the wastewater treatment processes (Daughton andTernes, 1999; Fent, 2006; Richardson, 2011).

Many PPCPs are ubiquitous and persistent in the environment. Some are capable of bioconcentration and many of those investigated are biologically active compounds. Some are suspected, or are recognized to be, endocrine disruptors, which could potentially influence environmental and human health. Additionally, they are continuously introduced into the environment; therefore even compounds with a low persistence might cause adverse effects in human and aquatic life (Kasprzyk-Hordern *et al.,* 2008).

The potential of ecological and environmental impacts associated with PPCPs are of particular concern because they continually penetrate the aquatic environment. Nonetheless, very little is known about the environmental fate

and ecotoxicological characteristics of these compounds on aquatic organisms (Kim *et al.,* 2009). One of the reasons has been the lack of analytical methods suitable to detection of these compounds at very low concentrations (ppb or ppt).

The latest analytical methods used for analysis of PPCPs in environmental matrices, in particular in aqueous matrices, utilise solid phase extraction (SPE) as a sample preparation and almost exclusively chromatography techniques (LC and GC) coupled with electrospray ionization tandem mass spectrometry (Petrovic, 2005; Kasprzyk-Hordern *et al.,* 2007).

Currently, most of the methods for the determination of PPCPs are directed to a single class of PPCPs, because it is very difficult to analyze simultaneously a broad range of compounds with different physical-chemical properties. The main challenge of this work was to determine simultaneously PPCPs belonging to different families.

In the present study novel multiresidue method for simultaneously determination of twenty-two selected PPCPs in different environmental matrices has been developed. The proposed analytical procedure combine SPE and DLLME techniques to perform the extraction (water)/purification (solid matrices) and the ultra-concentration of target PPCPs. An UHPLC-MS/MS multiresidue method was developed for the sensitive and selective quantification and confirmatory analysis of the analytes with different chemical characteristics.

Experimental parameters affecting SPE and DLLME efficiencies, such as type and volume elution solvent, volume of water, pH and ionic strength, were systematically investigated and optimized to achieve the best extraction efficiency and higher enrichment factor. Finally, the proposed methodology was validated for different aqueous matrices (tap water, sea water, river water and wastewater) and subsequently it was applied to real samples to evaluate the occurrence and environmental fate of selected PPCPs.

3.2. Pharmaceutical and Personal Care products (PPCPs)

PPCPs include thousands of chemical substances used by individuals for personal health or cosmetic reasons (Yan *et al*., 2010).

Pharmaceuticals are chemicals formulated into drugs for treatment of diseases (cure/mitigation), as chemo-preventatives, or those that enhance health or structural functioning of the human body (e.g., by use of steroids and hormones). They also include diagnostic agents (e.g., X-ray contrast media), illicit (recreational), and veterinary drugs.

Personal Care Products include cosmetics fragrances, detergents, soaps, insect repellents, skin anti-aging preparations, disinfectants and sunscreen agents.

PPCPS range from large, complex molecules to simple low molecular mass compounds, and from extremely bioactive molecules to inert compounds. Their levels in the environment depend on many factors: their consumption pattern, the flow of wastewater and the wastewater treatment processes. These features are characteristic of each country, although the worldwide trend in the use/consumption of major PPCPs tends to be similar due to the globalization of the chemical and pharmaceutical (Barral and Cohen, 1998).

PPCPs enter into the environment through individual human activity and as residues from manufacturing, veterinary, and agribusiness, hospitals and community uses. Individuals may add PPCPs to the environment through waste excretion or bathing, as well as by directly disposing of unused medications into septic tanks, sewers, or trash containers. Their presence has been identified and quantified in wastewater (Jelic *et al*., 2011), groundwater (Ellis *et al,* 2006), surface waters (Miege *et al.,* 2009), drinking water (Kumar *et al.,* 2010), agricultural manures (Motoyama *et al.*, 2011), biosolids (McClellan *et al.*, 2010), biota (Fromme *et al.,* 1999) and biological compartments (urine, plasma, human milk, adipose tissue) (Shlumpf *et al.,* 2010).

PPCPs environmental fate and transport is quite variable depending on the individual chemical. Some PPCPs are produced in small quantities, readily degradable and therefore occurring in ng L^{-1} or ppt quantities. Other PPCPs, manufactured in large quantities and resulting in continual environmental replacement, can be more easily analyzed because they occur in ppb quantities.

Many PPCPs are biologically active, showing estrogenic activity, and can potentially influence the environment and human health. Several studies have demonstrated adverse effects from longstanding, low-dose exposures in both aquatic and terrestrial wildlife, although human toxicity related to trace levels of PPCPs in the water supply remains unknown (Strauch, 2011).

PPCPs are of concern for potential environmental and ecological impacts because they may be active at extremely low concentrations, they are widespread and continuously released in large quantities, they may have unpredictable biochemical interactions when mixed, and at times these compounds may concentrate in the food chain and aquatic organisms (Poynton and Vulpe, 2009).

Some of the known potential impacts on organisms include delayed metamorphosis in frogs, delayed development in fish, and a variety of reactions including altered behaviour and reproduction (Daughton and Ternes, 1999).

3.2.1. Pharmaceuticals

Pharmaceuticals are primarily prescription and over-the-counter medications and dietary supplements, purposely designed to have biological effect at therapeutic concentrations (Seiler, 2002; Andreozzi *et al.,* 2003).

The primary routes for pharmaceuticals into the environment are through human excretion, manufacturing residues, hospitals, disposal of unused products and landfill disposal and subsequent leaching (Poynton and Vulpe,

2009; Kot-Wasik *et al.,* 2007). Pharmaceuticals are introduced not only by humans but also through veterinary use for poultry, livestock, and fish farming. Various drugs are commonly given to farm animals to prevent disease and to increase mass and weight of the animals.

Most pharmaceutically active compounds are polar compounds in order to make them orally available. Their molecular weights range typically from 200 to 500/1000 Da. These are the ones currently being researched and detected in the environment. They are often complex molecules with different functionalities and physical-chemical and biological properties (Kummerer, 2011).

The most commonly used pharmaceuticals are analgesics and non steroid anti-inflammatory drugs (NSAIDs), which are readily available, being non-prescription drugs. They have been assessed in many countries at μg L^{-1} and ng L^{-1} concentrations in wastewater and surface water, respectively. No proof of toxic effect of pharmaceutical residues on living organisms in the natural environment has been demonstrated (Kot-Wasik *et al.,* 2007). However, several pharmaceuticals are found in wastewater, usually at very low concentrations (below 1 μg L^{-1}). Many of these compounds are not removed by secondary wastewater treatment. An extensive study conducted in Germany on the effectiveness of purification treatment, showed that the rate of removal is around 7% for carbamazepine and other antiepileptic drugs, with high level values for propranolol (96%) and other β-blockers (Daughton and Ternes, 1999). This is of particular concern for drinking-water, in fact, different drugs and their metabolites were found in the German drinking water with concentrations up to ng L^{-1}, such as diclofenac, clofibric acid, phenazone, carbamazepine and benzofibrate (Daughton and Ternes, 1999).

Certain pharmaceuticals persist in the environment and several of them are considered resistant to biodegradation. However, even if the compounds break

down, they are continually being introduced into the environment, and the effect is as if they were persistent (Kot-Wasik *et al.,* 2007).

The risk to aquatic life of long-term exposure to very low concentrations of pharmaceuticals is essentially unknown. It is not always possible to evaluate the harmful effects of these compounds on living organisms due to the low concentrations at which they mostly occur. Therefore, it is very important to assess the type of pollutant and its concentration in water. Also, the human risk of long-term exposure to very low concentrations of pharmaceuticals in drinking water is essentially unknown. Because pharmaceuticals are purposely designed to have a biological effect at prescribed doses, the potential exists for unexpected impact at low levels. There is potential concern specifically for infants, foetuses and people with enzyme deficiencies. Research is continuing in this area (Kot-Wasik *et al.*, 2007).

Another concern for pharmaceuticals regards the release of antibiotics into the environment. It has been found that in the wastewater, in correspondence of hospital discharges, there are very high concentrations of some antibiotics, particularly penicillins, sulfonamides, quinolones (Hirsch *et. al.,* 1999).

The intake of these compounds by drinking water is suspected to cause the development of bacterial resistance. Also, it is believed that the antibiotics will decrease biodegradation of leaf and other plant materials, which serve as the primary food source for aquatic life in rivers and streams (Richardson, 2010). In this contest, very worrying is the environmental risk due to the diffusion of antibiotics in surface water and groundwater. Many aquatic ecosystems (and not only) are regulated by bacteria in many crucial mechanisms (denitrification, nitrogen fixation, degradation of organic matter, etc.), which are likely to be hindered or blocked by inhibition of bacterial colonies. The same may occur in wastewater treatment systems that are based in part on the degradation behaviour of bacteria (Costanzo *et al.,* 2005).

3.2.2. Personal Care Products

Whereas pharmaceuticals have been extensively studied in recent years and have been shown to occur widely, until recently, less attention has been paid to the presence of personal care products (PCPs) in environmental compartments. PCPs are a diverse group of compounds included in different products widely used in daily human life (e.g., gels, soaps, lotions, cosmetics, sunscreens, toothpaste and even food), so considerable amounts of PCPs are used every day (Pedrouzo *et al.,* 2011a)

PCPs include disinfectants, fragrances, insect repellents, preservatives and UV filters. PCPs are products intended for external use on the human body and thus are not subjected to metabolic alterations, unlike pharmaceuticals, which are intended for internal use. As a consequence, large quantities of PCPs enter into the environment unaltered through wastewater (Ternes *et al.,* 2004). Many of these compounds are used in large quantities, and recent studies have indicated that many are bioactive, environmentally persistent and they have the potential for bioaccumulation (Peck *et al.,* 2006; Mackay and Barnthouse, 2010). PCPs are among the most commonly detected compounds in surface water throughout the world (Peck *et al.,* 2006); however, in comparison to pharmaceuticals, relatively little is known about PCPs toxicity (Daughton and Ternes, 1999).

The literature has classified PCPs into five groups: antimicrobials, preservatives, musk fragrances, organic UV filters and insect repellents. Moreover, siloxanes were also recently classified as PCPs. Not only PCPs but also their by-products need to be taken into account because, in some cases, these by-products are even more environmentally persistent (Pedrouzo *et al.,* 2011).

3.2.2.1. Antimicrobials

Compounds that kill micro-organisms (bactericides) or at least prevent their growth (bacteriostatics) are employed to meet hygienic standards in medicine and food processing as well as for the preservation of certain chemical products such as paints or glues (Russell *et al.,* 1992). Antimicrobial agents have received increasing attention because of their pronounced microbial and algal toxicity, and their potential for fostering antimicrobial resistance (Pedrouzo *et al.,* 2011).

Triclosan (TCS) and triclocarban (TCC) are biphenyl ethers widely used as antimicrobials in deodorants, soaps, toothpaste, skin creams, and plastics (McAvoy *et al.,* 2002). TCS and TCC are among top 10 most commonly organic compounds detected in wastewater for frequency and concentration (Kolpin *et al.,* 2002; Halden and Paull, 2005).

TCS has been detected in surface water worldwide at concentration in the ng-μg L^{-1} range. For all studies conducted to date, TCS has been detected in 57% of surface water samples with a median concentration of about 50 ng L^{-1} (Braush and Rand, 2011). TCC has also been observed in surface water at concentrations up to 6.75 μg L^{-1}. It is believed that TCC occurs as frequently in WWTP effluent and surface water as TCS. However, TCC has been detected at higher concentrations and more frequently in WWTP effluent and surface water than TCS after 2004 (Braush and Rand, 2011).

TCS and TCC are known to be endocrine disruptors, but the main concern about them is that they can turn into more toxic and persistent species, such as chlorinated phenols, polychlorinated biphenyl ethers, polychlorinated dibenzodioxins and mono- and dichlorinated anilines (Braush and Rand, 2011; Lores *et. al.,* 2005; Gonzalez-Marino *et al.,* 2009). Recently, EPA has been classified TCS as a toxic pollutant for human health and the environment.

3.2.2.2. Preservatives

Parabens (alkyl esters of *p*-hydroxybenzoic acid) are antimicrobial preservatives used in toiletries, cosmetics, pharmaceuticals, and food (Daughton and Ternes, 1999). There are currently seven different types of parabens in use (benzyl, butyl, isobutyl, propyl, isopropyl, ethyl and methyl). In 1987 over 7000 kg of parabens were used in cosmetics and toiletries alone (Soni *et al.,* 2005) and this number has been expected to increase over the last 20 years. According to recent estimates, the man is exposed to about 76 mg/day of parabens (Cashman and Warshaw, 2005).

To date only few studies have examined parabens concentrations in wastewater and surface water. Greatest concentrations of parabens have been identified in surface water with concentrations ranging from 15 to 400 ng L^{-1} (Braush and Rand, 2011). Certain studies have demonstrated that parabens can be absorbed systematically in human by topical application of parabens in cosmetic creams (Janjua *et al.,* 2007) and consequently they are detected in blood samples (Sandager *et al.,* 2011) and human milk (Schlumpf *et al.,* 2010).

Among the seven types of parabens currently in use, acute toxicity studies have shown that benzylparaben appears to be very toxic for short exposures, while methylparaben and ethylparaben turn out to be least toxic for all trophic groups examined (Braush and Rand, 2011). There is currently a lack of information on the chronic effects of parabens to aquatic organisms with only a few studies (Dobbins *et al.,* 2009; Stuart *et al.,* 2012), which revealed that benzylparaben and butylparaben were most toxic to invertebrates and fish whereas methylparaben and ethylparaben appeared least toxic. According to the studies of acute studies, these results indicate that chain length increases their toxicity (Braush and Rand, 2011).

The parabens can act as endocrine disruptors mimicking the action of estrogen, although their power is lower than the estrogen (Golden *et. al.,* 2005)

and for this reason it has been suggested their correlation with the onset of breast cancer (Darbre, 2004). Similarly, in vivo studies have shown a link between exposure to the parabens and some alterations of the reproductive system (Darbre, 2002).

3.2.2.3. Musk Fragrances

Synthetic musk compounds are use in a wide-range of products including soaps, detergents and deodorants. Musk fragrances are the most widely studied class of PCPs and are believed to be ubiquitous contaminants in the environment (Daughton and Ternes, 1999). Synthetic musks are either nitro musks, which are used since 1800, or polycyclic musks, introduced in the 1950s (Daughton and Ternes, 1999).

The EU decided to limit the use of nitro musk fragrances due to concern about the toxicity for environment and humans (Pedrouzo *et al.,* 2011). Polycyclic musks are currently used in higher quantities than nitro musks, with galaxolide (HHCB), toxalide (AHTN) and celestolide (ABDI), and used most commonly (Daughton and Ternes, 1999). Nitro and polycyclic musks are water soluble, even if they have a high octanol/water coefficients (logP = 3.8 for nitro musk and 5.4–5.9 for polycyclic musks) (Schramm *et al.,* 1996; Balk and Ford, 1999) this indicates a high potential for bioaccumulation in aquatic species (Winkler *et al.,* 1998). This potential was evidence by numerous studies that reported high concentrations of the synthetic musks in the lipid fraction of molluscs and fish (Schramm *et al.,* 1996). Regard to environmental water compartments, in all studies conducted on the determination of fragrances, nitro musks have been detected in 83–90% of wastewater and approximately in 50% of surface waters.

Certain studies have demonstrated that nitro musk transformation products are highly toxic to aquatic organisms (Daughton and Ternes, 1999). Polycyclic

musks are more acutely toxic than nitro musks based on literature (Braush and Rand, 2011).

3.2.2.4. UV Filters

UV filters are used in sunscreen products and cosmetics to protect from UV radiation. UV filters enter in environment in two ways, either indirectly via wastewater or directly emitted into surface water after application when people go bathing and swimming.

Typical organic compounds used in sunscreens are benzophenones (BPs), benzhydrol, 4-hydroxybenzophenone, 2-hydroxy-4-methoxybenzophenone (HMB), 2,4-dihydroxybenzophenone (DHB), 2,20-dihydroxy-4-methoxybenzophenone, and 2,3,4- trihydroxylbenzophenone.

They have high lipophilicity (logP up to 7) and a relative stability to biodegradation. These properties make them environmental dangerous, because they tend to bioaccumulate in aquatic organism at levels similar to PCBs and DDT. In fact, UV filters have been found in adipose tissue of fish in concentrations up to 2 ppm with bioaccumulation factors of greater than 5000 in fish (Braush and Rand, 2011).

The UV filters detected more frequently and in higher concentrations in various environmental matrices are: 2-ethyl-hexyl-4-trimetossicinnamato (EHMC), benzophenone-3 (BP3), 4-methyl-benzidilene-camphor (4MBC) and octocrylene (OC), with mean concentrations of 0.035-4.4 $\mu g\ L^{-1}$ in surface waters, 0.9-2.7 $\mu g\ L^{-1}$ in wastewater and 0.17-1.8 $\mu g\ g^{-1}$ in fish (Braush and Rand, 2011).

Recent studies have indicated that UV filters act as endocrine disruptors because they are able to bind to specific receptors, target of some hormones, especially the thyroid system and the reproductive system, altering the functionality (Schlumpf *et al.*, 2008).

3.2.2.5. Insect Repellents

N,N-diethyl-m-toluamide (DEET) is the most common active ingredient in commercial insect repellent formulations (Costanzo *et al.,* 2007). DEET is currently registered for use in 225 products in the US and it is estimated annual usage exceeds 1.8 million kg (USEPA, 1998).

DEET has been routinely detected across the world in a wide range of water matrices, including groundwater, surface water and drinking water (Pedrouzo *et al.,* 2011). This repellent agent was detected both in effluents of sewage treatment plants and in surface (Kolpin *et al.,* 2002) with an incidence of 95% in the first and 65% in the last, and an average concentration of 0.2 μg L^{-1} and 55 ng L^{-1}, respectively (Braush and Rand, 2011).

To date very little data exist pertaining to acute toxicity of DEET to aquatic organisms and available data indicate that DEET is only slightly toxic to aquatic organisms (Braush and Rand, 2011). Little is known also on the long-term effects of DEET in aqueous environments (Pedrouzo *et al.,* 2011).

3.2.3. Analytical methods for determination of PPCPs in environmental matrices

For the analysis of PPCPs, a substantial number of analytical procedures have been developed.

Concentrations of PPCPs in environmental matrices are mostly in the μg - ng L^{-1} range, so they need to be extracted and concentrated prior to instrumental analysis. Solid-phase extraction (SPE) is the most widely used extraction and enrichment technique (Gros *et al.,* 2006; Pietrogrande *et al.,* 2007) for water samples and has replaced traditional liquid-liquid extraction (LLE). SPE cartridges are packed with different sorbents, but cross linked polymer hydrophilic-lipophilic balanced has been the most widely employed adsorbent. This sorbent provides the best conditions for the extraction of compounds with

a wide range of polarity (Gros *et al.,* 2006), a pre-requisite for multi-residue analysis of different organic contaminants.

Other extraction techniques used to extract PPCPs in waters are solid phase microextraction (SPME), stir-bar sorptive extraction (SBSE), or recycling continuous liquid liquid extraction (R-CLLE) (Jingming *et al.,* 2010).

The analytical methods for detecting and quantifying of PPCPs are generally based on gas chromatography (GC) for less polar and volatile or volatilizable compounds or liquid chromatography (LC) for polar and less volatile ones, coupled with mass spectrometry (MS) or tandem MS (MS^2).

In many works these analytical techniques have been applied, for example, Togala et al. used the GC-MS, mode selective ion mode (SIM), for the determination of 18 drugs, which include anti-inflammatory, anti-depressants and hypolipidemic, after derivatization with N-methyl-N-(trimethylsilyl) trifluoroacetamide (Togola and Budzinski, 2008). Cuderman and Heath developed a method for the determination of several UV filters and two common disinfectants antimicrobial, triclosan and the clorofene, using the SPE as sample preparation and the GC-MS preceded by derivatization for instrumental analysis (Cuderman and Heath, 2007).

Schultzt and Furlong reported a multi-residue method for the determination of antidepressants, present in traces in water, using the SPE as sample preparation and liquid chromatography coupled to a mass spectrometer with electrospray source (LC-ESI-MS-MS), with the limits of detection (LOD) for each antidepressant, ranging from 0.19 to 0.45 ng L^{-1} (Schultzt and Furlong, 2008). Van De Steene and Lambert developed a multi-residue method LC-ESI-MS/MS coupled SPE as sample preparation for the simultaneous determination of nine basic drugs in surface waters, with limits of detection (LOD) and quantification (LOQ), comprised between 0.05-1 ng $L^{-1}$and 0.05-10 ng L^{-1}, respectively, obtaining a good precision and recoveries (Van De Steene and Lambert, 2008).

Therefore, the growing concern over occurrence of PPCPs in environment necessitates more rapid and automated procedures to take into account the constant increase in the number of samples to be tested. For this purpose, Viglino et al. reported a method based on the use of SPE on-line, associated to LC-ESI-MS/MS (electrospray ionization in positive mode), for the determination of some drugs, pesticides and their metabolites in drinking water and surface water. The method requires around 20 minutes and it has LOD, between 2-24 ng L^{-1} (Viglino *et al.,* 2008).

Recently, for the chromatographic separation was used the ultra high performance liquid chromatography (UHPLC), this technique has many advantages: good resolution, speed and lower consumption of solvent. Conley et al have been developed a multi-residue method UHPLC-MS/MS for the determination of drugs and their metabolites in surface water in concentrations of the order of ng L^{-1} (Conley *et. al.,* 2008).

As regards the determination of the drugs and their metabolites, several papers have been published regarding the application of mass spectrometers hybrids, such as TOF-MS, QqTof and QqLIT (Jingming *et al.,* 2010). Ibanez et al. have developed a method for the determination of organic pollutants in water using such a system UPLC-TOF-MS (Ibanez *et al.,* 2008).

3.3. Results and Discussion

The occurrence of PPCPs in the aquatic environment has become a topic of scientific and public debate. It is noteworthy that the consumption of commonly used PPCPs is sometimes as high as that of the pesticides and other organic compounds. In Europe, draft guidelines for environmental risk assessments (ERAs) are available since 2005 and a key change in these guidelines is the requirement for chronic, rather than acute, ecotoxicity testing, recognizing that most environmental contaminants are not acutely toxic but may have long-term chronic effects at low levels.

One of the reasons for the lack of information about the environmental fate, the toxicity on aquatic organisms and the human health of PPCPs has been the shortage of analytical methods suitable to detection of these compounds at very low concentrations (ppb or ppt).

In this context, it is important to design analytical procedures for monitoring specific environmental compartments and to provide the basis for drawing conclusions about the occurrence, the persistence and hazard of PPCPs in the environment.

Currently, there is no single analytical method to detect all PPCPs and most of the developed methods are directed to a single class of PPCPs. Simultaneous identification of the widest possible spectrum of pollutants, with different physical-chemical properties, in the aquatic environment is very difficult. Analysis of a variety of contaminants in complex matrices at ultra-trace levels has always been a great challenge for analytical chemistry. In most cases, the enrichment and the separation of analytes from matrix is a prerequisite for reaching a prescribed limit of detection (LOD), thus a proper sample preparation significantly influences the accuracy and sensitivity of overall analytical procedures and determines the correctness of measurements obtained.

In this study a novel multiresidue method for the simultaneously determination of twenty-two selected PPCPs, spanning a large range of physical-chemical properties, in different environmental matrices has been developed. The main goals were to obtain a rapid and easy ultraconcentration of the analytes and to develop a highly sensitive method in order to investigate the occurrence of the selected PPCPs in aqueous environmental compartments at ultratrace levels. For this purpose, SPE and DLLME techniques were combined to perform the extraction, the purification and the ultra-concentration of the analytes and an UHPLC-MS/MS multiresidue method was developed for their sensitive and selective quantification and confirmatory.

The study was organized in foive main steps:

1. Selection of a representative pool of PPCPs taking into account of their persistence, their intake of environment, their toxicity and their resistance to degradation;
2. Development of a selective and sensitive UHPLC-MS/MS method;
3. Study and optimization of the experimental parameters affecting the sample preparation efficiency;
4. Validation of the analytical procedure for different environmental water matrices.
5. Analysis of real samples.

3.3.1. Selection of PPCPS

In this study, a representative pool of PPCPs was selected based on their potential to cause known or suspected adverse ecological or human health effects. Compounds with known environmental persistence (resistant to the wastewater treatment plants and the biodegradation, capable to bioaccumulate), toxicity and continuously introduced into the environment were considered.

A detailed study of the literature data allowed to select twenty-two PPCPs: 4 pharmaceuticals, 2 antimicrobial agent, 5 preservatives, 10 UV filters and 1 insect repellent (Braush and Rand, 2011; Onesios *et al.,* 2009).

Pharmaceuticals: naproxen (NPX), sulfamethoxazole (SFMT), ibuprofen (IBU) and carbamazepine (CBZ).

NPX and SFMT are defined "pseudo-persistent" because, although they are relatively degradable, they can reach biologically active concentrations (Zuccato, 2000). Their concentration range found in wastewater is 300-5200 ng L^{-1}, respectively (Andreozzi *et. al.,* 2003; Sedlak *et. al.,* 2005). In addition, SFMT can give antibiotic resistance, altering the balance of the ecosystem (Costanzo *et al.,* 2005). IBU is one of the most widely used pharmaceuticals and for this reasons it the most frequently detected in the environment (50-320 ng·L^{-1}) (Sedlak *et. al*., 2005). CBZ, instead, has been chosen for its high persistence and high concentrations (110-6300 ng L^{-1}) detected in environmental compartments (Metcalfe, 2004).

Antimicrobials: triclosan (TCS) and trichlorocarbanilide (TCC).

TCS and TCC are among top 10 most commonly detected organic wastewater compounds for frequency and concentration (Kolpin *et al.,* 2002; Halden and Paul, 2005). According to EPA the TCS is a toxic pollutant for human health and the environment and it contributes to the presence of dioxins in the environment. In the last decade, TCC showed similar environmental occurrence and persistence of TCS. Both antimicrobials interfere with the endocrine system of rats and amphibians.

Preservatives: methylparaben (MeP), benzylparaben (BZP), butylparaben (BuP), ethylparaben (EtP) and propylparaben (PrP).

According to some estimates, the man is exposed to about 76 mg/day of parabens. Maximum concentrations of parabens have been identified in surface water with concentrations ranging from 15 to 400 ng L^{-1} depending on paraben (Braush and Rand, 2011). The parabens exert a weak oestrogenic

activity and are capable to produce immunologically mediated systemic hypersensitivity reactions. Some data on environmental toxicity reported that butylparaben and benzylparaben should be classified as toxic substance whereas methyl-, ethylparaben and propylparaben are harmful (Stuart, 2012; Pedrouzo *et al.,* 2011).

UV Filters: 4-methylbenzylidene camphor (4MBC), 2,4-dihydroxybenzophenone (BP1), 2,2',4,4'-tetrahydroxybenzophenone (BP2), 2-hydroxy-4-methoxybenzophenone (BP3), 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid (BP4), 4-hydroxy benzophenone (4HB), octocrylene (OC), octylsalate (OS), homosalate (HMS) and octylmethoxycinnamate (OMC).

These compounds have a high lipophilicity and they are resistant to biodegradation. These characteristics make them dangerous for the environment because they tend to bioaccumulate, they were found in adipose tissue fish in concentrations up to 2 g g^{-1}. Recent studies have shown that these substances can act as endocrine disruptors (Braush and Rand, 2011).

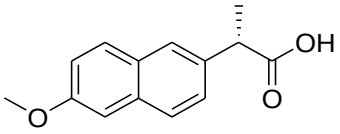
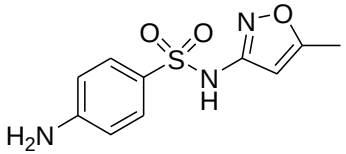
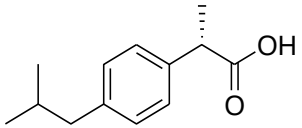
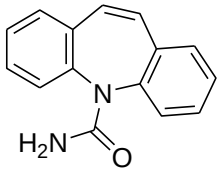
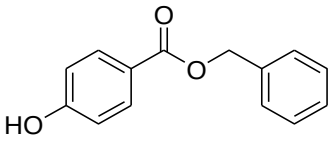
Although UV filters are used at high levels and are likely to enter into aquatic environments, very little is known about their environmental concentrations due to a lack of analytical methods. The extent of risk of UV filters in WWTP effluent and surface water is currently unknown based on the scarcity of environmental concentration data.

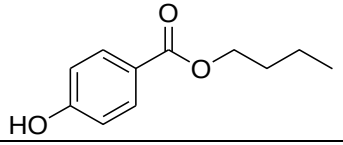
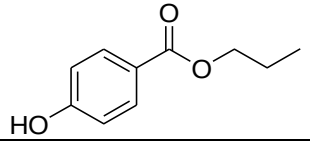
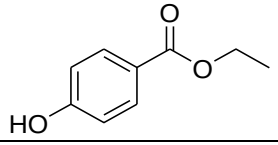
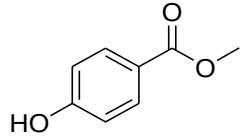
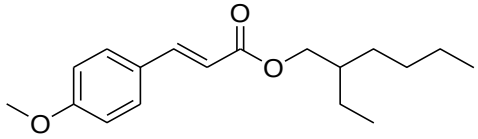
Insect repellents: N,N-dietil-m-toluamide (DEET).

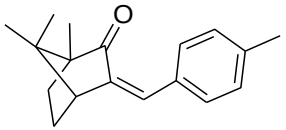
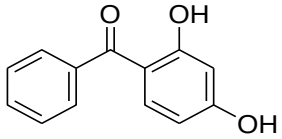
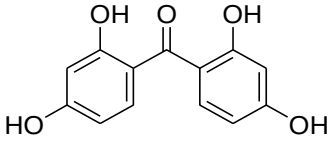
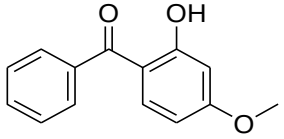
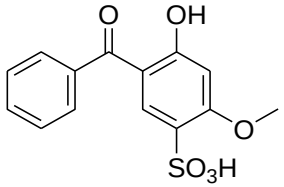
DEET, the most widely used compound in insect-repellent products, was selected due to its ubiquitous distribution and to high concentrations (55-356 ng L^{-1}) found in surface water and groundwaters (Pedrouzo *et al.,* 2011; Costanzo *et. al.,* 2007). To date little is known on the acute toxicity of DEET to aquatic organisms and of its long-term effects in aqueous environments (Pedrouzo et al., 2011).

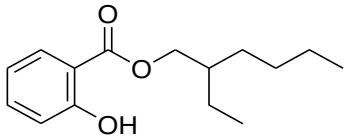
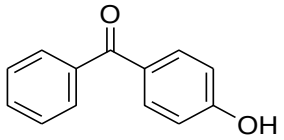
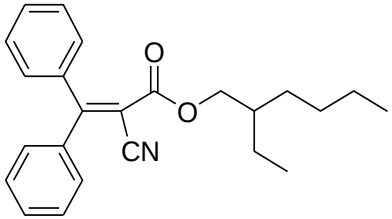
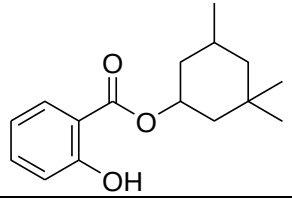
Table 3.1 reported characteristics of the selected PPCPs.

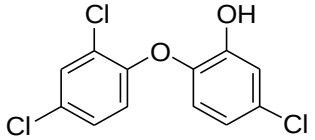
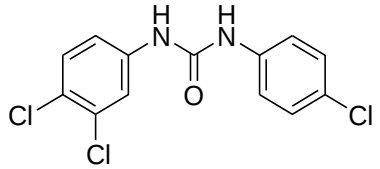
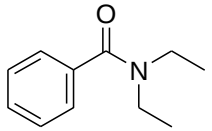
Table 3.1. *Selected PPCPS*

Code	Compound	Structure	Type	MW	LogP[a]
NPX	Naproxen		Pharmaceutical	230.26	2.876
SFMT	Sulfamethoxazole		Pharmaceutical	253.28	0.659
IBU	Ibuprofen		Pharmaceutical	206.28	3.502
CBZ	Carbamazepine		Pharmaceutical	236.27	1.895
BzP	Benzylparaben		Preservative	228.24	3.568

Code	Compound	Structure	Type	MW	LogP[a]
BuP	Butylparaben		Preservative	194.23	3.410
PrP	Propylparaben		Preservative	180.08	2.901
EtP	Ethylparaben		Preservative	166.06	2.391
MeP	Methylparaben		Preservative	152.05	1.882
OMC	Octylmethoxycinnamate		UV Filter	290.19	5.921

Code	Compound	Structure	Type	MW	LogP[a]
4MBC	Methylbenzylidene camphor		UV Filter	254.17	3.385
BP1	2,4- Dihydroxybenzophenone		UV Filter	214.06	3.152
BP2	2,2',4,4'-tetrahydroxybenzophenone		UV Filter	246.05	3.091
BP3	2-Hydroxy-4-methoxybenzophenone		UV Filter	228.08	3.995
BP4	2-Hydroxy-4-methoxybenzophenone 5-sulfonic acid		UV Filter	308.04	0.993

Code	Compound	Structure	Type	MW	LogP[a]
OS	Octylsalate		UV Filter	250.16	4.362
4HB	4-Hydroxybenzophenone		UV Filter	198.07	2.924
OC	Octocrylene		UV Filter	361.22	6.361
HMS	Homosalate		UV Filter	262.16	5.947

Code	Compound	Structure	Type	MW	LogP[a]
TCS	Triclosan		Antimicrobial	287.95	5.343
TCC	Trichlorocarbanilide (Triclocarban)		Antimicrobial	313.98	6.073
DEET	N,N-diethyl-m-toluamide		Insect repellent	191.13	2.419

[a]Physical-chemical data obtained from SciFinder Scholar Database 2013 (predicted properties).

3.3.2. UHPLC-MS/MS analysis

In LC-MS/MS an efficient separation is desired to minimise the matrix effects and improve the sensitivity. In order to achieve the best chromatographic performance (reduction of peak tailing and better resolution) and the most intense ionization of the analytes, several solvents combinations and buffer compositions (methanol/water and acetonitrile/water with 0, 1, 1.5 and 2.5 mM of acetic acid, formic acid and ammonium acetate), together with the ionization mode, were investigated. The analysis of the chromatograms showed that when buffers were added to the mobile phase a reduction of the formation of Na-adduct at advantage of the ionization of the analytes was observed. Globally, acetonitrile and 1.5 mM ammonium acetate provided a better response and chromatographic resolution, so that this solvent system was selected as mobile phase for the chromatographic analysis of target analytes. Gradient elution reported in the section 3.5.5 was able to separate the PPCPs in only 13 min.

For the detection, both ionization modes (NI and PI) were selected and the solvent- and flow rate-dependent source parameters were optimised at the chosen chromatographic conditions.

According to the 2002/657/EC regulation, which requires two different MS/MS transitions to confirm the identity of target analytes, the product ion spectra of $[M + H]^+$ or $[M - H]^-$ ions were studied to select at least two characteristic product ions for each analytes to be monitored in Selected Reaction Monitoring (SRM) mode. Quantification of the target analytes was carried out by adding both SRM transitions. The ratio between the signal intensities of two SRM transitions (transitions ratio) was used to confirm the identity of the analytes and to fulfil the EU regulation. Table 3.2 reported the experimental UHPL/ESI-MS/MS parameters used for the determination of selected PPCPs.

Table 3.2. *UHPLC/ESI-MS/MS parameters for the analysis of selected PPCPs and analytical performance*

PPCPs	t_R (min)	Precursor ion	SRM transitions (*m/z*)	Collision energy (V)	$I_1/I_2 \pm tol$[b]
BP4	0.7	$[M-H]^-$	307.1 → 211/227	25-38	1.37 ± 1.1
NPX	2.8	$[M-H]^-$	229.1 → 170/185	18-5	2.79 ± 0.8
MeP	3.3	$[M-H]^-$	151.1 → 136/92	15-21	1.22 ± 1.4
MeP-$^{13}C_6$[a]	3.3	$[M-H]^-$	157.31→ 98	21	-
BP2	5.0	$[M-H]^-$	245.1 → 135/109	16-23	1.71 ± 0.9
EtP	5.8	$[M-H]^-$	165.1 →137/92	14-24	1.16 ± 1.3
EtP-$^{13}C_6$[a]	5.8	$[M-H]^-$	171.07 → 98	24	-
4HB	6.8	$[M-H]^-$	197.1 → 92/120	35-25	5.24 ± 2.2
PrP	7.1	$[M-H]^-$	179.1 → 92/136	27-17	2.02 ± 1.2
PrP-$^{13}C_6$[a]	7.1	$[M-H]^-$	185.11 → 98	27	-
BP1	8.0	$[M-H]^-$	213.1 → 135/91	29-20	1.38 ± 0.7
BuP	8.7	$[M-H]^-$	193.1 → 92/136	25-18	1.21 ± 0.5
BuP-$^{13}C_6$[a]	8.7	$[M-H]^-$	199.14 → 98	25	-
BzP	8.8	$[M-H]^-$	227.1 → 92/136	26-16	1.11 ± 1.2
IBU	9.2	$[M-H]^-$	205.2 → 134/161	22-7	66.52 ± 3.5
TCC	9.6	$[M-H]^-$	313.1 → 126/160	18-18	25.26 ± 4.2
TCS	10.0	$[M-H]^-$	289.0 → 35/37	8	2.2 ± 1.2
OS	12.4	$[M-H]^-$	248.9 → 137/93	18-17	1.22 ± 0.7
HMS	12.6	$[M-H]^-$	261.2 → 137/93	18-28	1.19 ± 1.7
SFMT	3.2	$[M+H]^+$	253.9 → 156/108	23-14	1.34 ± 1.2
CBZ	5.9	$[M+H]^+$	236.9 → 137/193	21-35	3.15 ± 2.2
DEET	7.0	$[M+H]^+$	192.1 → 119/91	30-16	1.49 ± 0.9
BP3	9.4	$[M+H]^+$	229.0 → 151/77	19-17	4.52 ± 3.8
4MBC	10.8	$[M+H]^+$	255.1 → 105/171	29-14	1.9 ± 2.9
OMC	12.0	$[M+H]^+$	291.1 → 161/133	31-14	2.12 ± 2.5
OC	12.1	$[M+H]^+$	362.2 → 250/232	32-10	1.79 ± 0.9

[a] Internal standards; [b] intensity ratio SRM1/SRM2 ± maximum tolerance.

To improve the accuracy and the precision of the proposed method, methylparaben-$^{13}C_6$, ethylparaben-$^{13}C_6$, propylparaben-$^{13}C_6$ and butylparaben-$^{13}C_6$ were used as surrogate internal standards for MeP, EtP, PrP and BuP, respectively. ISs were added after the sample preparation procedure to compensate only the matrix effects.

Table 3.3 summarises some data related to the performance of the optimized UHPLC-MS/MS method. Linearity was excellent (R^2>0.995) in the concentration range of 0.5-1000 ng $mL^{-1}$for BP2, 4HB,PrP, BP1, BuP, BzP, CBZ and DEET; 1-1000 ng mL^{-1} for EtP, TCC and TCS; 5-1000 ng mL^{-1} BP4, NPX, MeP, IBU, SFMT, BP3 and 4MBC; 50-10000 OMC and OC; OS and HMS have a linear range of 2070-100000 ng mL^{-1}, due to the poor ionization of these compounds in ESI probe. The instrumental detection and quantification limits (IDL and IQL) ranged between 0.08-12.5 and 0.26-41.25 ng mL^{-1}, respectively, except for OS and HMS (IDLs of 625 ng mL^{-1}).

Table 3.3. *Performance of UHPLC-MS/MS method*

PPCPs	Linearity range (ng mL^{-1})	R^2	IDL (ng mL^{-1})[a]	IQL (ng mL^{-1})[b]	% RSD[c]
BP4	5 - 1000	0.995	1.25	4.13	7.8
NPX	5 - 1000	0.997	0.31	1.03	2.4
MeP	5 - 1000	0.998	0.31	1.03	5.4
BP2	0.5 - 1000	0.998	0.08	0.26	6.2
EtP	1 - 1000	0.999	0.16	0.51	3.7
4HB	0.5 - 1000	0.998	0.08	0.26	3.2
PrP	0.5 - 1000	0.998	0.08	0.26	3.1
BP1	0.5 - 1000	0.997	0.08	0.26	0.9
BuP	0.5 - 1000	0.999	0.08	0.26	4.2
BzP	0.5 - 1000	0.996	0.08	0.26	3.1
IBU	5 - 1000	0.995	0.31	1.03	0.6
TCC	1 - 1000	0.996	0.16	0.51	4.4
TCS	1 - 1000	0.997	0.16	0.51	1.3
OS	2070 - 100000	0.998	625.00	2062.50	3.1
HMS	2070 - 100000	0.997	625.00	2062.50	1.8
SFMT	5 - 1000	0.996	1.25	4.13	1.1
CBZ	0.5 - 1000	0.999	0.08	0.26	6.8
DEET	0.5 - 1000	0.999	0.08	0.26	1.3
BP3	5 - 1000	0.996	0.31	1.03	4.1
4MBC	5 - 10000	0.998	0.78	2.57	5.9
OMC	50 - 10000	0.996	12.50	41.25	1.4
OC	50 - 10000	0.996	12.50	41.25	4.0

a Instrumental detection limit, S/N = 3 in acetonitrile/water 1:1, v/v, standards solution; [b] Instrumental quantification limit, S/N = 10 in acetonitrile/water 1:1, v/v, standards solution; [c] Standards solution at the 100 ng mL^{-1} level (n = 10).

3.3.3. Optimization of SPE-DLLME procedure

According to one of the main aims of this study, the combination of SPE and DLLME techniques in the sample preparation procedure has been chosen to obtain a rapid and easy ultraconcentration of target analytes and consequently a high sensitivity of the analytical method. In the experimental plan, the SPE eluate of first sample preparation step was directly used in the second step, DLLME process.

Parameters affecting both SPE and DLLME procedures, such as type and volume SPE elution solvent, volume ionic strength and pH of DLLME aqueous phase, were systematically investigated and optimized to achieve the best extraction efficiency and higher enrichment factor.

The experiments for the optimization of SPE-DLLME process were carried out in triplicate and using 500 mL of spiked ultrapure water at concentrations of 100 (1000 of 4MBC, OMC and OC and 10000 of OS and HMS) ng $L^{-1}$of each PPCPS. The final extracts were reconstituted with 200 μL of MeCN/water, 1:1 v/v and analyzed by UHPLC-MS/MS. The ISs (50 ng mL^{-1}) were added after the SPE-DLLME step to compensate only the variations due to analytes ionization efficiency or injection volume. In this way, it was possible to evaluate the absolute recovery of SPE-DLLME technique.

3.3.3.1. Preliminary experiments

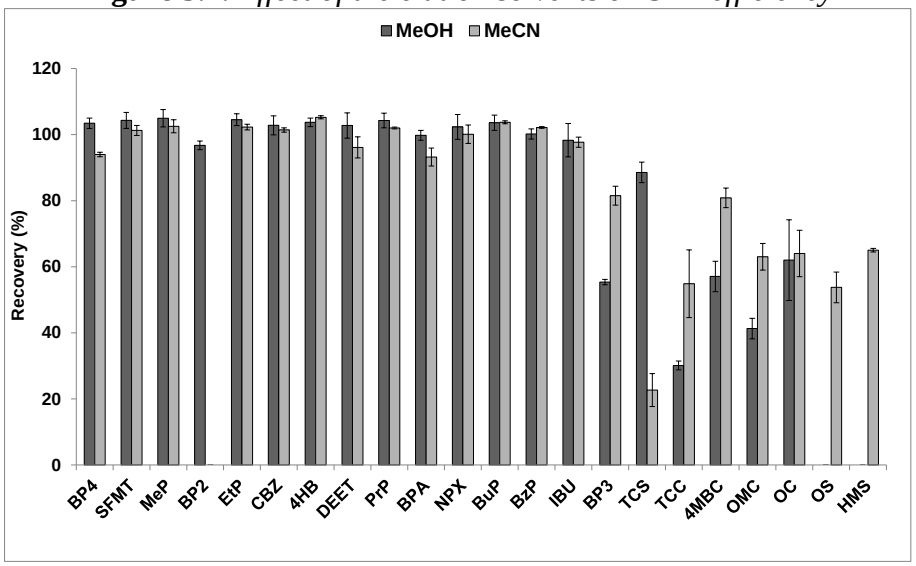
The choice of SPE stationary phase has been based on the properties of the selected analytes. The latter belong to different chemical classes and they have very different physical-chemical properties. Therefore, to allow the simultaneous analysis of a group of compounds with a wide range of polarity (logP 0.657-6.361), a polymeric reversed-phase sorbent with a good hydrophilic-lipophilic balance (Oasis HLB) was selected. This sorbent type, constituted by two monomers, N-vinylpyrrolidone and divinylbenzene, is a

universal polymeric sorbent that was developed for the extraction of a wide range of acidic, basic, and neutral compounds.

Initially, several experiments were carried out to select the solvents of SPE and DLLME processes. Since, the elution solvent of SPE should be used as DLLME disperser, it was chosen taking into account the properties required to DLLME disperser.

Among the most common DLLME disperser solvents, MeOH and MeCN were tested as SPE eluent. The results, illustrated in Figure 3.1, indicated that both solvents showed good recoveries for most analytes, with a better extraction efficiency of MeCN than MeOH. However, for the most lipophilic compounds (TCS, TCC, OMC, OC, OS AND HMS) an exhaustive extraction was not observed.

Figure 3.1. *Effect of the elution solvents on SPE efficiency*



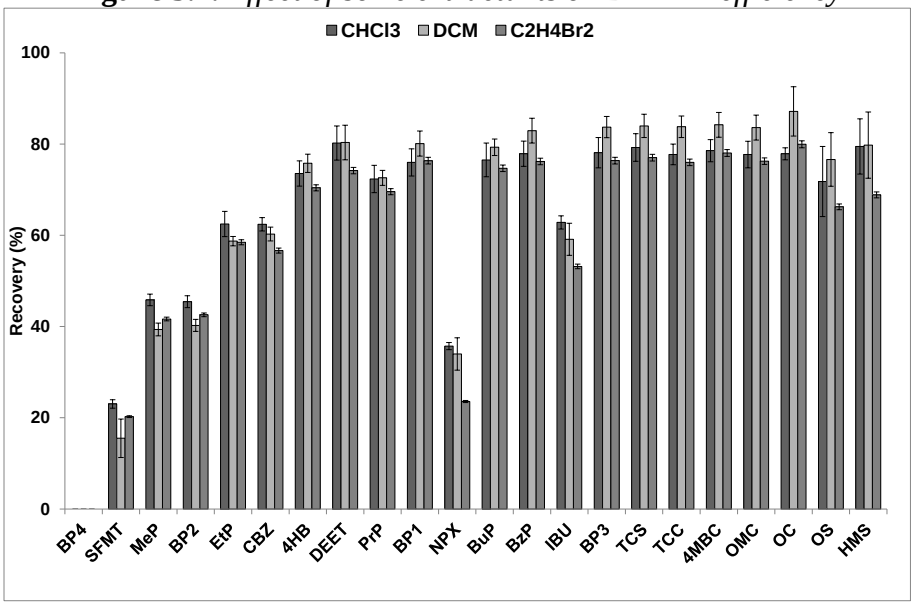
Experimental conditions: HLB, 200 mg, volume of elution solvent, 3 x 3 mL (n = 3).

Therefore, it was necessary to use a greater volume of MeCN or to increase the eluotropic strength of SPE elution solvent to improve the recoveries of these PPCPs. The first approach would decrease the enrichment factor of sample preparation procedure, thus the increase of the eluotropic strength of

SPE eluent was chosen, using directly the mixture extractant-disperser solvents of DLLME to elute the analytes from SPE.

In the next step, the main experimental parameters affecting DLLME efficiency, such as extractant type, ionic strength and pH, were evaluated. The selection of an appropriate extractant is the most important aspect for the DLLME process, and different chlorinated and brominated solvents ($CHCl_3$, CH_2Cl_2, and $C_2H_4Br_2$) with density higher than water and different polarities were tested to obtain good PPCPs extraction. The three solvents showed similar extraction efficiency (Figure 3.2); the DCM shows better recoveries for all analytes and it was chosen like DLLME extractant.

Figure 3.2. *Effect of some extractants on DLLME efficiency*



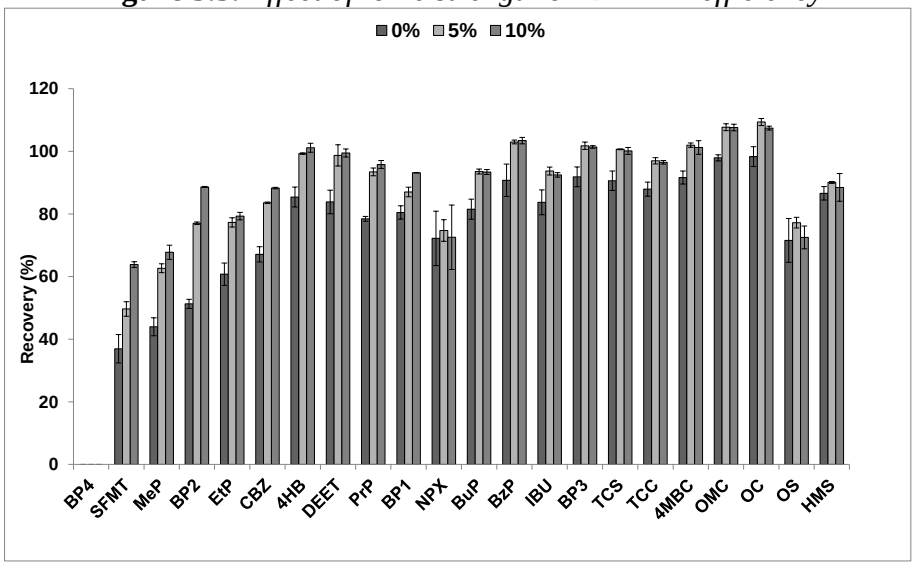
Experimental conditions: water volume, 5 mL; disperser solvent, MeCN 800 µL; Volume extraction solvent, 200 µL (n= 3).

Recovery data of Figure 3.2 highlighted a lower DLLME efficiency for more hydrophilic PPCPs (SMFT, MeP, BP2, EtP e CBZ) and for ionizable ones (BP4, NPX e IBU), due to their hydrophilic character than the rest of analytes.

So, in further experiments, the ionic strength and the pH were assessed to reduce the solubility in the aqueous phase of the more hydrophilic compounds and improve DLLME efficiency.

For this purpose, the effect of ionic strength of DLLME aqueous solution on the yield of DLLME process was investigated considering three different concentrations of NaCl (0; 5 and 10% w/v). Figure 3.3 compares the recoveries obtained with different percentage of NaCl. The addition of salt significantly improved the DLLME efficiency for all analytes, particularly for more hydrophilic ones. Consequently, an aqueous solution with NaCl 5% (w/v) was chosen.

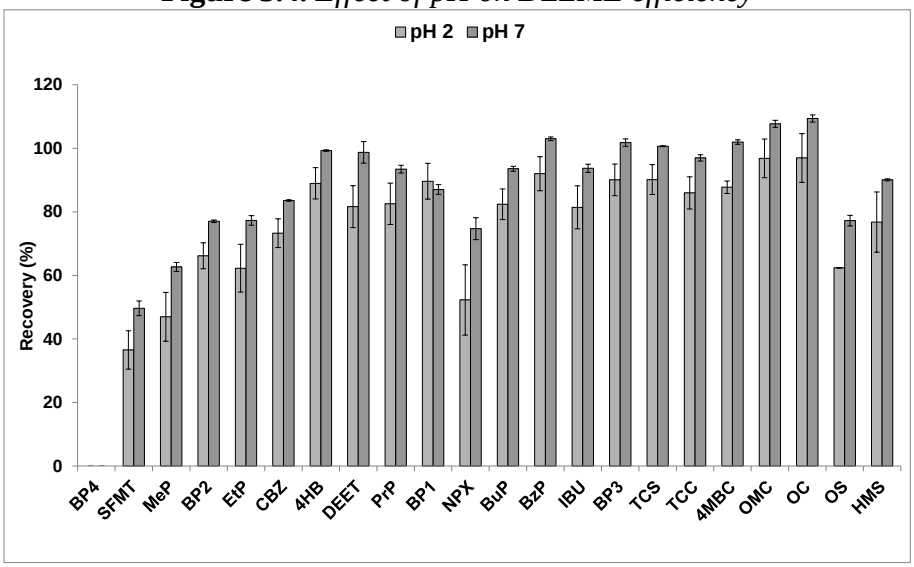
Figure 3.3. *Effect of ionic strength on DLLME efficiency*



Experimental conditions: water volume, 5 mL; disperser solvent, MeCN 800 µL; Volume extraction solvent, 200 µL (n= 3).

Regarding the effect of pH of the DLLME aqueous solution, an acidic phase (pH 2) was evaluated to improve the recovery of acid analytes (BP4, NPX and IBU), but no significant improvement in DLLME efficiency was observed (Figure 3.4). On the basis of this behaviour, no pH adjustment of DLLME aqueous solution was adopted.

Figure 3.4. *Effect of pH on DLLME efficiency*



Experimental conditions: aqueous phase NaCl 5% (w/v), 5 mL; disperser solvent, MeCN 800 µL; Volume extraction solvent, 200 µL (n= 3).

3.3.2.2. Multilevel experimental factorial design

Once the selection of the optimal DLLME solvents (extractant, DCM; disperser, MeCN; aqueous solution, NaCl 5%, w/v), the effect of SPE eluent volume (mixture MeCN/DCM) and of DLLME extractant percentage (DCM) were analyzed to improve the efficiency of SPE-DLLME procedure.

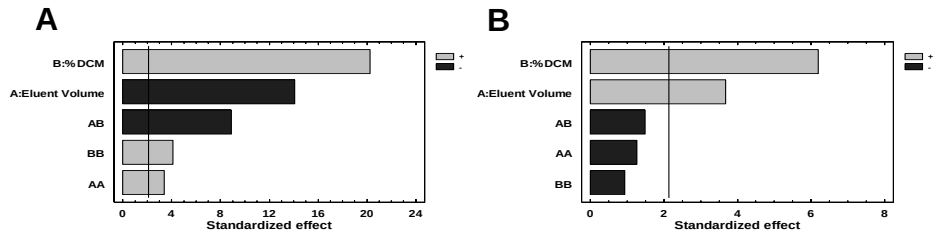
These variables can affect the process directly or indirectly, interacting each other, and hence their effects were simultaneously studied using a *multilevel experimental factorial design*. SPE eluent volume (A) and the DLLME extractant percentage (B) were set as independent variables and their low and high levels (A, 2 and 6 mL; B, 20-40% DCM) were established by preliminary experiments. As response factors were considered the geometric mean of recovery (R) and enrichment factor (EF) of analytes. The study consisted of a 3-level factorial design 3^2 with 15 degree of freedom, two block replicates of 11 randomized experiments and three centre points for block (Table 3.4). Statistical significance of the independent variable contributions, and their first-order interactions, was determined by analysis of variance (ANOVA).

Table 3.4. *Experimental conditions of multilevel factorial experimental design and experimental values of R and EF*

Indipendent variables			**Level**		
			Low	**Medium**	**High**
A: SPE Eluent volume (mL)			2	4	6
B: DCM (%)			20	30	40
	Variables		**Response**	**Factors**	
Run	**A**	**B**	**R (geometric mean)**	**EF (geometric mean)**	
1	4	30	65.0	16.3	
1	2	20	37.8	18.9	
1	4	20	61.5	15.4	
1	2	40	69.1	34.5	
1	2	30	57.5	28.8	
1	6	40	65.6	10.9	
1	4	30	64.2	16.3	
1	6	30	74.1	12.4	
1	6	20	58.6	9.8	
1	4	40	66.2	16.6	
1	4	30	65.2	16.3	
2	4	30	68.4	17.1	
2	2	20	44.6	22.3	
2	4	20	54.1	13.5	
2	2	40	65.3	32.7	
2	2	30	59.9	30.1	
2	6	40	78.2	13.2	
2	4	30	59.1	14.7	
2	6	30	70.5	11.7	
2	6	20	55.7	9.3	
2	4	40	83.4	20.8	
2	4	30	63.1	15.8	

The estimated standardized effects for the geometric means of R and EF and their first order interactions are summarized in the Pareto charts (Figure 3.5). Vertical line in the graphs defines the 95% confidence level.

Figure 3.5.*Pareto charts of standardized effects for EF (A) and R (B).*

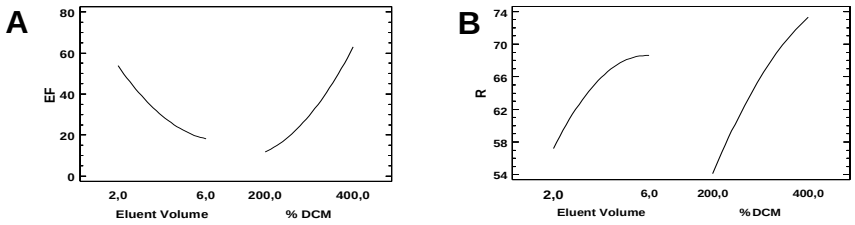


Globally, the independent variables A and B affected significantly both response factors, EF (Figure 3.5A) and R (Figure 3.5B).

In detail, a statistically significant influence of the variables A and B on EF was observed for all selected PPCPs (data not shown). Regarding to the effect on R, A and B affect differently the response depending on the analyte (data not shown): the percentage of DCM (B) influences significantly the recoveries of all analytes, whereas the volume of SPE eluent volume (A) did not show a statistically significant influence on the recoveries of BP3, 4MBC, OMC, OC, OS and HMS (data not shown). Probably, for these lipophilic compounds a strong eluotropic strength than the eluent volume was required to elute them from SPE stationary phase.

The main effect plots reported in Figure 3.6 show the trend of the geometric means of EF and R as a function of each independent variable, A and B, with a line draw between the low and high levels. The length of the line is proportional to the effect magnitude of the eluent volume (A) and DCM percentage (B).

Figure 3.6. *Main effect plots of A e B on EF (A) and R (B)*



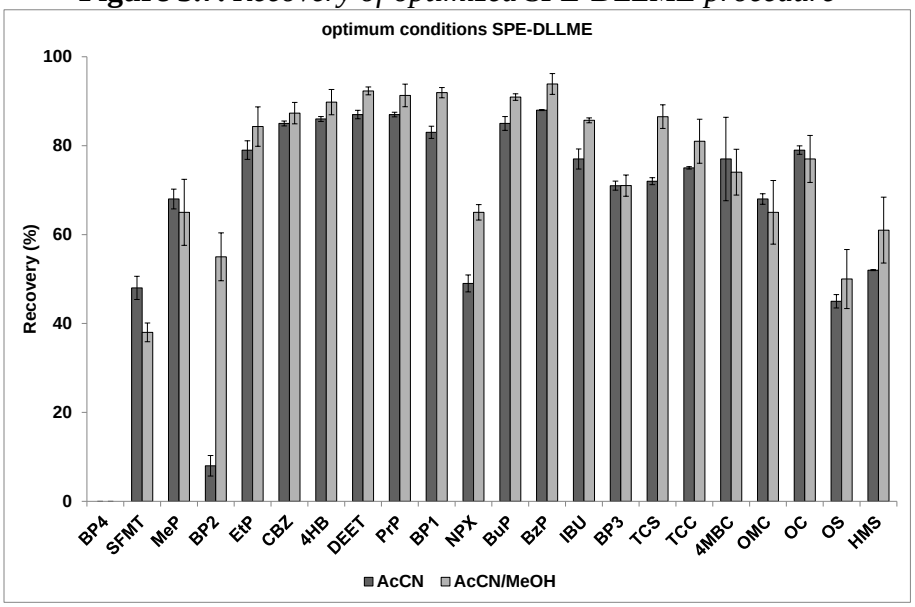
The eluent volume (A) negatively affected EF (EF decreased whit the enhancement of A) (Figure 3.6A) due to dilution of the analytes in the SPE eluate and consequently in the aliquot subjected to DLLME. On the other hand, DCM percentage (B) had a positive effect on EF (Figure 3.6A), because a greater DCM percentage increases the eluotropic strength of SPE eluent reducing consequently its volume.

The percentage of DCM (B) positively affected also the recoveries (R) of the analytes (Figure 3.6B). This variable simultaneously improves the eluotropic strength of SPE eluent and the partition coefficient of extractant/water of the analytes in DLLME process. In fact, a positive effect of the extractant volume has been frequently observed in the optimization of DLLME process: the higher the volume, the more the solubility of the analyte and, as a result, the higher the recovery. Also SPE eluent volume (A) significantly increased the geometric mean of R (Figure 3.6B) improving the efficiency of SPE.

The optimum conditions of A and B, extrapolated from chemometric analysis (SPE eluent volume, 5 mL; DCM percentage, 40%) showed a desirability level of 89%, calculated on the geometric mean.

These most favourable conditions were experimentally corroborated by recovery experiments and the results indicated acceptable recovery for almost all analytes (Figure 3.7) with a precision (expressed as relative standard deviation, RSD) of 0.3-7.4%.

Figure 3.7. *Recovery of optimized SPE-DLLME procedure*



Experimental conditions: SPE eluent, 5 mL MeCN/DCM 6:4, v/v or MeCN/MeOH/DCM 3:3:4, v/v/v; DLLME mixture extractant/dispersant, 2 mL SPE eluate; aqueous phase 10 mL 5% w/v NaCl; final extract volume DLLME, 200 µL (n = 6).

As shown in Figure 3.7, the optimized conditions of SPE-DLLME procedure insufficient recoveries of BP2 and BP4. Therefore, further experiments were performed to improve the efficiency of the developed procedure.

MeOH was previously demonstrated an excellent SPE extraction efficiency for BP2 (Figure 3.1). Therefore, the ternary mixture MeCN/MeOH/DCM 3:3:4, v,v,v, was assessed as SPE eluent and, as a result, BP2 recovery was considerably improved (Figure 3.7).

Regarding BP4, DLLME step is responsible of its failed recovery. This compound, being a strong acidic molecule, is present as anion in DLLME aqueous phase. To overcome this drawback, the use of an ion pair (IP) reagent was evaluated to mask the sulfonic group of BP4 and increase its solubility in the organic phase (DLLME extractant). Usually, IP reagents are used in liquid chromatography for their ability to change the selectivity and increase retention of highly polar molecules on reverse-phase columns. Typical IP

reagents contain a lipophilic portion, such as a long chain aliphatic hydrocarbon, and a hydrophilic portion, such as an acid or base. The polar portion of the IP reagent interacts with the charged group of the analyte, forming an "ion-pair" (Carson, 2000).

Between IP reagents commonly used for basic analytes (Carson, 2000), tetrabutylammonium bromide (Bu_4NH_4Br) was tested as IP reagent to recover BP4 in DLLME step. Bu_4NH_4Br was added to DLLME aqueous phase at two different concentrations (5 and 10 mg L^{-1}) and spiked ultrapure water samples were processed under the optimal experimental conditions. Bu_4NH_4Br allowed an acceptable BP4 recovery (63% ± 3 at 5 mg L^{-1} and 79% ± 2 at 10 mg L^{-1}) at both tested concentrations. Based on these data, 10 mg L^{-1} was selected as Bu_4NH_4Br concentration in order to exhaustively extract BP4 by the developed procedure.

3.3.4. Analytical performance

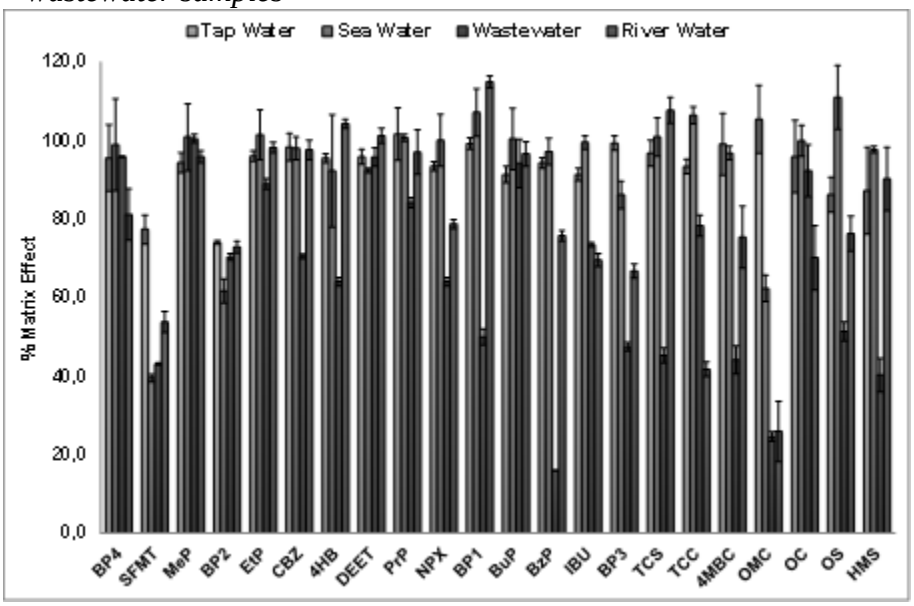
The developed method was applied to different types of water samples (tap water, seawater, river water and wastewater) and, according to the European Commission Decision 657/2002, it was validated, for the followed parameters: selectivity, linearity, sensitivity, accuracy and precision.

The developed method is highly selective because the data acquisition was performed in SRM mode with two characteristic transitions precursor ion → product ion of each analyte. So, it fulfilled EU guidelines with four identification points for the confirmation of analytes with LC–MS/MS detection. Additionally, the SRM1/SRM2 intensities ratio was used as additional identification criterion with a tolerance of less than 20% of the expected ratio.

An important issue in the development of quantitative LC-MS method is the possible occurrence of suppression or enhancement of the analyte response, the matrix effect (ME), due to co-eluting matrix constituents. The matrix effect

primarily influences the accuracy and precision of the method and therefore it should be evaluated carefully for each type of matrix (Niessen *et al.,* 2006). A simple way to assess ME is the comparison of the response obtained from a standard solution and that from a post-extraction spiked sample. To establish the best way to quantify PPCPs, absolute effect was investigated in different environmental water samples. Figure 3.8 displays the matrix effects of selected PPCPs in tap water, seawater, river water and wastewater spiked at the level of 100 (1000 of 4MBC, OMC and OC and 10000 of OS and HMS) ng L^{-1}.

Figure 3.8. *Matrix Effects for tap water, seawater, river water and wastewater samples*



The matrix effects observed for tap water and seawater samples were negligible (between 86 and 105% for tap water and 86 and 111% for seawater), with the exception of SFMT and BP2. These results proved that globally the proposed method did not suffer of matrix effects by virtue of the sample preparation efficiency. Thus, the quantification of PPCPs by external

standard calibration method may be adopted for these water matrices. Probably, SFMT and BP2 affected by the polar co-eluting matrix components (e.g. humic acids) being they among the most hydrophilic PPCPs analyzed. Several experiments were carried out with real water samples to reduce the matrix effects: an adjustment of pH of sample (pH 2) and a washing step (10 mL of MeOH/water 1:9, v/v) prior of SPE elution resulted provided the best results.

Conversely, the matrix effect of wastewater was considerable for many of the analytes due to the high complexity of the matrix. In the case of parabens MeP, EtP, PrP and BuP, the matrix effect was removed by the use of the correspondent labeled ISs (MeP-$^{13}C_6$, EtP-$^{13}C_6$, PrP-$^{13}C_6$ and BuP-$^{13}C_6$), as shown in Figure 3.8. However, their use failed to correct the matrix effects of the other PPCPs. Similar matrix effects were observed for river water. Thus, in the case of wastewater and river water, it is necessary the use of matrix-matched calibration curve or the standard addition method for the accurate determination of PPCPs.

The linearity of method was estimated by solvent- and matrix-matched standard calibration curves. The concentration ranges, reported in Table 3.3 and selected on the basis of instrumental sensitivity and the enrichment factor of the sample preparation procedure, were found to be linear ($R^2 > 0.995$) over the tested concentration range by analysis of variance (ANOVA).

Accuracy and precision were established processing different water samples (tap water, seawater, river water and wastewater), each spiked at the levels of 100 (1000 of 4MBC, OMC and OC and 10000 of OS and HMS) ng L^{-1}, by the optimised analytical procedure. The results of the accuracy (expressed as overall process efficiency, PE, and recovery of the sample preparation method, R%), and the precision (expressed as relative standard deviation, RSD) experiments (n = 4 independent analysis) are reported in Table 3.5.

Table 3.5. *Overall process efficiency (PE), Recovery of sample preparation method (R%), Enrichment factor (EF) and precision (RSD) of the proposed method in different environmental water matrices (n = 4)*[a]

	Ultrapure water		Tap water			Seawater			River water			Wastewater		
PPCPs	**R% (RSD)**	**EF**	**PE (RSD)**	**R% (RSD)**	**EF**	**PE (RSD)**	**R% (RSD)**	**EF**	**PE (RSD)**	**R% (RSD)**	**EF**	**PE (RSD)**	**R% (RSD)**	**EF**
BP4	71 (2)	707	55 (4)	57 (7)	546	50 (1)	50 (1)	499	49 (5)	61 (2)	492	3 (1)	3 (1)	26
SFMTX	38 (3)	220	33 (1)	43 (3)	332	8 (5)	21 (8.	83	27 (2)	51 (5)	272	32 (1)	75 (1)	322
MeP	71 (2)	708	46 (5)	49 (7)	463	56 (1)	55 (1)	560	71 (2)	74 (2)	705	95 (1)	95 (1)	952
BP2	57 (10)	238	34 (5)	46 (1)	343	32 (5)	52 (5)	320	83 (3)	113 (3)	826	44 (2)	62 (2)	438
EtP	95 (1)	946	68 (1)	71 (6)	680	81 (4)	79 (4)	805	95 (2)	96 (5)	946	82 (4)	92 (2)	817
CBZ	89 (2)	888	81 (1)	82 (2)	808	73 (2)	75 (2)	732	92 (1)	94 (4)	922	69 (2)	98 (1)	694
4HB	95 (1)	950	73 (4)	77 (2)	734	81 (1)	88 (1)	808	92 (3)	88 (4)	919	59 (3)	92 (3)	591
DEET	86 (1)	856	71 (1)	74 (2)	707	79 (5)	85 (7)	789	90 (4)	89 (2)	896	94 (1)	98 (2)	941
PrP	95 (2)	950	81 (2)	80 (4)	815	92 (1)	91 (1)	920	93 (2)	96 (3)	928	85 (2)	101 (1)	850
NPX	77 (2)	771	64 (5)	68 (8)	640	84 (2)	84 (1)	836	56 (4)	71 (4)	562	59 (3)	92 (3)	591
BP2	94 (2)	940	86 (1)	86 (2)	858	91 (1)	85 (3)	912	107 (5)	93 (2)	1074	53 (3)	106 (2)	529
BuP	93 (2)	930	87 (1)	95 (4)	866	96 (2)	95 (1)	958	85 (3)	88 (3)	848	109 (3)	115 (2)	1086
BzP	95 (3)	950	81 (2)	86 (1)	812	83 (1)	85 (2)	831	79 (2)	104 (3)	791	21 (1)	131 (3)	210
IBU	94 (1)	940	70 (4)	76 (3)	697	87 (3)	87 (4)	870	56 (3)	80 (4)	559	72 (5)	97 (3)	716
BP3	87 (8)	871	72 (5)	72 (1)	715	73 (2)	85 (1)	729	74 (2)	111 (3)	741	48 (4)	102 (1)	484
TCS	82 (3)	816	84 (1)	87 (2)	843	90 (5)	89 (5)	896	103 (2)	96 (2)	1033	58 (2)	127 (2)	578
TCC	95 (3)	950	70 (9)	75 (2)	699	87 (2)	82 (2)	875	38 (3)	92 (1)	383	90 (2)	115 (2)	898
4MBC	91 (1)	910	70 (3)	71 (1)	699	78 (1)	81 (1)	780	67 (1)	89 (2)	671	56 (4)	126 (4)	556
OMC	66 (4)	661	50 (2)	48 (10)	504	53 (3)	86 (3)	534	22 (2)	83 (3)	215	27 (5)	111 (5)	272
OC	71 (1)	712	50 (5)	52 (3)	498	65 (2)	65 (2)	653	61 (3)	86 (2)	606	112 (7)	121 (2)	1118
OS	56 (3)	556	51 (2)	59 (8)	512	79 (2)	72 (2)	794	60 (5)	79 (2)	603	44 (7)	86 (3)	443
HMS	78 (8)	776	64 (2)	73 (7)	637	66 (9)	67 (9)	656	78 (6)	86 (29	777	34 (9)	85 (4)	343

[a] PE and R% were calculated according to equations reported in the section 3.5.6.

Extraction efficiency of optimized SPE-DLLME procedure, evaluated with spiked ultrapure water, was good for most of the target analytes (R% > 71), except for SFMTX, BP2, OMC and OS. The poor extraction efficiency of DLLME technique towards very hydrophilic compounds is responsible of the low recoveries of SFMTX and BP2; whereas, slightly lower values, for OMC and OS, the most lipophilic of the compounds, probably due to adsorption to SPE cartridge and glass materials.

PE and R% obtained for spiked tap water and seawater samples were generally comparable, indicating that no occur matrix effects in the processing of these aqueous matrices. On the other hand, difference between PE and R% is particularly noticeable in wastewater sample for many PPCPs. Also for river water the matrix effects affect the accuracy of the proposed method for many analytes. Regard to extraction efficiency, In general, Table 3.5 shows that an adequate accuracy was observed for the majority of target compounds in different environmental aqueous samples, with the exception of the more polar PPCPs (BP4, SFMTX, MeP and BP2), which suffer both the low efficiency of DLLME and the matrix effects. The latter has often been observed in LC-MS in the analysis of very hydrophilic compounds, and it is attributed to salts and polar components of the sample overloading column capacity and eluting early in the chromatogram (Petrovich *et al.,* 2006).

The Table 3.5 also lists the enrichment factors (EF) of the proposed analytical procedure both ultrapure water and the environmental water matrices (tap water, seawater, river water and wastewater). The developed method shows very high enrichment factors for all water matrices that greatly improve the sensitivity of the analytical procedure.

The sensitivity of analytical procedure for each water matrix was expressed as method detection limit (MDL) and method quantification limit (MQL). The limits reported in Table 3.6 show a very high sensitivity with MDL and MQLs at ng L^{-1} levels (ppt). Only OS and HMS had limits at μg L^{-1} levels. However,

for most analytes (sixteen of the twenty-two target PPCPs) MDLs and MQLs were below 3 and 10 ng L^{-1}, respectively.

These low MQLs make the method useful for the determination of very low levels of PPCPs in the aqueous environment.

Table 3.6. *MDLs and MQLs for each PPCP in ultrapure water (UW), tap water (TW), seawater (SW), river water (RW) and wastewater (WW)*[a]

PPCPS	MDLs (ng L^{-1})					MQLs (ng L^{-1})				
	UW	TW	SW	RW	WW	UW	TW	SW	RW	WW
BP4	3.5	4.6	5.0	5,1	191.7	11.7	15.1	16.5	16,8	632.8
SFMT	11.3	7.5	30.2	9,2	15.5	37.4	24.9	99.8	30,3	51.3
MeP	0.9	1.3	1.1	0,9	1.3	2.9	4.4	3.7	2,9	4.3
BP2	0.7	0.5	0.5	0,2	0.7	2.2	1.5	1.6	0,6	2.4
EtP	0.3	0.5	0.4	0,3	0.8	1.1	1.5	1.3	1,1	2.5
CBZ	0.2	0.2	0.2	0,2	0.4	0.6	0.6	0.7	0,6	1.5
4HB	0.1	0.2	0.2	0,2	0.5	0.5	0.7	0.6	0,6	1.7
DEET	0.2	0.2	0.2	0,2	0.3	0.6	0.7	0.7	0,6	1.1
PrP	0.2	0.2	0.2	0,2	0.4	0.5	0.6	0.6	0,6	1.2
NPX	0.8	1.0	0.7	1,1	2.1	2.7	3.2	2.5	3,7	7.0
BP1	0.2	0.2	0.2	0,1	0.6	0.5	0.6	0.6	0,5	1.9
BuP	0.2	0.2	0.2	0,2	0.3	0.5	0.6	0.5	0,6	0.9
BzP	0.2	0.2	0.2	0,2	1.5	0.6	0.6	0.6	0,7	4.9
IBU	0.6	0.9	0.7	1,1	1.7	2.1	3.0	2.4	3,7	5.8
BP3	0.7	0.9	0.9	0,8	2.6	2.4	2.9	2.8	2,8	8.5
TCS	0.4	0.4	0.3	0,3	1.1	1.3	1.2	1.1	1,0	3.6
TCC	0.3	0.4	0.4	0,8	0.7	1.1	1.5	1.2	2,7	2.3
4MBC	1.7	2.2	2.0	2,3	5.6	5.7	7.4	6.6	7,7	18.5
OMC	37.8	49.6	46.8	116,1	183.7	124.8	163.6	154.4	383,1	606.3
OC	35.1	50.2	38.3	41,2	44.7	115.8	165.5	126.4	136,1	147.6
OS	2247	2441	1574	2074,	5644	7415	8055	5194	6844	18624
HMS	1611	1963	1904	1609	7284	5315	6477	6284	5309	24036

[a]MDL and MQL were calculated according to equations reported in the section 3.5.6.

3.3.5. Real samples analysis

In order to evaluate the occurrence and environmental fate of studied PPCPs, the proposed analytical procedure was applied to the analysis of real samples. Particularly, influent (IWW) and effluent (EWW) wastewaters of treatment plant of Avellino (Italy) and river water (RW) collected from the River *Sabato*, located near to exit of the wastewater treatment plant, were analyzed. Detected analytes were quantified by matrix-matched calibration method. The

quantitative data are presented in Table 3.7 and agree with those previously reported (Braush and Rand, 2011; Pedrouzo *et al.*, 2011a).

Of the targeted 22 compounds, 14 PPCPs were detected at levels above the MQL in IWW, 10 in EWW and 13 in RW. The highest levels were found in IWW sample with IBU and NPX occurring in µg L^{-1} (ppb) quantities. This is due to their wide consumption and continuous release into wastewater. Of PPCPs detected in IWW, only 4HB, TCC and TCS were efficiently eliminated by treatment plant process. These analytes are very hydrophobic compounds and probably they were adsorbed by sludge, whereas in the case of other PPCPs, an ineffective removal were observed. The presence of PPCPs detected in RW was directly related to their resistance to the wastewater treatments.

Table 3.7. *Concentration (ng L^{-1}) of targeted PPCPs in Influent Wastewater (IWW), Effluent Wastewater (EWW) and River Water (RW)*

PPCPs	IWW	EWW	RW
	ng L^{-1} ± SD (n=3)		
BP4	258.3 ± 8.1	308.6 ± 4.2	133.1 ± 5.2
SFMT	104.5 ± 7.4	53.4 ± 5.4	10.7 ± 7.5
MeP	n.d.	n.d.	17.5 ± 3.7
EtP	n.d.	n.d.	14.3 ± 1.5
CBZ	232.5 ± 8.4	189.7 ± 6.7	49.9 ± 3.2
4HB	20.1 ± 3.2	n.d.	2.6 ± 1.2
DEET	78.5 ± 2.2	8.9 ± 3.7	21.2 ± 4.4
PrP	3.4 ± 3.2	5.3 ± 2.1	25.6 ± 3.3
NPX	998.6 ± 9.4	183.4 ± 5.4	198.8 ± 4.3
BP1	86.9 ± 6.5	17.6 ± 4.2	19.4 ± 6.2
BuP	n.d.	4.6 ± 1.1	4.8 ± 1.4
IBU	1818.2 ± 13.2	86.9 ± 2.4	240.1 ± 5.1
BP3	102.8 ± 8.2	18.2 ± 1.2	6.9 ± 3.2
TCS	2.5 ± 3.2	< MQL	< MQL
TCC	53.4 ± 2.2	n.d.	< MQL

n.d., not detected corresponding to < MDL.

3.4. Conclusion

In this study, a novel analytical procedure, based on the sequential application of SPE and DLLME before instrumental analysis by UHPLC-MS/MS, was successfully developed for the analysis of twenty-two PPCPs in environmental matrices. The whole procedure was validated using different water matrices (ultrapure water, tap water, sea water, river water and wastewater) and its analytical performance fulfils the criteria required for methods of analysis from the European Commission Decision 2002/657/EC.

The developed method showed acceptable recoveries for almost all analytes, very high enrichment factors and limits of quantification at ng L^{-1} level, in all evaluated aqueous matrices.

The SPE-DLLME procedure allows a sensitive and selective determination of selected PPCPs and it can be applied to more or less complex matrices. In addition, it compared to conventional methods of sample preparation, offers numerous advantages such as: the simplicity of operation, rapidity, a high enrichment factor and the high environmental sustainability.

For these reasons, the developed method is suitable for monitoring and studies of occurrence of PPCPs in different environmental compartments, as demonstrate in application to real samples.

3.5. Experimental

3.5.1. Standards and materials

Standards of methylparaben-$^{13}C_6$ (MeP-$^{13}C_6$), ethylparaben-$^{13}C_6$ (EtP-$^{13}C_6$), propylparaben-$^{13}C_6$ (PrP-$^{13}C_6$) and butylparaben-$^{13}C_6$ (BuP-$^{13}C_6$) (internal standards, ISs), NPX, SFMT, IBU, CBZ, BzP, BuP, PrP, EtP, MeP, OMC, 4MBC, BP1, BP2, BP3, BP4, OS, 4HB, OC, HMS, TCS, TCC and DEET were obtained from Sigma-Aldrich (Milan, Italy). Full names of these target analytes are compiled in Table 3.1. Stock solutions of analytes and IS with concentrations of 1 mg mL^{-1} were prepared in acetonitrile, stored in amber glass vials at 5°C. PPCPs mixed standard solution was prepared at 5 (50 of 4MBC, OMC and OC and 500 of OS and HMS) μg mL^{-1} in acetonitrile. This solution was used for spike of the samples and for the preparation of reference solutions.

Acetonitrile (MeCN), chloroform ($CHCl_3$), 1,2-dibromoetane ($C_2H_4Br_2$), dichloromethane (DCM) and methanol (MeOH) were obtained from Carlo Erba (Milan, Italy) and Sigma-Aldrich. Sodium chloride (NaCl), tetrabutylammonium bromide (Bu_4NH_4Br), MS-grade ammonium acetate, MS-grade acetic and formic acids were provided by Sigma-Aldrich. HPLC-grade acetonitrile and water were purchased from Romil (Cambridge, UK). Ultrapure water (18MΩ) was prepared by a Milli-Q purification system (Millipore, Bedford, USA). Oasis 200 mg HLB cartridges were purchased from Waters (Waters, UK).

3.5.2. Water samples

24 h composite samples of influent (IWW) and effluent (EWW) wastewaters were collected at the urban wastewater treatment plant of Avellino (Italy). River water (RW) were collected from River *Sabato* (Avellino, Italy). The

sampling site was localized at 100 mt from the exit of the wastewater treatment plant. The sampling of IWW, EWW and RW was carried out for three consecutive days in January 2014. The tap water samples (grab sample) were collected from aqueduct of Salerno. The seawater samples (grab sample) were collected in Vietri (Salerno, Italy). Samples were collected in amber glass bottles previously rinsed with methanol and ultrapure water and stored at -20°C. Prior of extraction, the samples were filtered by 2.7 μm glass fibre filters (Millipore).

3.5.3. SPE-DLLME procedure

After filtration, pH of aqueous samples was adjusted at pH 2 with 1 N HCl. Volumes of 500 mL (ultrapure water, tap water, river water and sea water) or 250 mL (wastewater) were passed through SPE cartridge (Oasis HLB), previously preconditioned with 5 mL MeOH and 5 mL water, at a flow rate 5 mL min^{-1}. Then, the column was washed with 10 mL MeOH/water 1:9, v/v. After being vacuum dried for 30 min, the cartridges were eluted with 5 mL of MeOH/MeCN/DCM (3:3:4 v/v/v) at a flow rate 2 mL min^{-1}. Subsequently, 2 mL of eluate (DLLME extractant/disperser mixture) was injected rapidly into the 10 mL of aqueous phase (5% NaCl, 10mg L^{-1} Bu_4NH_4Br) and the mixture was gently shaken for few second. A cloudy solution, stable for a long time, was formed into test tube. Then, the mixture was centrifuged for 5 min at 6000 rpm. After removing most of the supernatant with a Pasteur pipette the volume of the settled phase was quantitatively transferred to 2 mL Eppendorf vial using a 500 μL microsyringe. After the addition of ISs (MeP-$^{13}C_6$, EtP-$^{13}C_6$, PrP-$^{13}C_6$ and BuP-$^{13}C_6$), the organic phase was dried under a gently nitrogen flow and the residue was reconstituted with 200 μL MeCN/H_2O 1:1, v/v.

3.5.4. Experimental Design

In order to obtain the best extraction parameters the Statgraphic Centurion XVI version 16.1 from Statistical Graphics (Rockville, USA) was used for experimental design analysis and statistical data processing.

3.5.5. UHPLC-MS/MS analysis

Analyses were performed on a Platin Blue UHPLC system (KNAUER GmbH, Berlin, Germany) consisting of two ultra high pressure pumps, an autosampler, and column temperature manager, coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a heated electrospray ionization (H-ESI) probe. UHPLC separation was achieved with a Kinetex C18 (100 x 2.1 mm I.D., 2.6 μm) column protected by a C18 Guard Cartridge (4x3 mm I.D.), both from Phenomenex (Torrance, CA, USA) held at 30 °C. The mobile phase consisted of water (A) and MeCN (B), both containing 1.5 mM ammonium acetate. The following elution gradient was used: 0-1 min, 20% B; 1-5 min, 20-40% B; 5-6.5 min, 40% B; 6.5-6.7 min, 70% B; 6.7-8.1 min, 70% B; 8.1-9.2 min, 80% B; 9.2-13 min, 80% B. After each injection, the column was washed with 95 % B for 4 min and re-equilibrated (5 min). The flow rate was 0.3 mL min^{-1} and the injection volume was 10 μL using the full loop injection mode.

The operative parameters of the mass spectrometer and solvent- and flow rate-dependent source parameters were optimised at the chosen chromatographic conditions by injecting PPCPs standard solution 10 ng mL^{-1}. The optimised conditions were spray voltage, 3,5 V; capillary temperature, 300°C; vaporizer temperature, 150°C; sheath and auxiliary gas pressure, 20 and 10 units, respectively; collision gas pressure, 1 bar. Nitrogen (99.9% purity) was used as the auxiliary and sheath gas in the ESI source and argon (99.9999% purity) as the collision gas in the collision cell. For identification and quantification of PPCPs, selected reaction monitoring (SRM) mode was applied using two

characteristic SRM transitions (Table 3.2). The SRM values for all scan transitions were scan width (m/z), 0.200; scan time (ms), 20; Q1 and Q3 resolution (FWHM), 0.7. Excalibur software version 2.2 was employed to collect and process the data.

3.5.6. Validation of analytical procedure

For the validation and analysis of PPCPs, MeP-$^{13}C_6$, EtP-$^{13}C_6$, PrP-$^{13}C_6$ and (BuP-$^{13}C_6$ were used as ISs to compensate matrix effects of corresponding unlabeled compounds (MeP, EtP, PrP and BuP).

Linearity of the solvent- and matrix-matched curves was estimated in the working range of 0.5-1000 ng mL^{-1} for BP2, 4HB,PrP, BP1, BuP, BzP, CBZ and DEET; 1-1000 ng mL^{-1} for EtP, TCC and TCS; 5-1000 ng mL^{-1} BP4, NPX, MeP, IBU, SFMT, BP3 and 4MBC; 50-10000 OMC and OC; OS and HMS had a linear range of 2070-100000 ng mL^{-1}, with ten calibration levels, each injected in triplicate. Calibration solutions were prepared by diluting appropriate volumes of PPCPs mixed standard solution with MeCN/H_2O (1:1, v/v) (solvent curve) or with the SPE-DLLME extracts (matrix-matched curves).

Matrix effect (ME), recovery of sample preparation procedure (%R) and overall process efficiency (PE) were established according to Niessen (Niessen et al., 2006). For each aqueous matrix (ultrapure water, tap water, seawater, river water and wastewater), four sample sets were processed, in quadruplicate, by the SPE-DLLME procedure. The first sample set (standard solution) consisted of the analytes and the ISs in mobile phase (MeCN/H_2O 1:1, v/v), the second sample set consisted of post-extraction spiked samples (matrix-matched standard), the third set of pre-extraction spiked samples (spiked real sample) and the last set of real samples (unspiked real sample). ISs were added to the matrix-matched standard, spiked and unspiked real sample over SPE-DLLME extract to compensate the matrix effects of MeP,

EtP, PrP and BuP. The spiked level corresponded to the concentration of 100 (1000 of 4MBC, OMC and OC and 10000 of OS and HMS) ng L^{-1} in aqueous sample. From the responses (peak area analyte/IS ratio for MeP, EtP, PrP and BuP and peak area for the other PPCPs) acquired for these sets, ME, %R and EP can be calculated from the following equation:

$$ME = 100 \times \frac{\text{(Response post-extraction spike – Response unspiked)}}{\text{Response of standard}}$$

$$\%R = 100 \times \frac{\text{Response pre-extraction spike}}{\text{Response post-extraction spike}}$$

$$PE = 100 \times \frac{\text{(Response pre-extraction spike – Response unspiked)}}{\text{Response of standard}}$$

UHLC–MS/MS instrumental detection limits (IDL) and quantification limits (IQL) were experimentally determined using signal-to-noise approach through analysis of a series of low concentration standards. IDL and IQL were selected as the concentrations that gave a S/N 3:1 and 10:1, respectively. Method detection limits (MDL_{calc}) and method quantification limits (MQL_{calc}) for different aqueous matrices were calculated using the following equations:

$$MDL_{calc} = \frac{IDL \times 100}{PE \times CF} \qquad MQL_{calc} = \frac{IQL \times 100}{PE \times CF}$$

Where CF is the concentration factor, which in this method denotes 500 for wastewater and 1000 for ultrapure water, tap water, seawater and river water. Quantification of PPCPs detected in real samples was performed by the matrix-matched calibration method, spiking the final extracts to four levels in the range of MQL-1000 (10000 for OMC and OC; 100000 for OS and HMS)

ng mL^{-1}. ISs were added at 50 ng mL^{-1} level. Responses of analytes (peak area analyte/IS ratio for MeP, EtP, PrP and BuP and peak area for the other PPCPs) in matrix-matched calibration levels were corrected by subtraction of the responses measured in the unspiked final extracts.

Chapter 4

Liquid chromatography quadrupole time-of-flight mass spectrometryquantification and screening of organophosphate compounds in sludge

Celano R., Rodriguez I., Cela R., Rastrelli L., Piccinell A.L, (2014) *Talanta*, 118, 312-320.

4.1. Introduction

Organophosphate esters (OPEs) are high production volume chemicals, mainly used as plasticizers and flame retardants additivesin furniture, upholstery and building materials. Consequently, they have been reported in air and particulate matter from indoor environments (Reemtsma *et al.,* 2008; Bergh *et al.,* 2012). OPEs are also ubiquitous in the aquatic media, where they are introduced through urban sewage water (Reemtsma *et al.,* 2008). Some OPEs (e.g. trichloroisopropyl phosphate, TCPP) display limited biodegradation at sewage treatment plants (STPs) and can bioccumulated in sludge (Olofsson *et al.,* 2013). Assuming that around 50% of the sludge generated at STPs is disposed as a fertilizer in agriculture fields (Macherius *et al.,* 2012), evaluation of OPEs discharges in the environment requires not only determining their dissolved concentrations, at the outlet stream of STPs, but also addressing their levels in sludge. This latter issue becomes particularly concerning after having reported i) significant uptakes of polar OPEs by vegetable roots and ii) their capability to migrate from roots to leaves (Eggen *et al.,* 2013); thus, the risk of OPEs introduction in the human food web through livestock animals and vegetables is not negligible.

Additionally, the phase out of polybrominated diphenyl ethers (PBDEs) might entail an increase in the amounts of OPEs incorporated in upholstery and building materials to meet regulated flammability standards (Stapleton *et al.,* 2012; van der Veen and Boer, 2012).

Most of the OPEs are amenable to gas chromatography (GC) separation, with very low limits of quantification (LOQs) provided by the nitrogen-phosphorus detector (NPD); on the other hand, the sensitivity of this system largely varies depending on the state of the active element in the NPD detector, which requires frequent replacement (Quintana *et al.,* 2008). GC–MS, using electron ionization (EI), has also some drawbacks such as i) the excessive

fragmentation of trialkyl OPEs, which lead to ions with low m/z ratios resulting in a limited selectivity, and ii) the poor ionization of tributoxyethyl phosphate (TBEP) (Quintana *et al.,* 2008). The problems mentioned above have been overcome with positive chemical ionization (PICI), combined with single MS (Quintana *et al.,* 2007), or tandem mass spectrometry (MS/MS) (Bergh *et al.,* 2012). Another option for OPEs analysis is liquid chromatography (LC) followed by MS/MS, based on triple quadrupole (QqQ) mass spectrometers. LC–MS/MS allows the determination of tri- and di-substituted OPEs, attaining very low detection limits for sewage water analysis (Quintana *et al.,* 2006b; Wang *et al.,* 2011); however, its performance has not been evaluated with sludge samples.

With regard to the sample preparation process, approaches for OPEs extraction from sludge should provide high extraction yields and enough selectivity to avoid interferences and matrix effects in the MS determination step. Usually, such problems are related to variations in the efficiency of the injection process, between pure standards and extracts from complex matrices, and changes in the yield of electrospray ionization. The proposed sample preparation strategies for OPEs determinationin sludge involve a hard extraction step (using high temperatures, pressures and multiple cycles), based on non-selective pressurized liquid extraction (PLE) (Marklund *et al.,* 2005) or Soxhlet (Bester, 2005), followed by extensive clean-up of the raw extract with normal-phase sorbents plus gel permeation chromatography, ending with GC–EI-MS detection. Although effective in terms of recoveries, these approaches are time and solvent consuming.

In collaboration whit the *Laboratory of Analytical Chemistry of the University of Santiago de Compostela*, a novel and advantageous analytical procedure, suitable to investigate the presence of OPEs residues in sludge samples, was developed. Matrix solid-phase dispersion (MSPD) was selected as an extraction technique considering its low cost, reasonable selectivity (Capriotti

et al., 2010; Zuloaga *et al.,* 2012) and previous successful applications dealing with emerging compounds extraction from sludge (Sànchez-Brunete *et al.,* 2007; Capriotti *et al.,* 2013). Although MSPD has been already proposed for OPEs extraction from dust (Garcìa *et al.,* 2007) and biota (Campone *et al.,* 2010), its performance for the most complex sludge matrix has not been investigated, yet. OPEs were determined by LC using, for the first time, a hybrid quadrupole time-of-flight (QTOF) MS system, as an alternative to QqQ instruments. The quantitative possibilities of such system for targeted OPEs determination in sludge samples are discussed. Furthermore, the information contained in accurate, scan MS spectra were used to screen the presence of additional OPEs, which had not been included in the quantitative method, in sludge samples. The reliability of tentative identifications derived from this post-target analysis strategy, without using reference standards, and its capability to detect residues of novel organophosphorus flame retardants in sludge, are also discussed.

4.2. Organophosphate esters

Organophosphorus flame retardants (PFRs) comprise a broad and heterogeneous group of chemical compounds in terms of organic substituents, polarities, vapour pressures and industrial applications.

OPEs are industrially produced by reacting phosphorus oxychloride ($POCl_3$) with various reactants. Structurally, they are derivates of phosphoric acid that can be divided into three groups: trialkyl-, alkyldiaryl- and triaryl phosphates. Generally, OPEs are semi-volatile compounds with low or moderate solubility in water and a relatively high affinity to particles. Nevertheless, the various substituents confer to the compounds different physico-chemical properties (Marklund, *et al.,* 2005).

PFRs can be divided in two broad groups: chlorinate and non chlorinate compounds and are mainly triesters of phosphoric acid (OPEs), with the exception of monoester mono-2-ethylhexyl phthalate (MEHP) and diestersdi-n-butyl phthalate (DnBP) and diethylhexyl phosphate (DEHP). Other PFRs and plasticizers used to a minor extent are bisphosphates, phosphonates and phosphinates.

OPEs are the PFRs most common used, in fact, in Europe it has been estimated an increase of their use, 2.5% (2001-2005) and 7.1% (2005-2006) (EFRA, 2007).

First studies concerning the OPEs date back to 1970s and thereafter in the 80s, Muir et al. conducted several studies on their possible bioaccumulation and biodegradability in the different environmental compartments (Muir *et al.,* 1981; Muir *et al.,* 1983). Subsequently, these studies were abandoned; insofar the aryl and alkyl phosphates initially considered resulted degradable in the environment. Thereafter, in the second half of the 90s the research on OPEs were resumed, because many of them were found in indoor (Carlsson et al., 1997), and the chlorinated-alkyl phosphates were included in the 2nd (1995)

and 4th (2000) European Union (EU) priority lists (EU, 1995; EU, 2000) for risk assessment and many of them were considered persistent in the environment (Kawagoshi *et al.,* 2002). Thus, they may be classifiedas ‘‘re-emerging’’ rather than emerging contaminants. The term emerging contaminants is generally used to refer to compounds that have not been included in regulatory systems and they are continuously introduced into the environment due to their use anthropogenic (Reemtsma *et al.,* 2008). For this reason, a chemical substance, to be called "emerging contaminant" should not necessarily be new, but a substance that have the potential to cause known or suspected adverse ecological or human health effects.

Their release into the environment is due mainly to extensive use and the mode in which the OPE are employed, because the OPEs are not chemically bonded with products that this are added.

Furthermore, their distribution in the environment, (water, air, particulate samples), possible accumulation and bioaccumulation strongly depend on their physical-chemical properties. Their physical-chemical properties are rather variable and depend upon the alcohol moieties esterified to the phosphoric acid (Table 4.1) (Reemtsma *et al.,* 2008).

Table 4.1.*Physical-chemical properties of the most relevant OPEs.*[a]

Compound name	Code	Formulae	Log*P*[a]	Vp (Torr)[a]
Tri-methyl phosphate	TMP	$C_3H_9O_4P$	- 0.65	8.50 x10^{-1}
Tri-ethyl phosphate	TEP	$C_6H_{15}O_4P$	0.80	3.93 x10^{-1}
Tri-propyl phosphate	TPrP	$C_9H_{21}O_4P$	1.87	4.33 x10^{-3}
Tri-isobutyl phosphate	TiBP	$C_{12}H_{27}O_4P$	3.60	1.28 x10^{-2}
Tri-(2-chloroethyl) phosphate	TCEP	$C_6H_{12}Cl_3O_4P$	1.44	6.13 x10^{-2}
Tri-butoxyethyl phosphate	TBEP	$C_{18}H_{39}O_7P$	3.75	2.50 x10^{-8}
Tri-phenyl phosphate	TPhP	$C_{18}H_{15}O_4P$	4.59	6.28 x10^{-6}
Tri-cresyl phosphate	TCrP	$C_{21}H_{21}O_4P$	5.11	6.00 x10^{-7}
Tri-n-butyl phosphate	TnBP	$C_{12}H_{27}O_4P$	4.00	1.13 x10^{-3}
Tri-(2-ethylhexyl) phosphate	TEHP	$C_{24}H_{51}O_4P$	9.49	8.45 x10^{-8}
Tri-(dichloropropyl) phosphate	TDCP	$C_9H_{15}Cl_6O_4P$	3.65	7.36 x10^{-8}
Tri-(chloropropyl) phosphate	TCPP	$C_9H_{18}Cl_3O_4P$	2.59	2.02 x10^{-5}
2-Ethylhexyl diphenyl phosphate	EHDPP	$C_{20}H_{27}O_4P$	6.54	6.49 x10^{-7}
Di-n-butyl phosphate	DnBP	$C_8H_{19}O_4P$	2.29	4.26 X 10^{-9}

[a]Physical-chemical data obtained from Syracuse Research Corporation database of physic-chemical propierties.

4.2.1. Occurrence

OPEs do not occur naturally in the environment, but only as a result of anthropogenic activity. They have been detected in both indoor and outdoor environments. In indoor environments, OPEs have been found mainly in air and dust. The detection in indoor environments of these compounds is due to their presence in building, materials, electric appliances and upholstery (Reemtsma *et al.,* 2008). The sources of OPEs in the samples of air are many, for example computer screens and televisions are major sources of emissions of tri-phenyl phosphate (TPhP), while floors polishing contribute strongly to the presence of tributoxyethyl phosphate (TBEP) in indoor.

Addition of TCPP to upholstery and plastic components has led to levels 10–100 times higher for this compound in cars and public transport vehicles than in private houses (Reemtsma *et al.,* 2008). The OPEs are normally found at mg kg^{-1} levels in dust and at ng m^{-3} or μgm^{-3} levels in indoor air (Andresen *et al.,* 2004; Marklund *et al.,* 2005).

Unlike, of data reported for air samples, less information are available in relation to the presence of OPEs in dust. Several studies conducted on dust samples have shown that for almost all the OPEs studied tri-n-butyl phosphate (TnBP), tri (2-chloroethyl) phosphate (TCEP), TCPP, tri (dichloropropyl) phosphate (TDCPP), tributoxyethyl phosphate (TBEP) and TPhP the concentration remained over 5 μg g^{-1} level, with the highest levels corresponding to 147 μg g^{-1} for TBEP (Reemtsma *et al.,* 2008). Detection of several OPEs in particulate matter and different environmental samples (pine needles, rain water and snow) from remote areas confirms the contribution of air transport to the ubiquitous distribution of these pollutants in the environment (Reemtsma *et al.,* 2008).

In outdoor environments, OPs have been found in diverse compartments, including riverwater, groundwater (Fries and Püttmann, 2003) wastewater (Fries and Püttmann, 2003; Fries and Püttmann, 2001; Bester, 2005; Rodil *et al.,* 2005). Since the 1980s, their detection in surface waters, in groundwaters and in drinking water were reported (Reemtsma *et al.,* 2008). A study conducted in wastewater treatment plants (WWTPs) in several European countries (Kim et al., 2007; Reemtsma et al., 2006) has shown that TCEP and TCPP are routinely detected in their effluents, at concentrations of ng L^{-1}. In a other study conducted on 29 polar trace pollutants, TCPP was among the 10 with no significant elimination in WWTP, whereas TCEP showed slight removal (only 20%) (Reemtsma et al., 2006). WWTPs are considered the major source of OPEs in surface waters (Fries and Püttmann, 2003). Emission into groundwater may occur via landfill leachate (Paxeus, 2000) and release into the marine environment from dump sites (Kawagoshi *et al.,* 2002). Therefore, OPEs are ubiquitous contaminants in the aqueous environment.

To date, information about the impact of OPEs on biological and human samples are quite scarce in literature. he OPes were detected for the first time in human adipose tissue in 1983 (LeBel and Williams, 1983). In this study

TBEP, tributyl phosphate (TBP) and TDCPP have been detected in adipose tissue at levels up to 260 ng g^{-1}. TDCPP has also been found in human seminalplasma at concentrations ranging from 5 to 50 ng g^{-1}(Hudec *et al.*, 1980).

4.2.1.1. Sludge

In the last 20 years the development of new strategies for the treatment of wastewater in depuration plants, have given, on the one hand, the improvement of water quality, but the other, the increased production of sewage sludge. Today it is estimated that about 8.5 million tons were produced and their disposal has become a national and international problem (Rulkens, 2004). The method most common of disposal/utilization of sludge is the reuse in agriculture. For this reason, the presence of pollutants such as OPEs in this matrix can cause damage to the environment and human health, because these compounds can accumulate in the vegetable foods and arrive at man (Spinoza, 2001).

According to data reported in the literature, OPEs were have been detected in sludge samples at high concentrations (μg g^{-1}). Markludnd et al. have determined in 11 sludge samples various OPEs, TCPP (61-1900 ng g^{-1}), TBEP (5-1900 ng g^{-1}), tri-iso-butyl phosphate (TiBP) (27-2700 ng g^{-1}), TBP (39-850 ng g^{-1}), TPP (52-320 ng g^{-1}), TDCPP (3-260 ng g^{-1}) and TCEP (6.6-110 ng g^{-1}) (Marklund *et al.*, 2005). In Germany, Bester et al. have detected levels of TCPP between 1700 and 2200 ng g^{-1} in sludge samples with 60% of aqueous content (Bester *et al.*, 2005). In USA Harrison et al. have detected TBP and TPP at concentration of 2.4 and 1.9 μg g^{-1}, respectively (Harrison *et al.*, 2006).

4.2.2. Toxicity

Due to their chemical diversity and consequently their physicochemical properties, the toxicities of OPEs are quite different.

According to several studies, the TCEP is toxic to aquatic organisms and it may cause chronic adverse effects. TCEP is carcinogenic for animals (WHOEHC, 1998), it has been shown to induce adverse reproductive effects in rats (Chapin *et al.,* 1997) and it is a neurotoxin in rats and mice (Tilson *et al.,* 1990; Umezu *et al.,* 1998).

TCPP is considered to be potentially carcinogenic (Ni *et al.,* 2007). The dermal and the inhalative toxicity have been tested in rats (Leisewitz *et al.,* 2000). TDCP is harmful when inhaled and it can enter the body, where it easily can enter the blood stream (ATSDR, 2009). Tumors were observed in the kidneys, liver and testes of rats which were fed with TDCP for 2 years (ATSDR, 2009). According to Andresen et al. and the WHO the TDCP is carcinogenic (Andresen *et al.,* 2004; WHOEHC, 1998).

In the case of non-chlorinated OPEs, trimethyl phosphate (TMP) was recognized as genotoxic (OECD, 1996) and neurotoxic effects were found for TnBP and TPhP (WHOEHC, 1991), whereas TBEP is also a suspected carcinogenic compound (WHOEHC, 2000).

Nevertheless, in terms of toxicity, persistence and mobility, TCEP, TPhP, TCPP, and TDCP are the more concerning compounds. In fact, chlorinated OPEs pass through conventional urban wastewater treatment plants without undergoing significant degradation. Furthermore, they are not removed by other treatments such as ozonization or the use of multilayer filters and consequently, they might reach surface and drinking water (Reemtsma *et al.,* 2008).

The indoor areas (private houses, work places and other confined areas) represent the main source of human exposure to these pollutants through oral ingestion and inhalation of particulate dust and matter. In such environments, the most volatile OPEs are found in the gas phase, whereas other OPEs are mainly associated with airborne particulate matter and dust (Reemtsma *et al.,* 2008).

4.2.3. Analytical methods

The development of an analytical method for trace analysis of OPEs is very difficult, since these compounds from a chemical point of view are very different among them. They have different physical-chemical properties from very polar and volatile to very hydrophobic and non-volatile compounds (Quintana *et al.,* 2008). Determination of OPEs in the environmental samples is normally obtained by extraction and concentration steps, sometimes followed by a clean-up procedure, with a final determination by gas chromatography (GC) or liquid chromatography coupled with mass spectrometry (LC-MS) (Quintana *et al.,* 2008).

Currently several methods for the analysis of OPEs in environmental and biological matrices were developed, the choice of samples preparation and determination method used, mainly depend on type of matrix.

Generally, the determination of OPEs in water samples is carried out by a concentration step followed by chromatographic analysis. The sample preparation techniques most commonly employed are the liquid-liquid extraction (LLE) (Andresen and Bester, 2006) and the solid-phase extraction (SPE) (Rodil *et al.,* 2005; Rodriguez *et al.,* 2006; Bacaloni *et al.,* 2007). Several SPE sorbents have been used for the dermination of OPEs in water, including disks (DVB-hydrophobic Speedisks) (Meyer and Bester, 2004) and cartridges (C18) (Knepper *et al.,* 1999) and divinylbenzene (DVB) polymers (Hydrophilic DVB polymer, Baker bond Speedisk, Bond Elut PPL and Oasis HLB) (Rodil *et al.,* 2005, Rodriguez *et al.,* 2006; Bacaloni *et al.,* 2007). Most of these sorbents provide satisfactory recoveries from filtered surface water and wastewater samples (0.1–5 L). Elution can be obtained by different solvents, the choice of the solvent depends on the determination technique and the target analytes, including acetone, ethylacetate, methanol and acetonitrile.

Unfortunately, there are some drawbacks in SPE procedure, the samples with suspended matter cannot be extracted without filtration and during

concentration step the OPEs can be lost, ue to their volatility (Martinez-Carballo *et al.,* 2007, Bacaloni *et al.,* 2007).

Although SPE and LLE are the most used techniques for determination of OPEs in water samples, recently, the microextraction techniques play an important role in the determination of OPEs, becose they present many advantages (, reduction of organic solvents and improvement in extraction selectivity)(Quintana *et al.,* 2006a). Solid-phase microextraction (SPME), membrane-assisted solvent extraction (MASE) (Quintana *et al.,* 2006b) and dispersive liquid-liquid microextraction (DLLME) (Garcia-Lopez *et al.,* 2007) have been successfully applied to the determination of OPEs in water samples.

Regarding the sample preparation methodologies for the determination of OPEs in solid matrices are usually, analysed by extraction with medium polarity solvents (edichloromethane, ethyl acetate and acetone), followed by one or more clean-up steps. The extraction efficiency of process can be enhanced using high pressures, high temperatures or a large number of extraction cycles. Quintana et al. reported selective isolation of OPEs from urban dust using pressurized liquid extraction (PLE) (Quintana *et al.,* 2007).

GC is the most common technique in the determination of triesters, because the most OPEs are sufficiently volatile, and GC coupled with selective detector, as NPD or MS, provides good selectivity and sensitivity. The chromatographic separation of OPEs can be achieved by a DB-5 (95% methyl, 5% phenyl polysiloxane) capillary column, with the exception of TBEP and TPhP (Quintana *et al.,* 2008).

Mostly, detection is performed with NPD (Garcia-Lopez *et al.,* 2007; Staaf and Östman, 2005; Marklund*et al.,* 2005) and MS (by electron impact ionization; EI) (Marklund *et al.,* 2005; Hartmann *et al.*, 2004; Carlsson *et al.,* 1997). GC-EI-MS suffers from unfavorable fragmentations, in particular for the aliphatic triesters. For this reason, GC-NPD is employed for routine

operation, while GC-EI-MS is commonly used as a confirmation technique, (Quintana *et al.,* 2008).

The use of LC-MS for the determination of OPEs is less frequently because most of the phosphoric-acid triesters are analyzed by GC-MS. An LC-MS method was first developed for the determination of nine trialkyl and triaryl phosphates in human blood samples (Amini and Crescenzi, 2003). In this study, atmospheric pressure chemical ionization (APCI) in positive mode was prefered over electrospray ionization (ESI) because matrix effects were reduced. Successively, a method for the determination of triesters by LC-MS/MS from aqueous samples was published in 2005 (Rodil *et al.,* 2005).

In this study nine trimesters, two bisarylphosphate flame retardants and TPPO were analysed by ESI in positive ionization mode.

Direct determination from aqueous samples was possible for concentrations around 1 μg L^{-1}, thus providing a fast screening procedure for many wastewater samples. This sensitivity is sufficient for the most widely used triesters in untreated municipal wastewaters. Furthermore, some triesters, TCEP and TCPP, were detected by direct injection in effluent of wastewater treatment plant.

However, considering the information above mention, it is clear that GC-NPD will remain the favourite techniques for the determination of phosphoric acid triesters in water and air samples. However, lower limits of determination, higher selectivity and feasibility to determine tri-alkyl OPEs, as well as diesters and monoesters, are the major advantages of LC-MS/MS versus GC based techniques (Quintana *et al.,* 2008).

4.3. Results and Discussion

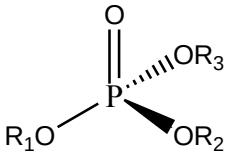
The OPEs are generally utilized as flame retardants and plasticizers. The broad application range of OPEs and the fact that they are utilized as additives may result in their diffusive spreading into the environment by leaching, volatilization and abrasion. The toxicity studies for these compounds have shown evidence of neurotoxicity and carcinogenicity (WHOEHC, 2000) The OPEs can be transferred to STPs by the sewage system of households, industrial sites, and drainage of storm water (Marklund *et al.,* 2005). These compounds show limited biodegradation at STPs andthey can be accumulated in sewage sludge (Olfsson *et al.,* 2013). Nowadays, the sewage sludges are utilized as a fertilizer in agriculture fields (Macherius *et al.,* 2012) and consequently the OPEs can migrate from sludge to vegetables and consequently they may be introduced in the human food. For these reasons, it is very important know the concentration, fate and toxicity of this substance in sewage sludges. In collaboration with *Laboratory of Analytical Chemistry of the University of Santiago de Compostela* an innovative analytical method for the determination of eight OPEs in sludge samples was developed. For the first time, we assess the performance of liquid chromatography (LC) quadrupole time-of-flight (QTOF) mass spectrometry (MS) for the selective quantification of OPEs, used as plasticizers and flame retardant additives, in sludge from urban sewage treatment plants. Moreover, the usefulness of accurate, full scan MS and MS/MS spectra to screen and to confirm thepresence of additional OPEs, without using reference standards, in sludge samples is discussed. Matrix solid-phase dispersion (MSPD) was used as a sample preparation technique.

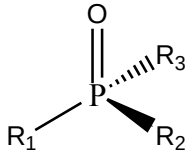
The study was organized in following steps:

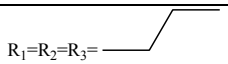
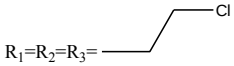
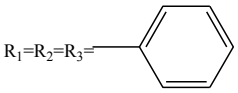
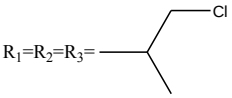
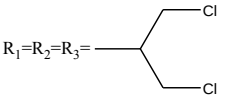
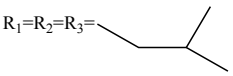
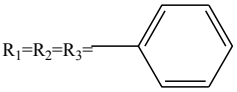
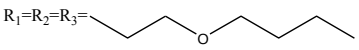
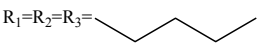
1. Development of a method LC-QTOF-MS for the specific and sensitive detection of selected analytes;

2. Optimization of MSPD conditions to obtain clear extracts and quantitative recoveries for OPEs;
3. Validation method for different sludge samples (primary and biological sludges);
4. Application of the developed method to real samples;
5. Post-target-screening of additional OPEs.

Table 4.2. *Structures and physical-chemical data of selected OPEs. (A) fosforic acid and (B) triphenylphosphine oxide*

(A) 

(B) 

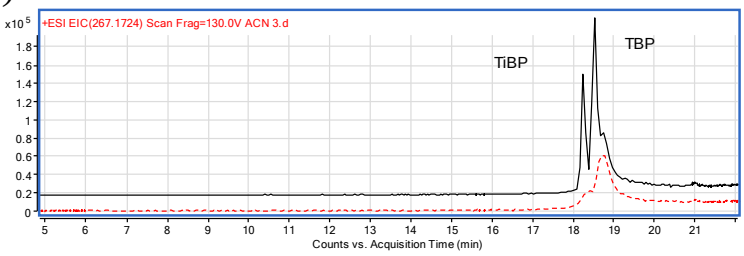
OPEs	Compound name	MW	Sobstituents
TPrP[a]	Tripropylphoshate	224.12	
TCEP	Tris (2-chloroethyl) phosphate	283.95	
TPPO	Triphenylphosphine oxide	278.09	
TCPP	Tri (cloroisopropyl) phosphate	326.01	
TDCP	Tri (dicloroisopropyl) phosphate	429.88	
TiBP	Tri-iso-butyl phosphate	266.16	
TPP	Triphenyl phosphate	326.07	
TBEP	Tributoxyethyl phosphate	398.24	
TnBP	Tri-n-butyl phosphate	266.16	

4.3.1. LC-QTOF-MS analysis

LC conditions were optimized to achieve the best possible resolution between the two isomeric OPEs, TiBP and TBP. In addition to the mobile phase gradient, the peak shapes of OPEs and the resolution between TiBP and TBP were affected by the injection solvent. Fronting peaks for the earlier eluting compounds (TCEP and TPPO) and co-elution of tributylphosphate isomers were observed for standards in MeCN; thus, an MeCN/H_2O 1:1, v/v, mixture was selected as injection solution. ESI and MS/MS parameters were evaluated in order to i) maximize the responses for the $[M+H]^+$ ion of each OPEs and ii) obtain, at least, two intense product ions in their MS/MS spectra. Optimal LC–QTOF-MS determination conditions are compiled in Table 4.3.

The LC–MS extracted chromatogram of s for a standard solution (50 ng mL^{-1}) of tributyl phosphates (m/z 267.1724) displayed a significant baseline disturbance at the retention time of TBP, which was also noticed in the LC–MS/MS mode (Figure 4.1).

Figure 4.1. *Overlay of LC-MS chromatograms (mass window 20 ppm) for a 50 ng mL^{-1} standard solution (continuous line) and a simulated injection (dotted line)*



Such a disturbance was observed even for simulated injections of empty vessels (Figure 4.1), and it was noticed with different LC columns. Replacement of ACN by methanol and formic acid by ammonium acetate as organic mobile phase and modifier, respectively, did not overcome the problem. Thus, the origin of TBP contamination was attributed to ultrapure

water, as previously reported (Matuszewski *et al.,* 2003). However, the intensity of the TBP contamination remained mostly unchanged after passing the mobile aqueous phase through a C18 solid-phase extraction (SPE) membrane and varied only slightly among different ultrapure water samples. Thus, leaching of TBP from plastic components (e.g. pipes between phase reservoir sand pumps) in the LC system cannot be excluded. Although identificationof the TBP contamination source requires a deeper study, a small column, placed before the injection valve, might serve to retainTBP coming from LC pipes and/or pumps.

The instrumental quantification limits (IQLs) of the LC–QTOF-MS system, operated in the MS/MS mode, were established as the concentration of each compound providing a peak area 10 times higher than the standard deviation of the chromatographic baseline for an injection blank. They varied between 0.5 and 4 ng mL^{-1}, except in the case of TBP (IQLs 15 ng mL^{-1}) (Table 4.3). These IQLs are 5–10 times higher than those previously reported for same compounds using a triple quadrupole LC–MS/MS system (Garcìa-Lòpez *et al.,* 2010). On the other hand, they remained below IQLs reported for GC–EI-MS (20–50 ng mL^{-1}) and GC–PICI-MS/MS (4–200 ng mL^{-1}) (Bergh *et al.,* 2010).

Linearity of LC–QTOF-MS responses was evaluated in the range of concentrations from 5 ng mL^{-1} (20 ng mL^{-1} for TBP) to1000 ngmL^{-1}, using TPrP as a IS (300 ng mL^{-1}). Determination coefficients (R^2) of the obtained graphs stayed above 0.994 (Table 4.3).

Table 4.3. *LC-MS/MS determination parameters, linearity and Instrumental quantification limits (IQLs) of the LC-QTOF instrument*

OPEs	Rt(min)	$[M+H]^+$ (m/z)	Quantification ion (m/z)	Other Product ion (m/z)	Collision energy (eV)	[a]Linearity R^2	IQLs (ng mL^{-1})
TCEP	7.72	284.9615	124.9995	98,9843; 160,9761	12	0.998	4
TPPO	10.55	279.0934	201.0934	173,0510; 77,0392	27	0.993	0.5
TCPP	14.30	327.0086	98.9843	174,9919; 250,9997	8	0.999	3
TDCP	17.93	430.8884	98.9843	208,9527; 320,9187	15	0.999	3
TiBP	18.36	267.1724	98.9846	155,0446; 211,1091	10	0.994	5
TPP	18.56	327.0781	215.0254	153,0690; 77,0392	30	0.995	2
TnBP	18.58	267.1724	98.9846	155,0446; 211,1091	10	0.998	15
TBEP	19.24	399.2514	199.0723	98,9845; 143,0102	27	0.996	4
TPrP (IS)	12.21	225.1252	98.9843	141.0310; 183.0789	12	-	-

[a]Linearity (5-1000 ng mL^{-1})

4.3.2. Optimization of MSPD procedure

Starting MSPD conditions were adopted from previous studies dealing with emerging contaminants extraction from sludge (Capriotti *et al.,* 2013; Negreira *et al.,* 2005).

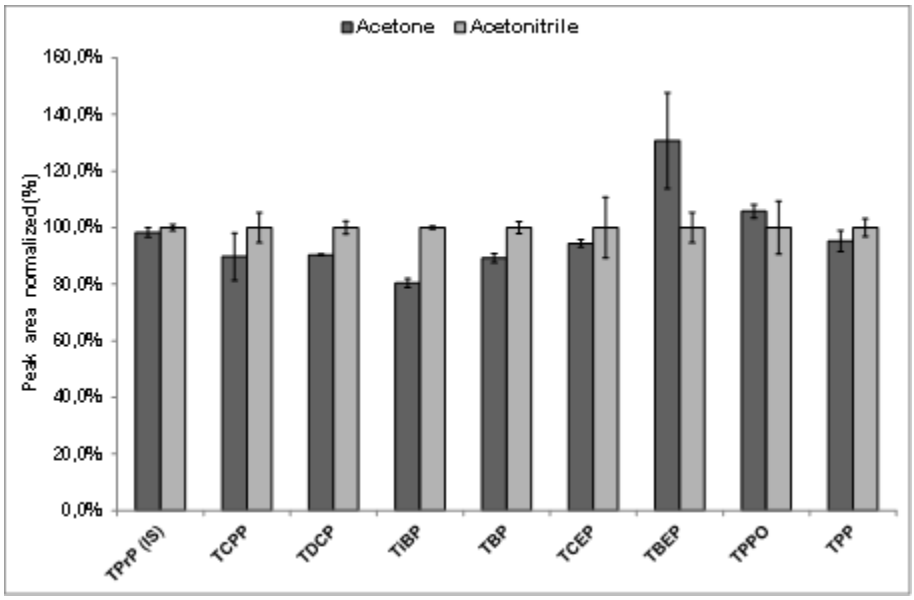
In brief, lyophilized samples (0.5 g) were dispersed with 2 g of diatomaceous earth and loaded into a polypropylene syringe containing PSA (1 g) followed by graphitized carbon (0.25 g), as clean-up sorbents. Acetonitrile and acetone (25 mL) were selected as elution solvents based on their affinity for OPEs (Garcìa *et al.*, 2007). Extracts in acetonitrile were concentrated to 1 mL and diluted with1 mL of ultrapure water before LC–QTOF-MS analysis. Those in acetone were evaporated to dryness and reconstituted with 2 m of MeCN/H_2O 1:1, v/v.

Under above conditions, TPP could not be recovered from the spiked pooled sludge matrix. Removal of the carbon clean-up layer overcame the above problem, at the expense of increasing the visual complexity (color) of the extracts. Thus, the potential benefit of introducing a washing step in the extraction protocol was evaluated (Campone *et al.,* 2010; Canosa *et al.,* 2007). To this end, MSPD cartridges were first rinsed with 10 mL of n-hexane, and then analytes eluted using either acetone or acetonitrile. Rinsing and elution fractions were injected in the LC–Q-TOF-MS system after solvent exchange when required. Although OPEs were not eluted in the rising fraction, this extra step exerted a minor improvement in the selectivity of the extraction; furthermore, exhaustive drying of the MSPD syringe was required after n-hexane rinsing and before acetonitrile elution due to the immiscibility of both solvents. Therefore, neither the washing step nor the carbon clean-up layer was included in the MSPD extraction protocol.

Comparison between the relative extraction efficiencies of acetone and acetonitrile revealed similar responses for most targeted OPEs (Figure 4.2); however, acetonitrile extracts displayed a less intense color than those in

acetone. Taking this observation into account, and considering that dryness evaporation was not required for acetonitrile extracts, this solvent was selected to continue with optimization of extraction conditions.

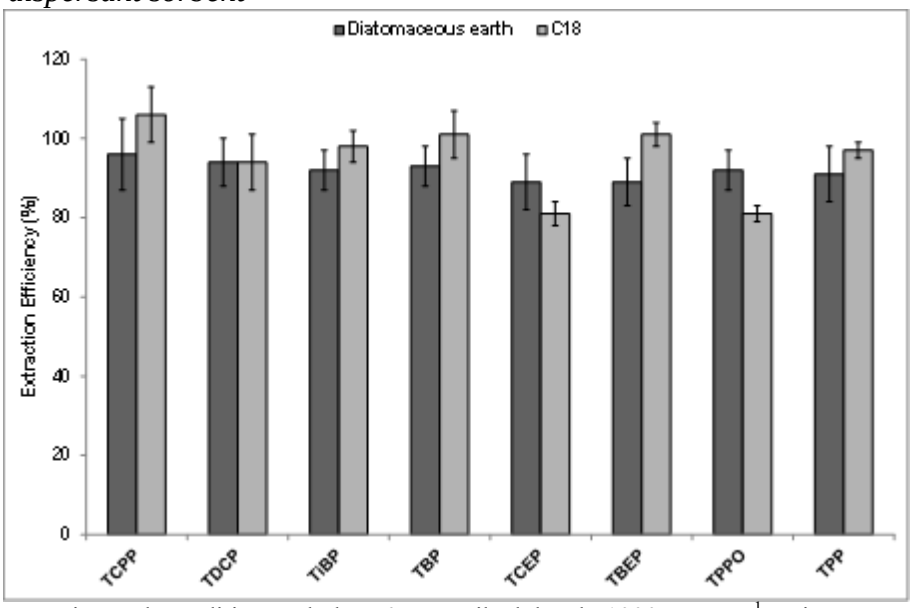
Figure 4.2. *Relative MSPD extraction efficiencyas function of the elution solvent*



Experimental conditions: sludge, 0.5g; spiked level, 1000 ng mL^{-1};Dispersant sorbent,2 gdiatomaceous earth; elutionsolventvolume, 15 mL; n = 4.

The effect of the dispersant in the performance of the extraction was assessed in terms of efficiency and selectivity. Diatomaceous earth, used in the initial extractions and regarded as an inert material allowing to mechanically disrupt the sample and to increase the surface of sludge in contact with the elution solvent, was compared to C18, the original dispersant reported by Barker et al. (Baker *et al.,* 1989) for MSPD. The alkyl chains in the C18 sorbent are supposed to solubilize and to retain certain components of the sludge matrix, improving the efficiency and the selectivity of the process (Baker *et al.,* 1989). As shown in Figure 4.3,equivalent extraction efficiencies, ranging from 89% to 105%, were obtained in both cases.

Figure 4.3. *Efficiency of MSPD extraction as function of the dispersant sorbent*



Experimental conditions: sludge, 0.5g; spiked level, 1000 ng mL^{-1}; Dispersant sorbent, 2 g; eluent solvent, 15 mLacetonitrile; n = 4.

Matrix effects (ME), calculated as defined in Section 4.5.5,varied from 82 to 126% for samples dispersed with diatomaceous earth; whereas, they stayed between 84 and 108% for C18 (Table 4.4).

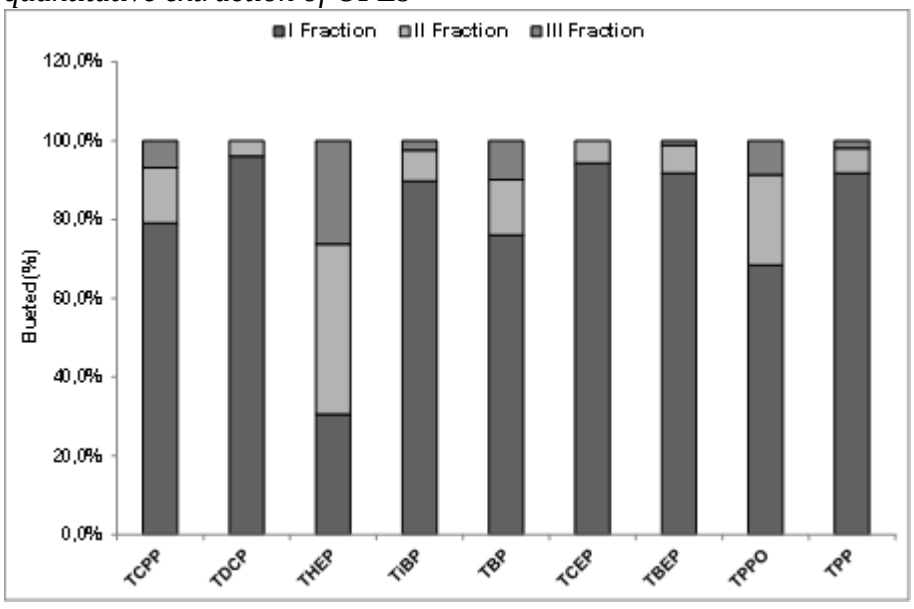
Table 4.4. *Evaluation of matrix effects (ME, %) as function of the dispersant sorbent*

OPE	ME (%) ± SD (n=6)	
	Diatomaceous earth	**C18**
TCEP	100 ± 2	104 ± 6
TPPO	110 ± 2	103 ± 6
TCPP	114 ± 9	92 ± 3
TDCP	96 ± 7	91 ± 4
TiBP	126 ± 5	93 ± 2
TPP	91 ± 5	84 ± 3
TBP	82 ± 10	99 ± 2
TBEP	126 ± 3	108 ± 2

Completely transparent extracts were obtained with C18, whereas, those from samples dispersed with diatomaceous earth displayed a pale yellowish appearance and a slight turbidity after water dilution, requiring a filtration step before injection in the LC–QTOF-MS system. Likely, in case of C18 dispersion some lipophilic components of sludge remained, within the MSPD syringe, trapped due to interactions with C18 chains, which resulted in cleaner extracts and lower matrix effects during ESI ionization process. Thus, C18 was used as a dispersant in further extractions.

Finally, the minimum volume of acetonitrile required for the quantitative extraction of targeted compounds was established by collecting consecutive fractions (5 mL each) from the MSPD cartridge (Figure 4.4). Above 75% of the responses measured for all targeted compounds corresponded to the first fraction; however, some compounds were still noticed in the third fraction. Thus, 15 mL was adopted as the working acetonitrile extraction volume.

Figure 4.4. *Minimum volume of acetonitrile required for the quantitative extraction of OPEs*



Experimental conditions: sludge, 0.5g; spiked level, 1000 ng mL^{-1}; Dispersant sorbent, 2 g C18; eluent solvent, acetonitrile 5 mL (x3); n = 4.

4.3.3. Analytical performance

The overall extraction efficiency (PE) of the optimized procedure was evaluated with primary and biological sludge samples. Providing that i) MSPD achieved quantitative extraction yields (Figure 4.3) and ii) the efficiency of ESI ionization underwent small variations between pure standards and sample extracts (Table 4.4), absolute recoveries were assessed against standard solutions, prepared in MeCN/H_2O 1:1, v/v. For each sludge sample, unspiked (n=3) and spiked (n=4) fractions, at two different concentration levels, were processed. The attained overall recoveries are compiled in Table 4.5.

Table 4.5.*Overall recoveries of the developed method, limits of quantification (MQLs, ngg^{-1}) of the analytical procedure referred to lyophilized sludge.*

	Recoveries (%) ± SD				
OPEs	**Primary sludge**		**Biological sludge**		**MQLs (ng g^{-1})**
	1000 ng g^{-1}[a]	300 ng g^{-1}[a]	1000 ng g^{-1}[a]	100 ng g^{-1}[a]	
TCEP	86 ± 3	101 ± 6	92 ± 3	95 ± 9	16
TPPO	94 ± 2	85 ± 4	95 ± 3	88 ± 2	2
TCPP	87 ± 7	93 ± 12	87 ± 7	123 ± 5	12
TDCP	70 ± 5	113 ± 7	82 ± 1	102 ± 6	12
TiBP	96 ± 4	111 ± 3	89 ± 1	85 ± 4	18
TPP	76 ± 2	81 ± 3	84 ± 2	69 ± 3	6
TBP	96 ± 5	83 ± 1	100 ± 2	96 ± 3	50
TBEP	109 ± 5	117 ± 13	110 ± 7	n.d.[b]	18

[a]spike level; [b] Not evaluated

In the case of primary sludge, they varied from 70%, for TDCP, to 117%, for TBEP, with SDs remaining below 13. For biological sludge, recoveries ranged from 69%, for TPP, to 123%, for TCPP, with SDs below 9. For this latter matrix, the recovery for TBEP at the lower spiked level (100 ng g^{-1}) could not be evaluated since its native concentration in the matrix (around 1800 ng g^{-1}) was significantly higher than the added level. Recoveries compiled in Table 4.5 are better than those reported by Chen and Bester (Chen and Bester, 2009) (from 57 to 96%) for same compounds, considering PLE extraction followed

by a multistep clean-up approach, requiring around 200 mL of different organic solvents per sample, versus 15 mL used in this research.

The limits of quantification (MQLs) of the overall method varied from 2 ng g^{-1}, for TPPO, up to 50 ng g^{-1}, for TBP (Table 4.5). Procedural blanks did not contain traces of OPEs, with the exception of the already commented contamination problem for TBP, which cannot be attributed to the sample preparation process. Thus, for the rest of OPEs, the attained MQLs were controlled by sensitivity of the LC–QTOF-MS instrument, the sample intake and the final extract volume. In the previous studies, the achieved MQLs varied from 10 ng g^{-1}, for TPP, to 100 ng g^{-1}, for TCPP, using PLE followed by GC–MS (Chen and Bester, 2009). Following a very similar methodology, Marklund et al. (Marklund *et al,* 2005) calculated LOQs in the range of values from 0.5 to 15 ng g^{-1}; nevertheless, they highlighted the presence of TBP and TiBP at the 20 ng g^{-1} level in procedural blanks.

4.3.4. Real samples quantification

Table 4.6 summarizes the concentrations of targeted OPEs in 11 freeze-dried sludge samples and a reference material of the same matrix (BCR-088). With regards to sludge obtained from STPs (codes1-11) located in Galicia (Spain), TCPP, TBEP and TPP were quantified in all samples, withmaximum levels above 1000 ng g^{-1} for the first two congeners. Inthe case of TPP, the measured concentrations remained below150 ng g^{-1}. Their arithmetic mean concentrations (sample codes 1-11) were 758 ± 379 ng g^{-1} (TCPP), 744 ± 437 ng g^{-1} (TBEP) and 67 ± 30 ng g^{-1} (TPP), which are similar to the levels reported in sludge samples from Sweden (n = 11 STPs) (Marklund *et al,* 2005). On the other hand, the mean concentration of TCPP is lower than 5000 ng g^{-1}, reported as the average value of this flame retardant in sludge from several (n = 20) German STPs (Bester, 2005). The BCR-088 sludge material contained similar levels of most OPEs to the rest of samples compiled in Table

4.6. The exception was TCEP, which was present at higher level in BCR-088. The concentrations of TCPP, TBEP and TPP in samples from years 2005 to 2010 (codes 1-6) were similar to levels measured in sludges collected in 2013 (codes 7-11). However, TDCP and TiBP were more frequently detected in the latter group of samples (Table 4.6). This trend, which requires additional confirmation, might be a consequence of the phase out of other flame retardants, such as PBDEs, and agrees with the proposed increase in OPEs consumption (van derVeen and de Boer, 2012; Dodson *et al.,* 1989).

Table 4.6. *Concentration (ng g^{-1}) of targeted OPEs in freeze-dried sludge samples, n= 3 replicates*

Code	Type	Year	Concentration (ng g^{-1}) ± SD							
			TCEP	**TPPO**	**TCPP**	**TDCP**	**TiBP**	**TPP**	**TBP**	**TBEP**
1	P.S.	2005	n.d.	n.d.	1184 ± 83	n.d.	n.d.	54 ± 1	n.d.	909 ± 45
2	B.S.	2005	n.d.	n.d.	396 ± 36	n.d.	137 ± 3	83 ± 2	n.d.	1786 ± 36
3	P.S.	2010	n.d.	3 ± 1	700 ± 200	32 ± 6	n.d.	66 ± 13	n.d.	810 ± 110
4	B.S.	2010	n.d.	n.d.	780 ± 180	n.d.	n.d.	52 ± 3	n.d.	527 ± 16
5	B.S.	2010	n.d.	n.d.	583 ± 35	n.d.	n.d.	54 ± 5	n.d.	213 ± 19
6	B.S.	2010	n.d.	n.d.	270 ± 35	n.d.	n.d.	47 ± 2	n.d.	516 ± 19
7	M.S.	2013	22 ± 1	20 ± 0.1	381 ± 14	25 ± 3	115 ± 14	58 ± 1	n.d.	1200 ± 250
8	M.S.	2013	n.d.	21 ± 0.1	919 ± 38	13 ± 1	58 ± 2	38 ± 3	n.d.	736 ± 45
9	M.S.	2013	n.d.	n.d.	888 ± 77	n.d.	49 ± 6	86 ± 10	n.d.	391 ± 16
10	M.S.	2013	n.d.	n.d.	670 ± 80	n.d.	41 ± 36	50 ± 6	n.d.	562 ± 6
11	M.S.	2013	n.d.	22 ± 0.2	1570 ± 80	40 ± 6	55 ± 6	144 ± 6	n.d.	532 ± 32
12	BCR-088		1650 ± 150	6 ± 1	517 ± 15	n.d.	48 ± 2	117 ± 26	124 ± 3	800 ± 48

n.d., not detected; P.S., primary sludge; B.S., biological sludge; M.S., mixture of primary and biological sludge.

4.3.5. Post-target screening of additional OPEs

In addition to the product ion (MS/MS) spectra of preselected targeted OPEs, the LC–QTOF-MS instrument acquires and records full scan, accurate MS spectra throughout LC chromatograms. These spectra allow searching for additional pollutants, not included in the quantitative procedure, providing that they are co-extracted from sludge together with target analytes. Hence, this latent information can be useful to detect the use and potential accumulation of novel OPEs in the previously processed sludge samples. Tentative identifications derived from this post-target strategy require additional confirmation, using product ion scanMS/MS spectra (which are obtained in a 2nd injection, preferably considering different collision energies), and/or retention time comparison with pure standards, when available (Diaz *et al.,* 2013).

In order to assess the reliability of this strategy, a database (Table 4.7) with the empirical formulae and the exact molecular weights of nine OPEs, non-included in the quantitative method but previously reported in environmental samples (Bergh *et al.,* 2012; Stapleton *et al.*, 2012; Dodson *et al.,* 2012) was built. It is highlighted that reference standards of these compounds were not injected in the LC–QTOF-MS system.

Table 4.7. *Database of OPEs investigated in sludge using a post-target screening strategy*

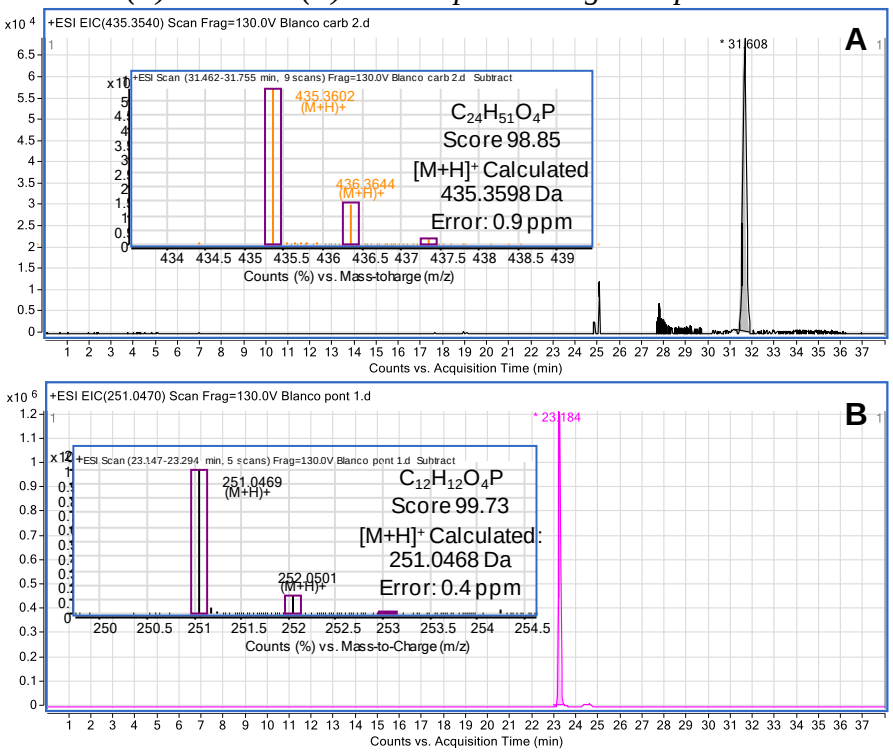
Name	Code	Formula	Exact MW
Tri(4-butylphenyl) phosphate	TTBPP	$C_{30}H_{39}O_4P$	494.2586
Tri(4-methylphenyl) phosphate	TMPP	$C_{21}H_{21}O_4P$	368.1177
Tri(2,3-dibromopropyl) phosphate	TDBPP	$C_9H_{15}BrO_4P$	691.5808
2-ethylhexyl-diphenyl phosphate	EHDPP	$C_{20}H_{27}O_4P$	362.1647
Tripentylphosphate	TPP	$C_{15}H_{33}O_4P$	308.2116
Trihexylphosphate	THP	$C_{18}H_{39}O_4P$	350.2586
Diethylhexylphosphate	DEHP	$C_{16}H_{35}O_4P$	322.2273
Diphenylphosphate	DPP	$C_{12}H_{11}O_4P$	250.0395
Tri(2-ethylhexyl) phosphate	TEHP	$C_{24}H_{51}O_4P$	434.3525

The Mass Hunter software was used to search for their $[M+H]^+$ ions (automated search of sodium and ammonium adducts is also possible) in the LC–MS chromatograms of samples compiled in Table 4.6, within a mass interval of 10 ppm around their theoretical values. This software extracts the accurate LC–MS chromatograms and compares the experimental MS spectra of detected peaks with the theoretical (calculated) ones. Then, a normalized score (0–100), which combines mass accuracy, isotopic pattern and spacing among ions in the $[M+H]^+$ cluster, is calculated. A score of 100 represents a perfect match between the empirical and the theoretical spectrum.

LC–MS chromatograms for all samples compiled in Table 4.6 contained a well-defined peak at m/z 435.3598 Da (retention time31.61 min), and half of them showed also a signal at m/z251.0468 Da (retention time 23.18 min). The MS spectra of both peaks fitted (calculated scores above 95%) with the theoretical ones of TEHP and DPP, respectively. Figure 4.5 shows the extracted ion LC–MS chromatograms and the experimental MS spectra (average peak spectrum after background correction) for both peaks in unspiked sludge samples. The superposed boxes represent the calculated spectra of TEHP and DPP (Figure 4.5). Differences between calculated and experimental masses of the most intense ion in MS spectra remained below 1 ppm in both cases.

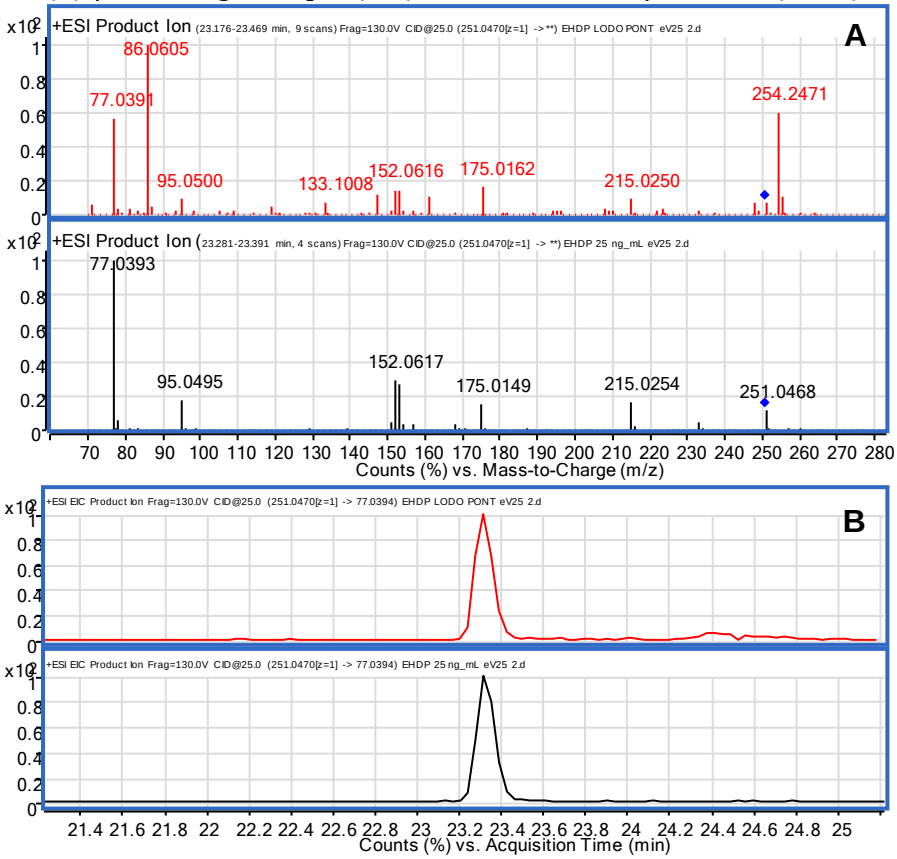
The identity of TEHP was confirmed from its experimental MS/MS spectrum, and by injection of a pure standard of this compound. The MS/MS spectrum of the peak at 23.18 min was also coherent with the structure of DPP; however, its retention time did not agree with the relatively high polarity, and thus, poor retention expected for DPP in C18 LC columns (Gàrcìa-Lòpez*et al.,* 2010). In fact, the retention time for a pure standard of DPP, under conditions reported in section 4.5.4, turned to be 4.2 min.

Figure 4.5.*Extracted LC–MS chromatograms and detail of empirical MS spectra for compounds tentatively identified as TEHP (A) and DPP (B) in non-spiked sludge samples*



The second possibility was that the peak at 23.18 corresponds to EHDPP, assuming that during ESI ionization the 2-ethylhexyl moiety (C_8H_{16}) is replaced by one atom of hydrogen. In such a case, the MS spectrum of EHDPP will render the $[M+H-C_8H_{16}]^+$ ion ($C_{12}H_{12}O_4P$, 251.0468 Da), instead of the $[M+H]^+$ one ($C_{20}H_{28}O_4P$, 363.1720 Da). This second hypothesis was confirmed with the MS/MS spectrum and the retention time for a standard solution of EHDPP (Figure 4.6).

Figure 4.6. *MS/MS spectra (A) and LC-MS/MS chromatograms (B) for a sludge sample (red) and a standard of EHDPP (black)*



Although the performance of the MSPD procedure was not validated for TEHP and EHDPP, a semi-quantitative evaluation of their levels in sludge was performed assuming that, as occurred for the targeted OPEs, quantitative recoveries are attained for both compounds and that their ESI ionization efficiencies were similar between standards and sludge extracts. Under these considerations, TEHP levels ranged between 20 and 100 ng g^{-1}, whereas EHDPP varied from not detected up to 30 ng g^{-1}.

4.4. Conclusion

LC–QTOF-MS, in the MS/MS mode, provides instrumental LOQ slow enough to allow the quantification of eight OPEs in sludge from urban STPs, with accurate product ion spectra permitting the unambiguous identification of these targeted OPEs. When LC–QTOF-MS detection is combined with the mild extraction conditions employed in the optimized MSPD method, quantitative recoveries and limited ESI matrix effects were observed. Consequently, targeted analytes could be quantified by comparison against pure standard solutions. Another significant advantage of the described procedure is the reduction in the consumption of organic solvents versus previously published methodologies.

Accurate full scan MS spectra, provided by the LC–QTOF-MS instrument, render valuable clues to investigate the presence of additional OPEs, not considered during method development, in real samples. However, preliminary identifications derived from this post-target screening approach require additional confirmation using authentic standards, since some OPEs might undergo insource fragmentation and then, their molecular ions are not obtained.

4.5. Experimental

4.5.1. Standards and materials

Standards of TPrP (internal standard, IS), TiBP, TBP, TCEP, TDCP, TBEP, TPP and TPPO were obtained from Sigma–Aldrich (Milwaukee, WI, USA). TCPP, as technical mixture of isomers, was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Full names of these targeted analytes are compiled in Table 4.2. Tri-(2-ethylhexyl) phosphate (TEHP), 2-ethylhexyl-diphenyl phosphate (EHDPP) and diphenylphosphate (DPP) standards, also purchased from Sigma–Aldrich, were used to confirm their tentative identification in sludge, derived from accurate MS and MS/MS spectra (post-target analysis). However, they were not considered during the optimization of the quantitative analytical procedure. Individual standards of each compound were prepared in methanol and stored at -20°C. Diluted solutions and mixtures of OPEs were made in acetonitrile and acetone. Calibration standards, containing increasing concentrations of eight targeted OPEs and a fixed amount of the IS (300 ng mL^{-1}), were prepared in MeCN/H_2O 1:1, v/v, and used for a maximum of one week.

Formic acid, acetonitrile (HPLC gradient quality), n-hexane and acetone (trace analysis grade) were supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained in the laboratory from a Milli-Q Gradient A-10 system (Millipore, Billerica, MA, USA).

Diatomaceous earth and the C18 sorbent were provided by Sigma–Aldrich. Silica bonded to ethylenediamine-N-propyl groups (PSA) and graphitized carbon were purchased from Supelco (Bellefonte, PA, USA). All sorbents were employed as received, without any further clean-up. Empty polypropylene syringes (15 mL capacity) and 20 μm polyethylene frits were acquired fromInternational Sorbent Technology (Mid Glamorgan, UK).

4.5.2. Sludge samples

Non-digested sludge samples (primary, biological and mixturesof both) were obtained from different STPs located in Galicia (Northwest Spain). After reception at the laboratory, they were maintained at -20°C and lyophilized at the beginning of this study. Freeze-dried samples were stored, at 4°C, in amber glass vessels. Their total carbon and nitrogen contents varied between 20–40% and 2–7%, respectively. A reference material of sludge, BCR-088, was purchased from the Institute for Reference Materials and Measurements (Geel, Belgium).

4.5.3. MSPD procedure

MSPD conditions were optimized with a pool of primary and biological sludges (TOC 30%) fortified with targeted OPEs at the1000 ng g^{-1} level. Spiked samples were prepared by mixing an accurately weighed amount of sludge with a standard solution of OPEs in acetone. The slurry was manually blended and left in the hood for 2 days (protected from direct exposure to sun light) in order to allow acetone removal. The spiked samples were stored for 5–6 days, at 4°C, before extraction.

Freeze-dried sludge samples (0.5 g) were mixed and dispersed with 2 g of C18 in a glass mortar, with a pestle, for 5 min. Then, the blend was transferred to a polypropylene syringe containing 1 g of PSA as clean-up sorbent. Analytes were recovered passing 15 mL of acetonitrile through the packed syringe. After the addition of TPrP (IS), the extract was evaporated (a gentle stream of nitrogen at room temperature was used) and adjusted to a final volume of 1 mL. The concentrated acetonitrile extracts were diluted with ultrapure water (1:1) before injection in the LC–QTOF-MS system.

4.5.4. LC-MS/MS analysis

Compounds were determined using a LC–ESI–QTOF-MS system acquired from Agilent (Wilmington, DE, USA). The LC instrument was an Agilent 1200 Series, consisting of an autosampler, two isocratic high pressure mixing pumps, a vacuum degasser unit and a chromatographic oven. The QTOF mass spectrometer was an Agilent 6520 model, furnished with a Dual-Spray ESI source. Compounds were separated in a Luna C18 column (100 mm x 2 mm, 3 mm) acquired from Phenomenex (Torrance, CA, USA) and connected to a C18 (4 mm x 2 mm) guard cartridge from the same supplier. Ultrapure water (A) and acetonitrile (B), both 0.1% in formic acid, were used as mobile phases applying the following gradient: 0–2 min, 35% B; 17 min, 85% B; 18–30 min, 100% B; 31–38 min, 35% B. The mobile phase flow was 0.2 mL min^{-1}, the injection volume for standards and sample extracts was 10 μL and the column temperature was set at 30°C.

Nitrogen (99.999%), provided by a high purity generator (ErreDue srl, Livorno, Italy), was used as nebulizing (35 psi) and drying gas (330°C, 10 L min^{-1}) in the ESI source. The QTOF instrument worked in the 2 GHz Extended Dynamic Range resolution mode (mass resolution 5000 at m/z values of 120) and compounds were ionized in positive ESI, applying a capillary voltage of 3500 V. A mass reference solution (Agilent calibration solution A) was continuously infused in the source of the QTOF system, through the second nebulizer, employing the ions with m/z 121.0509 (purine) and 922.0098 (HP-921) for recalibrating the mass axis. The Mass Hunter Workstation software was used to control the LC-ESI–QTOF-MS system and to process the obtained data.

Precursor ($[M+H]^+$) ions for targeted compounds were obtained using a fragmentor voltage of 130 V. Collision energies were optimized with the aim of generating several products from each precursor. Accurate product ion scan (MS/MS) spectra were acquired in the range of m/z values from 70 to 500

units, considering a time window of 3 min centered in the retention time of each analyte. Fullscan MS spectra (m/z range 100–1700 units) were simultaneously acquired to the MS/MS ones. Acquisition rates in MS and MS/MS modes were set at 1.4 spectra s^{-1}, with each spectrum being the combination of 9600 transients. Selective LC–MS and LC–MS/MS chromatograms were extracted with a mass window of 20 ppm around the $[M+H]^+$ and the most intense product ion of each OPEs, respectively. The MS/MS mode was employed for quantification purposes, whereas LC–MS chromatograms were used, in the post target analysis strategy, to screen the presence of nine additional OPEs in real-life sludge samples.

4.5.5. Matrix effect evaluation

Potential matrix effects (ME) occurring in the ESI source were calculated as follows:

$$ME = \frac{(A\ post\text{-}spike - A\ unspiked)}{A\ standard} \times 100$$

where A post-spike is the response (peak area without IS correction) measured for a targeted compound in the spiked MSPD extract from sludge, A unspiked is the response for the same compound in an unspiked MSPD extract of the same sludge, and finally, A standard is the response for a standard solution containing the same concentration of the analyte. Thus, ME values around 100% point out to little differences between the efficiency of ESI ionization for sludge extracts and standard solutions.

4.5.6. MSPD extraction efficiency and samples quantification

Recoveries, provided by the optimized MSPD method, were evaluated with individual samples of primary and biological sludges spiked at different concentration levels.

The yield of the MSPD extraction was calculated as the ratio between the IS corrected responses (analyte peak area/IS peak area) measured for spiked sludge samples and extracts from the same matrix fortified after the extraction step, multiplied by a factorof 100.

The overall recoveries (R) of the procedure were defined as follows:

$$R=\frac{(Cs-Cb)}{Ct} \times 100$$

Being Cs the concentration measured in the extract from a spiked sample, Cb is the concentration in the extract from a non-spiked fraction of the same sludge and Ct is the concentration added to the sample. Cs and Cb were determined using calibration curves obtained for standard solutions prepared in MeCN/H_2O 1:1, v/v. As discussed further, the MSPD procedure provided overall recoveries above 70% for eight targeted analytes; therefore, their levels in sludge were calculated by comparison with calibration solutions containing increasing concentrations of these OPEs (5–1000 ng mL^{-1}) and TPrP (300 ng mL^{-1}) as IS.

Chapter 5

Determination and measurement of illicit drugs in urban wastewater

5.1. Introduction

The use of drugs of abuse (DAs) is increasing worldwide; data provided by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) estimate that, in the last year, 22.5 million Europeans smoked cannabis, 12 million consumed cocaine, 12.5 million have tried amphetamines and 9.5 million used ecstasy (EMCDDA, 2011).

DAs enter in wastewater as unalterated drug and/or their active metabolites by human excretion after illegal consumption or by accidental or deliberate disposal from clandestine drug laboratories. They are released into surface waters because their removal during sewage treatments is often incomplete (Pedrouzo *et al.,* 2011b; Postigo *et al.,* 2010) and consequently they can even reached drinking water sources (Boleda *et al.,* 2011; Boleda et al., 2009).

In last decade, the determination of the concentration of DAs in environment (water, air, soil, sediment) has been used as an indirect tool to estimate the community level consumption of DAs. Moreover, the determination of DAs in the raw wastewater provided an objective and useful approach to estimate and monitor the consumption of DAs in different countries (Postigo *et al.,* 2010; Terzic *et al.,* 2010; Boleda *et al.,* 2009). This analytical approach, named *Sewage Epidemiology*, is based on the assumption that the concentration of drug residues in raw wastewater is proportional to the amount of drug consumed by the local population from which the wastewater originated (Zuccato *et al.,* 2005).

The widespread use of DAs causes not only a well-known serious social problem but also concern as environmental pollutants, and recently DAs and their metabolites have been identified as the latest group of emerging contaminants (Richardson, 2012). There are few available data on the presence of DAs in the environment, and although the environmental concentrations are not very high, they can potentially impact the human health and ecosystem

functioning by chronic low level exposure. The available data on the ecotoxicity of DAs in the literature are scarce and not systematic. Nowadays, only few reports are available on the ecotoxicity of amphetamine, cocaine, and morphine on aquatic organisms (Lilius *et al.,* 1994; Binelli *et al.,* 2012; Gagné *et al.,* 2006). The presence of DAs and their metabolites needs attention from an ecotoxicological point of view because their possible negative effect on aquatic organisms, biota and the ecosystem might be comparable with therapeutic drugs (van Nuijs *et al.,* 2011).

Knowledge of environmental occurrence and contamination levels of aqueous matrices, hence, is a topic of growing concern for ecological health and for estimate levels of community consumption.

In this study, a multi-residue method for the determination of eighteen analytes, corresponding to a wide range of DAs and their major metabolites, in wastewater samples has been optimized and validated.

The method is comprised of a selective SPE step with a mixed-mode sorbent (Oasis MCX), to concentrate and achieve higher selectivity and sensitivity for extracting basic compounds with cation-exchange mode, followed by UHPLC-MS/MS analysis with isotope dilution assay for the identification and the accurate quantification of DAs in raw wastewater.

In collaboration with "*Società Alto CaloreServizi S.p.A",* the developed method was applied to 24 h composite untreated wastewater samples collected from five different inlet sites of sewage treatment plant (STP) of the province of Avellino, each monitored for one week period. Subsequently, a sewage epidemiology approach, using levels of DA residues detected in wastewater, was applied to evaluate and monitor in near real time the collective use of the DAs in local community.

5.2. Drugs of abuse

The term ‘emerging pollutants’ has been defined as substances that are not presently known to cause damage in environmental compartments but have characteristics such as the persistence in the environment, toxicity and ability to bioaccumulate that suggest that they could adversely affect the ecosystem (Boles and Wells, 2010). DAs (both synthetic drugs and plant derived) are the latest group of emerging contaminants identified in the aquatic environment demanding attention (Boleda *et al.,* 2009; Kasprzyk-Hordern *et al.,* 2010).

DAs are those for which nonmedical use is prohibited by the national and international laws (Hall *et al.,* 2008). DAs fall into the categories of cocaine opiates, amphetamine-like compounds, cannabinoids, and LSD (Hall *et al.,* 2008; UNODC, 2007). The methods of administration of DAs are generally oral, intranasal, by needle injection, or by inhaling smoke. The environment is the ultimate destination for all these compounds: a large proportion of drugs is excreted unchanged and/or as metabolites in human urine and feces and are released to the environment by wastewater (EMCDDA, 2008; NIDA, 2008). Furthermore, they enter into environmental compartments by accidental or deliberate disposal of illicit drugs and associated compounds.

The most of DAs and their metabolites are very polar and therefore, they are not easily absorbed to soils and sediments, consequently they may come into surface or ground waters (Pal *et al.,* 2013). Whereas, cannabinoids are highly hydrophobic and they were detected in sewage sludge, istead methamphetamine was found to be relatively persistent with half-life in soil up to 502 days. Generally, water compartments are the most susceptible environmental matrices for contamination by DAs (Pal *et al.,* 2013).

DAs group of environmental concern includes not only the substances used as illicit drugs, but also their human metabolites. The latter in many cases are more abundant than the parent compounds.

It is reported that in urine, a large proportion of cocaine (80%) can be accounted in the form of benzoylecgonine (35-54%) and ecgonine methyl ester (32–49%) and only 1-9 % of a single cocaine dose is excreted in urine unchanged. Heroin is primarily excreted as morphine (42%) and 6-acetylmorphine (1.3%), the latter is its minor but exclusive metabolite (Baselt, 2008). About 70 % of a dose of Δ9-tetrahydrocannabinol (THC) is excreted within 72 h in feces and urine mainly as metabolites (11-carboxy-THC and 11-hydroxy-THC), while unchanged form is present only in traces in urine. Lysergic acid diethylamide (LSD) is excreted as 2-oxo-3-hydroxy-LSD in urine (20%) and in feces (80%). Methadone is mainly excreted in urine as 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, (50%). Amphetamine group compounds are primarily excreted as the unchanged drug (methamphetamine, unaltered 43–62%; amphetamine, unaltered 30–40%; MDMA, unaltered 65%) (Boles and Wells, 2010), but the excretion rate can change with the urine pH of users, route of intake and dose (van Nuijs *et al.,* 2011). Although methamphetamine and amphetamine are excreted mainly as unchanged drugs, metabolites of these drugs are also excreted and several additional metabolites may form during sewage treatment processes (Boles and Wells, 2010).

5.2.1. Occurrence

The presence of DAs (parent drugs and their metabolites) in aquatic environments is of significant interest to environmental chemistry. Unlike legal drugs, comprehensive information on the presence of illicit drugs in the aquatic systems is still very scarce. Nowadays, a number of studies have revealed the presence of illicit drugs in wastewaters in different countries of the world (Pal *et al,* 2013).

The literatures indicate that ecgonine methyl ester, benzoylecgonine, methamphetamine, MDMA, amphetamine and morphine are the most

abundant residues in wastewater and the levels of DAs and their metabolites are in the μg L^{-1}-ng L^{-1} range.

According to literature data on the occurrence in wastewater, the cocaine and its major metabolite benzoylecgonine were found in higher concentrations in Italy, Spain, and Switzerland, while the lowest concentrations were recorded for USA, Australia, and France (Pal *et al,* 2013). Morphine was reported at relatively high concentrations in both influents and effluents from Switzerland. The high morphine concentration in the sewage treatment plants of Switzerland cannot be interpreted directly as heavy consumption of heroin, as the usage of opiate alkaloids in pain management treatments, cough suppression preparations, over the counter analgesics and poppy seeds used in bakery products might have been contributed (Berset *et al.,* 2010). In line with cocainics and opiates, the higher concentrations of amphetamine in influents were reported from Spain and UK, methamphetamine from USA, and MDMA from Spain (Bijlsma *et al.*, 2009). As regard LSD and its metabolite, 2-oxo-3-hydroxy-LSD, absence or very low concentrations have been found in influent samples (Huerta-Fontela *et al.,* 2007; Postigo *et al.,* 2010). Results agree with the low doses needed to produce an effect compared to those needed for other drugs (μg vs mg), since LSD is the most potent psychoactive drug known so far. The presence of THC in sewage waters has been observed to be less significant than that of its metabolites due to its extensive metabolization before excretion. 11-carboxy-THC and 11-hydroxy-THC have been found at levels below 100 ng L^{-1} and 50 ng/L^{-1}, respectively (Postigo *et al.,* 2010).

DAs, like pharmaceuticals, reach surface waters unaltered or slightly transformed from wastewater treatment plants (Boleda *et al.,* 2011). Wastewater treatment is only partially effective in removing pharmaceutically active compounds. Therefore, efforts have been made to improve sewage treatments with a tertiary step to efficiently remove all the organic contaminants by ozonolysis, advanced oxidization process, osmosis, etc.

Nevertheless, most of the treatment plants do not include the treatment due to the high cost, this causes the release of illicit drugs and metabolites in surface water and sometimes in drinking water (Pedrouzo *et al.,* 2011b; Terzic *et al.,* 2010). Hence, DAs and their metabolites are detected in the aquatic environment (lakes, rivers, and groundwater) due to their incomplete removal during wastewater treatment and/or by discharge of manufacturing residues (Al-Rifai *et al.,* 2007; Roberts and Thomas, 2006).

Several studies have been conducted in different country of the world to determinate concentration levels of illicit drugs in surface waters. The presence of cocaine and benzoylecgonine in surface waters was reported at relatively higher concentrations from Belgium, Spain, and Italy. The methamphetamine was higher in USA, while the concentrations of MDMA and amphetamine were detected higher in the surface waters from Spain. Although the concentrations of different drugs and their metabolites in surface waters are in the few ng L^{-1} range, their possible effects on ecosystem and human health cannot be ignored (Valcárcel *et al.,* 2012).

The removal efficiency in conventional drinking water treatment showed complete removal for almost all the illicit drugs and metabolites with few exceptions for benzoylecgonine, methadone, and EDDP (Huerta-Fontela *et al.,* 2008; Boleda *et al.,* 2009). In fact, cocaine, benzoylecgonine, methadone and its metabolite EDDP were frequently detected in tap water (Boleda *et al.,* 2011; Valcárcel *et al.,* 2012).

5.2.2. Toxicity

DAs are continuously released into the aquatic environment due to their high and constant consumption (Valcárcel *et al.,* 2012). Their ecotoxicity has received less attention than the legal drugs with particular attention to potential adverse effects on aquatic systems, or their bioconcentration in biota (Binelli *et al.,* 2012; Daughton, 2011).

According to the few studies reported in the literature, morphine, amphetamines and MDMA have potent pharmacological activity and their presence as complex mixtures in surface waters can be toxic to aquatic life and human health, and this cannot be ignored (van Nuijs*et al.,* 2011).

Some studies have been conducted to evaluate the toxic effects of DAs on fishes. Darland and Dowling observed that repeated exposure of cocaine induced in zebra fish (*Danio renio*) slowing of movements, reduced visual sensitivity, excitation and increased aggression. Moreover, in embryos of the same species the concentrations above 2 mg L^{-1} of THC caused the death of numerous individuals within 24 h of exposure (Darland and Dowling, 2001).

The lethal concentration (LC_{50}) of amphetamine determined for *Daphnia magna* varied between 60.4 and 265.3 mg L^{-1} after an exposure of 24 h. The THC present a LC_{50} for rainbow trout (*Oncorhynchus mykiss*, 96 h) and European carp (*Cyprinus carpio*, 48-h) of 19 and 36 mg L^{-1}, respectively and for *Daphnia magna* it present a LC_{50} of 24,5 mg L^{-1} after an exposure of 48 h (Guilhermino *et al.,* 2000).

Some studies have shown that these substances degrade very quickly in the environment and they may give rise to potentially toxic by-products. Postigo et al. have investigated the phototransformation of methadone in aquatic environment and the generated byproducts showed no significant toxicity (Postigo *et al.,* 2011b). Recently, Gonzàlez-Marino et al. have studied the stability of the main metabolite of cannabis, 11-carboxy-THC, during water chlorination. 11-carboxy-THC was degraded in few seconds following a pseudo-first order kinetics and seven by-products were identified. The software predicted toxicity of these products towards Daphnia magna indicates that they are expected to have toxicity values similar or higher than its parent compound (Gonzàlez-Marino *et al.,* 2013).

5.2.3. Analytical methods

Nowadays, it is very important to develop sensitive and accurate analytical methods for the determination of DAs in environmental matrices, both to assess their distribution in the environment and to estimate the consumption of drugs in the population.

The most of the published studies for the determination of DAs in water samples are based on off-line SPE. The most of used SPE sorbent to extract and concentrate the DAs and metabolites from water samples are: reversed-phase hydrophilic-lipophilic polymeric solid phases (e.g., Oasis HLB (Boleda *et al.,* 2007; Gheorge *et al.,* 2008;) and PLRPs (Postigo *et al.,* 2008), as well as mixed mode cation exchange sorbents (e.g., Oasis-MCX (Castiglioni *et al,* 2006; Kasprzyk-Hordern *et al.,* 2008; Gonzàlez-Marino *et al.,* 2012) and Strata-XC (Bones *et al.,* 2007).

Generally, in off-line SPE procedure the extract before analysis by LC must be concentrated; this step can entail losses of target analyses by volatilization or thermal decomposition. Moreover, the sample preparation is a crucial step to remove matrix components that may compete with the target analytes in the ionization process in LC-MS analysis and consequently to influence the analyte recoveries and the limits of detection and/or quantification. This drawback can be overcome by using isotopically labeled analogues as surrogate standards (van Nuijs *et al.,* 2011).

As an alternative to in off-line SPE procedure Postigo et al. have developed the first method based on on-line SPE–LC-MS/MS for the determination of DAs and their metabolites in sewage waters, opening the possibility for a full-automated analysis (Postigo *et al.,* 2008). This strategy has allowed to overcome the common limitation of off-line SPE procedure: ow and variable analyte recovery, time-consuming and high costs (Chiaia et al., 2008).

Another alternative method to eliminate the expenditure of time and to speed up the analyses was developed by Chiaia et al. These authors have developed a

Large-Volume Injection (LVI) LC–MS/MS procedure using a special injector kit for the determination of Das in wastewater. In this technique the sample preparation was completely eliminated, the sample is introduced directly into the chromatographic system (Chiaia *et al.,* 2008).

Successively, González-Mariño et al. have evaluated SPE with commercially available amphetamine class-selective molecular imprinted polymers (MIPs), to extract and concentrate amphetamine drugs from wastewater samples. According to this study the MIPs compared to Oasis MCX and HLB cartridges, in terms of sensitivity, selectivity, precision and accuracy, was better. The main drawbacks of this approach were a higher time requirement for the sample preparation step and a lower load capacity compared to Oasis SPE (González-Mariño *et al.,* 2009a).

As regard the detection of DAs the most of the method employed for the analysis of these compounds are based on LC-MS/MS, only few methods GC-MS are reported in literature (Gonzàlez-Marino *et al.,* 2010; Mari *et al.,* 2009).

Usually, the separation in LC is achieved by reversed-phase columns, using a moderately polar mobile phase consisting of a mixture of water and an organic solvent. However, the performance of hydrophilic interaction liquid chromatography (HILIC) has been evaluated for the separation of cocaine and its metabolites (Gheorghe *et al.,* 2008; van Nuijs *et al.,* 2009), the opiates, the amphetamine-like compounds, methadone and its metabolite (van Nuijs *et al.,* 2009). The HILIC separation involves a polar stationary phase (porous silica microspheres) and a highly organic mobile phase consisting of methanol or acetonitrile in which water is introduced as the eluting solvent. This type of chromatography has been shown to resolve better the polar ecgonine methyl ester that is poorly retained in reversed-phase columns (Gheorghe *et al.,* 2008; vanNuijs *et al.,* 2009).

Usually, the ionization of DAs and their metabolites is performed by electrospray (ESI). However, these compounds are mainly ionized in positive

mode, except the cannabinoid as they show good response in both positive (Boleda *et al.,* 2007; Bijlsma *et al.,* 2009) and negative ionization modes (Castiglioni *et al.,* 2006; Postigo *et al.,* 2008; Hogenboom *et al.,* 2009). The main drawback of the ESI interface is its susceptibility to matrix effects (suppression or enhancement of the analyte ionization signal). Several studies have shown that the ionization of DAs and their metabolites in aqueous environmental samples decreases considerably with increase of matrix complexity. For example, co-eluting matrix constituents of wastewater reduce analyte ionization between 30 and 94%. Furthermore to a selective sample extraction process, most of the developed methodologies include isotopically labeled analogues as surrogate standards to compensate for matrix effects in wastewater matrices (van Nuijs *et al.,* 2011).

Generally, the developed methods to determine DAs and their metabolites in water use as analyzers the triple quadrupole (QqQ), the ion trap (IT) and hybrid technologies that combine quadrupole and linear IT analyzers (QLIT) or quadrupole and time of fly (QToF) (van Nuijs *et al.,* 2011). Usually, these analyzers operate in the selected reaction monitoring (SRM). This mode of acquisition provides good selectivity and sensitivity, when two SRM transitions are recorded per compound, four identification points are obtained as required by the European Union (EU) (2002/657/CE) for identification and confirmation of banned substances (Postigo *et al.,* 2008).

5.3. Results and Discussion

DAs and their metabolites are among the most recent ECs of concern, particularly regarding the environmental aqueous matrices. Their presence in aquatic compartment, even at low concentrations, together with the residues of many therapeutic pharmaceuticals and other organic compounds, may lead to unexpected pharmacological interactions causing toxic effects to aquatic organisms. In addition, they may cause a wide variety of environmental and health problems (Pal *et al.,* 2013).

Apart from the environmental impact, the determination of DAs in raw wastewater can also be used to monitor their consumption in a specific location. This approach, named "*Sewage Epidemiology*", was applied for the first time in 2005 by Zuccato et al. Urban wastewaters entering an sewage treatment plant is an accessible, economical source of real-time, pooled epidemiologic information and they can provide valuable evidence of the amount and type of any common product consumed by a population (Zuccato *et al.,* 2005).

Hence, monitoring of DAs in environmental water bodies is very useful from two perspectives: i) epidemiologists can assess the nature and magnitude of drug abuse (Rieckermann and Christakos, 2008) and information on changes in drug abuse trend (Terzic *et al.,* 2010); and ii) environmental scientists and policy makers can implement control strategies to protect the environment from biologically active substances.

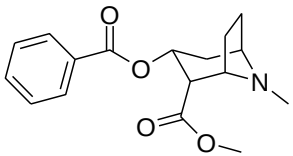
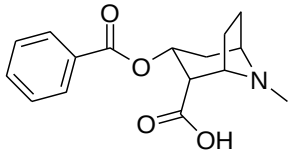
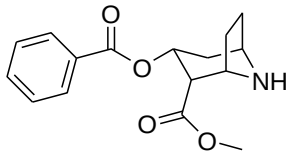
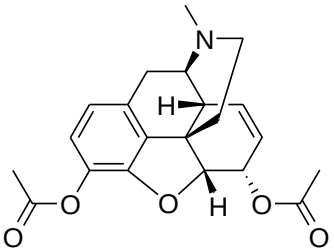
However, there is no current regulation demanding the determination of occurrence of these emerging contaminants in treated wastewater, surface and drinking waters. Thus, critical investigation on distribution pattern of this new group of emerging contaminant and their potential harmful impact on the environment needs immediate attention. For this purpose, several analytical procedures have been developed for DAs determination in different

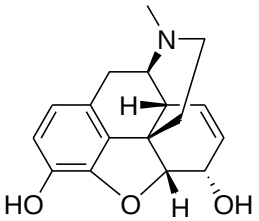
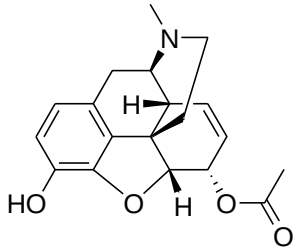
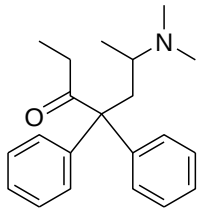
environmental compartments to estimate both their occurrence and the levels of community consumption (Postigo *et al.,* 2008). Liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) is the method of choice for the analysis of DAs, due to the high sensitivity and the selectivity offered. Nowadays, the use of UHPLC and smaller particle size columns, provide well-established advantages in terms of sensitivity, speed of analysis and resolution of analytes compared to HPLC (Baker and Kasprzyk-Hordern, 2011). In regards to sample preparation, solid-phase extraction (SPE) is the preferred technique. Analytes are concentrated using either the hydrophilic reversed-phase or mixed-mode (reversed phase plus cation-exchange) materials and then recovered using an organic solvent or mixture of solvents compatible with further LC separation (Posigo *et al,* 2008). This technique provides very good selectivity and sensitivity in trace analysis of DAs, fundamental requirement in the analysis of environmental emerging contaminants.

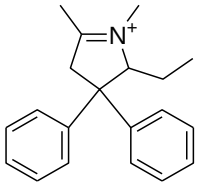
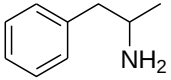
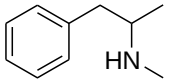
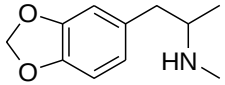
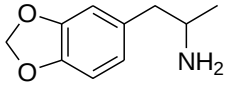
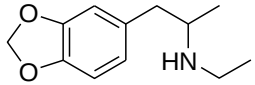
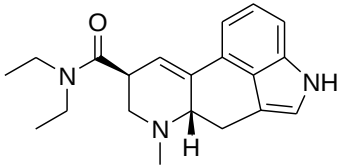
In this study, a simple multiresidue method for the simultaneous determination of 11 DAs and their metabolites in raw wastewater has been reported. Target drugs (Table 5.1) were selected based on the levels reported in wastewater (van Nuijs *et al.,* 2011) and recent abuse trends according to the United Nations Office of Drugs and Crime (UNODC) and EMCDDA (UNODC, 2011 EMCDDA, 2011).

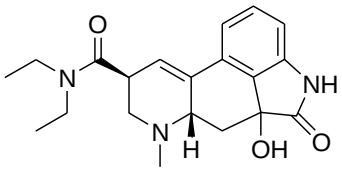
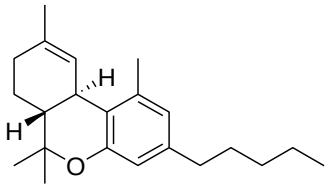
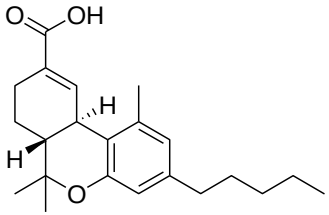
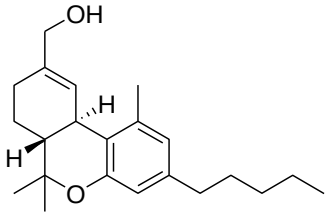
The main goals were to obtain i) a simple but selective and accurate method for determination of the target DAs at very low levels (ppt) in wastewater and ii) a robust analytical tool to investigate the occurrence of DAs in untreated wastewater of STP from Avellino province (Italy) and to estimate the use of the DAs in local community by sewage epidemiology approach.

Table 5.1. *Selected DAs*

Code	Compound	Structure	Type	MW	LogP[c]
CO	Cocaine		Cocainics[a]	303.15	2.275
BE	Benzoylecgonine		Cocainics[b]	289.13	2.263
nor-CO	Norcocaine		Cocainics[b]	289.33	3.111
HER	Heroin		Opiates[a]	369.16	1.580

Code	Compound	Structure	Type	MW	LogP[c]
MOR	Morphine		Opiates[a]	285.14	0.872
6AM	6-acetylmorphine		Opiates[b]	327.15	1.322
METH	Methadone		Opiates[a]	309.21	3.930

Code	Compound	Structure	Type	MW	LogP[c]
EDPP	2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine		Opiates[b]	277.18	5.363
AM	Amphetamine		Amphetamine-like[a]	135.10	1.789
MA	Methamphetamine		Amphetamine-likea	149.12	2.202
MDMA	3,4-methylenedioxymethamphetamine		Amphetamine-likea	193.11	2.050
MDA	3,4-methylenedioxyamphetamine		Amphetamine-likea	179.09	1.637
MDEA	3,4-methylenedioxyethamphetamine		Amphetamine-likea	207.13	2.337
LSD	Lysergic acid		LSD[a]	323.20	2.821

Code	Compound	Structure	Type	MW	LogP[c]
O-H-LSD	2-oxo-3-hydroxy-LSD		LSD[b]	355.19	1.874
THC	Δ^9-tetrahydrocannabinol		Cannabinoids[a]	314.22	6.838
THC-COOH	11-nor-9-carboxy-Δ^9-THC		Cannabinoids[b]	344.20	5.250
OH-THC	11-hydroxy-THC		Cannabinoids[b]	330.47	5.357

[a]Parent drug;[b]metabolites;[c]Physical-chemical data obtained from SciFinder Scholar Database 2013 (predicted properties).

The reported analytical procedure is based on SPE with a mixed reversed-phase/cation-exchange stationary phase (Oasis-MCX) combined to UHPLC-MS/MS multiresidue analysis. The stable isotope dilution assay (SIDA) was used to compensate the matrix effects and losses of sample preparation, and to ensure a high accuracy and precision to the method. The use of the stable isotopically labelled standard is the better approach to make the LC-MS analysis of DAs more accurate and precise (Nissen, 2006).

The study was organized in following steps:

1. Development of a highly accurate and sensitive UHPLC-MS/MS multiresidue method for the specific determination of DAs in wastewater;
2. Optimization of a simple and selective SPE procedure;
3. Validation of analytical method for wastewater;
4. Application of the developed method to real samples and estimation of DAs usage (sewage epidemiology).

5.3.1. UHPLC-MS/MS analysis

In LC-MS/MS an efficient separation is desired to minimise the matrix effects and improve the sensitivity. In order to achieve the best chromatographic performance (reduction of peak tailing and better resolution) and the most intense ionization of the analytes, several solvents combinations and pH of mobile phases (methanol/water and acetonitrile/water with pH ranged between 4.5-8.5), together with the ionization mode, were investigated.

The analysis of the chromatograms showed that methanol and 5 mM ammonium acetate provided a better response and chromatographic resolution, so this solvent system was selected as mobile phase for the chromatographic analysis of target analytes. Then, the chromatographic conditions were adjusted to favour the negative or positive ionization of target analytes and their separation. To this end, the organic phase was acidified to an apparent pH of 4.5, whereas the aqueous phase buffer was made to a pH of 8.5. In this way, basic compounds could be

effectively retained in the C18 column at low organic content and, at the same time, the organic content gradient was accompanied by a pH gradient, increasing the retention of THCCOOH and decreasing the retention of basic drug so that they could be separated into two well-defined segments. Gradient elution reported in the section 5.5.4 was able to separate the DAs in only 13 min.

The optimization of MS and MS/MS parameters was carried out by infusing individual solution of the analytes (1 μg mL^{-1}). All DAs showed maximum sensitivity operating in the positive ionization (PI) mode, except THC-COOH that present slightly more abundant ionization in negative (NI) mode.

Solvent- and flow rate-dependent source parameters were optimized at the selected chromatographic conditions.

According to the 2002/657/EC regulation, which requires two different MS/MS transitions to confirm the identity of target analytes, the product ion spectra of $[M + H]^+$ or $[M - H]^-$ ions were studied to select at least two characteristic product ions for each analytes to be monitored in Selected Reaction Monitoring (SRM) mode. Quantification of the target analytes was carried out by adding both SRM transitions. In addition, the ratio between the signal intensities of two SRM transitions (transitions ratio) was used to confirm the identity of the analytes and to fulfil the EU regulation. Table 5.2 reported the experimental UHPL/ESI-MS/MS parameters used for the determination of selected PPCPs.

To improve the accuracy and precision of overall analytical quantitative method, the stable isotope dilution assay was adopted as internal standard method, for almost all analytes (except OH-THC and OH-LSD), to compensate the analyte losses during clean-up and the ion suppression or enhancement matrix effects during the ionization process in the ion source.

Table 5.2. *UHPLC/ESI-MS/MS parameters for the analysis of selected PPCPs and analytical performance*

DAs	t_R (min)	Precursor ion	SRM transitions (*m/z*)	Collision energy (V)	$I_1/I_2 \pm tol$[b]
	4.30	$[M + H]^+$	290.1 → 168/105	22-34	3.8 ± 7.1
BE-d_3[a]	4.30	$[M + H]^+$	293 → 171	18	
OH-LSD	5.12	$[M + H]^+$	356.3 → 237/222	25-38	3.5 ± 5.2
MOR	5.20	$[M + H]^+$	286.1 → 152/165	35-30	1.4 ± 6.7
MOR-d_3[a]	5.20	$[M + H]^+$	289.1 → 152	35	
AM	5.53	$[M + H]^+$	136.1 → 91/119	8-21	2.3 ± 4.4
AM-d_6[a]	5.53	$[M + H]^+$	142.1 →125	20	
MDA	5.55	$[M + H]^+$	180.1 → 133/105	20-22	1.1 ± 6.6
MDA-d_5[a]	5.55	$[M + H]^+$	185.1 → 138	20	
MDMA	5.77	$[M + H]^+$	194.1 → 163/133	12-20	3.2 ± 1.4
MDMA-d_5[a]	5.77	$[M + H]^+$	199 → 165	12	
MA	5.78	$[M + H]^+$	150.2 → 91/119	12-21	2.6 ± 1.8
MA-d_5[a]	5.78	$[M + H]^+$	155.2 → 92	12	
MDEA	5.80	$[M + H]^+$	208.3 → 133/163	13-22	3.1 ± 2.1
MDEA-d_5[a]	5.80	$[M + H]^+$	213.1 → 163	15	
6-AM	5.98	$[M + H]^+$	328.1 → 165/211	15-25	1.1 ± 2.1
6-AMd_6[a]	5.98	$M + H]^+$	334.1 → 334	8	
CO	6.45	$[M + H]^+$	304.2 → 182/82	21-32	5.1 ± 7.1
CO-d_3[a]	6.45	$[M + H]^+$	307.1 → 185	20	
LSD	6.69	$[M + H]^+$	324.1 → 223/208	26-35	2.3 ± 5.4
LSD-d_3[a]	6.69	$[M + H]^+$	327.1 → 226	25	
nor-CO	6.86	$[M + H]^+$	290.3 → 136/168	18-22	1.5 ± 7.1
nor-CO-d_3[a]	6.86	$[M + H]^+$	293.1 → 171	18	
HER	7.10	$[M + H]^+$	370.3 → 165/268	30-35	2.9 ± 3.3
HER-d_9[a]	7.10	$[M + H]^+$	379.1 → 272	30	
EDDP	7.59	$[M + H]^+$	278.3→ 249/234	25-35	2.2 ± 5.7
EDDP-d_3[a]	7.59	$[M + H]^+$	281.1 → 234	25	
METH	9.03	$[M + H]^+$	310.3 → 265/105	18-26	2.7 ± 1.1
METH-d_3[a]	9.03	$[M + H]^+$	313.1 → 268	18	
THC-COOH	9.54	$[M - H]^-$	343.1 → 299/327	15-27	8.9 ± 7.4
THC-COOH-d_3[a]	9.54	$[M - H]^-$	346.1 → 302	15	
OH-THC	9.74	$[M + H]^+$	331.3 → 313/193	16-26	7.5 ± 6.4
THC	11.30	$[M + H]^+$	315.3 → 193/123	19-32	1.1 ± 5.6
THC-d_3[a]	11.30	$[M + H]^+$	318.1 → 196	20	

[a] Internal standards; [b] intensity ratio SRM1/SRM2 ± maximum tolerance.

UHPLC-MS/MS method showed a good linearity from level close to IQL at 1000 ng mL^{-1} ($R^2 < 0.996$). The instrumental detection and quantification limits (IDL and IQL) ranged between 0.10-3.12 and 0.32-10.30 ng mL^{-1}, respectively (Table 5.3).

Table 5.3. *Performance of UHPLC-MS/MS method*

DAs	Linearity range (ng mL^{-1})	R^2	IDL (ng mL^{-1})[a]	IQL (ng mL^{-1})[b]	RSD[c]
BE	1-1000	0.998	0.19	0.63	6.7
OH-LSD	0.5-1000	0.996	0.10	0.32	1.2
MOR	5-1000	0.996	0.78	2.57	5.7
AM	5-1000	0.998	0.78	2.57	1.7
MDA	5-1000	0.997	0.78	2.57	3.3
MDMA	0.5-1000	0.998	0.10	0.32	3.8
MA	5-1000	0.999	0.39	1.29	5.5
MDEA	0.5-1000	0.997	0.10	0.32	3.7
6-AM	5-1000	0.997	0.78	2.57	1.6
CO	1-1000	0.998	0.19	0.63	5.4
LSD	0.5-1000	0.997	0.10	0.32	6.3
nor-CO	1-1000	0.998	0.19	0.63	7.6
HER	5-1000	0.998	0.78	2.57	4.4
EDDP	5-1000	0.996	0.78	2.57	3.5
METH	5-1000	0.997	0.78	2.57	2.8
THC-COOH	15-1000	0.998	3.12	10.30	4.8
OH-THC	5-1000	0.997	1.56	5.15	3.8
THC	15-1000	0.998	3.12	10.30	4.6

a Instrumental detection limit, S/N = 3 in acetonitrile/water 1:1, v/v, standards solution; [b] Instrumental quantification limit, S/N = 10 in acetonitrile/water 1:1, v/v, standards solution; [c] Standards solution at the 100 ng mL^{-1} level (n = 10).

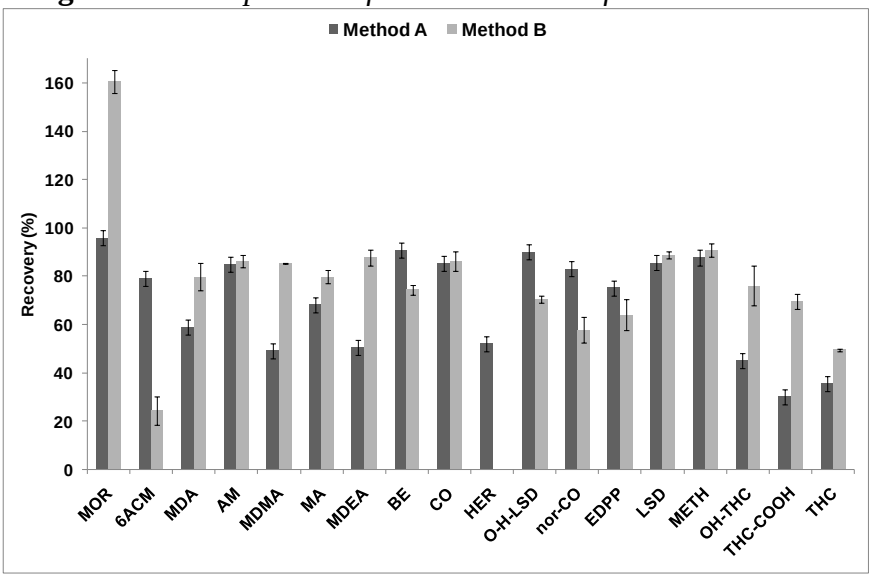
5.3.2. Optimization of SPE procedure

SPE on reversed phase plus cation-exchange stationary phase (MCX sorbent) was selected as sample preparation procedure to concentrate and purify the target DAs in wastewater samples. According to literature data, this technique provides a good selectivity in the trace analysis of Das together to adequate enrichment factors (Postigo *et al.,* 2008).

In the SPE optimization study, two methods previously reported in literature, A (Castiglioni *et al.,* 2006) and B (Gonzàlez-Marino *et al.,* 2012), were used as start points. The method A is one of the first methods (SPE-LC-MS/MS) used for the simultaneous analysis of a wide variety of DAs and their metabolites in wastewater. Whereas, the method B is a recent SPE version developed to reduce the matrix effects. Both methods use the mixed-mode Oasis MCX sorbents for the pre-concentration of the analytes, but differ in the elution protocol. In fact, the method B, unlike method A, uses a fractionated elution strategy: the MCX cartridges are eluted first with 2 mL of MeOH (cannabinoids and interfering matrix constituents) and finally with 4 mL of MeOH/NH_4OH (95:5) (remaining basic DAs). Both eluates are collected separately and analyzed in two different LC-MS/MS injections. This strategy improves the selectivity and MDLs for basic drugs of the method (Gonzàlez-Marino *et al.,* 2012). Furthermore, the two method differ also for the pH of sample (A, pH 2; B, pH 4.5) and for the percentage of NH_4OH in the second elution (A; MeOH/NH_4OH 98:2, v/v; B; MeOH/NH_4OH 95:5, v/v).

Initially, both methods were evaluated in terms of extraction efficiency of SPE procedure (SPE recovery, evaluated adding ISs after SPE). As displayed in Figure 5.1, the method B provided better recoveries than the method A for all cannabinoids and this was very relevant because these analytes are the only target analytes affected by a lack selectivity of the method. Additionally, this method allowed to reduce the matrix effects for basic DAs and consequently to improve the sensitivity of the overall analytical procedure (Gonzàlez-Marino et al., 2012). Nevertheless, the method B showed different drawbacks regarding the opiates: the complete loss of HER, a recovery unsatisfactory of 6-AM and an over-estimation of MOR (Figure 5.1). This behaviour was related to the strongly basic pH of the second elution that caused the conversion of HER and 6-AM in MOR by hydrolysis of the acetyl groups in position 3 and 6 in HER and in position 6 in 6-AM.

Figure 5.1. *Comparison of SPE recoveries of A and B methods*

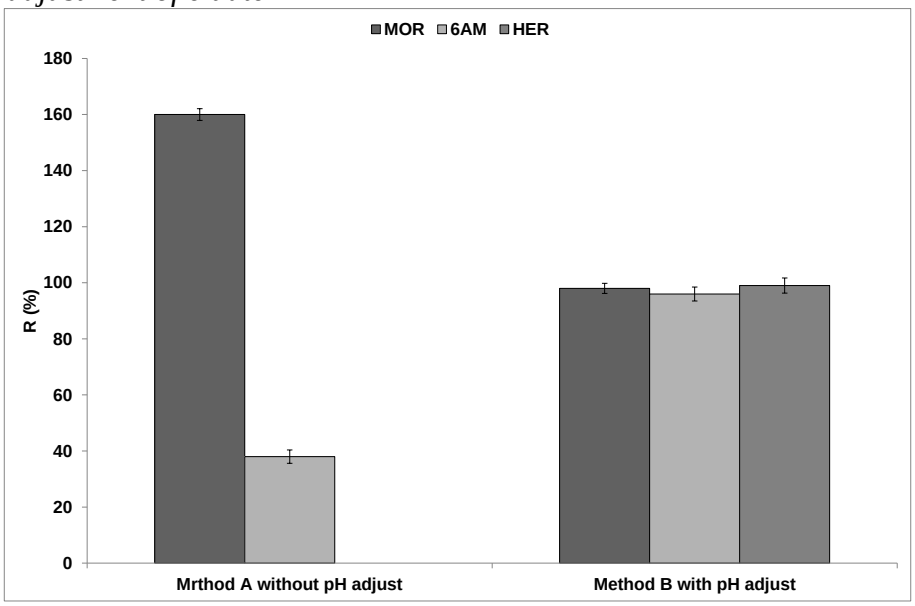


SPE extraction efficiency evaluated adding ISs after SPE.
Experimental conditions: 200 mL ultrapure water spiked at 100 ng L^{-1} level (n= 3).

These drawbacks were overcome neutralizing immediately the pH elution fraction by collection of eluate in a tube containing an amount of acetic acid able to adjust its pH to around 5.5. So, HER and 6-AM resulted stable and accurate recoveries of opiates were obtained (Figure 5.2). The stability of HER and 6-AM was established in subsequent experiments on SPE extract of spiked ultrapure and real water samples. No significant losses of these analytes were observed in 24 hours at 4°C and seven days at -20°C.

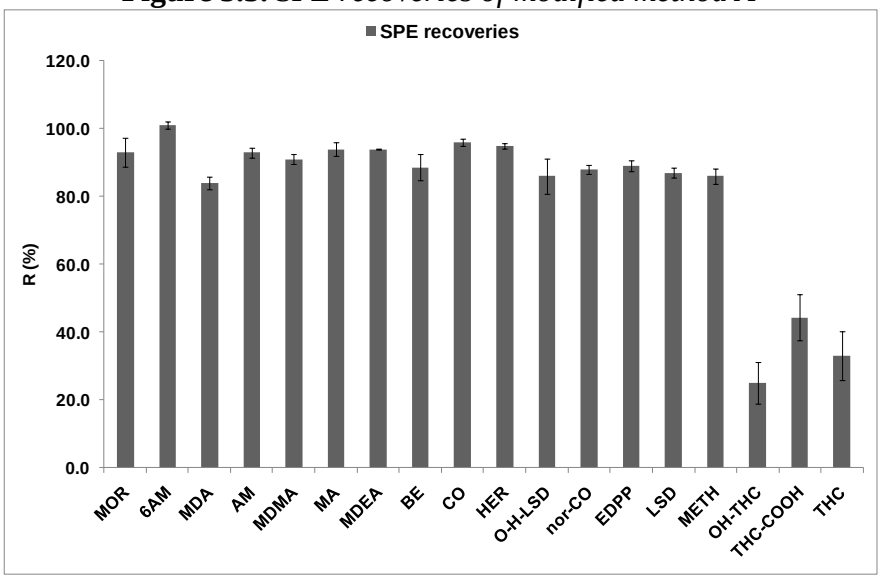
SPE extraction efficiency of the modified method A (sample volume, 200 mL; SPE sorbent, Oasis MCX 150 mg; I elution, 2 mL MeOH; II elution, 4 mL MeOH/NH_4OH 95:5, v/v) was experimentally validated with ultrapure water spiked at 100 ng L^{-1} level. Excellent SPE recoveries (Figure 5.3) were obtained for almost all analytes, with the exception of cannabinoids (25-44%). The precision of procedure (expressed as RSD) was less than 9%).

Figure 5.2. *SPE recoveries of MOR, 6AM and HER after pH adjustment of eluate*



SPE extraction efficiency evaluated adding ISs after SPE.
Experimental conditions: 200 mL ultrapure water spiked at 100 ng L^{-1} level (n= 3).

Figure 5.3. *SPE recoveries of modified method A*



SPE extraction efficiency evaluated adding ISs after SPE.
Experimental conditions: 200 mL ultrapure water spiked at 100 ng L^{-1} (n= 6).

5.3.3. Analytical performance

The method was validated for ultrapure water and wastewater according to CE guidelines (657/2002/CE). The parameters investigated were: selectivity, linearity, sensitivity, accuracy and precision.

The developed UHPLC-MS/MS method recorded two SRM transitions for each analytes (Table 5.2), in this manner, the method fulfilled EU guidelines with four identification points for the confirmation of analytes with LC–MS/MS detection. Additionally, the SRM1/SRM2 ratio was used as an additional identification criterion with a tolerance for relative on intensities of less than 20 % of the expected ratio.

The linear dynamic range of the mass spectrometer was estimated from standard calibration curves obtained plotting the analyte/labelled IS areas ratio versus the concentration. The concentration ranges, close to IQL–1000 ng mL^{-1} (Table 5.3), selected based on instrumental sensitivity and enrichment factor of SPE procedure, were linear ($R^2 > 0.995$) in the studied ranges by analysis of variance (ANOVA).

Accuracy and precision of the analytical procedure were determined processing ultrapure and wastewater samples, spiked at the level of 100 ng L^{-1} with target DAs and ISs, by the optimized SPE procedure. The results of the accuracy (expressed as overall internal standard corrected process efficiency, PE) and the precision (expressed as relative standard deviation, RSD) experiments (n = 3 independent analysis) are reported in Table 5.4. The extraction process was highly accurate and precise, with PE > 90% and RSD <9. The excellent accuracy of method was related to the use of isotopically labelled standards, which corrected the matrix effects and the losses of analytes occurring in SPE process. Only PE of OH-THC was low because its isotopically labelled analogues were not available.

Finally, the sensitivity of method was evaluated by the estimation of MDLs and MQLs. In this case, the effective extraction efficiency (PE estimated without ISs correction) was considered (see equation of 5.5.6 section). As shown in Table 5.4, the estimated MQLs of the whole method varied from 2 to 61 ng L^{-1} in ultrapure

water and 2 to 89 ng L^{-1}. This method offers good sensitivity for the quantification of DAs at trace level, it is comparable with other methods reported in literature by SPE-LC-MS/MS (van Nuijs et al., 2011).

Table 5.4. *Overall internal standard corrected process efficiency (PE), precision (RSD) and MQLs of the proposed method in ultrapure water and wastewater*[a]

	PE (RSD)		MQLs ng L^{-1}	
DA	**Ultrapure water**	**Wastewater**	**Ultrapure water**	**Wastewater**
MOR	102 ± 5	109 ± 6	14	12
6AM	99 ± 2	95 ± 7	14	18
MDA	98 ± 1	91 ± 7	15	27
AM	101 ± 1	92 ± 5	14	29
MDMA	95 ± 2	102 ± 4	2	3
MA	97 ± 2	108 ± 6	7	11
MDEA	91 ± 1	90 ± 5	2	2
BE	95 ± 1	117 ± 7	4	4
CO	92 ± 2	100 ± 3	4	5
HER	105 ± 5	112 ±5	14	20
O-H-LSD	97 ± 9	101 ± 4	2	2
nor-CO	93 ± 2	94 ± 6	4	5
EDPP	102 ± 1	101 ± 3	14	20
LSD	90 ± 1	92 ± 4	2	2
METH	99 ± 2	93 ± 4	14	18
OH-THC	67 ± 8	64 ± 8	45	44
THC-COOH	95 ± 4	90 ± 8	61	78
THC	96 ±4	92 ± 9	60	89

[a] PE and MQL were calculated according to equations reported in the section 5.5.6; (n = 3).

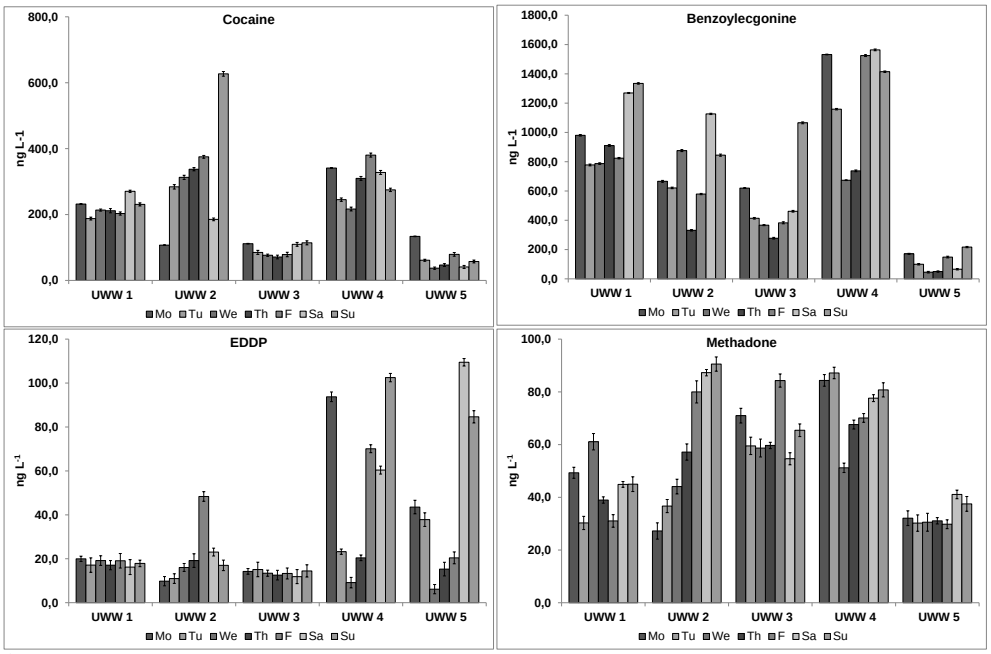
5.3.4. Application to the real wastewater samples and estimation of DAs collective use in Avellino province

To test the reliability of the reported analytical procedure, untreated wastewater (UWW) samples were analyzed following the described protocol. UWW sampling was carried out in collaboration with "*Società Alto CaloreServizi S.p.A"* and it was applied to five different inlet sites of sewage treatment plant (STP) of Avellino. DAs analyses were performed on 24-h composite samples and each sampling site was monitored for seven consecutive days.

CO and its major metabolite, BE, were detected in all UWW samples of five sampling sites with concentration ranges of 30-600 ng L^{-1} for CO and 40-1250 ng L^{-1} for the BE (Figure 5.4). Also METH and its main metabolite, EDDP, were found in all sampling sites with concentrations between 20 and 90 ng L^{-1} and 10 and 100 ng L^{-1}, respectively (Figure 5.4). The obtained concentrations are in the same range as recent results from the literature (Postigo *et al.,* 2008; Zuccato *et al.,* 2008).

Clear differences were observed in CO, BE, METH and EDPP concentrations the different UWW sampling sites, but also between water samples collected from the same UWW site at different dates. Several parameters may account for these observed differences: i) the water debit through the WWTP; ii) the day of collection; (iii) the number of people which are really served by the WWTP; (iv) seasonal effects (e.g. summer–winter variation, rainfall and temperature), (v) the fate of drugs (e.g. degradation, stability, sorption and partition), (vi) human metabolic patterns of the investigated drugs (van Nuijs *et al.,* 2011).

Figure 5.4. *Weekly trend of cocaine, benzoylecgonine, methadone and EDDP concentrations in the five Untreated WasteWater (UWW) of Avellino STP* [a]



[a] Week day: Mo, Monday; Tu, Tuesday; W, Wednesday; Th, Thursday; F, Friday; Sa, Saturday; Su, Sunday.

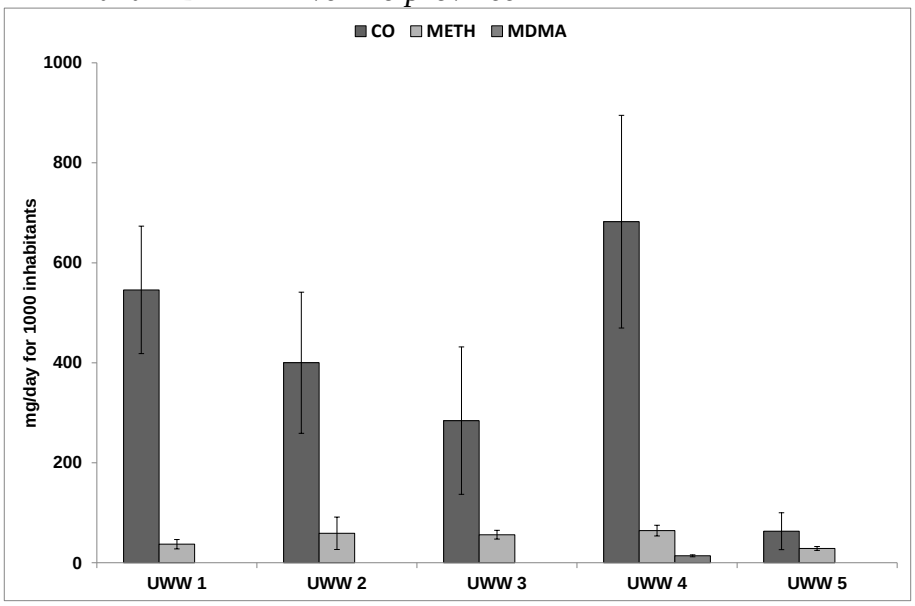
As regard the other target DAs, MDMA was found only in IV UWW sampling site during the week-end, with a mean concentration of 40 ng L^{-1}. The presence of MDMA in these UWW samples can be justified from the presence of a disco in the vicinity of the sampling site.

As proposed by Daughton and Ternes (Daughton, 2001b) and Zuccato et al. (Zuccato *et al.,* 2005), the measured concentrations of DAs and metabolites may serve to estimate the illicit drug usage at the community level, using the sewage epidemiology approach. Therefore, the levels of DAs detected in analyzed UWW samples were used to back-calculate the mass loads of the parent drugs and/or metabolites, by Zuccato's method (Zuccato *et al.,* 2005). This method allows to estimate the consumption of DAs (g day^{-1}) simply by knowing the metabolism of drugs and their mode of excretion. In addition, it is possible to express the data

obtained in g day^{-1} per 1000 inhabitants, taking into account the population served by the sampling site and the flow rate.

Particularly, the collective consumption of CO and METH was estimated. As shown in Figure 5.5, a CO consumption rate between 63 and 680 mg day^{-1} per 1000 inhabitants was estimated. For METH this data varied between 29 and 64 mg day^{-1} per 1000 inhabitants, and for MDMA was 14 mg day^{-1} per 1000 inhabitants.

Figure 5.5. *Estimated consumption rates (weekly average) of CO, METH and MDMA in Avellino province*



The data show that, within the same province, the consumption of DAs can be very diverse.

The average consumption of in Avellino is comparable with other Italian cities and it is similar to the consumption of Palermo and Florence (Zuccato and Castiglione, 2012). The METH consumption is constant, because it is used in medicine as a medical substitute of heroin in anti-addictive treatment.

5.4. Conclusion

A rapid and simple SPE-UHPLC-MS/MS method to analyze DAs and their metabolites in wastewater at ultra-trace levels was reported and validated. The SPE procedure was optimized having as reference the Gonzàlez-Marino method (Gonzàlez-Marino *et al.,* 2012). The analytes were concentrated using mixed-mode Oasis MCX sorbent and they were recovered by a fractioned elution strategy. In this way the selectivity and sensitivity of the method were improved. The use of highly selective and sensible UHPLC-MS/MS method in combination with isotope dilution assay provided a precise and accurate quantification and confirmatory method that fulfils the analytical criteria required by EU Regulation concerning the performance of analytical methods and the interpretation of results (2002/657/EC 2002).

In collaboration with "Società Alto Calore Servizi S.p.A" the developed method was used to monitor the occurrence of DAs in untreated wastewater and, subsequently, to estimate the drug consumption rate in the population of Avellino by sewage epidemiology approach.

The developed SPE-LC-MS/MS method due to its simplicity, sensitivity and accuracy, is extremely suitable for drug monitoring campaigns and in addition to the classic socio-epidemiological studies it can be used for the identification of illicit drug consumption trend.

5.5. Experimental

5.5.1. Standards and materials

Standards of AM, MA, MDA, MDMA, MDEA, CO, BE, nor-CO, LSD, OH-LSD, MOR, 6-AM, HER, METH, EDDP, THC, THC-COOH, OH-THC were obtained from LGC Standards S.r.L. (Milan, Italy) as 1 μg mL^{-1} solutions in acetonitrile or methanol. Deuterated compounds AM-d_6, MA-d_5, MDA-d_5, MDMA-d_5, MDEA-d_5, CO-d_3, BE-d_3, nor-CO-d_3, LSD-d_3, MOR-d_3, 6-AM-d_6, HER-d_9, METH-d_3, EDDP-d_3, THC-d_3 and THC-COOH were also obtained from LGC standard (0.1 μg mL^{-1} in MeCN or MeOH) and used as surrogated internal standards (ISs) for the quantification of their analogue native analytes. Full names of target analytes and ISs are compiled in Table 5.2. LSD-d_3 and THC-COOH-d_3 were selected as ISs of OH-LSD and OH-THC, respectively, based on structural and chromatographic similarities. DAs and ISs mixed standard solutions, were prepared in MeCN/H_2O at 5 μg L^{-1} and 1 μg L^{-1}, respectively, and stored in the dark at −4 °C. Acetonitrile (MeCN), methanol (MeOH), ammonium hydroxide (NH_4OH) solution (33%), hydrochloric acid (HCl) (37%), and acetic acid (CH_3COOH) were obtained from Sigma-Aldrich (Milan, Italy). HPLC-grade methanol and water were purchased from Romil (Cambridge, UK). Ultrapure water (18MΩ) was prepared by a Milli-Q purification system (Millipore Corp.). Oasis 150 mg MCX cartridges were purchased from Waters (Waters, UK).

5.5.2. Wastewater samples

Untreated wastewater (UWW) samples were collected from five different inlet sites of sewage treatment plant (STP) of the province of Avellino, in the period September-October 2013. The first sampling site serves a population of 95461 inhabitants with a mean flow rate of 22741000 L day^{-1}; the second serves 3340 inhabitants with a mean flow rate of 796000 L day^{-1}; the third serves 16092

inhabitants with a mean flow rate of 3833000 L day^{-1}; the fourth serves 23801 inhabitants with a mean flow rate of 5670000 L day^{-1}; and the fifth serves 500 inhabitants with a mean flow rate of 119000 L day^{-1} For each sampling site, one 24-h composite samples, obtained by pooling water collected every 20 min by automatic sampling devices, was collected. Each sampling site was monitored for seven consecutive days. Water samples (1-2 L each) were stored in dark glass bottles at -20 °C for a maximum of 3 days before of the analysis.

5.5.3. SPE procedure

Untreated wastewater samples were filtered on a 2.7 μm glassfibers filters (Millipore Corporation, Billerica, MA, USA). The filtrate were adjusted to pH 4 with 0.1 N HCl and spiked with ISs (50 ng each) prior of SPE process.

The samples (200 mL) were passed through Oasis MCX cartridges (10 mL min^{-1}) previously washed with 2 mL of MeOH/NH_4OH 95:5, v/v, and then conditioned with 2 mL of ultrapure water at pH 4. Immediately after loading, SPE cartridges were washed with 10 mL of ultrapure water (pH 4) and dried by a continuous nitrogen stream for 30 min. Finally, analytes were eluted in two separated fractions: cannabinoids (together with neutral/acidic matrix components) were first eluted by 2 mL of MeOH (first fraction); the basic DAs were recovered with 4 mL of MeOH/NH_4OH 95:5, v/v, (second fraction) and collected in a test tube with 200 μL of CH_3COOH. Both fractions were concentrated down separately to 0.5 mL with a gentle stream of nitrogen (99.999%) and adjusted to a final volume of 1 mL with H_2O.

5.5.4. UHPLC-MS/MS analysis

Analyses were performed on a Platin Blue UHPLC system (KNAUER GmbH, Berlin, Germany) consisting of two ultrahigh pressure pumps, an autosampler, and column temperature manager, coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a heated

electrospray ionization (H-ESI) probe. UHPLC separation was achieved with a Kinetex C18 (100 x2.1 mm I.D., 2.6 μm) column protected by a C18 Guard Cartridge (4x3 mm i.d.), both from Phenomenex (Torrance, CA, USA) held at 35 °C. The dual eluent system consisted of (A) 5 mM of ammonium acetate (NH_4OAc) in ultrapure water adjusted to pH 8.5 with NH_4OH and (B) 5 mM of NH_4OAc in MeOH made to an apparent pH of 4.5 (by adding the equivalent amount of acetic acid to have such a pH in an aqueous solution). The following elution gradient was used: 0-1 min, 2% B, 3 min, 50% B, 3-12 min, 98% B. After each injection, the column was washed with 95% B for 4 min and re-equilibrated (5 min). The flow rate was 0.3 mL min^{-1} and the injection volume was 10μL using the full loop injection mode.

The operative parameters of the mass spectrometer and solvent- and flow rate-dependent source parameters were optimized at the chosen chromatographic conditions by injecting DAs standard solution (10 ng mL^{-1}). The optimized conditions were spray voltage, 3000 V; capillary temperature, 250 °C; vaporizer temperature, 50 °C; sheath and auxiliary gas pressure, 20 and 5 units, respectively; collision gas pressure, 1 bar. Nitrogen (99.9 % purity) was used as the auxiliary and sheath gas in the ESI source and argon (99.9999 % purity) as the collision gas in the collision cell. For identification and quantification of DAs, selected reaction monitoring (SRM) mode was applied using the characteristic SRM transitions (Table 5.2). The SRM parameters for all scan transitions were scan width (m/z), 0.200; scan time (ms), 20; Q1 and Q3 resolution (FWHM), 0.7. Excalibur software version 2.2 was employed to collect and process the data.

5.5.5. Quantification and method validation parameters

Each compound was quantified by SRM using the two most abundant precursor/product ion transitions. Retention times were also compared with reference standards to identify the compounds. Each analytes was quantified by isotope dilution assay using its corresponding deuterated analogue as IS (except for

THC-OH and OH-LSD, which were quantified using THC-COOH-d_3 and LDS-d_3, respectively).

Linearity of each DAs was estimated in the working range of IQL (level close to IQL, Table 5.2) – 1000 ng mL^{-1}, with ten calibration levels, each injected in triplicate. Calibration solutions were prepared by diluting appropriate volumes of DAs mixed standard solution with ultrapure water and adding a fixed amount (100 ng mL^{-1}) of each IS. Ten-point calibration curves were generated plotting analyte/IS area ratio versus the concentration (ng mL^{-1}).

UHPLC-MS/MS instrumental detection limit (IDL) and instrumental quantification limit (IQL) were experimentally determined using signal-to-noise (S/N) approach through analysis of a series of low concentration standard solutions. IDLs and IQLs were calculated by extrapolation of the lowest concentrations giving a S/N of 3 and 10, respectively.

Recoveries of SPE procedure (R) were assessed spiking 200 mL of ultrapure water at 100 ng L^{-1} DAs level (pre-extraction spiked sample) and calculated by the equation:

$$\%R = 100 \times \frac{\text{Area analyte of pre-extraction spike}}{\text{Area analyte of standard}}$$

Overall IS corrected extraction efficiencies (PE) of the whole procedure were evaluated with water samples (ultrapure water and raw wastewater) spiked at 100 ng L^{-1}level of analytes and ISs. Responses of analytes (DA/IS area ratio) in spiked real sample were corrected by analysis of unspiked real samples processed with the same procedure. PE was calculated according to equation:

$$PE = 100 \times \frac{\text{(DA/IS area ratio pre-extraction spike – DA/IS ratio unspiked)}}{\text{Area analyte of standard}}$$

Matrix effects (ME) were determined by the fortification of SPE extract of raw wastewater (post-extraction spiked WW sample) and calculated by the equation:

$$ME = 100 \times \frac{\text{(Area analyte of post-extraction spike – Area analyte of unspiked)}}{\text{Area analyte of standard}}$$

Method detection limits (MDL_{calc}) and quantification limits (MQL_{calc}) for ultrapure water and wastewater were calculated using the following equations:

$$MDL_{calc} = \frac{IDL \times 100}{ME \times R \times EF} \qquad MQL_{calc} = \frac{IQL \times 100}{ME \times R \times EF}$$

Where ME and R are the matrix effect and the recovery of SPE procedure, respectively, determined without considering the contribution of ISs. EF is the enrichment factor, which in this method denotes 200.

5.5.6. Back calculation

In this study, the drug consumption was estimated by sewage epidemiology approach, devised and implemented by Zuccato et al. (Zuccato *et al.,* 2005).

This approach is based on the assumption that the concentration of drug residues in wastewater, before treatment, is proportional to the quantity of drug consumed by the local population from which the wastewater originated (Zuccato et al., 2005).

Drug consumption (g day^{-1}) was calculated by an equation based on concentration (C) of parent compound or major metabolite (ng L^{-1}), the water flow rate (F) (L day^{-1}) and a conversion factor, which takes into account the molecular mass ratio of metabolite/parent drug and excretion rates.

$$\text{drug (g day}^{-1}\text{)} = \text{C (ng L}^{-1}\text{)} \times \text{flow rate (L day}^{-1}\text{)} \times \text{conversion factor}$$

In the case of CO, its metabolite BE is selected as consumption indicator. The factor conversion is 2.33, because it take into account the BE/CO molar mass ratio (1.05) and the mid-range excretion percentage of 45% as BE (Baselt, 2008). The consumption indicators used for METH and MDMA are the concentration of parent drug, and the conversion factors are 4.46 and 1.5, respectively (van Nuijs*et al.*, 2011).

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