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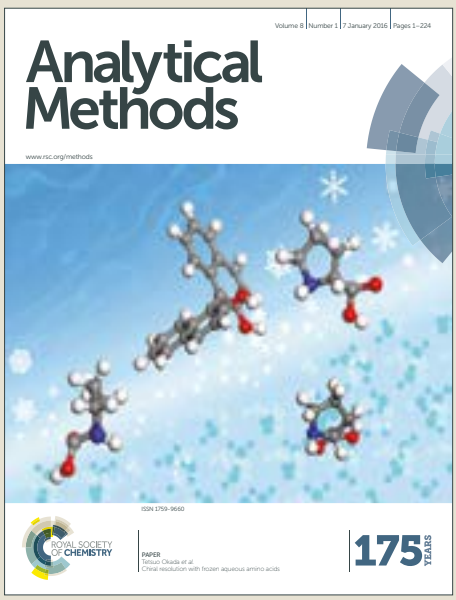

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Methodologies for the analysis of pesticides and pharmaceuticals in sediments and plant tissue

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Abstract

Eco-technologies that utilize natural processes involving wetland vegetation, soil and their associated microbial assemblages are increasingly used for the removal of contaminants of emerging concern (CECs) from polluted water. However, information on removal processes in these systems is not always available, possibly due to the lack of simple and robust methodologies for analysis of CECs in complex matrices such as sediment and plant tissue. The aim of the present study was to use a simple and fast procedure based on ultrasonic extraction (USE) and reduced clean-up procedures to analyse 8 pesticides and 9 pharmaceuticals by high-performance liquid chromatography (HPLC) coupled with diode array detector.

The established methods demonstrated suitable sensitivity and reliability, and proved fit-for-purpose in quantifying multiple classes of pesticides and pharmaceuticals. For sediments, extraction with methanol/acetone (95:5, v/v) followed by a simple evaporation to dryness and redissolution (water:methanol 50:50) provided acceptable recovery (50 - 101%) and RSD < 14%. The complex matrix of plant samples posed specific problems resulting in individualized approaches for pesticides and pharmaceuticals in the final optimized conditions. Pesticides were extracted with *n*-hexane followed by saponification (KOH), pH

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adjustment and solid-phase extraction; while pharmaceuticals were extracted with methanol:acetone (95:5), supernatant cleaned with activated carbon, evaporated to dryness and redissolved (water:methanol 50:50) prior to HPLC injection. Final method characteristics, with a few exceptions, showed acceptable recovery (> 64%) with RSD < 22% determined using different types of wetland plants.

The methodology has been successfully applied in different studies on the fate of emerging contaminants in water treatment eco-technology systems.

Keywords: emerging contaminants; biological sample; environmental matrix; constructed wetlands; water treatment

1. Introduction

Emerging contaminants are a new class or classes of unregulated chemicals previously known to be present in the environment but showing new documented environmental impacts [1]. Many of these emerging contaminants are detected in the aquatic environment at low μg/L to ng/L levels, including trace organic pollutants [2], referred to as contaminants of emerging concern (CECs). Examples of CECs are pharmaceuticals, personal care products, plasticizers, surfactants and biocides that are discharged to the environment as a consequence of human activity.

Major sources of the discharge of most of these CECs into the environment are usually the wastewater treatment plants (WWTPs) [3]. Discharge of CEC with unknown potential adverse effects and/or bioaccumulation into the environment may pose a risk to humans considering their uptake either via the food chain or via drinking water [4]. Therefore, there is an increasing interest in the development of more efficient wastewater treatment technologies capable of dealing with CECs [5]. Among these, eco-technologies such as constructed wetland systems (CWs) or phytoremediation engineered systems, that utilize natural processes involving wetland vegetation,

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soil and their associated microbial assemblages to treat polluted water, have been pursued.

Studies along the last decade have shown that these eco-technologies are able to degrade CECs [6]. However, in spite of promising results [7], detailed information on the removal processes is lacking. In fact, analysis of sediment/substrate and plant tissues samples is crucial to be able to perform flow studies and total mass balances in wastewater treatment systems[8, 9]. In several of the applied studies on CWs, sediments and plant levels have not been studied, or when studied, the methodology used is not always sufficiently described. Sediment is already considered a complex matrix with different organic and inorganic fractions as well as biomass, and humic compounds. Plants present even greater challenges in terms of matrix interferences due to their high contents of pigments and fatty or waxy materials [10]. In addition to the compounds/matrix interactions, the large variety of CECs combined with the normally very low concentrations of the target compounds pose difficult challenges to their detection and analysis [11]. There is a clear need for simple but reliable and robust methodologies concerning CECs analysis in sediment and plant tissue.

The analytical procedures usually comprise three steps, which are followed by detection and data analysis: i) sampling, ii) compound extraction and iii) clean-up of the extract that contains the compound [12]. In general, solid samples will go through a series of steps for preservation (freezing, lyophilizing, chemical drying) followed by homogenization (blending, chopping, grinding, milling, etc.). Homogenization with a mortar and pestle is one of most common procedures for sediment [13]. Considering the analytical procedures for the determination of CECs in crop plants a recent review by Matamoros, Calderon-Preciado [14] has covered the major achievements and drawbacks. Several extraction techniques have been tested for both sediment and plant tissue samples, including accelerated solvent extraction (ASE) also called pressurized liquid extraction (PLE), ultrasonic extraction (USE), sea sand disruption method (SSDM), microwave assisted extraction (MAE), “Quick, Easy, Cheap, Effective, Rugged, and Safe” method (QuEChERS), and matrix solid-phase dispersion (MSPD) in combination with pressurized fluid extraction (PFE) [10, 15, 16]. Classical Soxhlet extractions have been phased out for techniques allowing for higher throughput such as PLE, USE and QuEChERS. Independently of the extraction

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technique used, these primary extracts of multi-residue methods need to be cleaned up before final measurements. In the early days liquid-liquid partitioning (LLP) between an aqueous and organic solvents (such as acetone or dichloromethane), at modulated pH was often performed for pesticide analysis [10, 16, 17], followed by laborious and extensive procedures for condensation, particles removal, gel permeation chromatography (GPC) more commonly referred to as size exclusion chromatography (SEC) and polarity fractionation previous to chromatographic analysis[18]. More recently a typical approach after the extraction of solid samples is the use of solid-phase extraction (SPE), where several different adsorbents can be used and solvents use reduced. SPE and *n*-hexane washing for sample clean-up methods, however, either lack good sensitivity or have considered just a few target analytes [17]. While research on pesticides has historically been more important due to the need for monitoring their levels in food matrices, interest in the analysis of pharmaceuticals in environmental samples has recently risen [14]; therefore very little information on clean-up applications focused on pharmaceuticals analysis is available [19]. The clean-up steps are important to reduce co-extracted compounds that may compromise the chromatographic run avoiding further laborious and/or expensive quantification procedure such as the use of matrix matched[20] or standard addition calibrations and surrogate and internal standards (often isotopically labelled compounds).

In spite of the different available extraction techniques for sediment and plant extracts, recoveries reported are generally variable [14, 21]. On the other hand, several published articles focused on environmental studies, due to different final aims, only briefly report the methodology used without a complete description of optimization and/or validation details. Plant matrices present added difficulties as lipids and pigments which can interfere with analytical procedures are co-extracted with the analytes, resulting in critical ion-enhancement or ion-suppression during MS analysis in HPLC-MS [22]. Therefore, development of simple clean-up steps is important. Simple and fast, but reliable analytical methods are necessary to monitor and control the distribution of CECs in different environmental matrices.

In this work a method for the analysis of triclosan and pesticides (referred further as pesticides group) and pharmaceuticals (Table S1) in sediment and plant tissue samples was developed. The compounds selected are

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known to be present in wastewater and comprise different families and chemical characteristics (molecular weight and log K_{ow}). Ultrasonic extraction (USE) was selected due to the wide availability of the equipment and its easy operation. Following extraction, the need for a simple clean-up procedure prior to sample analysis was evaluated. The compounds were analysed by high-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD).

2. Experimental

2.1 Material and Reagents

Methanol, acetone and *n*-hexane (SupraSolv ®) and formic acid (98 %, reagent ACS) were purchased from Merck (Darmstadt, Germany). High purity grade triclosan (by Dr. Ehrenstorfer GmbH Augsburg, Germany) and the analytical standards of the pesticides carbendazim, benzoisothiazolinone, imazalil, terbutryn, diuron, and mecoprop were supplied by Sigma–Aldrich (Schnelldorf, Germany) and tebuconazole by Dr. Ehrenstorfer GmbH (Augsburg, Germany). High purity grade analytical standards of the pharmaceuticals iopamidol, iohexol, iomeprol, iopromide, propranolol and diclofenac were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) and carbamazepine, naproxen and ibuprofen by Sigma–Aldrich (Schnelldorf, Germany). Other solvents and reagents used were analytical grade. Water used in this study was ultrapure water (18.2 MΩ cm^{-1}, Milli-Q plus system).

Individual standard solutions of each pharmaceutical and pesticide (1000 mg L^{-1}) were prepared in methanol. A standard working solution of the mixture of all compounds in methanol, at a concentration of 60 mg L^{-1}, was prepared weekly. This solution was used to prepare daily calibration standard solutions in Milli-Q water and for the sample (sediment and plant tissue) spiking. All standard solutions were kept at 5 °C in a refrigerator (light protected from photo-degradation).

For decontamination purposes all plastic and glassware used were rinsed with soap, water, deionized water, soaked overnight in 4.5 % (v/v) hydrochloric acid (technical -30% purity, VWR BDH Prolabo), rinsed with deionized water again and dried at 60 °C. Procedural blanks were used to control material cleanliness.

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2.2. Sample collection and preparation

Samples were selected in order to provide real environmental matrices for method development and performance check. Sediment (anaerobic, TOC 3%-7%) and plant tissue samples (*Typha latifolia* and *Berula erecta*) were both collected in a stormwater pond designed for urban-runoff treatment near Skoldhoejvej, Aarhus, Denmark.

Plants were cleaned with deionized water and the plant material divided into roots and leaves. The sediment and plant tissue were frozen at -4 °C and subsequently lyophilized (Christ Alpha 1-4 LSC Freeze Dryer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). Before proceeding to the extraction, the lyophilized plant material was finely ground (< 2 mm) using a rotor mill (Retsch KG, Haan, Germany), while the sediment material was homogenized with mortar and pestle and sieved (particle size < 2 mm).

Spiked samples were prepared by addition of a methanolic standard solution mixture of either pesticides or pharmaceuticals (representing an added volume of 0.5 mL) to the lyophilized and ground samples (0.2 g for plant tissue and 2 g for sediment) into a glass vial (20 mL) per individual sample for future extraction. The mixture was shaken and let to dry overnight in the hood, light protected. The target levels for method optimization and validation ranged between 0.5 to 5 μg $g_{dry\ sediment}^{-1}$ and 0.5 to 100 μg $g_{dry\ plant\ material}^{-1}$ of the individual compounds, as observed before[15, 23, 24]. The pesticides and pharmaceuticals studies were performed in separate batches.

Method optimization and further characterization was carried out using spiked samples of both sediment and plant material. Once real sediment and plant material were used for spiking, non-spiked samples were also analysed to control background levels. All results further presented along both optimization and method validation report means and standard deviation of at least 3 replicates.

2.3. Sample extraction

Optimization of the sample extraction was performed using ultrasonic solvent extraction (USE). The first parameter to be tested was the selection of extraction solvent. For that, six different solvents methanol, *n*-hexane,

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dichloromethane, methanol:formic acid (96:4, v:v), methanol:acetone (95:5, v:v) and acetonitrile:formic acid (99:1, v:v) were tested keeping a fixed solvent volume (10 mL) and a fixed sample mass, 0.2 g for plant material and 2 g for sediment. Each spiked sample was mixed with the different solvents and further placed in an ultrasonic bath (Metason 120, Struers, Denmark) for 30 min.

After extraction, the samples were centrifuged (3000 rpm for 10 min; Sigma 3-18K Centrifuge, Laborzentrifugen GmbH, Osterode, Germany) and supernatants collected. For direct analysis, the supernatants were filtered through nylon filter (0.45 µm) (Frissenette, Knebel, Denmark), while for pre-concentration the supernatants were evaporated to dryness under a nitrogen stream at 35°C, further dissolved in 1.0 mL of methanol and filtered through nylon filters 0.45 µm. All extracts analysis was processed by HPLC-DAD (see section 2.5). Filters were previously tested in terms of blanks as well as sorption, to ensure that the filtration step would not affect the results.

In the optimized operating conditions, for both pesticides and pharmaceuticals, 2 g of sediment samples were extracted with 10 mL of methanol/acetone (95:5, v/v) for 30 min in the ultrasonic bath. The resulting samples were centrifuged and the supernatant evaporated to dryness. Residues were dissolved in 1 mL of methanol and subsequently the solution was filtered and injected into the HPLC system. No clean-up procedures were required for the sediment extracts.

Regarding plant material, in the optimized operating conditions, for pesticides, 0.2 g of plant tissue samples were extracted with 10 mL of *n*-hexane for 30 min in the ultrasonic bath. For pharmaceuticals, 0.2 g of plant tissue samples were extracted with 10 mL of methanol/acetone (95:5, v/v) for 30 min in the ultrasonic bath. Optimization of the clean-up for plant tissue extracts for pesticides and pharmaceuticals is further discussed in section 2.4.

2.4. Clean-up procedure

Extracts obtained by USE generally require an additional clean-up step, such as solid-phase extraction (SPE) which is one of the most common techniques [25]. In the present study a clean-up based on reversed phase approach using Phenomenex Strata-X SPE columns (200 mg / 6 mL) and a normal phase approach using a Supelclean™ LC-Florisil® (1 g / 6 mL) were

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tested. SPE cartridges were processed accordingly to the technical indications (described in the SI).

SPE eluted samples were then evaporated to dryness under a nitrogen stream at 35°C and the residues dissolved in 1.0 mL of methanol prior to HPLC injection.

Plants pigments, mainly chlorophylls and carotene, are highly hydrophobic and co-extracted together with the micropollutants. A saponification step with KOH suggested by Dugay, Herrenknecht [26] to improve PAHs recovery from plant material was investigated. For that, 5 mL of KOH solution 1 mol L^{-1} (methanol:water (4:1, v/v)) was used to dissolve dried residues (after extraction solvent evaporation) and the obtained solution further sonicated for additional 30 min.

In the optimized clean-up conditions, plant slurry samples for pesticide analysis were centrifuged and the supernatant evaporated to dryness. Afterwards, saponification was performed by dissolving the residues in 5 mL of KOH solution (methanol:water (4:1, v/v)) and sonicating the sample for 30 min. Then, samples were filtered, diluted with MilliQ water (MeOH content < 5%), acidified to pH 5.5 (HCl addition) and further processed through SPE (Strata-X) prior to HPLC analysis.

For pharmaceuticals, in the clean-up step optimized conditions, plant slurry samples were centrifuged, pellet discarded and the supernatant passed to a clean vial to which 0.25 g of activated charcoal was added and the solution sonicated for 30 min. After an additional centrifugation, supernatants were filtered, evaporated to dryness and the residues were then dissolved in 1.0 mL of methanol prior to HPLC analysis.

2.5. High performance liquid chromatography conditions

Analytes separation was performed using a HPLC Thermo Scientific Dionex UltiMate 3000 equipment with automatic sampler, column oven and diode array detector (DAD). The analytes were separated on a Synergy 4µ Polar 80 Å column (150 mm × 2.0 mm ID) using a linear gradient program with two eluents, water (0.2% formic acid) and methanol (0.2% formic acid). The linear gradient program used was: 100 % of eluent A (water), keeping isocratic conditions for 2 min, followed by a 2 min linear gradient to 35 % of eluent A (65

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% of eluent B (methanol)), followed by a second slower 9 min linear gradient to 0 % of eluent A which was held afterwards for 3 min. Finally, initial conditions (100 % of eluent A) were reached again in 1 min, with a re-equilibration time of 3 min to restore the column. Flow rate gradient started with 0.25 mL min^{-1}, maintained for 16 min, followed by a 1 min linear gradient to 0.3 mL min^{-1}, which was held for 1 min and another linear gradient along 1 min back to the initial 0.25 mL min^{-1}. The two groups of micropollutants (i.e., a) pesticides plus triclosan and b) pharmaceuticals were quantified separately using a 6 points external calibration. The Chromeleon® 7.1 software (Thermo Scientific, Germany) was used for data integration of chromatograms. The sample injection volume was set at 10 µL, sampler temperature at 8 °C, column oven at 20 °C and the detector signal was acquired simultaneously in 3 channels, for quantitation at 220 nm and 240 nm, and a 3D-field in the λ range 190 to 800 nm (bunch width of 5 nm). These two wavelengths provide a suitable compromise to obtain acceptable sensitivity for the detection of all compounds. The instrument (HPLC-DAD) basic analytical figures of merit (LOD, LOQ, linearity and RSD) are presented in Table S2.

2.6 Analysis of Real Samples

The here described optimized and validated methodology has been efficiently applied by the authors on different works focused on the removal of micropollutants from water through the use of constructed wetland systems. Plant samples from an uptake study in spiked hydroponic medium (10 mg L^{-1} level) where both the above and below ground tissues were analysed, as well as for the quantification of the accumulated amount of micropollutants in the substrate of constructed wetland bed mesocosms along a 9 months trial. Fully described experimental setups can be found elsewhere [27, 28].

2.7. Statistical analysis

Statistically significant differences between samples were evaluated through Student's t-test (*p*-value cut-off: 0.05).

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3. Results and discussion

3.1 Extraction optimization

The solvents tested were chosen based on typical applications for extraction of solid matrices for a variety of organic contaminants. Ultrasonic extraction (USE) was chosen due to its fast and easy to use approach, besides being attractive because the equipment necessary is widely available and the extraction can be done using a reasonably small amount of sample (0.1 – 2 g) and volume of solvent (5 – 25 mL) [25]. Furthermore, this method has a short extraction time compared to those of classical liquid extraction methods.

Sediment samples

Recovery percentages obtained for both pesticides and pharmaceuticals in spiked sediment extracts with the different solvents (methanol, *n*-hexane, dichloromethane, methanol:formic acid (96:4, v:v), methanol:acetone (95:5, , v:v), acetonitrile:formic acid (99:1, v:v)) were compared in order to identify the best solvent/mixture to be further optimized (Figure 1). In general, methanol or methanol mixtures presented better recoveries, although some low recoveries were observed for the pesticides carbendazim, BIT, imazalil and for the iodinated X ray contrast agents. A careful look on methanol-based extracts showed higher recovery efficiency for mixture with either formic acid or acetone. Once the recoveries for methanolic extracts were very similar among themselves, the next step to choose the best solvent passed by visually study the quality of the different chromatograms. The interpretation of the signal to noise ratio based on chemical noise (Typical chromatogram shown in Figure S1) was used to evaluate chemical background effects and interferences, and also the reproducibility of the two most promising mixtures.

An extraction with methanol:aqueous formic acid resulted in higher chemical background noise than acetone. For the pesticides, triclosan and tebuconazole were affected by the background noise resulting in recovery rates exceeding 100%. On the other hand, with acetone good recoveries were obtained for all pesticides except BIT and carbendazim. For pharmaceuticals, the mixture methanol:acetone also provided better resolved peaks. The final decision was in favour of methanol:acetone (95:5, v:v) for both pesticides and pharmaceuticals as a compromise for lower recoveries but having chromatograms with less

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background noise, less interference peaks and well defined target compound peaks.

The introduction of a condensation/evaporation step is a common practice along extraction procedures, typically due to solvents change or as a pre-concentration step. Thus, differences in recovery using methanol:acetone (95:5, v:v) were also accessed with direct analysis of the extract or using a pre-concentration step by drying and redissolution (in water:methanol 50:50, v:v) in order to achieve a 10x concentration factor, Table 1. For pesticides, there were no differences in the recovery (carbendazim, BIT, mecoprop) or there was a significant negative effect on the recoveries (imazalil, terbutryn, diuron and triclosan) and a significant increase in the recovery of tebuconazole. Due to the significant decrease of triclosan recovery, the use of the concentration step needs to be careful evaluated depending on the target analytes of most interest for specific studies. However, for pharmaceuticals drying and redissolving improved significantly the recovery rate of the iodinated pharmaceuticals, without impact on the other compounds. The evaporation step resulted in precipitation of particles that were not redissolved by the mixture water:methanol (50:50). These particles most probably worked as a sink for the more hydrophobic compounds present in the extract. This co-precipitation explains both the reduced recovery for some moderately hydrophobic target compounds ($\log_{Kow}$ 2.67 – 4.66) and the decrease in background noise in the chromatogram. Therefore, there was increased S/N of the target peaks rather than a true recovery improvement.

Once sample extracts resulted in clean chromatograms and similar or better recoveries than the existing techniques (PLE, MAE) [29-31], the use of sequential extraction (commonly used) or further extract clean-up were not considered in order to ensure a fast and simple method.

Plant samples

For the optimization stage, only leaf material was used. As leaf extracts were expected to show higher backgrounds, they were not analysed directly, but only after the evaporation to dryness and a redissolution (in water:methanol 50:50) step. The recovery percentages of the pharmaceuticals and pesticides were evaluated for the most promising solvent/mixture (methanol, *n*-hexane,

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dichloromethane, methanol:formic acid (96:4, v:v), methanol:acetone (95:5, v:v), acetonitrile:formic acid (99:1, v:v)) (Figure 2).

Main results considering both pesticides and pharmaceuticals are that either some compounds show low recovery efficiencies (< 50%) or recoveries are higher than 120% as a consequence of high background influence on results (typical chromatogram shown in Figure S2). For pesticides, independently of the solvent used, the chemical background noise in the first part of the chromatographic run resulted in poor recovery for carbendazim, benzoisothiazoline and imazalil. As for the sediments, x-ray contrast agents had lower recoveries also in plant extracts, while the propranolol peak was overlapping with the background noise. Additional solvents (acetone, ethanol) and mixtures of solvents in different proportions (dichloromethane:methanol, *n*-hexane:acetic acid) were tested without noticeable improvements (results not shown) to reduce the background influence while providing acceptable recovery rates. Therefore, optimization of a clean-up step was further pursued.

A commonly used technique for environmental samples clean-up is the employment of Florisil in the form of SPE cartridges, for a variety of organic contaminants such as organochlorine pesticides or PAHs. For the pesticides included in this study clean-up by Florisil presented a general improvement in the results by reducing the matrix effect considerably. However, the extracts still contained too much background to analyse carbendazim and benzoisothiazoline. Regarding the Florisil step in itself, benzoisothiazoline and mecoprop also showed reproducibility problems that could not be overcome by optimizing the elution solvent. For pharmaceuticals, the Florisil SPE step results (not shown) revealed the occurrence of strong sorption to the sorbent, not only of the chemicals responsible for the background but also the target compounds. The obtained extracts provided chromatograms with reduced background, but low recoveries. Possibly there were problems eluting the target analytes. Therefore, the use of Florisil SPE cartridges was further discarded.

The next option chosen for both pesticides and pharmaceuticals was a typical reverse phase SPE approach for water samples. For that, extracts (after drying) were re-dissolved in water and processed in polymeric SPE orthogonal to the separation column (i.e., Strata-X cartridges) as water samples. Although the improvement in the chromatographic run was noticeable as for Florisil cartridges, it was still not enough to eliminate the chromatogram background,

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masking the results mainly for carbendazim and benzoisothiazoline (pesticides) and the x-ray contrast agents (pharmaceuticals). Use of SPE in these conditions would not ensure the quantification of all the compounds.

Therefore, a less commonly used but promising approach for sample clean-up tested was pigments saponification [26]. Chlorophylls and carotenes, are present in high concentrations in plants and will interfere in the analysis because they are extracted into the organic solvent. The saponification step addresses a base hydrolysis (at pH 13) of chlorophylls by cleavage of the two-ester bonds present in the chlorophylls. Nevertheless, it does not affect carotenes in the solution. Results revealed an improvement in the background removal showing clear chromatograms. For pesticides, the introduction of this saponification step resulted in less background and consequently in improved recovery (Figure 3) for the first pesticides of the run (early retention times) for all solvents, especially carbendazim and imazalil, and in general less co-eluted peaks with the target compounds. In fact, at this stage, *n*-hexane extraction followed by the saponification step was the most effective choice considering the amount of compounds and acceptable recoveries obtained. However, for pharmaceuticals, saponification was not as promising as for the pesticides (results not shown). Although showing chromatograms with less background, it was still not enough to reduce the interferences with the x-ray contrasts agents, as well as the last compound of the chromatographic run, diclofenac.

For the clean-up step, the use of less commonly applied materials was further considered. Activated carbon[32], Sephadex LH-20® or LRA (Lipid Removal Agent) media® have been previously employed on environmental samples for clean-up procedures [33]. Preliminary tests using methanolic plant extracts (5 mL) spiked with the target compounds, mixed with the different materials (0.25 g) in an ultrasonic bath for 30 min, revealed (results not shown) a general improvement in the chromatogram background, after the analysis of the supernatant. Especially for activated carbon, the typical green colour of the plant extracts was completely removed. Nevertheless, for pesticides this also resulted in strong sorption of the pesticides to the activated carbon causing lower recoveries. For the other tested materials, LRA and Sephadex, the improvement in the chromatograms were still not sufficient to completely remove the background. For the pharmaceuticals, activated carbon was the most promising material, especially because it allowed the quantification of some of

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the x-ray contrast agent compounds. Further tests were performed by adding the activated carbon to the extracts obtained with the six solvents under screening (Figure 3). Although allowing an acceptable analysis of the x-ray contrasts agents, it resulted in lower recovery efficiency than previously observed with for instance SPE for the remaining compounds, especially naproxen and diclofenac.

Considering the advantages and disadvantages of the previously tested steps, different procedural lines were further considered in order to clean-up the plant extracts. For pesticides, *n*-hexane at 100% was chosen as the most promising solvent for the extraction, and further efforts were placed in optimizing the saponification procedure, instead of working on improving the elution from activated carbon. For pharmaceuticals, activated carbon was considered to be more promising than the saponification step for improved recoveries of the iodinated compounds.

Final procedures establishment for pesticides was conducted by checking the pH influence in the SPE after the saponification step. The crude extract after evaporation to dryness was re-dissolved in methanolic KOH solution, ultrasonicated for 30 min, then the pH adjusted with hydrochloric acid (no adjustment, 2, 4, 5.5, 7) and further processed by SPE. A general improvement in recovery, except for imazalil, was observed when the pH of the KOH solution was adjusted to 5.5 before the SPE step, by comparison with no adjustment (Table 2).

Regarding pharmaceuticals, the last optimization step was to check which of the most promising solvents (Figure 3), methanol or methanol:acetone mixture (95:5, v:v) followed by the activated carbon clean-up step would provide the best and most reproducible results (Table 2). There were no significant differences in recovery between solvents, nevertheless the methanol:acetone mixture was chosen as it provided the highest recovery values. It should be noted that some of the recovery values obtained after the optimized clean-up step are lower than the methanolic (solution obtained by direct extracts evaporation to dryness and redissolution) extracts analysis. However, the existence of background noise on the extract analysis raises doubts on the reliability of this method when used as a routine for a high number of samples. In the present work, the choice of a multi approach overcomes individual best recoveries optimization for all compounds. Therefore, extraction with methanol:acetone mixture (95:5, v:v) followed by the activated carbon clean-up

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was selected for the improvement in the reliability of iodinated compounds analysis compromising recovery efficiency of diclofenac and naproxen.

The final optimized procedures selected were (Figure 4):

a) for sediments, samples were extracted with methanol:acetone (95:5, v/v) in an ultrasonic bath for both pesticides and pharmaceuticals. The extract was evaporated to dryness and dissolved in methanol prior to HPLC injection;

b) for plant tissue, pesticides were extracted with *n*-hexane followed by saponification (KOH), pH adjustment and SPE (Strata X) steps; while pharmaceuticals were analysed after extraction with methanol:acetone (95:5, v:v), supernatant cleaning with activated carbon and drying and re-dissolving in methanol/water prior to HPLC injection.

3.2 Method characteristics and testing

Precision, limits of detection (LOD) and quantification (LOQ), were assessed for the final method. The HPLC instrument LOD and LOQ were determined based on the signal-to-noise ratio (S/N) of 3 and 10, respectively, and further confirmed by injection of decreasing concentrations of standards (Table S2). The overall methodology limits were calculated based on samples mass used for extraction and further confirmed by assessing S/N of spiked matrix extracts in the calculated limits range. Overall methodology precision was based on extracts analysis.

Sediment samples

In sediment, the LODs and LOQs were calculated considering the extraction of 2 g of sediment sample. LODs ranged from 5 to 100 ng g^{-1} for the pesticides and 15 to 50 ng g^{-1} for the pharmaceuticals, while LOQ ranged from 25 to 250 ng g^{-1} for the pesticides and 50 to 150 ng g^{-1} for the pharmaceuticals (Table 3). The characteristics of the method are consistent with the analysis of different organic contaminants in sediments using different extraction procedures (Table S3). The LODs for sediment samples were higher than those obtained for pesticides in sediment samples by LC-MS/MS (0.01 – 17 ng g^{-1}) [13, 15, 29, 34] or GC-MS (0.01 to 2 ng g^{-1}). For example, a direct comparison of specific compounds across studies showed that the present LODs for terbutryn and

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diuron (5 ng g^{-1}), mecoprop and tebuconazole (50 ng g^{-1}), and triclosan (40 ng g^{-1}) were higher than those reported for PLE-LL-LC-HRMS/MS (0.05, 0.31, 0.4, 0.24 and 0.89 ng g^{-1}, respectively)[13] and PLE-SPE-LC-MS/MS (diuron 0.06 and mecoprop 4.17 ng g^{-1})[40]. For pharmaceuticals, the present LODs for sediment samples were higher than those obtained by LC-MS/MS (0.01 – 10 ng g^{-1}) [13, 15, 21, 35, 36] or GC-MS (0.3 – 6 ng g^{-1}) [30, 37] and similar to pharmaceuticals determination in sediments by DAD (LOD < 167 ng g^{-1} [11] and LOQ of 1 -187 ng g^{-1} [38]). For example, the comparison for propanolol showed that the present LOD (15 ng g^{-1}) was higher than that reported for USE-SPE-HPLC-DAD/FL (2 ng g^{-1})[38], USE-SPE-LC-MS/MS (0.9 ng g^{-1})[15] and PLE-LL-LC-HRMS/MS (0.03 ng g^{-1})[13]. Main differences in LOD performance are related to the use of more powerful detector such as MS or MS/MS, and less to the extraction techniques.

The overall precision of the methodology was determined based on the intermediate precision (i.e., replicates analysed by HPLC-DAD on various working days) of the extraction recovery of 6 spiked sediment samples, including both 0.5 and 5 μg g^{-1} level. This precision, reported as a relative standard deviation (RSD), was lower than 14 % (except for benzoisothiazoline 30%) (Table 3). Overall methodology recoveries (Table 3) ranged between 50 to 98% for the pharmaceuticals and 53 to 101% for the pesticides. For the pharmaceuticals, naproxen, and for the pesticides, benzoisothiazoline and triclosan, were the more affected compounds by the background noise resulting in poorer recoveries. Nevertheless, the obtained results are similar to previous published methodologies (Table S3) for sediment analysis of pesticides using simple solid-liquid extraction (40-125%) [39], PLE followed by SPE (67 – 118%)[40], USE followed by SPE (68 – 102%) [15], QuEChERS (46 – 102%) [34] or even MAE (81 – 112%) [37, 41]. For example, a direct comparison for carbendazim across studies showed that the present recovery (79%) is similar or higher to that reported for QuEChERS-LC-MS (61-80%) [34] and SLE-LC-MS (68%) [39]. Similarly, the current methodology recovery for pharmaceuticals is higher than obtained by Wagil, Maszkowska [35] (98 – 103%) and in the same range of previous works using MAE (25 – 81%) [11, 30] PLE (< 57 – 139 %) [13] or even USE followed by SPE (< 10 – 343%) [15, 21, 36, 38] (Table S3). For example, a comparison for carbamazepine showed that the present recovery (98%) was higher than that reported for USE-SPE-HPLC-DAD/FL (95%)[38], MAE-HPLC-DAD (78%)[11] and PLE-LL-LC-HRMS/MS (72%)[13].

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Plant samples

For plant tissue, LODs and LOQs were calculated considering the extraction of 0.2 g of sample. Values ranged from 0.05 to 1 μg g^{-1} for LOD and from 0.25 to 2.5 μg g^{-1} for LOQ for both the pesticides and the pharmaceuticals (Table 4). The overall methodology limits were higher than those obtained for pesticides in plant samples (Table S4) by LC-MS/MS (LOD of 3 ng g^{-1} [29], LOQ of 10 ng g^{-1} [42, 43]) and GC-MS/MS (LOQ of 10 ng g^{-1} [44]). For example, a direct comparison for tebuconazole across studies showed that the present LOQ (2 μg g^{-1}) was higher than that reported for dispersive-SPE-LC-MS/MS (100 ng g^{-1})[42]. For pharmaceuticals, the present limits for plant material were higher than those (Table S4) by LC-MS (LOD 2 – 13 ng g^{-1}) [14], LC-MS/MS (LOD of 0.5 to 8 ng g^{-1} [14, 45]) or GC-MS (7 – 58 ng g^{-1}) [14]. For example, a comparison for carbamazepine showed that the present LOD (0.25 μg g^{-1}) was higher than that reported for buffer extraction followed by SPE-GC-MS (10-20 ng g^{-1})[14], PLE-SPE-GC-MS (19 ng g^{-1})[14], QuEChERS-LC-MS/MS (0.7 ng g^{-1})[45] or PLE-SPE-LC-MS (0.17 ng g^{-1})[14]. Again, the main differences in LOD performance are related to the use in other works of a powerful detector such MS and less to the extraction and clean-up technique.

For the optimized conditions, recoveries (Table 4) ranged between 9 to 99% for the pharmaceuticals and 56 to 103% for the pesticides. The proposed methodology is not appropriate for iopamidol (25 %), propranolol (31%), naproxen (9%) and diclofenac (46%) quantification in plant tissue samples. The recoveries of the remaining pharmaceuticals were above 65%. For the pesticides, acceptable recoveries for this type of matrix (above 75%) were determined with the exception of benzoisothiazoline (56% recovery). The obtained recoveries are generally similar or higher than those previous published (Table S4) for pesticides in plant tissue samples using dispersive-SPE (72 – 104%) [42], solid-liquid extraction followed by salting out and SPE steps (10 – 120%) [44] and QuEChERS (80 - 136%) [43]. For example. a comparison for tebuconazole across studies showed that the present recovery (92%) was similar to the one reported for dispersive-SPE-LC-MS/MS (94%)[42]. Similarly, the current methodology recovery for pharmaceuticals are generally similar or higher than obtained using buffer extraction followed by SPE (15 – 98%) [14], USE followed by SPE (73 – 192%) [14], PLE with[14] or without [45] SPE (46 – 176%) and QuEChERS (70 –

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119%) [45] (Table S4). For example, a comparison for carbamazepine showed that the present recovery (82%) was higher than that reported for buffer extraction followed by SPE-GC-MS (15-61%)[14], PLE-SPE-GC-MS (75%)[14], and similar or lower than QuEChERS-LC-MS/MS (84-96)[45] and PLE-SPE-LC-MS (110)[14].

The overall precision of the methodology was determined as the intermediate precision (i.e., replicates analysed by HPLC-DAD on various working days) of the extraction of different spiked plant tissue (*Typha* and *Berula* n=2) parts (leaves n=3 and roots n=3), including both 2.5 and 5 μg g^{-1} level. This precision, reported as a relative standard deviation (RSD), was lower than 21% (except for iopromide, 38%). These results suggest good method repeatability, even considering different type of plant tissue (leafs and roots). In fact, in previous works the RSD for pharmaceuticals has been considered matrix-dependent [10]. The RSDs presently obtained (6-38%) is within the range previously found for pesticides and pharmaceuticals determination in plant tissue [43-45].

The use of the standard addition method could improve the overall quality of the proposed methodology for both sediment and plant analysis. However, that would have negative impact on simplicity and sample throughput. Since the objective was to establish a reliable but fast and simple method, the standard addition methodology was disregarded in the present study. Another option especially interesting for MS detectors would be the use of stable isotope labelled internal and/or surrogate standards, although Zhou, Ying [36] showed that even the addition of internal standards does not always overcome the matrix effects obtained for sediment samples. The sensitivity of HPLC–MS/MS is very dependent on the chemical ionisation procedure that is conditioned by the sample, the analyte, the eluent and the ion source design [22]. The use of matrix-matched calibration can be an interesting approach to minimize the matrix effects[20]. However, to match the matrix of the calibration standards with all individual plant samples (i.e., standard addition technique) can result in extended number of injections and consequently instrument time. Therefore, for MS detectors the use of internal standards is preferred over matrix-matched calibration[46]. Application of the methodology should be accompanied by recovery tests on the specific substrate to ensure a proper quality assurance and control.

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3.3 Application to real samples

The optimized and validated methodology has subsequently been used in different studies focused on removal of micropollutants from water by constructed wetland mesocosm systems. As an example of the method applicability, the quantification of the total accumulation of imazalil in a constructed wetland mesocosms substrate/sediment continuously run over 9 months under various hydraulic loading rates and imazalil concentrations of both 10 and 100 μg L^{-1}) (Figure S3) [28], as well as ibuprofen accumulation in plant tissue (roots and leaves) after exposure to an initial spike of 10 mg L^{-1} in the hydroponic media (Figure S4) [27]. In a recent work by the authors, studying an initial exposure of *Phragmites australis* to 10 μg L^{-1} of imazalil in hydroponic solution, plant extracts obtained with the present methodology were successfully analysed by HPLC-MS/MS for quantification of imazalil enantiomers and screened for transformation products with success [47]. The intra-equipment deviation for control samples (n≥8) analysed by both HPLC-DAD and HPLC-MS/MS were below 15% for the quantification of imazalil in plant tissue [47].

The validated methodologies proved fit-for-purpose in quantifying multiple classes of pesticides and pharmaceuticals in complex matrices. However, a broader application of the current methodology should be approached carefully. The use of a non-selective (non-confirmatory) DAD detector is only recommended when dealing with systems studied under controlled conditions. The application to field-samples should always be coupled with a confirmation step, or in alternative, the current extraction and clean-up steps can also be coupled with LC-MS. Nevertheless, as discussed before, the coupling to LC-MS, needs to be validated prior to full application specialty to assess matrix-effects and ion suppression in the detector. The range of compounds studied was broad and the methods may be applied for other compounds from the same family, chemical properties. But such application will always require a validation step.

The proposed USE methodology is a fast, easily accessible and effective alternative to the most advanced PLE or MAE methods (Table S3 and S4). Sample preparation time will be grossly similar across platforms. However, USE (presently, 24 samples in 30 min) and MAE (typically 24 samples in 40 min)

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allow the simultaneous extraction of samples being faster than PLE, which implies a sequential process (typically 20 min per cell, resulting in 24 samples in 8 hours). USE extraction is done in disposable glass vials, while MAE and PLE require additional clean-up and decontamination of the Teflon vessels or cells after use. PLE and MAE require an additional programming of an extraction/sequence procedure. Therefore, sample throughput is larger for USE. It should be noted that as drawback, USE does not have any automated control over the extraction process, as can be achieved by MAE and PLE. The difference in cost and accessibility to a simple ultrasonic bath that can be used for USE and the more advanced and dedicated equipment for MAE or PLE with the respective dedicated consumables is distinct.

4. Conclusions

The here established USE methods with the different optimized clean-up and pre-concentration steps coupled to HPLC-DAD analysis demonstrated suitable sensitivity and reliability, and proved fit-for-purpose in quantifying multiple classes of pesticides and pharmaceuticals in complex matrices such as sediment and plant tissue. For sediments, an acceptable extraction efficiency (50 - 101%) and RSD < 14% (except for benzoisothiazoline) were achieved without performing any clean-up step. The complex matrix of plant tissues poses specific problems, especially for improving the methodology recoveries. Thus, the final optimized method implies individualized approaches for the extraction of pesticides and pharmaceuticals. The established final method shows in general an acceptable extraction efficiency (> 46%) (except for iopamidol, propranolol and naproxen) with RSD < 21% (except iopromide) for different type of wetland plant tissues.

Compared with the existing methods in the literature, the proposed USE methodology is a fast, easily accessible, and effective alternative to PLE or MAE for extracting emerging contaminants from sediment and plant tissue samples. The methodology was successfully applied in different studies on the fate of pesticides and pharmaceuticals in water treatment eco-technology systems.

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