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Analysis and study of the distribution of polar and non-polar pesticides in wastewater effluents from modern and conventional treatments

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ABSTRACT

The analysis of a wide range of pesticides in wastewaters (WWs) undergoing different treatments (both modern and conventional) has been studied. The need for optimizing specific extraction methods for each WW effluent based on their physico-chemical characteristics has been considered. A distribution study was performed to establish if the filtration step before extraction is a correct procedure since pesticides can be more prone to be in the aqueous or the solid phase, depending on their hydrophobicity. This evaluation demonstrated that pesticides are distributed between the aqueous phase and the suspended particulate matter (SPM; e.g. pyrethroids are only found in the SPM). The proposed methodologies involved the determination of 39 polar and 139 non-polar pesticides using solid-phase extraction (SPE) and pressurized-liquid extraction (PLE) for the extraction of the aqueous phase and the SPM, respectively. Ultra high pressure liquid chromatography and gas chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS, GC–MS/MS) were used in the determination stage. WW samples from four different technologies were evaluated: membrane bioreactor, extended aeration, maturation pond and anaerobic pond. Validation data for the four effluents studied were generated, obtaining adequate precision values (estimated as % relative standard deviation, RSD) in almost all cases (<25%). The methods showed limits of detection at 0.01–0.20 $\mu g\,L^{-1}$ and limits of quantification from 0.02 to 0.50 $\mu g\,L^{-1}$. The proposed methods were applied to the analysis of real samples collected from an experimental WW treatment plant, detecting non-polar and polar pesticides at concentrations in the range 0.02–1.94 $\mu g\,L^{-1}$ and 0.02–0.33 $\mu g\,L^{-1}$, respectively.

1. Introduction

The re-use of urban wastewaters (WWs), mixture that includes both industrial and domestic WWs, which have been previously treated in WW treatment plants (WWTPs), is currently the most employed strategy in several countries to deal with the water shortage problem. However, the occurrence of contaminants and residues in WW effluents can be a cause of concern when they are re-used. Thus, the use of treated WWs in Spain was regulated by the establishment of minimum quality criteria according to their usage [1]. The treated WWs can be utilized in agricultural irrigation [2], for municipal and industrial purposes, for environmental aims, such as the recharging of aquiferous or they can be directly discharged into rivers or the sea.

Certain groups of contaminants (e.g. pesticides) are listed as priority pollutants by the European Union (EU) [3–6] and the United States Environmental Protection Agency (US-EPA) [7,8]. Consequently, and bearing in mind the possible re-use of WWs, these compounds need to be determined and controlled in WW effluents in order to assure their quality.

In general, WWTPs consist of a line of WW treatments composed on a pre-treatment and consecutives primary, secondary and tertiary treatments, employing both conventional and modern/recent technologies. Conventional treatments include extended aeration (EA), maturation pond (MP) and anaerobic pond (AP), which are characterized by a relatively high amount of suspended solids or suspended particulate matter (SPM). At present, several conventional treatments are being replaced by emerging techniques, such as membrane bioreactors (MBRs), which are a combination of a membrane process like microfiltration or ultrafiltration with a suspended growth bioreactor that can produce effluents of high quality. Recent technical innovation and significant membrane cost reduction have pushed MBRs to become an established process

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option to treat WWs [9]. Nevertheless, the high cost of the modern technologies complicate their implementation in developing countries, and thus, conventional systems must still be taken into account.

Depending on the treatment that WWs have undergone, they have different amounts of SPM. Most of the analytical methods found in literature for the analysis of pesticides in WW are only based on the analysis of the aqueous phase obtained after sample filtration, without regarding to the SPM that is retained in the filters [10,11]. This methodology is also applied for the determination of other contaminants, such as phenols or polycyclic aromatic hydrocarbons (PAHs) in WWs [12,13]. In such cases, aqueous contaminant concentrations reported for filtered samples (freely dissolved fraction) may deviate considerably from the total concentration in the sample. Another fact to be considered is that, normally, WW analyses are performed without carrying out a previous characterization of the different effluents. Bearing in mind that, depending on the treatment, the final effluents have different physico-chemical characteristics, the matrixes are therefore different. Consequently, the extraction process can be affected by the type of effluent and this must be characterized in order to apply the most suitable extraction process.

It is well-known that liquid chromatography (LC) coupled to mass spectrometry (MS) detection [14–16] and gas chromatography (GC) coupled to mass spectrometry (MS) detection [11,15,17] are widely implemented for the determination of pesticides and other organic contaminants and residues in WW and water analysis. Besides, the use of liquid–liquid extraction (LLE) with solvents such as *n*-hexane [18] or dichloromethane [19,20] is still generalized for the extraction stage, despite some drawbacks such as solvent consumption and analysis time. Furthermore, public institutions, such as the US-EPA, propose official methods for the analysis of organochlorine pesticides [21] in WW that involve the utilization of LLE with dichloromethane. Other methodologies are based on solid phase extraction (SPE). In literature, polar and non-polar pesticides are extracted by SPE, separately in groups [22–24] or simultaneously [11,25,26]. However, it is important to point out that in many of the reported studies, LLE and SPE have been employed for the analysis of a small number of pesticides (e.g. 25 [11], 17 [19], and 19 compounds [22]). Furthermore, most of the studies carried out in WWs are mainly based on the monitoring of polar pesticides. However, the Water Framework Directive [6] indicates that it is also necessary to analyze non-polar pesticides in the case of drinking water.

A well-known critical point in the analysis of WW is matrix effect. In order to minimize it, and taking into account the difficulty of finding blank samples, different calibration methods such as matrix-matched calibration [27,28], standard addition [28,29] and the use of isotope-labelled internal standards [11,26] have been employed. In this study, two of these calibration methods (matrix-matched calibration and isotope-labelled internal standards) have been evaluated with the aim of achieving a more accurate quantification. Furthermore, it is possible to quantify by using both simultaneously, taking into account the nature of these complex samples.

Bearing all this in mind, the purpose of this study is to develop a general protocol for the determination of >100 pesticides covering a wide range of polarity and families (including polar and non-polar pesticides) in WW effluents. The selected compounds include pesticides currently applied and other pesticides whose use is now forbidden. Four types of WW effluents have been evaluated with the aim of establishing a classification depending on their physico-chemical characteristics in order to develop a specific extraction method for each of them, if necessary. In this sense, a study of the distribution of the compounds between the aqueous phase and the SPM has been performed, which is, up to our knowledge, the first approach described in this topic. For the aqueous phase, LLE and SPE have been evaluated, whereas a pressurized liquid extraction (PLE) method has been developed for the SPM. Finally, the instrumental analyses have been carried out by GC coupled to triple quadrupole MS (GC–QqQ-MS/MS) and ultra-high-pressure LC (UHPLC) coupled to triple quadrupole mass spectrometry (UHPLC–QqQ-MS/MS) for non-polar and polar pesticides determination, respectively.

2. Materials and methods

2.1. Chemicals and reagents

Pesticide analytical standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), as well as the isotopically labelled pesticides 4,4′-DDE-d_8, parathion ethyl-d_{10}, pirimicarb-d_6, permethrin-d_6, *trans*-cypermethrin-d_6, [$^{13}C_6$]-hexachlorobenzene and [^{13}C]-caffeine, which were used as internal standards (ISs) for non-polar compounds, and simazine-d_5 and diuron-d_6, which were employed as ISs for polar pesticides. Single standard stock solutions of the analytes (with concentrations ranging from 173.1 to 1903.2 mg L^{-1}) and the ISs (concentration between 10 and 296 mg L^{-1}) were prepared by exact weighing of powder or liquid and dissolution in acetone (non-polar pesticides) or methanol (MeOH) (polar pesticides), and stored in a freezer (-20 °C). Two multi-compound working standard solutions, containing 2 mg L^{-1} of each compound, were prepared by further dilution of the individual stock standard solutions with acetone (non-polar pesticides) or MeOH (polar pesticides). Both solutions were stored under refrigeration at $T \leq 4$ °C. Working standard solutions of all the ISs (20 mg L^{-1}) were prepared by appropriate dilution of the stock solutions with acetone or MeOH and stored under the aforementioned conditions. 4,4′-DDE-d_8 and *trans*-cypermethrin-d_6 were prepared by dilution of the commercial standard solutions (100 mg L^{-1}) with acetone (final concentration: 10 mg L^{-1}). These solutions were also stored under refrigeration at $T \leq 4$ °C.

Ethyl acetate (EtOAc), *n*-hexane and MeOH were supplied by J.T. Baker (Deventer, Holland). Acetone and cyclohexane were purchased from Fluka (Steinheim, Germany) and dichloromethane (DCM) was obtained from Riedel-de Haën (Seelze, Germany). All organic solvents were of analytical grade. Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA). Formic acid (purity >98%) was purchased from Panreac (Barcelona, Spain). Sodium chloride (NaCl), anhydrous sodium sulfate (Na_2SO_4) and hydrochloric acid (HCl, purity 37–38%) were obtained from J.T. Baker.

47-mm glass microfibre filters from Whatman (Maidstone, England, UK) and 0.45-μm HNWP nylon membrane filters from Millipore (Carrigtwohill, County Cork, Ireland) were also available for filtration stages.

For SPE extractions, C_{18} Sep-Pak cartridges (500 mg, 6 cm^3) as well as Oasis HLB (200 mg, 6 cm^3) cartridges, obtained from Waters (Milford, MA, USA), were employed. Florisil SPE cartridges were purchased from Varian (Habour City, CA, USA).

30-mm cellulose filters (Whatman) and Hydromatrix (Varian) were used for PLE extractions.

2.2. Instrumentation

Non-polar pesticide analyses were carried out using a GC system Varian 3800 (Varian Instruments, Sunnyvale, CA, USA) equipped with electronic flow control (EFC). Samples were injected into an SPI/1079 split/splitless programmed-temperature injector, utilizing the large volume injection (LVI) technique and a Combi Pal (CTC Analytics AG, Zwingen, Switzerland) autosampler, using a 100-μL syringe. The glass liner was equipped with a plug of

carbofrit (Resteck, Bellefonte, PA, USA). A fused-silica untreated capillary column (2 m × 0.25 mm i.d.) from Supelco was used as pre-column connected to a Factor Four Capillary Column VF-5ms (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The carrier gas was helium (99.9999%) at a constant flow rate of 1 mL min^{-1}. The GC was interfaced to a 1200 L QqQ mass spectrometer (Varian Instruments) operating in electron ionization (EI) at 70 eV. Argon (99.999%) was used as collision gas. The mass spectrometer was calibrated every four days with perfluorotributylamine. Varian Workstation software was used for instrument control and data analysis.

Polar pesticide analyses were performed in an Acquity UPLC system using an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm), with a 1.7 μm particle size (both from Waters). Chromatographic separations were carried out using gradient elution with eluent A, being MeOH, and eluent B, consisting of an aqueous solution of formic acid (0.01%, *v*/*v*). MS analysis was carried out using a Waters Acquity TQD QqQ mass spectrometer (Waters, Manchester, UK). The instrument was operated using positive electrospray ionization (ESI+). Data acquisition was performed using MassLynx 4.0 and QuanLynx software (Waters).

The horizontal shaker used in the distribution study was obtained from P-Selecta (Selecta, Barcelona, Spain).

PLE was performed using an ASE 100 Accelerated Solvent Extraction system (Dionex, Sunnyvale, CA, USA) equipped with 34-mL stainless steel extraction cells.

The ProStar gel permeation chromatography (GPC) system used (Varian) consisted of a 410 autosampler with a 24-vial (10 mL) tray, a 230 solvent delivery module, a 325 UV–vis detector with dual wavelength operation (λ = 254 nm), a 704 fraction collector, and two on-line connected Envirogel GPC clean-up columns from Waters packed with polystyrene–divinylbenzene (150 mm × 19 mm i.d. and 300 mm × 19 mm i.d., respectively).

2.3. WW collection

Urban WW effluents from four different treatments, namely, MBR, EA, MP and AP (ordered from low to high SPM content) were collected from the Foundation Centre for New Water Technologies ("Centro de las Nuevas Tecnologías del Agua", CENTA) located in Seville, Spain. This WWTP has an area of 41,000 m^2 and currently holds more than 20 systems with different technologies. WW samples were stored at 4 °C and processed within 24 h after the collection. Due to the difficulty of finding real blank WW samples, during the optimization and validation stage, non-spiked samples were used and they are named "blank" samples throughout the text, despite pesticide traces were found in some cases.

2.4. Distribution study

Non-filtered WW samples were spiked with 4 μg L^{-1} of the target pesticides, and then they were shaken overnight at a rate of 100 oscillations per min to allow a thoroughly interaction between the compounds and the SPM. After this, the samples were filtered to separate and analyze both phases. The aqueous phase was extracted by SPE, whereas for the SPM, a PLE process was carried out. The distribution of the compounds between the phases was determined as the percentage of them present in each phase.

2.5. Analysis of non-polar pesticides by GC–QqQ-MS/MS

WW samples were filtered consecutively using two different pore-size filters (47-mm glass microfibre filters and 0.45-μm nylon membrane filters). The filters containing the SPM were stored at 4 °C until their analysis by PLE, and the aqueous phase was extracted by SPE.

2.5.1. Strategy applied during the optimization stage

Two sets of samples of each effluent were extracted. In one set, samples were spiked at a concentration of 4 μg L^{-1}, and in the second set, SPE sample extracts were spiked at 500 μg L^{-1} (corresponding to 4 μg L^{-1} in samples) with the same target compounds after the SPE process as one-point calibration for quantification purposes. For the analysis of SPM, in a first set, filters were spiked with 1 μg of the non-polar pesticides to obtain 500 μg L^{-1} as final concentration after the extraction process, regardless the amount of SPM present in the filters. In the other set, PLE sample extracts were spiked after the PLE procedure at 500 μg L^{-1}, and it was used for quantification purposes as one-point calibration.

2.5.2. Extraction of the aqueous phase by SPE

WW samples were processed according to the following procedure: 250 mL of each filtered water sample were adjusted to pH 3.0 with 2 N HCl (all samples showed pH > 7), and 2.5 g of NaCl was added in order to adjust the conductivity to 50 mS. An organic modifier (MeOH) was added (1%, *v*/*v*) before performing the SPE procedure in order to avoid possible analyte adsorptions in the glass material. This protocol was also applied during the optimization of the extraction method. The C_{18} cartridges were previously conditioned with 3 mL of EtOAc, followed by 3 mL of MeOH and 3 mL of ultrapure water without allowing the cartridges to dry out. Then, the WW samples were passed through the cartridges under vacuum at a flow rate of 10 mL min^{-1}. The cartridges were dried for 3 h and the pesticides were eluted with 5 mL of EtOAc. The extracts were evaporated with a vacuum rotary evaporator at 45 °C, and the residues were redissolved adding 25 μL of parathion ethyl-d_{10} (500 μg L^{-1}) and EtOAc (final volume: 2 mL) before chromatographic analysis.

2.5.3. Extraction of the SPM by PLE

The filters obtained in Section 2.5 containing the SPM were dried and submitted to the PLE extraction. Briefly, a cellulose filter was placed at the bottom of a 34-mL stainless steel extraction cell. Filters with the SPM were cut into small pieces and placed into the cell mixed with Hydromatrix up to filling it. The extraction was performed using EtOAc:MeOH (3:1, *v*/*v*) under the PLE conditions described by Martínez-Vidal et al. for the extraction of pesticides in agricultural soils [30]. After that, a clean-up step was carried out by using Florisil SPE cartridges, which is a methodology also described by the US-EPA method 3620C [31]. The cleaned extracts were then evaporated and redissolved as explained for the SPE samples. A clean-up methodology based on GPC was also assessed (but finally discarded) in the optimization process following the procedure described below.

2.5.4. GPC clean-up procedure

After the PLE process, the final extract was evaporated in the rotary evaporator at 45 °C and redissolved with 5 mL of EtOAc:cyclohexane (1:1, *v*/*v*). The re-dissolved samples were transferred into a 10-mL vial and then 2 mL were injected in the GPC system. EtOAc:cyclohexane (1:1, *v*/*v*) was used as the mobile phase at a column flow rate of 5 mL min^{-1}. The representative fraction containing the target pesticides was collected from 14 to 22 min (approximately 45 mL). The GPC fraction was evaporated to dryness and the residue was redissolved adding 25 μL of parathion ethyl-d_{10} (500 μg L^{-1}) and EtOAc (final volume: 2 mL) before chromatographic analysis.

2.5.5. GC–QqQ-MS/MS analysis

Aliquots of 10 μL of sample extract were injected into the GC system operating at a syringe injection flow rate of 10 μL s^{-1}. The injector temperature program was as follows: 70 °C (hold for 0.5 min) → 310 °C (100 °C min^{-1}, hold for 10 min). The injector split

Table 1
Average value ± range of variation of several physico-chemical parameters measured during one year for the four effluents evaluated ($n = 23$).

Parameter	MBR[a]	EA[a]	MP[a]	AP[a]
Ammonium (mg L^{-1} N)	5 ± 4	34 ± 8	25 ± 9	50 ± 11
BOD (mg L^{-1} O_2)	12 ± 10	45 ± 15	48 ± 16	272 ± 39
COD (mg L^{-1} O_2)	35 ± 12	114 ± 39	209 ± 42	503 ± 54
Phosphate (mg L^{-1} P)	5 ± 3	5 ± 4	5 ± 3	6 ± 3
Total Phosphorus (mg L^{-1} P)	6 ± 3	7 ± 5	7 ± 4	7 ± 3
Nitrates (mg L^{-1} N)	34 ± 10	7 ± 5	7 ± 2	8 ± 3
Total suspended solids (mg L^{-1})	9 ± 6	38 ± 14	59 ± 17	99 ± 21
pH	7 ± 1	7 ± 1	8 ± 1	7 ± 1
Conductivity (μS cm^{-1})	1223 ± 214	1364 ± 173	1237 ± 152	1386 ± 166
T (°C)	18 ± 6	21 ± 5	21 ± 5	20 ± 6
Dissolved oxygen (mg L^{-1} O_2)	6 ± 2	2 ± 2	4 ± 3	1 ± 1

[a] *Abbreviations*: MBR: membrane bioreactor; EA: extended aeration; MP: maturation pond; AP: anaerobic pond; BOD: biochemical oxygen demand; COD: chemical oxygen demand.

ratio was initially set at 10:1. Splitless mode was switched on at 0.5 min until 3.5 min. At 3.5 min, the split ratio was 100:1 and at 10 min, 20:1. The column oven program was: 70 °C (hold for 3.5 min) → 180 °C (35 °C min^{-1}) → 300 °C (10 °C min^{-1}, hold 7 min). Cryogenic cooling with CO_2 was applied when the injector temperature was 170 °C. The total running time was 25.6 min.

The QqQ mass spectrometer was operated in the selected reaction monitoring mode (SRM). In certain cases, the single-ion monitoring mode (SIM) was also applied. The temperatures of the transfer line, manifold and ionization source were set at 300, 40 and 280 °C, respectively. The electron multiplier voltage was set at a voltage value +100 V above the optimal value indicated by the software instrument. The optimal values for the scan time ranged from 0.264 to 0.504 s. The peak widths set in the first and third quadrupoles were m/z 2.0 and 1.5, respectively, and the analysis was performed with a filament-multiplier delay of 5.5 min. The total running time was 25.6 min. The specific MS/MS conditions for the selected pesticides are shown in Table S-1.

2.6. Analysis of polar pesticides by UHPLC–QqQ-MS/MS

250 mL of WW sample were filtered and pH and conductivity adjustments were performed as described for the analysis of non-polar pesticides. In this case, only the aqueous phase was analyzed (see Section 3) by applying an SPE-based method. Depending on the type of WW effluent, two conditioning/elution conditions were utilized. Oasis HLB cartridges were conditioned with 5 mL of EtOAc (for MBR, EA and MP effluents) or DCM (for AP samples) followed by 5 mL of MeOH and 5 mL of ultrapure water. The cartridges were dried for 3 h and the pesticides were eluted with 5 mL of MeOH, followed by 5 mL of EtOAc (MBR, EA and MP) or DCM (AP). The extracts were evaporated with a vacuum rotary evaporator at 45 °C, and the residues were redissolved adding 25 μL of simazine-d_5 (500 μg L^{-1}) and a mixture of MeOH/aqueous solution of formic acid 0.01% (50:50, v/v) to a final volume of 2 mL before chromatographic analysis. Finally, the extract was transferred into a vial and 5 μL were injected into the UHPLC–QqQ-MS/MS system.

2.6.1. UHPLC–QqQ-MS/MS analysis

The elution started at 10% A and then was linearly increased up to 90% A in 5 min, keeping constant for 2 min before being returned to the initial conditions in 0.5 min. Finally, the total run time, including the conditioning of the column to the initial conditions was 9.0 min. The flow rate was 0.35 mL min^{-1} and the column temperature was maintained at 35 °C. All pesticides were detected using ESI+. The ionization source parameters were: capillary voltage 3 kV, extractor voltage 2 V, source temperature 120 °C, desolvation temperature 350 °C, cone gas flow 80 L h^{-1} and desolvation gas flow 600 L h^{-1} (both gases were N_2). Collision-induced dissociation (CID) was performed using argon as the collision gas at a pressure of 4×10^{-3} mbar in the collision cell. The specific MS/MS parameters for each pesticide are shown in Table S-2.

2.7. Validation study

Because of the impossibility of obtaining blank samples, WW samples were previously analyzed to check the occurrence of the compounds under study. In positive samples, this presence was taken into account in the quantification stage by subtracting the blank area. In order to assure the reliability of the proposed methods, the validation requirements were as follows: (1) to minimize matrix effects, matrix-matched calibration curves are used and determination coefficients (R^2) must be ≥0.98; (2) intraday precision expressed as relative standard deviation (% RSD) must be ≤25%; (3) trueness, expressed as recovery can be in the range 50–120% and (4) limits of detection (LODs) and limits of quantification (LOQs) must be obtained in the same concentration range than the limits established for water intended for human consumption, although they can accepted slightly higher due to the complex nature of the samples under study and their destination.

3. Results and discussion

One of the aims of this work is the development of adequate extraction methods for the determination of polar and non-polar pesticides in urban WW effluents. Samples were obtained from a WWTP (CENTA), which employs more than 20 different WW treatments. Four of which were selected as the most representative and interesting technologies, considering their current utilization and covering a wide range of physico-chemical properties. Table 1 shows several parameters of the treated WWs under study. It is important to notice the different amounts of SPM observed, finding that the treatment that generated the effluent with higher amount of SPM was AP (99 ± 21 mg L^{-1}), followed by MP (59 ± 17 mg L^{-1}), EA (38 ± 14 mg L^{-1}) and MBR (9 ± 6 mg L^{-1}), which contained a minimal amount of solids. Consequently, besides their origin, WW sample nature depends on the treatment applied, so it is necessary to evaluate the performance of the used analytical methods in the different WW types. This protocol is well-known, for instance, in the determination of pesticides in food analysis, where the methods are validated for the different reference matrixes (e.g. high water content) and verifications are carried out within the same group of matrixes (e.g. pepper, cucumber, eggplant, etc.). However, the reported methods usually do not describe the type of WW effluent or a verification demonstrating that they can be applied to a variety of WW samples. For this reason, the four selected WW effluents were analyzed in order to investigate the presence of the target compounds and to develop a specific extraction methodology for

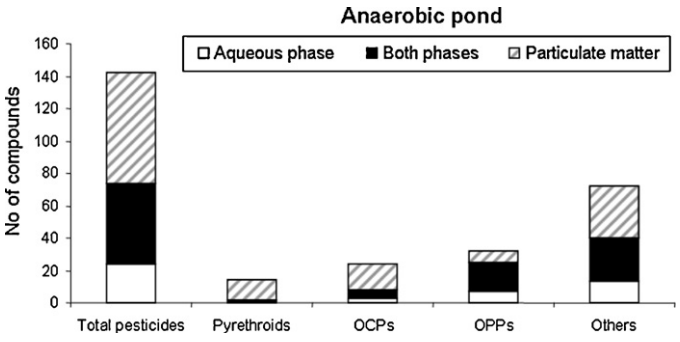

Fig. 1. Distribution of the non-polar pesticides found in each phase with a rate higher than 75%, as well as those distributed between both phases with rates between 25% and 75%, in the AP effluent. *Abbreviations*: OCPs: organochlorine pesticides; OCPs: organophosphorus pesticides.

each one depending on the treatment that they have undergone. Another relevant objective of this work was also to study matrix effects in every of the sample types analyzed.

Finally, it must be indicated that during the optimization procedure, blank samples of each effluent were also processed to subtract the levels of possible target compounds.

3.1. Distribution study

Because of the different physico-chemical properties, pesticides might be found distributed in the two phases composing WW samples: aqueous phase and SPM. A previous study of their distribution between these phases was carried out, using the procedure described in Section 2. Fig. 1 shows the results obtained for the non-polar pesticides in the AP effluents. It can be observed that depending on the hydrophobicity (established as the logarithm of the octanol–water partition constant value, $K_{o/w}$) the compounds are mainly distributed in the aqueous phase or SPM. The same trend was observed irrespective of the type of effluent (see Table S-3). Thus, for non-polar pesticides it was observed that most of the compounds were distributed in both the aqueous and the "solid" phases, except for pyrethroids and organochlorine pesticides, which were more prone to remain in the SPM. This fact must be adduced as a result that demonstrates the need for analyzing both phases, the aqueous and the "solid phase", which is normally discarded. On the contrary, for polar pesticides, the fraction of analytes bound to particles was insubstantial, and they were mainly found in the aqueous phase. Therefore, the analysis of SPM was not necessary when monitoring polar pesticides. Furthermore, as the content of SPM of the different effluents increased, a higher number of compounds mainly retained in the solids were observed, indicating that WW samples do not show a unique analytical behavior.

3.2. Development of the extraction methods

At this point, two strategies were proposed for the development of the extraction methods for non-polar pesticides: the analysis of the phases separately (by using different extraction methods) or the development of a method capable of extracting simultaneously the analytes dissolved in the liquid phase of the sample and those adsorbed into the SPM. In this sense, several extraction methods were tested in order to develop the best methodologies that can be able to extract the target compounds from the four different types of effluents, bearing in mind their different physico-chemical characteristics.

3.2.1. Analysis of non-polar pesticides

LLE, SPE and PLE were the methodologies evaluated for the extraction of the target compounds. Experiments were done at 4 $\mu g\,L^{-1}$ throughout all the optimization process and, due to the fact that WWs are biologically active matrixes, they were previously acidified to pH 3.0 and conductivity was adjusted to 50 mS with NaCl, in order to stop the possible degradation of the target compounds and to normalize all the procedures.

3.2.1.1. Simultaneous analysis of the aqueous phase and SPM by LLE. Initially, an LLE procedure was applied to a mixture of non-filtered WWs in order to carry out the simultaneous extraction of the compounds from both phases in a single step, reducing the cost and analysis time. Although in literature the use of DCM as extraction solvent is commonly employed, in this study other organic solvents have been used due to the toxicity of DCM. Therefore, 20 mL of *n*-hexane were added to 200 mL of the spiked sample. The mixture was shaken in a rotary agitator for 30 min. Then, the organic phase was collected and dried with anhydrous Na_2SO_4. The recovery results were not adequate for a high percentage of compounds (data not shown). Higher extraction times (30, 60, 120 min), as well as two extractions of 30 min each one, were then tested using 20 mL of a mixture of *n*-hexane:EtOAc (50:50, *v*/*v*) as extraction solvent. As it can be seen in Fig. S-1, the best results were obtained when two consecutive extractions of 30 min were performed, although the results were still not completely adequate. Another fact to be considered is the formation of emulsions in certain samples (normally EA, MP and AP), which encumber the extraction process and could produce analyte losses. Finally, taking into account all these problems and the poor recoveries obtained with this methodology, the second strategy was therefore evaluated.

3.2.1.2. Separated analysis of the aqueous phase: optimization of the SPE stage. Initially, an SPE method based on a previous procedure for the extraction of pesticides in water samples [32] was employed, using C_{18} cartridges. Different organic solvents (EtOAc, cyclohexane, toluene and DCM) and elution volumes (3 and 5 mL) were tested to optimize the elution step. Elution using 5 mL of EtOAc and DCM provided better recovery values for a larger number of compounds. Since both solvents showed very similar results (Fig. 2), EtOAc was chosen due to its lower toxicity, independently of the effluent analyzed. Then, another parameter tested included the washing of the cartridges with ultrapure water. As it was seen that recoveries did not improve, this step was discarded.

Therefore, for the extraction of the aqueous phase, the same methodology was carried out for the four effluents since their behaviors were the same independently of the treatment of the WW. Finally, Fig. 3 shows a representative chromatogram obtained when the optimized extraction method was applied to spiked WW samples from the four effluents (50 $\mu g\,L^{-1}$). It can be observed that

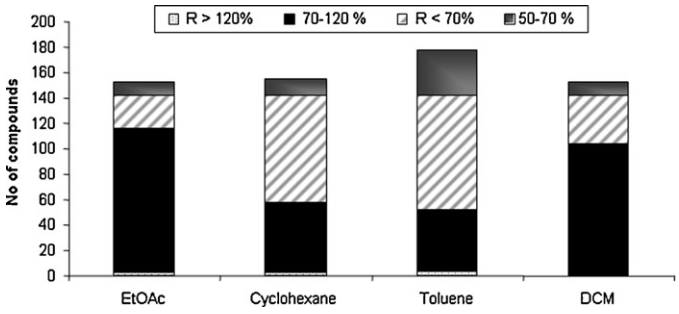

Fig. 2. Effect of type of solvent used in the SPE process on the number of non-polar pesticides recovered in a mixture of WW effluents (anaerobic pond and membrane bioreactor).

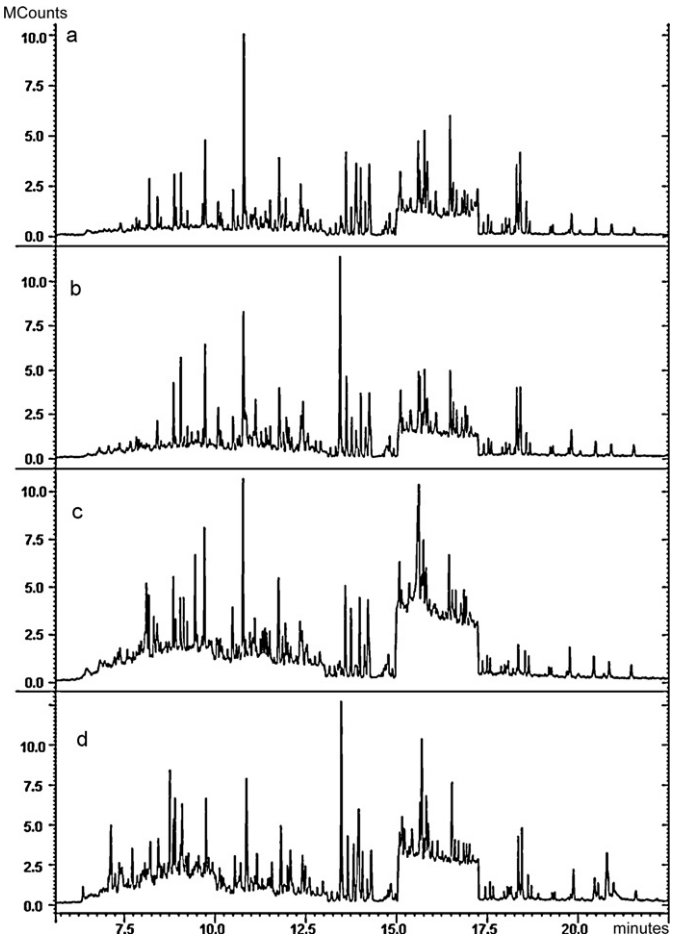

Fig. 3. Total ion chromatogram (TIC) of different WW effluents spiked with the selected non-polar pesticides at 50 μg L^{-1}: (a) membrane bioreactor; (b) extended aeration; (c) maturation pond; and (d) anaerobic pond.

the background noise increases as does the amount of SPM of the effluents, fact that demonstrated that depending on the treatment, the WW samples can present different analytical behaviors.

3.2.1.3. Separated analysis of the SPM: optimization of the PLE stage. Due to the complexity of WW samples, the SPM obtained after filtration could be consider as a 'special soil' with a high percentage of organic matter. For this reason, a previous extraction method developed for polar and non-polar pesticides in agricultural soils [30] was evaluated for the analysis of the SPM of the four selected effluents. As it can be observed in Table S-4, satisfactory results were obtained for all of the effluents, although for AP (the "dirtier" effluent) the number of compounds with adequate recoveries was slightly lower. Due to the complexity of the matrix, a clean-up step was necessary after the PLE for all the extracts. SPE using Florisil and GPC were evaluated and similar results were obtained when both methods were applied. Therefore, SPE with Florisil was chosen since it was faster, required less solvent consumption and increased sample throughput.

3.2.2. Analysis of polar pesticides

As previously commented in Section 3.1, only the extraction process of the aqueous phase was optimized for the polar pesticides. Due to the difficulty of finding an organic solvent immiscible with the water sample but with the sufficient polarity to extract the target compounds, LLE was discarded and an SPE method was optimized for the determination of the analytes in all the selected effluents.

3.2.2.1. Analysis of the aqueous phase: optimization of the SPE procedure. Initially, an SPE method based on the extraction of herbicides in water samples [33] was employed, using Oasis HLB cartridges. The first step in the optimization process was the evaluation of the elution organic solvent, trying to avoid the use of DCM due to its well-known toxicity. For this, DCM and EtOAc were tested for all the effluents. The results shown in Fig. 4 indicate that when the amount of SPM in the effluent increases (from MBR to AP) a higher number of compounds were extracted when DCM was used, probably due to this solvent is more selective than EtOAc, showing the obtained results of individual analyte recoveries in Table S-5. Furthermore, DCM provided cleaner extracts for the AP effluent than EtOAc. Consequently, depending on the type of effluent, EtOAc or DCM can be used as eluent solvent.

At this point, two possibilities can also be considered: the use of one solvent depending on the type of effluent or the development of a common method for all the effluents using both solvents sequentially (first DCM and later EtOAc). The two possibilities offered similar results (data not shown), and the first one was chosen as optimized methodology in order to reduce the use of DCM. Thus, for the effluents from MBR, EA and MP treatments, the elution step was performed with EtOAc as organic solvent, while DCM was employed only for the elution of the AP effluent. This demonstrated that the same extraction process cannot be the most suitable for all the effluents when polar pesticides are determined.

3.3. Assessment of the matrix effects

Matrix effects from the four effluents under study were evaluated for polar and non-polar pesticides by comparing the peak area of known amount of a standard solution (A) with that from a sample extract spiked with the same amount of analyte after extraction (B). The ratio (B/A × 100) is defined as absolute matrix effect (ME%) [34]. In the case of non-polar pesticides, for the EA and AP effluents signal suppression (ratio <100) was typically found, while for the MBR and MP effluents both signal suppression and enhancement effects (ratio >100) were observed. However, for polar pesticides, almost all compounds presented signal enhancement in the four different effluents. Firstly, the aim was to select one of the treatments under study as representative matrix of all of them. For this, the different types of effluents were analyzed after spiking samples at different concentration levels and the obtained slopes were compared. Since significant differences between the different matrixes were observed, it was not possible to use a representative matrix during routine analysis. This fact demonstrated that WW matrixes do not show the same behavior, and therefore they cannot be treated in the same way without a previous verification.

The heterogeneous behavior observed for the different effluents when determining multi-class analytes, makes rather difficult matrix effect correction. Bearing in mind the nature of the matrix under study and thus the difficulty of obtaining blank samples, methodologies such as matrix-matched calibration must be used with other alternative quantification method. In this respect, the

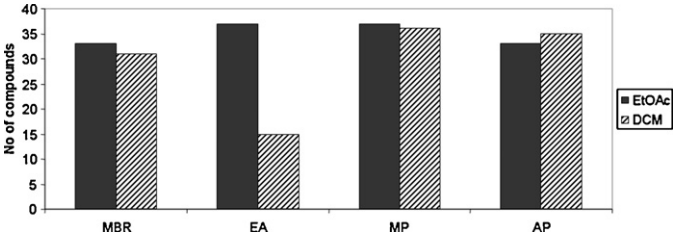

Fig. 4. Effect of type of solvent used in the SPE process on the number of polar pesticides recovered with rates between 50% and 120% in the different WW effluents.

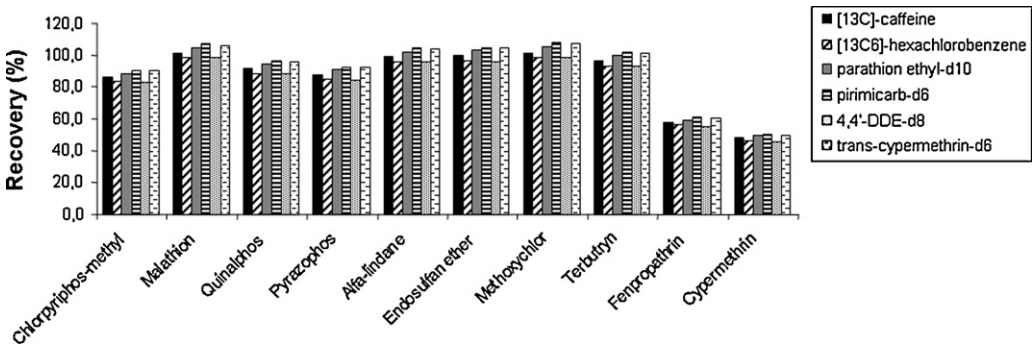

Fig. 5. Recovery values for non-polar pesticides from different families depending on the IS used for quantification purposes in the extraction of the aqueous phase of the extended aeration effluent.

use of isotopically labelled ISs seems to be the best solution [26]. However, in a multi-component analysis, as well as the prohibitive cost that it supposes, finding the own isotope-labelled molecule for each analyte may be rather difficult or impossible. For these reasons, only a few isotope-labelled molecules (4,4′-DDE-d_8, parathion ethyl-d_{10}, pirimicarb-d_6, *trans*-cypermethrin-d_6, [$^{13}C_6$]-hexachlorobenzene and permethrin-d_6 for non-polar pesticides, and simazine-d_5 and diuron-d_6 for polar pesticides) were tested as ISs in this study, which were selected as a function of the retention times of the compounds and taking into account the different pesticide families evaluated. In spite of this, in GC–MS/MS it can occur that an isotope-labelled molecule presents the same transitions that its corresponding non-labelled compound, being impossible to distinguish them, such in the case of permethrin-d_6, which was not used as IS. A structural analogue such as [$^{13}C_6$]-caffeine, used in some reports as pesticide IS [30,32,35] was also utilized for non-polar pesticides. The application of isotope-labelled ISs as surrogates was tested with the aim of correcting losses in the extraction step. By comparison of these results with those obtained when the ISs were added after the extraction process, it was concluded that the use of these compounds as surrogates did not improve the general recovery values. This fact is in accordance to other studies where multi-class pesticides were determined in WW samples [26]. Consequently, due to the fact that it was not the best alternative, in further experiences the isotope-labelled compounds were always added after the extraction process. For non-polar pesticides, it was obtained that independently of the IS used and the retention time of the compounds, similar recovery results were observed for the four studied effluents. Hence, although the ISs belonged to different specific families, each one was able to provide results similar to the others, being valid any of them for determination of multi-class analytes (Fig. 5). Parathion ethyl-d_{10} was then chosen as IS for quantification purposes due to its higher sensitivity. In the case of polar pesticides, no differences were observed when simazine-d_5 or diuron-d_6 was employed, and thus, simazine-d_5 was finally selected as IS.

In relation to the quantification process, and considering the lack of blank samples and representative matrices, an interesting alternative could be the use of standard addition methodology. However, it is time consuming because a calibration set must be prepared for each sample. In some cases it is possible to use one-point calibration in order to increase sample throughput [36], but when this approach is used, two points (sample and fortified sample) must still be injected. Therefore, in this work a matrix-matched calibration approach was used, subtracting the signal of the blank.

3.4. Validation

During the method optimization, experiments were done at relatively high concentration levels (4 $\mu g\,L^{-1}$). However, to evaluate the feasibility of generic extraction procedures at low levels, the methods were validated in the aqueous phase for each type of effluent at 0.1 and 1.0 $\mu g\,L^{-1}$ for trueness (recovery) and repeatability (intraday precision) studies. These low levels were selected considering the current legislation in water for human consumption [37] and surface water [4] due to the lack of reference levels in WWs. The method was then validated in the aqueous phase and in the SPM (only for non-polar pesticides) for each WW effluent.

The linearity of the method was studied by means of matrix-matched standard calibration (10, 50 and 200 $\mu g\,L^{-1}$), preparing one calibration curve with each effluent. The linearity across the studied range was excellent, with $R^2 \geq 0.98$ for all the studied compounds. Trueness was estimated in terms of recovery, by evaluating two different spiking levels (0.1 and 1.0 $\mu g\,L^{-1}$ for the aqueous phase and 25 and 250 ng for the SPM). The lowest level was chosen bearing in mind the value legislated for pesticides in water intended for human consumption in the EU [37]. However, up to our knowledge, there is not any legislation or rule in the EU or US about levels of pesticides in WWs so a higher concentration level was also evaluated. Three blank samples of each effluent were spiked with the studied compounds at each fortification level. Although the EU criteria from the field of pesticide residue analysis demands an average recovery between 70% and 120%, bearing in mind the nature of the samples under study, it is possible to increase the recovery range to 50–120%, providing that the RSD values are <25% (Tables S-6, S-7 and S-8). Several compounds shown in Tables S-1 and S-2 do not appear in the validation data since adequate analytical performance were not obtained (propoxur, dimethoate, thiometon, disulfoton, fenpropidin, propargite, iprodione and furathiocarb in the aqueous phase; 2-phenylphenol, propoxur, dimethoate, thiometon, disulfoton, fenpropidin, pendimethalin, methidathion, propargite and furathiocarb in the SPM for non-polar pesticides; desmediphan in the case of polar pesticides).

Finally, LODs and LOQs were determined as the lowest concentration giving a signal-to-noise ratio (S/N) of three and ten times, respectively, using matrix-matched standards of each effluent (Tables S-9, S-10 and S-11). For non-polar pesticides, LODs ranged from 0.01 to 0.20 $\mu g\,L^{-1}$ and LOQs ranged from 0.02 to 0.50 $\mu g\,L^{-1}$ in the aqueous phase of the four studied effluents, as well as for polar pesticides. LODs ranged from 2 to 50 ng in the SPM (volume of filtrated WW employed: 250 mL). Most of the determined pesticides showed LODs lower than the limits established for water intended for human consumption in the EU [37] (US-EPA legislation establishes higher limits [8]). Although some of the compounds showed higher limits than these established values, bearing in mind that pesticides levels in WWs are not legislated, it is not so necessary to reach such low levels. Thus, 0.2 $\mu g\,L^{-1}$ can be considered as an adequate value, taking into account the matrix under study. In Fig. S-2, a comparison of a chromatogram of a MBR

sample spiked at the lowest validation level (0.1 $\mu g\,L^{-1}$) with the corresponding blank sample (without addition of compounds) is shown, indicating the selectivity of the developed method for the determination of pesticides in WWs.

3.5. Analysis of real samples

The validated methods were applied to the analysis of five WW effluent samples from each of the four selected treatments, which were collected in different days. Several internal quality controls were carried out in order to guarantee that the measurement process was under statistical control. A reagent blank was obtained by performing the whole process without sample. This sample eliminated possible false positives produced by contamination in the instrument or solvent used. A spiked sample at the second calibration level was used to control the extraction efficiency. Calibration curves were prepared daily obtaining $R^2 \geq 0.98$.

Several non-polar compounds were detected at concentration levels in the range from 0.02 to 1.94 $\mu g\,L^{-1}$ (results are shown in Tables S-12 and S-13). The most abundant compounds were quintocene and isophenphos, which appeared in 10 samples. The highest number of pesticides was detected in an AP sample, which is also one of the effluents showing more SPM (Table 1). Some polar pesticides were detected ranging from 0.02 to 0.33 $\mu g\,L^{-1}$; diuron and terbuthylazine were frequently found in all the analyzed samples. Besides, other polar and non-polar pesticides were also found at trace levels (<LOQ).

The SPM obtained for each sample was also analyzed. As expected from the distribution study (Fig. 1), pyrethroids were mainly found. Cypermethrin was detected in MP and AP effluents (47 and 1000 ng, respectively). Permethrin and cyfluthrin were detected in the SPM of an AP effluent (37 and 78 ng). Other non-polar pesticides, namely mirex, tebufenpirad, fluacipop-butyl, hexachlorobenzene (detected at trace level) and tolcophos methyl (9 ng) were detected in a MBR effluent. It must be remarked that some of these compounds were not detected in the aqueous phase, such as pyrethroids, fact that indicates the importance of analyze both phases in order to have knowledge about the total concentration of the target compounds in WW samples (volume of WW employed: 250 mL).

4. Conclusions

Four different treated WWs have been analyzed in order to establish the methodology appropriated for the extraction of a high number of compounds (including polar and non-polar pesticides) bearing in mind the different physico-chemical characteristics of each effluent. Although the SPM is not usually analyzed, a previous study of the distribution of the non-polar pesticides between the aqueous phase and the SPM has revealed the need for analyzing both phases to consider the total concentration in the sample. However, for polar pesticides this analysis does not provide significant information, due to this type of pesticides are mainly found in the aqueous phase.

For the extraction of non-polar pesticides from the aqueous phase, two extraction techniques (LLE and SPE) have been evaluated, resulting the SPE method as the most adequate, regardless the type of treatment. SPE was also adequate for the extraction of polar pesticides from all the effluents studied, but using different elution conditions for the AP treatment. For the SPM, a PLE process has been employed. Both extraction techniques have been validated for the analysis of these contaminants. Validation parameters, such as trueness and precision, were satisfactory for approximately 80% of the target compounds in all the effluents studied. LODs were in the range from 0.01 to 0.20 $\mu g\,L^{-1}$. Bearing in mind that matrix effect is significant in these types of samples, isotope-labelled analytes were used as IS with the aim to correct this undesirable effect. Several ISs were studied and it has been observed that only one IS can be used and it can be added after the extraction procedure.

The developed methods were applied to analysis of 20 real WW samples. Non-polar pesticides were detected at concentrations levels in the range from 0.02 to 1.94 $\mu g\,L^{-1}$ in the aqueous phase and 9–1000 ng in the SPM. Pyrethroid pesticides were found only in the SPM. Polar pesticides were also detected at lower concentrations in the aqueous phase (0.02–0.33 $\mu g\,L^{-1}$).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.011.

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