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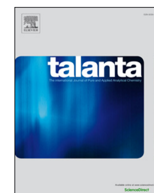

A novel sample preparation procedure for effect-directed analysis of micro-contaminants of emerging concern in surface waters

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ABSTRACT

A novel sample preparation procedure relying on Solid Phase Extraction (SPE) combining different sorbent materials on a sequential-based cartridge was optimized and validated for the enrichment of 117 widely diverse contaminants of emerging concern (CECs) from surface waters (SW) and further combined chemical and biological analysis on subsequent extracts. A liquid chromatography coupled to high resolution tandem mass spectrometry LC-(HR)MS/MS protocol was optimized and validated for the quantitative analysis of organic CECs in SW extracts. A battery of in vitro CALUX bioassays for the assessment of endocrine, metabolic and genotoxic interference and oxidative stress were performed on the same SW extracts. Satisfactory recoveries ([70–130]%) and precision (< 30%) were obtained for the majority of compounds tested. Internal standard calibration curves used for quantification of CECs, achieved the linearity criteria ($r^2 > 0.99$) over three orders of magnitude. Instrumental limits of detection and method limits of quantification were of [1–96] pg injected and [0.1–58] ng/L, respectively; while corresponding intra-day and inter-day precision did not exceed 11% and 20%. The developed procedure was successfully applied for the combined chemical and toxicological assessment of SW intended for drinking water supply. Levels of compounds varied from < 10 ng/L to < 500 ng/L. Endocrine (i.e. estrogenic and anti-androgenic) and metabolic interference responses were observed. Given the demonstrated reliability of the validated sample preparation method, the authors propose its integration in an effect-directed analysis procedure for a proper evaluation of SW quality and hazard assessment of CECs.

1. Introduction

Although contaminants of emerging concern (CECs) are generally found in the aquatic environment at the trace level their presence is of particular concern, due to the continued exposure of aquatic ecosystems and subsequent risks for ecological and human health [1,2]. Several studies have successfully identified major contributors to the toxicity of complex environmental samples, allowing the prioritization of hazardous CECs, by the combination of chemical and bioanalytical tools [3–11]. Besides, the European Commission considers the development of methodologies for the identification of such drivers of mixture toxicity a research priority [12]. Effect-directed analysis (EDA) may help to probe causal links between mixture occurrence and combined effects on biota and human health [13–15]. EDA aims to reduce complexity of environmental samples by bioassay directed fractionation so that relevant toxicants can be isolated and identified by further, focused, chemical screening analyses. The applicability of this approach has been successfully demonstrated in several instances [14,16–19].

Bioanalytical methods, such as in vitro bioassays, may be ideal screening tools for hazard assessment as they can detect a wide range of contaminants based on their biological activity rather than their chemical structures [13,20]. *In vitro* bioassays assess the mechanism-based effects, such as hormone receptor binding, of a complex environmental sample for the detection of several endpoints (e.g., toxicity, cytotoxicity, genotoxicity or estrogenicity) [21,22]. Due to their ease of use, sensitivity, robustness and cost-effectiveness in vitro bioassays, such as the CALUX bioassay, are suitable as screening tools for water samples [23].

Most of the existing methods for determination of CECs in environmental waters are target multi-residue methods based on liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). This technique allows the identification and quantification of target analytes at low concentrations, mostly using triple quadrupole (QqQ) and ion trap (IT) analyzers due to their excellent sensitivity and

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selectivity [24–27]. However, although quantitative data are highly useful, information on the possible presence of other potentially harmful compounds not included in the target list is missed [24,25,27]. For this reason, the use of accurate mass high resolution mass spectrometry (HR-MS), with e.g., Orbitrap and time-of-flight (TOF) instruments, has gained application for wide-scope screening purposes in the environmental field, since they are capable to perform both targeted as well as non-targeted analysis based on full-spectrum accurate mass acquisition at good sensitivity [25,27]. Advantages of HRMS are widely recognized in qualitative analysis [24,26]; The higher sensitivity and resolving power, wider linear dynamic range, and selectivity of new-generation LC–TOF or Orbitrap MS instruments have allowed quantification of several CECs in complex environmental samples [28].

Despite the increasing sensitivity of MS techniques, a sample preparation technique allowing analyte pre-concentration and sample clean-up is frequently required to detect CECs at their typical trace levels (ng/L or µg/L) in surface waters (SW) [29]. Solid phase extraction (SPE) is the most commonly used sample preparation technique due to the wide variety of sorbent materials with different modes of interactions and applications [30–32]. In the SPE procedure, the choice of sorbent is critical because it controls selectivity or specificity, affinity, and capacity and it is based on the characteristics of the target compounds (e.g. polarity) and sample matrix [32]. However, increased specificity of the sorbent means lower applicability to generic multi-residue analytical methods. Various protocols have been proposed to overcome the downsides of using a single sorbent material on a generic analytical method [15]. One procedure consists of the sequential extraction of diverse CECs from waters by the use of different SPE materials in cartridges assembled in series [33]. The second combines four different sorbent materials in a single mixed-bed multilayer cartridge providing high recoveries for a wide variety of compounds present in waters [34]. These SPE methods have improved recovery and selectivity for a wide range of CECs present in aqueous matrices.

In this context, the principal goal of this study was to develop and validate an SPE procedure for the enrichment of a large list of organic CECs, presenting widely different physicochemical properties, from SW and subsequent application of the same extracts for both chemical and hazard assessment by the combination of LC–HRMS/MS and a set of in vitro bioassays. To be able to link results from bioassays and chemical analysis directly, it is required that both analyses are performed within the same sample extract. These demands were implemented in a SPE-based sample preparation method, previously developed in our research group [35]. However, the procedure was only tested in SW spiked with 39 well-known CECs. Furthermore, the method was not validated for the analysis of a wide range of CECs with different physicochemical properties and biological activities in real SW samples. The procedure developed in the present work is directed towards obtaining the required recovery efficiencies and biochemical analytical quality performance for the quantification of a more extended group of CECs as well as biological activity in SW. To that aim: (i) 117 CECs characterized by a wide range of physicochemical and biological properties were selected as test compounds; (ii) an LC-HRMS/MS analytical procedure was optimized and validated for the analysis of selected test compounds in SW extracts; (iii) a sequential-based SPE sample preparation method was optimized and validated for the enrichment of test CECs from SW and further combined chemical analysis and in vitro bioassays of subsequent SW extracts; and (iv) the SPE-LC-HRMS/MS analytical procedure together with a battery of CALUX bioassays were applied to environmental SW in order to evaluate their applicability.

2. Materials and methods

2.1. Selection of compounds

A list of 117 CECs was selected as model compounds to test diverse sample preparation procedures. The selection criteria of the candidate substances were based on: (i) occurrence in SW, (ii) broad range of physicochemical properties and (iii) relevance to human health. Physicochemical properties were predicted by EPiSuite (www.chemspider.com) and ChemAxon (www.chemaxon.com) platforms. The properties estimated were: water solubility, acid dissociation constant (pKa), the octanol-water distribution coefficient log K_{OW} and the pH-dependent octanol–water distribution coefficient log D_{OW} at pH 2.3 and 6.5. Toxicity was searched in literature and the database PubChem (https://pubchem.ncbi.nlm.nih.gov/).

2.2. Reagents and chemicals

Acetone (HPLC grade), petroleum ether (ultraresi-analyzed), ethylacetate (ultraresi-analyzed), methanol (MeOH) (ultraresi- analyzed), acetonitrile (ACN) (HPLC grade) and ammonium acetate (NH_4Ac) (ACS grade) were purchased from Mallinckrodt Baker B.V. (Deventer, the Netherlands). The ultrapure water was obtained by purifying demineralized water in a Milli-Q-system (Millipore, Bedford, MA). Dimethylsulfoxide (DMSO) (purity of 99.9%, for spectroscopy) was purchased from Acros Organics (Geel, Belgium). Hydrochloric acid (HCl) (Suprapur®, 30%), formic acid (HFo) (Suprapur®, 50%), and ammonium hydroxide (NH_4OH) (Suprapur®, 28%) were obtained from Merck (Darmstadt, Germany). SPE columns HLB (200 mg, 6 mL) and MCX (150 mg, 6 mL) were purchased from Waters Corporation (Milford, MA, USA). In-house made mixed-bed multilayer cartridges were prepared by packing glass cartridges (6 mL) with a mixture of bulk materials: Oasis HLB (30 µm) provided by Waters (Etten-Leur, the Netherlands), Strata X-AW and Strata X-CW both from Phenomenex (Utrecht, the Netherlands) and Isolute ENV+ purchased from Biotage (Uppsala, Sweden). As shown in Table A.1 (in supplementary material), standards of compounds studied labeled with letters *a* to *l* were respectively purchased from Supelco, Sigma-Aldrich, Dr. Ehrenstorfer, Aldrich, Fluka, AK Scientific, TRC, Acros, IOOIC, Fagron, ABCR and Santa Cruz Technology. Stock solutions of individual compounds i.e. CECs analyzed and deuterated compounds used as internal standards (IS) were prepared at 1000 mg/L in acetonitrile (100%), methanol (100%)or in acetonitrile/methanol (50:50; v/v). Working solutions of analytes (0.01–1.0 mg/L) were prepared in a mixture of ultrapure water and acetonitrile (90:10; v/v). Working solutions of IS were prepared in a mixture of acetonitrile/methanol (50:50; v/v) at 1.0 mg/L. Stock solutions were stored at a temperature of − 18 °C and working solutions at 4 °C. Ultrapure water was obtained by purifying demineralized water in a Milli-Q system (Millipore, Bedford,MA).

2.3. Sample collection

For method optimization and validation purposes, SW was collected from the Lekkanaal (Nieuwegein, The Netherlands), which is a tributary of the river Rhine. Grab SW samples were collected in ultra-cleaned stainless steel containers, stored at 4 °C in the dark and delivered to the laboratory within 1 h. Samples were stored at 4 °C in the dark until treatment and analysis.

2.4. LC-HRMS/MS analysis

Test compounds were analyzed using an LTQ FT Orbitrap interfaced with a HPLC-pump and autosampler Accela (Thermo Electron GmbH, Bremen, Germany). The linear ion trap (LTQ) part of the hybrid MS system was equipped with an Ion Max Electrospray Ionization (ESI) probe operating in both positive and negative ionization modes ((+)-ESI and (−)-ESI, respectively). Full-scan accurate mass spectra (mass range from 50 to 900 Da) were obtained at a mass resolution of 30,000 FWHM (*m/z* 400). The ESI conditions were: capillary voltage 3.0 kV, heated capillary temperature 300 °C, capillary voltage 35.5 V, and tube lens 70 V. The mass spectrometer was operated in a data-

Table 1
Description of the three different SPE procedures tested: Method A, Method B and Method C.

	Method A	Method B	Method C
Sorbent Material	Oasis HLB (200 mg) + 1/1/1.5 Strata X-AW/Strata X-CW/Isolute ENV+ (350 mg)	Oasis HLB (200 mg)	Oasis MCX (150 mg)
Sample pH	6.5 (adjusted with NH_4Ac 1 M)	2.3 (adjusted with HCl 32%)	2.3 (adjusted with HCl 32%)
Conditioning	8 mL ACN	8 mL ACN	8 mL ACN
	8 mL MeOH	8 mL MeOH	8 mL MeOH
	8 mL ultrapure water at pH 6.5	8 mL ultrapure water at pH 2.3	8 mL ultrapure water at pH 2.3
Loading	1 L sample under vacuum	1 L sample under vacuum	1 L sample under vacuum
Washing	10 mL ultrapure water at pH 6.5	10 mL ultrapure water at pH 2.3	10 mL ultrapure water at pH 2.3
Dry	under vacuum	under vacuum	under vacuum
Elution	5 mL MeOH: ACN (20:80, v/v)	2.5 mL MeOH: ACN (20:80, v/v)	2.5 mL ACN (100%)
	6 mL NH_4OH 0.5% in MeOH: ACN (20:80, v/v)	2.5 mL MeOH: ACN (20:80, v/v)	2.5 mL NH4OH 5% in ACN (100%)
	4 mL HFo 1.7% in MeOH: ACN (20:80, v/v)	2.5 mL MeOH: ACN (20:80, v/v)	2.5 mL NH4OH 5% in ACN (100%)

dependent-acquisition (DDA) mode. Product ions with nominal mass were generated in the LTQ trap at normalized collision energy setting of 35% and using an isolation width of 2 Da. Data were acquired and processed using Xcalibur software version 2.1. Mass calibration was performed with Polytyrosine-1,3,6 solution ($[M+H]^+$ 182.01170/508.20783 and 997.39781 for (+)-ESI mode; and $[M\text{-}H]^-$ 180.06662/506.19327 and 995.38326 for (-)-ESI mode) at a flow rate of 10 $\mu L\ min^{-1}$.

Ten µL of final extract were injected into the LC system. Chromatographic separation was achieved using an X-Bridge C18 column (150 mm × 2.0 mm i.d.; 3.5 µm particle size) (Waters Milford, MA, USA) and an Aqua C18-Guard column (4.0 mm × 3.0 mm i.d.) (Phenomenex, Utrecht, the Netherlands). Both columns were maintained at a temperature of 21 °C in a column thermostat. For both polarity modes, a linear gradient of acetonitrile (3–100%) and ultra-pure water with 0.05% formic acid was used in 41 min and held at this composition for an additional 7 min. The analytical column was re-equilibrated for 10 min between consecutive runs. The flow rate of the mobile phase was 0.3 mL/min.

Identification and confirmation of target compounds was achieved by: (i) accurate mass measurements of the protonated and deprotonated molecular ions within a mass window of 5 ppm; (ii) retention time match (≤ 0.20 min) of analytes detected in samples with corresponding standards in calibration solution; (iii) match between fragmentation ions of analytes acquired at low resolution (nominal mass) previously identified in calibration solution and those determined in samples. Thus, the acquisition of a high resolution precursor ion in combination with at least one nominal product ion and the LC relative retention time met the minimum requirement of 4 identification points [36,37].

Fig. A.1 provides an example of identification of the flame retardant triphenylphosphan-oxid (a, b) and the artificial sweetener acesulfame (c,d) in a standard solution and a SW sample.

To correct for possible ion suppression/enhancement due to matrix effects, analytes were quantitatively determined by the IS calibration method using a calibration standard solution curve at concentrations ranging between 0.01 and 1000 µg/L. To that end, a final concentration of 50 µg/L of isotopically labeled compounds (deuterated analogues) were added to both the calibration curve solutions and extracts immediately before LC-HRMS/MS analysis. Analytes for which deuterated analogues were unavailable, either the closest deuterated structure, the deuterated analyte with a similar polarity or the compound with the closest retention time was selected for correction. Since matrix effects were only corrected in the ionization source after extraction, the relative recovery factor (see Section 2.5) of each compound was applied to concentrations determined in SW extracts in order to correct for possible loss of compounds of interest during extraction or potential matrix effects due to other compounds present in the sample co-eluting with compounds tested that might interfere with their ionization.

Laboratory blank samples of ultrapure water were used to prevent potential carryover after highly concentrated standards or samples. Ultrapure water spiked with IS was used to quantify potential carryover. Quality control samples (i.e. 2 blank SW spiked with known amounts of the analytes at two different levels of concentration) together with the calibration curve were ran between each batch of 20 samples, in order to routinely assess the signal stability and quality of the analytical performance in terms of precision (i.e. reproducibility) of the calibrators and quality control samples. Maximum 15% of imprecision was accepted.

2.5. Solid Phase Extraction procedures tested for selection of sample preparation method

A sequential-based SPE cartridge [34] was prepared in-house by filling an empty glass column (6 mL) with 350 mg of a mixture of Strata X-AW, Strata X-CW and Isolute ENV+ in a ratio of 1/1/1.5 (X-AW/X-CW/ENV+) at the bottom and 200 mg Oasis HLB on top. This SPE protocol, named hereafter as method A, was compared to other two methods previously developed in our research group [35], based on the use of single sorbents Oasis HLB (200 mg, 6 mL) (method B) and Oasis MCX columns (150 mg, 6 mL) (method C). Recovery experiments were carried out to compare the extraction efficiency of the different procedures tested. To this end, 1 L of SW sample was spiked (n = 5) prior to the extraction with standard mixtures containing the target analytes at two levels (10 and 100 ng/L). 1 L of non-spiked sample(n = 3) was extracted to correct recoveries. Procedural blanks consisting of 1 L of ultrapure water (n = 3) were also tested to correct recoveries for possible contamination of the sorbent materials. Relative recovery was calculated by comparing the measured concentration according to the regression equation with added concentration as a percentage ratio. The relative recovery for each matrix was calculated using the average of all spiked samples. In order to prevent clogging of SPE cartridges, samples were pre-treated before extraction step (filtered through sea sand, ~ 0.20 µm). A detailed description of the steps followed in the three different SPE procedures tested is provided in Table 1. The eluates were collected together in glass test tubes and evaporated under a gentle stream of nitrogen and reconstituted in 50 µL of DMSO as previously described [38]. For further chemical analysis, 10 µL of 20,000 times concentrated DMSO extracts were pipetted and diluted in 200 µL of ultrapure water, yielding 1,000-fold concentrated analytical samples with a 5% DMSO. Extracts were stored at − 18 °C until analysis.

2.6. Statistical tools

A one-way analysis of variance (ANOVA) was used to analyze the differences among the obtained recoveries of each speciation group (anionic, cationic and neutrals) from the three different SPE procedures tested. Speciation group on each procedure tested was set as fixed factor. Effects were analyzed post hoc with Tukey's b test. Data were first tested for normal distribution and homogeneity of variance. Correlation analyses between recoveries and chemical properties were performed using the product-moment Pearson coefficient. These analyses were performed with SPSS Version 15.0. For all the analyses

statistical significance was set at $p < 0.05$.

2.7. Assessment of quality performance

The performance of the method was evaluated through the assessment of the linearity, sensitivity in terms of instrumental limits of detection (ILOD) and method limits of quantification (MLOQ), matrix effects, extraction recoveries, as well as intra-day and inter-day instrument precision (repeatability and reproducibility, respectively).

Calibration curves were generated using linear regression analysis and acceptable linearity was achieved when the coefficient of correlation was at least 0.99, and the precision was < 20% relative standard deviation (RSD). Accuracy of the method was assessed in terms of instrument trueness and precision. Trueness was determined from seven repeated injections of a 50 ng/L standard mixture and calculated as the % of error between the measured value and the true value (expected concentration of reference standard). Intra-day and inter-day instrument precision were determined from seven repeated injections of a 50 ng/L standard mixture during the same day (repeatability) and in three successive days (reproducibility) and calculated as relative standard deviation (RSD). ILODs were estimated from the injection of a standard solution successively diluted until reaching the lowest concentrated point of each compound, with a detected peak matching the expected retention time and a detected exact mass matching the predicted exact mass of the corresponding molecule with a mass error below 5 ppm. MLOQ for each compound was determined on the basis of the lowest calibration standard meeting the linearity criteria, with a confirmed retention time and exact mass with mass error below 5 ppm and a correction factor for matrix effects [36]. To evaluate the degree of ion suppression or enhancement in the ESI source caused by the presence of matrix components in the LC eluent, the peak areas from the analysis of spiked SW extracts were compared with peak areas from matrix-free solutions spiked at the same concentration. The percentage of matrix effect (% ME) corresponding to each analyte was calculated as the ratio $(A)/B \times 100$ [39], where A is the peak area of the standard spiked after extraction into surface water extracts and B corresponds to the peak area obtained in a standard neat solution. The ratio was corrected for the blank values. Thus, the signal is enhanced in the MS instrument if the % ME > 100, whereas the signal is suppressed if the % ME < 100. Relative recoveries in SW were determined with samples spiked at two different concentrations as explained in Section 2.5 for the selection of the SPE procedure for sample preparation. Spiking of the analytes was performed before extraction, allowing the validation of all sample treatment steps after the filtration.

2.8. CALUX bioassays

Hormone and xenobiotic receptor activities for several endpoints: endocrine interference (i.e. estrogenic, ERα; glucocorticoid, GR; and anti-androgenic, anti-AR); metabolic interference (i.e. peroxisome proliferator-activated receptor, PPARγ and pregnane X receptor, PXR), oxidative stress (i.e. nrf2); and genotoxicity (i.e. p53 in presence of S9 metabolic mix; and p53 in absence of S9 metabolic mix) were measured in SW extracts and procedural blanks by a panel of CALUX bioassays (Table A.2). The ERα-, GR-, anti-AR-, PPARγ-, PXR-, nrf2-, p53-(+/-S9) CALUX bioassays were conducted as described elsewhere [40–45]. Briefly, human U2-OS osteosarcoma cell lines stably transfected with a luciferase reporter gene downstream of an ERα, GR, anti-AR, or PPR, PXR, nrf2, p53 + S9 or p53-S9 responsive element were exposed to SPE extracts.

Cells were seeded into 96 wells plates with Dulbecco's modified Eagle's medium and Ham's F-12(DF) medium (without phenol red) that was supplemented with dextran-coated charcoal (DCC) stripped serum (5%). After 24 h of incubation (37 °C, 7.5% CO_2), the medium was replaced by medium containing aliquots of the water extracts (maximally 0.1% DMSO) for activity testing. After 24 h of exposure in triplicate, the medium was removed and the cells were lysed in 30 μL of triton-lysis buffer. The amount of luciferase activity was quantified using a luminometer (Centro XSLB 90 luminometer (Berthold Technologies)). To rule out any confounding influences due to toxicity of the extracts, cells were monitored for signs of cytotoxicity by means of light microscopy. Only non-cytotoxic dilutions were used for quantification of the response. A nonlinear regression method was used to analyze the generated concentration–effect curves and express activity in model compound equivalents. On all plates, a dose-response curve of the reference compound was included for adequate quantification of the response to micro, nano or picograms of reference compound equivalents per liter (μg, ng or pg ref comp eq/L). Reference compounds used were 17 β-estradiol, dexamethasone, flutamide, rosiglitazone, nicardipine, curcumine, cyclophosphamide, and actinomycin D for the ERα-, GR-, anti-AR-, PPARγ-, PXR-, nrf2-, p53- (+/-S9) CALUX, respectively.

3. Results and discussion

3.1. Final list of target compounds and physicochemical properties

The final list of target compounds classified according to their different uses, namely: pharmaceuticals and metabolites (55); herbicides and metabolites (32); insecticides (6); fungicides (2); flame retardants (4); plasticizers (1); industrial chemicals (14); and artificial sweeteners (3).

Target compounds are listed in Table A.1, together with their applications and additional identifiers. Well known (e.g. caffeine) and less investigated CECs (e.g. acesulfame), as well as priority pollutants (e.g. atrazine) were included in the list. Furthermore, a few transformation products (TPs) (e.g. guanylurea) were investigated. Additionally, Table A.3 provides the physicochemical and biological properties of selected compounds for this study. Fig. 1 plots the mass of selected compounds, which ranged from 102 to 650 Da, against their log K_{ow}, ranging from −3.57 to 4.77. At pH 6.5 21 compounds are present as anionic species, 19 as cationic species and 77 as neutral species. At pH 2.3 3, 39 and 75 compounds were present as anionic, cationic and neutral species, respectively. Since their pK_a values were close to pH, a few compounds were considered to co-exist in equilibrium as two species (e.g. simazine is present at pH 6.5 as both its cationic and anionic forms; while metribuzin is present as both cationic and neutral species at pH 2.3, see Table A.3). Because of the dependence of compounds' speciation on pH conditions, log D_{OW} was additionally considered. These values ranged from −4.91 to 5.6 at pH 2.3 and from −4.93 to 5.6 at pH 6.5. While non-ionizable compounds in aqueous solution show the same log K_{ow} and log D_{ow} values, the different behavior of ionizable compounds tested under different pH conditions evidenced the need to include their speciation according to their hydrophobicity.

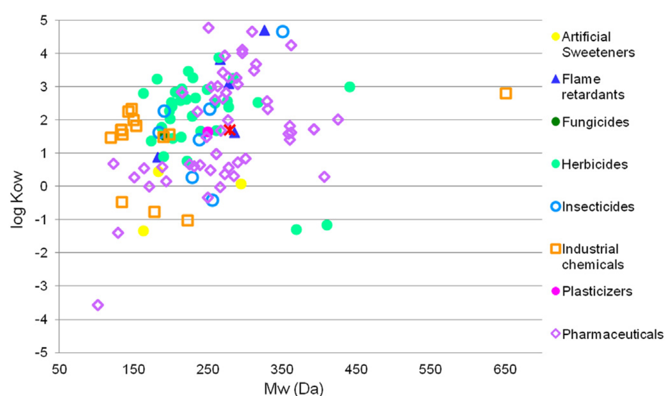

Fig. 1. Average mass (Da) and estimated log K_{ow} values of the compounds studied belonging to different classes of organic chemicals obtained from the platform EpiSuite (www.chemspider.com).

Regarding bioactivity, the selected compounds display several MoA, particularly: cytotoxicity (CG); genotoxicity (GT); mutagenicity (MT); thyroid activity (TRβ); anti-thyroid activity (anti-TRβ); estrogenicity (ER); androgenicity (AR); anti-androgenicity (anti AR); progestagenicity (PR); anti-progestagenicity (anti-PR); glucocorticoid activity (GR); oxidative stress activity (OS); peroxysome proliferator-activated nuclear receptor gamma activity (PPARγ-active); and pregnane X receptor activity (PXR-active) (see Table A.3).

3.2. LC-HRMS/MS analytical method performance

Table A.4 summarizes the optimized HPLC-(ESI)-HRMS/MS analytical conditions for the screening of 117 CECs in SW.

Of the 117 compounds studied, 106 analytes and 3 IS were only detected or showed higher response in the (+)-ESI mode; while the remaining 11 analytes and 12 IS (bentazone-d6 was used in both (+)-ESI and (−)-ESI modes) did so in the (−)-ESI mode. With the exception of 17-α-estradiol, 17-β-estradiol and 17-α-ethinylestradiol for which the pseudo-molecular ion $[M+H-H_2O]^+$ was selected, protonated and deprotonated molecules were selected as precursor ions for (+)-ESI and (−)-ESI modes, respectively.

The elution gradients optimized for (+)-ESI and (−)-ESI modes allowed the maximum separation and sensitivity for the target compounds (see Figs. A.1 and A.2). The chromatographic setup resulted in sharp peaks with baseline widths generally lower than 30 s and defined by 13–20 points. Retention times (RT) for 106 compounds analyzed in (+)-ESI mode were in the range of [1.7–31.9] min; while for 11 analytes in (-)-ESI mode they were [5.7–29.0] min.

Isotope-labeled ISs were available for 14 analytes. For the remaining 103 analytes, quantification was performed using the closest-matching IS according to structure and retention time. To demonstrate that a certain IS was suitable for compensating matrix effects on analyte signal, calibration curves prepared in SW extracts and ultrapure water (0.1–100 ng/L) were compared [39,46]. Similar slopes (see Fig. A.3) confirmed that calibration curves in pure water could be used for SW analysis when corrected by the signal intensity of the IS.

Maximum carryover rates were less than 1% after the injection of a 1000 ng/L standard, two solvents and two laboratory blanks.

3.3. Selection of sample preparation method

A mixture of sorbents packed in a self-made sequential-based multilayer extraction cartridge [34] was selected to be tested in method A. The mixture consisted of a combination of reversed-phase poly(styrene-divinylbenzene) polymeric sorbents with diverse functional groups in the structure allowing interaction with compounds in a wide range of hydrophobicity ($1 < \log K_{ow} > 1$) and speciation: (i) Oasis HLB (hydrophilic–lipophilic balance), providing hydrophilic (Nvinyl- pyrrolidone) and lipophilic (divinylbenzene-rings) interactions for retention of non-polar and polar compounds, (ii) Strata X-AW (weak anion exchange) and (iii) Strata X-CW (weak cation exchange) allowing ion exchange interactions for the retention of anions (di-amino ligands) and cations (carboxylic acid ligands) as well as hydrophobic interactions (phenyl rings and aliphatic chains together with pi–pi interactions at phenyl rings) and (iv) Isolute ENV+ enhancing interactions with very polar compounds (hyper cross-linked hydroxylated polystyrene-divinylbenzene copolymer with a high surface area). Sample pH, elution solvents, sequence and number of elution steps and solvent volumes were tested for optimal conditions. In order to cover the extraction of a large amount of ionic compounds, pH was properly adjusted (i.e. pH 6.5). To exploit the mixture of SPE sorbents used and given that compounds assessed presented different speciation forms in water at pH 6.5, the mixture of organic solvents chosen for elution (i.e. 20% methanol in acetonitrile) was applied sequentially as neutral, basic and acidic solution. Thus, the interaction of neutral and ionic compounds with the stationary phase was sequentially disrupted and the maximum variety and number of compounds were recovered.

Recovery tests were carried out for comparison of the different procedures tested. The protocol yielding highest recoveries was method A with the sequential-based cartridge.

3.4. Comparison of sample preparation method selected with other procedures applied

Afterwards, recoveries of method A were compared to those of the other methods, which are similar to those described previously in the literature for the analysis of a wide range of CECs in SW, i.e. B and C. The purpose of this comparison was to test whether the method presented in this work (method A) would represent a significant improvement compared to existing procedures. While Oasis HLB and MCX materials are reported to assure good recovery of compounds in a wide range of polarities [30,35], the use of additional sorbents providing extra interactions was expected to enhance the recovery of a large list of CECs presenting a wide range of physicochemical properties (i.e. K_{ow} values ranging between −4 and 5). Relative recoveries of method A ranged from 2% to 230% while for methods B and C the compounds studied were recovered at respective ranges of [2–431]% and [1–142]% (Table A.5). Fig. 2 shows the comparison of the three protocols tested in terms of percentage of recovery of the studied compounds. The higher average recovery obtained for method B pointed out this procedure as the more suitable. These results could be expected, since method B applied the generic sorbent HLB providing interactions for a wide range of hydrophobic and hydrophilic compounds as those included in this study. However, a substantial number of compounds showed non acceptable recoveries exceeding the 130% of recovery, probably due to co-elution of analytes with other compounds present in the matrix. On the other hand, Method C showed on average acceptable recoveries for all compounds. This improvement observed with Method C relative to Method B could be explained by the use of the mixed-mode sorbent MCX, that next to a HLB backbone with corresponding interactions, also provides specific interactions for cations and thus reduces the sorption of matrix components [23]. Nevertheless, the number of compounds that yielded satisfactory recoveries (70–130%) was higher for method A. In addition, a high percentage of compounds performed with good

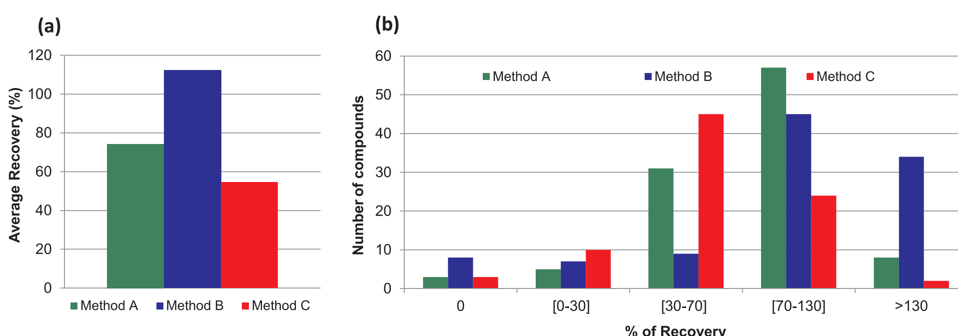

Fig. 2. Comparison of the three SPE procedures tested (i.e. method A using sequential-based multilayer cartridges; method B using HLB cartridges; and method C using MCX cartridges) for the extraction of 117 different compounds by means of: **(a)** average percentage of relative recovery of all compounds tested; and **(b)** number of compounds recovered at: 0%, [0–30] %, [30–70] %, [70–130] % and ≥ 130% with a maximum RSD of 30% in all cases.

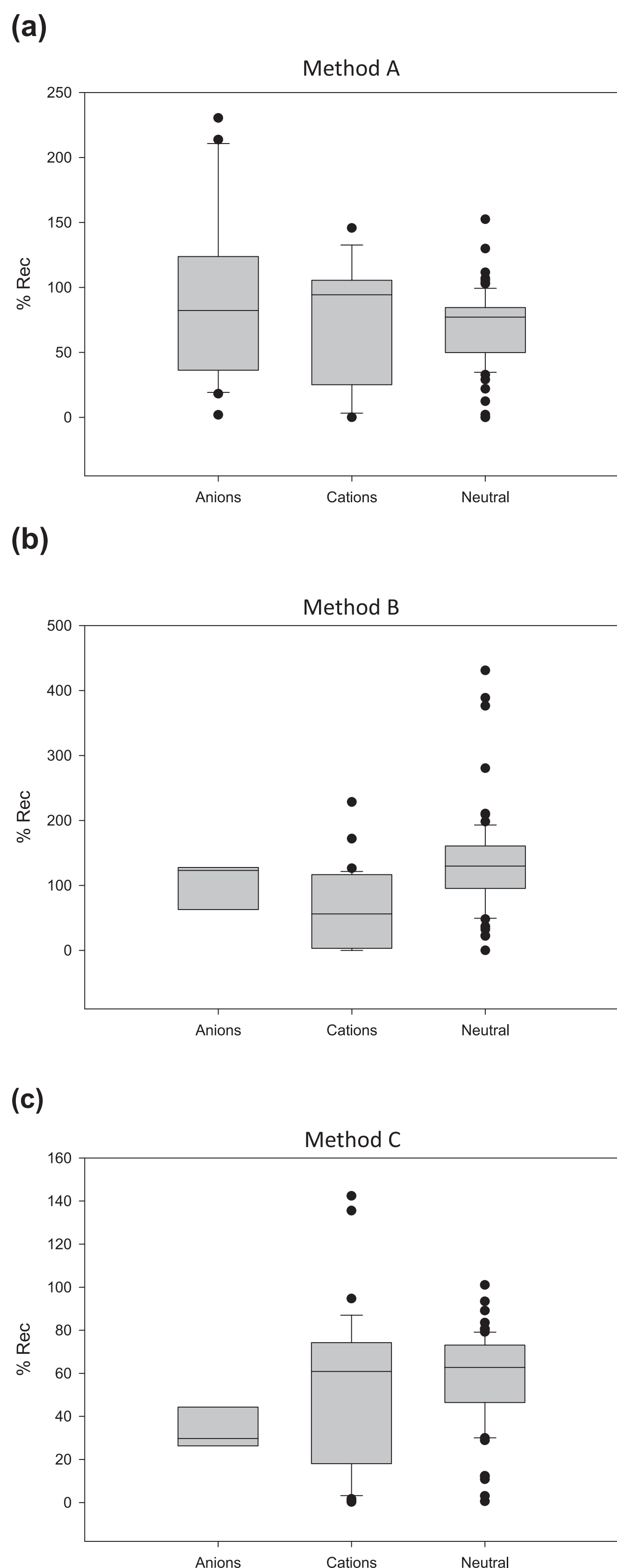

Fig. 3. Boxplots indicating recovery ranges of the compounds studied, classified by their speciation determined by the pH at which every SPE procedure was conducted. Method A (a) was conducted at pH 6.5 and methods B (b) and C (c) at pH 2.3. Each box corresponds to the percentage of recoveries of those compounds present in the aqueous phase in their anionic, cationic or neutral speciation form.

precision (RSD < 30%) when procedure A was applied. Moreover, for methods B and C a higher percentage of compounds that were not recovered at all and those with recoveries exceeding 130% was observed. Therefore, procedure A proved to be the more efficient SPE method for the extraction of the selected CECs from SW.

To better understand the variability of recoveries of the three different SPE procedures tested, the different method conditions (i.e. pH and type of sorbent material) applied and physicochemical properties of compounds (i.e. pK_a, log D_{ow} and solubility) were investigated. Fig. 3 shows boxplots indicating recovery ranges of test compounds classified by their speciation, which was determined by the pH conditions of every SPE procedure (i.e. pH 6.5 for method A, Fig. 3a; and pH 2.3 for methods B and C, Figs. 3b and 3c, respectively). Each box corresponds to the recoveries of those compounds present in the aqueous phase in their anionic, cationic or neutral speciation form. Recovery ranges and average for anionic compounds extracted by method A were [2–230]% and 89%, respectively; while for cationic compounds these were [0–146]% and 75%; and for neutrals [0–152] % and 769% (Fig. 3a). Thus method A proved to be highly efficient for the extraction of all speciation groups from SW. When method B was applied, recovery ranges and average for anionic compounds were [63–128]% and 105%; while for cationic compounds these were [0–228]% and 65%; and for neutrals [0–431] % and 139% (Fig. 3b). Although method B also showed high recoveries for all species, recoveries for many compounds, and especially for neutrals, exceeded the maximum accepted (i.e. 130%). Diversely, the same species extracted with method C showed respective recovery ranges and average of [26–44]% and 34% for anionic; [0–142]% and 52% for cationic; and [1–101]% and 60% for neutral compounds (Fig. 3c). Overall, method C proved to be moderately and equally efficient for the extraction of cationic and neutral compounds while for anionic species their recovery was reduced by half compared to method A.

In addition, statistical tests were applied in order to find correlations or significant differences between recoveries obtained for every speciation group (i.e. anionic, cationic or neutral) after application of each one of the SPE procedures tested and the hydrophobicity and solubility of compounds. For method A, no significant difference was observed among the different speciation groups in terms of recovery, and no correlation between recoveries and log D_{ow} or solubility of compounds was obtained. These results demonstrate that method A performance was the same for all compounds regardless of their speciation, log D_{ow} and solubility. For method B, significant differences ($p < 0.001$) were observed among species; and recoveries were positively correlated with log D_{ow} (Pearson $r = 0.434$; $p < 0.001$) and negatively correlated with solubility (Pearson $r = -0.256$; $p = 0.011$). These results showed that the recovery of method B was dependent on speciation of compounds. Besides, recoveries were observed to decrease when solubility increased, demonstrating that the more soluble in the aqueous phase compounds are, the less proportion is retained in the solid phase of the sorbent material. The increase of recoveries with increasing log D_{ow} values of compounds tested was also expected and proved that interaction of less hydrophilic compounds with HLB materials enabled high recoveries. For method C no significant difference was observed among the different speciation groups in terms of recovery. However, as in method B, a positive correlation was observed between recoveries and log D_{ow} (Pearson $r = -0.200$; $p = 0.0317$) and a negative correlation with solubility (Pearson $r = -0.265$; $p = 0.004$). Thus, the extraction efficiency of method C was also independent of the speciation and hydrophobicity of compounds, as for method A; but dependent on solubility of compounds as observed for method B.

3.5. (SPE)-LC-HRMS/MS method quality assurance

Table 2 shows quality performance parameters calculated for validation of the whole analytical procedure optimized for the analysis of 117 CECs in SW: Linearity (r^2) of the quantification method; trueness

Table 2
Method performance parameters: Linearity (linear correlation coefficient, r^2), trueness (% of error), repeatability (intra-day instrument precision) and reproducibility (inter-day instrument precision), instrumental limit of detection (ILOD), method limit of quantification (MLOQ), matrix effects (ME), average percentage of relative recoveries and relative standard deviation determined for the analysis of 117 compounds in surface water extracts.

Group Use / Target Compound	Linearity (r^2)	Trueness (% min-max error, n = 7)	Repeatability (RSD %, n = 7)	Reproducibility (RSD %, n = 3)	ILOD (pg injected)	MLOQ (ng/L)	ME (%)	REC (%)	RSD (%)
Artificial Sweeteners									
Acesulfame	0.9995	0–4	3	2	5	27	58	23	76
Aspartame	0.9999	0–7	2	7	3	5	97	18	23
Saccharin	0.9991	0–2	2	6	5	1	62	67	15
Flame retardants									
Tributyl phosphate	0.9997	30–41	3	9	3	5	182	84	17
Triethyl phosphate	0.9991	2–8	5	19	1	6	108	33	31
Triphenyl phosphate	0.9996	8–12	1	10	1	1	160	107	10
Triphenylphosphan-oxid	0.9996	1–5	3	9	1	5	110	79	11
Fungicides									
Carbendazim	0.9999	4–7	1	4	3	6	96	99	9
Metalaxalyl-M	0.9993	6–15	3	9	10	1	127	130	17
Herbicides									
2,4-Dichlorophenol	0.9997	1–6	4	4	6	1	52	68	9
2,4-Dichlorophenoxyacetic acid (2,4 D)	0.9999	1–4	2	4	6	1	55	214	10
2-methyl-4-chlorophenoxyacetic acid (MCPA)	0.9997	0–2	2	4	5	1	55	159	16
Aclonifen	0.9979		4	7	24	5	108	86	7
Amidosulfuron	0.9988	0–3	2	2	3	5	137	71	23
Atrazine	0.9995	0–4	2	8	1	1	93	47	12
Azinfos-methyl	0.9969	4–22	9	11	29	58	89	78	30
Bentazone	0.9970	2–6	2	1	1	1	63	97	11
Bromacil	0.9990	0–5	4	11	3	56	77	78	13
Chloridazon	0.9983	1–5	5	15	1	5	67	47	9
Chlorotoluron	0.9998	1–4	2	11	3	5	100	83	16
Dimethenamid-p	0.9997	1–5	2	6	3	6	128	77	20
Diuron	0.9988	3–10	3	5	3	6	110	78	8
DNOC	0.9967	3–8	2	1	1	1	36	42	15
Isoproturon	0.9998	0–4	3	10	1	1	102	98	17
Linuron	0.9997	1–8	2	8	12	1	112	103	7
Mecoprop (MCPP)	0.9998	2–5	1	3	5	1	61	199	13
Mepanipyrim	0.9994	0–2	1	4	3	1	100	83	8
Metamitron	0.9991	0–2	2	8	3	5	98	94	10
Metazachlor	0.9984	1–7	1	12	3	1	117	85	13
Metobromuron	0.9994	0–6	2	8	6	6	105	80	6
Metolachlor	0.9993	0–4	3	7	3	5	130	93	19
Metoxuron	0.9957	0–2	5	12	1	6	88	53	6
Metribuzin	0.9992	0–2	3	8	6	1	104	81	9
Monuron	0.9989	1–5	2	8	1	6	104	57	8
Nicosulfuron	0.9991	1–4	1	6	3	1	196	53	31
Simazine	0.9998	0–4	3	8	10	5	92	46	11
Tembotrione	0.9978	0–4	2	5	11	6	112	64	4
Terbuthylazine	0.9999	1–5	2	8	1	1	99	54	15
2-(Methylthio)benzothiazole	0.9991	1–7	5	6	11	5	111	45	17
6-Deisopropylatrazine (metabolite of atrazine)	0.9994	1–4	5	18	3	6	92	42	16
BAM (2,6-dichlorobenzamide)	0.9998	0–5	3	9	10	10	89	82	8
Desethylatrazine (metabolite of atrazine)	0.9992	0–4	3	10	1	1	102	46	14
Industrial chemicals									
1-H-benzotriazole	0.9994	0–5	3	12	6	58	108	29	113
2-Amino − 3-methyl-3H-imidazo (4,5-f)quinoline	0.9982	0–2	2	13	11	5	53	21	9
2′-aminoacetophenone	0.9997	1–4	3	12	3	1	99	12	17
2-Aminobenzothiazole	0.9996	0–4	2	5	3	6	101	81	12
2-naphthylamine	0.9991	0–3	2	10	11	6	79	2	34
3,5,3′-triiodo-thyronine	0.9987	1–11	5	12	5	5	64	0	0
4-Methyl-1H-benzotriazole	0.9998	1–23	3	7	3	5	150	22	139
4-Nitroquinoline 1-oxide	0.9932	0–5	4	5	26	10	84	27	6
5,6-Dimethyl-1H-benzotriazole monohydrate	0.9991	0–2	3	10	1	0.1	94	112	7
5-Chloro-1H-benzotriazole	0.9997	0–4	4	18	3	5	99	106	9
5-Methyl-1H-benzotriazole	0.9995	1–12	6	10	3	27	78	66	31
Diglyme	0.9973	0–10	8	9	25	49	147	0	0
Tetraglyme	0.9994	2–6	3	2	10	25	149	49	36
Triglyme	0.9998	0–5	3	5	24	10	176	35	13
Insecticides									
Thiacloprid	0.9994	0–5	3	7	5	10	91	87	11
Chlorpyrifos-ethyl	0.9995	19–36	2	17	11	5	162	47	19

(*continued on next page*)

Table 2 (*continued*)

Group Use / Target Compound	Linearity (r^2)	Trueness (% min-max error, n = 7)	Repeatability (RSD %, n = 7)	Reproducibility (RSD %, n = 3)	ILOD (pg injected)	MLOQ (ng/L)	ME (%)	REC (%)	RSD (%)
DEET	0.9998	0–4	3	11	3	5	105	74	24
Dimethoate	0.9972	0–3	3	17	1	11	90	50	18
Imidacloprid	0.9991	1–7	3	2	11	5	94	67	11
Pirimicarb	0.9999	2–6	2	8	3	1	105	82	14
Pharmaceuticals									
17-α-estradiol (-H2O)	0.9986	4–8	3	8	5	5	76	56	4
17-β-estradiol (-H2O)	0.9994	2–9	3	11	10	5	74	52	4
17-α-Ethinylestradiol (-H2O)	0.9980	1–8	8	17	12	58	88	57	12
Aminophenazone	0.9962	3–7	4	14	3	1	83	37	13
Atenolol	0.9996	2–6	2	4	1	1	111	94	10
Betamethasone	0.9998	2–12	6	10	3	6	103	93	8
Bezafibrate	0.9998	1–4	2	7	10	1	130	104	15
Caffeine	0.9997	2–5	5	16	3	5	100	78	11
Carbamazepine	0.9997	1–7	2	11	3	5	119	79	11
Ciclophosphamide	0.9991	1–3	3	19	5	5	117	84	9
Clenbuterol	0.9997	1–4	1	5	1	4	92	102	12
Clindamycin	0.9990	1–9	2	10	1	1	152	125	8
Cortisol	0.9998	0–3	3	8	3	5	97	62	26
Cortisone	0.9997	0–2	2	7	5	5	95	61	15
Dexamethasone	0.9995	1–12	4	6	6	6	109	86	10
Diclofenac	0.9992	1–6	2	7	5	5	114	106	11
Dihydrotestosterone	0.9999	0–4	3	6	3	1	110	93	27
Estrone	0.9992	0–7	4	11	6	6	85	152	58
Fluoxetine	0.9998	34–52	1	24	10	1	121	58	10
Furosemide	0.9991	0–4	1	2	5	5	37	142	11
Gemfibrozil	0.9977	2–11	3	2	25	5	40	31	139
Ifosfamide	0.9992	2–8	6	17	3	5	104	79	8
Ketoprofen	0.9992	0–5	2	11	3	1	106	101	15
Levonorgestrel	0.9996	1–3	2	4	3	1	116	98	29
Lincomycin	0.9978	0–6	3	5	9	5	110	60	13
Metformin	0.9996	1–6	3	14	5	5	64	0	8519
Metoprolol	0.9990	1–4	2	5	1	5	103	146	13
Metronidazole	0.9990	0–8	2	14	5	5	83	103	10
Nandrolone	0.9990	0–3	2	6	1	1	110	84	27
Niacin	0.9950	3–12	4	19	96	100	110	2	137
N-Methyl-N′-phenylacetohydrazide	0.9996	0–2	4	18	5	1	118	41	12
Paracetamol	0.9998	0–3	4	10	5	5	144	99	13
Paroxetine	0.9988	9–21	1	20	3	1	126	25	7
Pentoxyfyline	0.9997	1–5	2	3	5	5	102	82	8
Phenazone	0.9999	0–2	2	10	1	10	100	79	10
Pindolol	0.9997	0–4	1	4	3	1	78	19	45
Prednisolone	0.9992	2–4	3	9	10	10	105	70	30
Prednisone	0.9961	0–4	3	7	12	6	107	65	13
Progesterone	0.9993	1–4	3	5	3	1	141	88	30
Propranolol	0.9997	1–5	1	10	1	1	118	133	9
Salbutamol	0.9991	3–6	3	13	10	1	91	104	11
Sotalol	0.9997	0–3	2	5	49	5	95	99	11
Sulfadiazine	0.9997	1–8	4	9	3	5	82	45	15
Sulfamethoxazole	0.9992	0–4	3	4	5	5	103	84	15
Sulfaquinoxaline	0.9975	0–2	2	1	5	5	115	71	14
Sulphachloropyridazine	0.9990	0–3	2	2	10	5	99	61	14
Tertbutaline	0.9992	2–5	3	14	1	5	87	105	14
Testosterone	0.9991	0–5	2	5	5	5	104	73	29
Tramadol	0.9992	1–5	2	5	1	5	102	86	9
Trenbolone	0.9983	0–3	2	4	3	5	88	48	32
Trimethoprim	0.9999	0–5	4	15	1	1	102	100	9
Venlafaxine	0.9999	0–4	2	7	1	1	124	108	7
Clofibric acid (metabolite of clofibrate)	0.9981	3–35	11	8	5	1	63	230	8
Guanylurea (transformation product of metformin)	0.9994	1–11	3	13	52	26	12	3	224
Plasticizers									
4,4′-sulfonyldiphenol	0.9998	1–10	4	19	3	27	39	50	11

(% error), intra-day and inter-day precision (repeatability and reproducibility, respectively), ILOQs and MLOQs; ME (%), recoveries (%) and RSD (applying SPE method A) were determined.

Regarding quantitative performance in terms of dynamic range, the linear response generally covered three orders of magnitude. Linear calibration curves gave good fits ($r^2 > 0.99$) and achieved the linearity criteria over the established concentration points ranging from 0.1, 0.25 to 100 or 250 μg/L depending on the compounds.

As for instrument accuracy, trueness did not exceeded the 23% of error for the majority of compounds, except for tributyl phosphate, clorpyrifos-ethyl, fluoxetine and clofibric acid (up to 41%, 36%, 52% and 35%)(see Table 2). About precision, the RSD values achieved for

intra-day analysis (repeatability) were below 10% (see Table 2), with the exception for clofibric acid, showing a RSD of 11%. Regarding inter-day analysis (reproducibility), RSDs were ≤ 15% for the majority of compounds (see Table 2), with few exceptions (e.g. dimethoate, paroxetine or fluoxetine).

As for instrument sensitivity, ILODs ranged from 1 to 96 pg injected. MLOQs ranged between 0.1 and 100 ng/L.

Both ion enhancement and suppression were observed in the MS signals of compounds (Table 2), thus demonstrating the required co-analysis of structurally similar IS to correct for such matrix effects. Overall, no statistically significant relationship was found between ion enhancement or suppression and low or high recoveries; i.e. Pearson correlation between ME % and REC % values from method A (Table 2) was $r = 0.019$, $p = 0.845$. Similarly, for those compounds recovered at percentages above the quantitatively acceptance (RE% > 130), no relationship was found between recovery and ion enhancement (ME % > 100) (i.e. Pearson $r = -0.444$; $p = 0.271$). In fact, among these compounds, only propanolol and metoprolol did show non acceptable high recoveries in correlation with ion enhancement; while the others (6 compounds) experienced ion suppression (ME % < 100) although being recovered at very high percentages. Diversely, the majority of those CECs showing non quantitative recoveries (REC % < 30) did also undergo ion suppression; while only diglyme, 4-Methyl-1H-benzotriazole, paroxetine and 1-H-benzotriazole, out of 15 compounds poorly recovered, experienced ion enhancement. Nevertheless, there was not any statistically significant relationship between REC % and ME % among these compounds either (i.e. Pearson $r = -0.356$; $p = 0.193$). Since none of these correlations were statistically significant, the trends observed for the co-variation of recoveries and matrix effects could not be confirmed.

Relative recoveries (REC %) of the SPE method applied for the extraction of the 117 target CECs from SW ranged between 0% and 230% and RSDs were below 30% for the majority of compounds (see Table 2). Only six compounds were nearly (i.e. 0 < REC % < 15) or not recovered at all (i.e. REC% = 0) by SPE method A:5,3′-triiodo-thyronine, diglyme, metformin, niacin, 2-naphthylamine, guanylurea and 2′-aminoacetophenone, and 3). Other compounds that were poorly recovered (i.e. 15 < REC % < 30) were: aspartame, pindolol, 2-Amino-3-methyl-3H-imidazo(4,5-f)quinoline, 4-Methyl-1H-benzotriazole, acesulfame, paroxetine, 4-Nitroquinoline 1-oxide, and 1-H-benzotriazole. Such behavior could be explained by the high hydrophilicity of metformin, niacin and guanylurea (Table A.3), travelling through the cartridge with the solvent without any interaction with the sorbent material. However, good recoveries were obtained for imidacloprid or salbutamol, with similar solubility and hydrophobicity than those not recovered at all i.e. metformin, niacin and guanylurea. Such behavior could then be explained by differences in chemical structure and molecular weight of compounds. For example, small molecules, with less active functional groups and thus less prone to interactions with the stationary phase, such as metformin, niacin and guanylurea, could be expected to be less retained on the SPE cartridge. On the other hand, the same reason could be applicable to explain the nearly zero recovery of 2-naphthylamine compared to thiacloprid for example. Although being characterized by similar hydrophobicity and solubility, the elution capacity of solvent used could have not been enough to disrupt such an apolar interaction between 2-naphthylamine and the stationary phase; while the recovery of thiachloprid might have been favored by the more active functional groups of this larger molecule. Nevertheless, according to this explanation, 3,5,3′-triiodo-thyronine might show such a strong affinity (Table A.3) for the cartridge stationary phase, but given its numerous active functional groups throughout the molecule potential interactions with elution solvents used could be strong enough to recover the compound. Instead, 3,5,3′-triiodo-thyronine was not recovered at all. All in all, the particular behavior of compounds not recovered or poorly recovered, as just discussed, is a clear example of general trends observed for the whole set of CECs assessed (see Section 3.4). Overall, there was no relationship between physicochemical properties of compounds and recoveries obtained by method A. Thus if compounds not recovered by the method presented in this work are of particular interest for a given study, further tests should be conducted in order to optimize the recovery of these CECs from SW. According to the quantitative criteria explained in section 2.4 (i.e. REC < 130% and RSD < 30%), only 23 compounds (e.g. tetraglyme, mecoprop and metoprolol) showed values exceeding the limits settled.

Summarizing, 87 compounds (74%) out of the 117 CECs tested provided an acceptable quality performance for the optimized analytical method applied.

3.6. SPE-LC-HRMS/MS method validation

Levels of analytes were corrected by multiplying the concentration of each compound by its corresponding recovery factor. As mentioned in the previous section, 23 compounds exceed the quantitative criteria for matrix effects correction by the application of recovery factors (see also Section 2.4) and thus levels determined for these compounds were considered semi-quantitative (e.g. tetraglyme, mecoprop and metoprolol). Individual and total levels for all compounds classified by their different uses (see Table A.1) and determined in SW and procedural blank analyzed collected are provided in Table A.6. Levels of compounds detected in the SW sample varied from < 10 ng/L to 400 ng/L. The compounds present in the highest concentration (i.e. > 100 ng/L) were industrial chemicals 1-H-benzotriazole, 4-methyl-1H-benzotriazole and 5-methyl-1H-benzotriazole: followed by the flame retardant triphenylphosphan-oxid. Regarding the procedural blank samples, only paracetamol and saccharine were determined at levels above their MLOQs, while the total concentration of CECs analyzed was 22 ng/L.

3.7. Suitability of the SPE-LC-HRMS/MS validated for EDA of SW

Typically, HLB has been the sorbent used for sample preparation based on SPE, in multi-residue analysis, non-target screening and EDA studies of CECs in SW due to its reported good recovery efficiencies covering diverse hydrophobicities [23]. However, the use of a single sorbent requires a balanced compromise among all species present in the water sample at specific pH conditions [23,47]. Recent research, including the present study, has demonstrated that the combination of different sorbent materials, providing a wide variety of possible interactions with the different species present in waters, has widened the scope to a broader variety of CECs with different physicochemical properties from SW [15,23,33,34]. In fact, the sequential-based SPE protocol optimized in this work (Method A) showed acceptable recoveries for widely diverse organic CECs (58 out of 117 compounds tested) regardless of their physicochemical properties (i.e. speciation). Differently from many other studies combining chemical analysis and bioassays on environmental samples [3–11], the optimized conditions of the sample preparation step led to both required concentration factors (i.e. 1000 and 20,000 for chemical analysis and bioassays, respectively) and an appropriate solvent mixture (i.e. 5% DMSO in water for chemical analysis and 100% DMSO compatible with required bioassays medium conditions) for the combined determination of concentration of chemicals and in vitro toxicity in the same SW extracts. The response of the several CALUX bioassays to SW extracts and the procedural blank are provided in Table A.7. Endocrine interference (i.e. estrogenicity and anti-androgenicity) and metabolic interference in SW extracts were observed. Blank samples showed no responses in almost all tests, with the exception of relatively low responses (< LOQ) in the PPARγ-CALUX (< 60 ng rosiglitazone eq/L in C1); and high responses in the p53-CALUX both without metabolic activation (-S9) (i.e. 145 and 6.7 pg actinomycin D eq/L in C1 and C2, respectively) and with metabolic activation (+ S9), bioassays (i.e. 548 and 34 µg ciclophosphamide D eq/L in C1 and C2, respectively). Mutagenicity observed in

blank samples should be further investigated. Nevertheless, the SPE protocol developed in this work proved its versatility and suitability for effect-based studies (i.e. EDA) of unknown toxicants in SW. In order to reduce the complexity of potentially toxic mixtures present in the sample and successfully identify key toxicants in EDA, fractionation removes non- or less toxic fractions [14,15,25]. Collecting separately the subsequent eluates from the three different extraction steps of the validated SPE method A would allow a first fractionation step of SW samples. Fractionation of SW samples in EDA is typically based on preparative chromatography using the reversed phase ((RP)-LC) partition separation principle as a criterion [15]. Thus, the adjustment of the sample preparation method optimized in this work to a (RP)-LC fractionation protocol makes a significant breakthrough in EDA studies of organic CECs in SW. Besides, in the present study the main downside of (RP)-LC fractionation (i.e. use of diverse organic solvents, such acetonitrile- or methanol–water mixtures, often not compatible with bioassays) [15] is overcome, as was demonstrated by means of recovery tests of selected CECs. However, toxicity recovery tests should be performed in addition to compound recovery tests in order to evaluate the applicability of the fractionation method [15]. Recovery of toxicity tests consist of the analysis of a reconstituted sample, from recombination of fractionation aliquots, with the same nominal concentration as the parent sample. This test provides information regarding the sensitivity of the fractionation protocol as well as receptor antagonists that might be present in the sample and masking a certain agonistic activity. Nevertheless, the potential causes of insufficient toxicity cannot be identified by toxicity recovery tests alone (e.g. low solubility in mobile phase or irreversible binding to stationary phase). Importantly, these effects have been evaluated in this work by compound recovery tests of selected CECs expected to exert certain mode of action.

All in all, the method developed in the present work integrates several improvements respective to our previous study [35]: (i) a broader list of organic CECs with a wide range of physicochemical properties was selected for method optimization and validation (i.e. 39 compounds in the previous work while 117 compounds in the present one); (ii) overall better recoveries were obtained for all compounds tested (i.e. Methods B and C tested in previous research performed less efficiently than Method A, eventually selected for method validation in the present study); (iii) while in previous work only spiked SW samples were analyzed, the present study provides valuable data obtained from the combined chemical analysis and bioassays performed in non-spiked SW extracts from diverse Dutch river courses.

Although compounds included in this research were previously selected and thus a target analysis was conducted, the overall good performance of the SPE-LC-HRMS/MS analytical method developed and validated demonstrates its suitability to be applied to non-target and EDA studies. Under the chromatographic conditions applied, optimal separation of compounds studied was achieved, thus acceptable separation of CECs with a broad range of polarities would be expected. The use of the Orbitrap analyzer acquiring simultaneously accurate mass full scans and product ion scan spectrums would allow the semi-quantitative and qualitative determination of unknown toxicants or suspect CECs of widely different physicochemical properties in SW as it has been previously demonstrated in target studies [21,24].

The analytical method developed proved to be a reliable procedure for the combined assessment of the chemical and biological quality of SW intended for DW production.

4. Conclusions

117 organic CECs characterized by a wide range of applications, physicochemical properties and modes of action were selected as model compounds of tests performed along the study. An LC-HRMS/MS analytical method was optimized for the analysis of 117 CECs in SW extracts. A sequential-based SPE procedure was optimized for the sample preparation of SW and further combined chemical analysis and in vitro bioassays of subsequent extracts. The SPE protocol developed yielded acceptable recoveries for a wide variety of CECs in the log K_{ow} range from -4 to 5 expected to be present in SW. When compared to other SPE procedures relying on a mono-bed Oasis HLB and MCX materials, the optimized mixed-bed method resulted more efficient for the recovery of the wide scope of chemicals studied. The use of different sorbent materials on a single SPE procedure provided complementary properties that broadened its application possibilities for qualitative and quantitative analysis of CECs in complex environmental samples. The optimized SPE procedure was successfully applied for the recovery of tested CECs from SWs intended for drinking water supply. Chemical and biological quality of samples could be assessed by combined LC-HRMS/MS analysis and a battery of CALUX bioassays performed in subsequent SPE extracts. Therefore, the authors propose the integration of the SPE validated method in an EDA procedure for a proper evaluation of SW quality and the prioritization of CECs that are key toxicants of complex environmental samples.

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Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2018.04.058.

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