Talanta 115 (2013) 349–360

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

On-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry optimized for the analysis of steroid hormones in urban wastewaters

Paul B. Fayad [a], Michèle Prévost [b], Sébastien Sauvé [a,*]

[a] Department of Chemistry, Université de Montréal, Montreal, QC, Canada, H3C 3J7
[b] Department of Civil, Geological and Mining Engineering, École Polytechnique de Montréal, Montreal, QC, Canada

ARTICLE INFO

Article history:
Received 9 April 2013
Received in revised form
13 May 2013
Accepted 14 May 2013
Available online 28 May 2013

Keywords:
Endocrine disrupting compounds
On-line SPE
Liquid chromatography
Mass spectrometry

ABSTRACT

An analytical method based on on-line SPE–LC–APCI–MS/MS has been developed for the detection and quantification of eight selected estrogenic and progestagenic steroid hormones; estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol (EE2), levonorgestrel (LEVO), medroxyprogesterone (MEDRO), norethindrone (NORE) and progesterone (PROG) in wastewater matrices. The injection volume could range from 1 to 10-mL according to the expected concentration of steroid hormones in matrix. The method characteristics are: analysis time per sample (<15 min), acceptable recovery values (71–95%), good precision (RSD≤10%) and limits of detection at the low-nanogram per liter levels in affluent and effluent wastewaters (8–60 ng L^{-1}). In particular, a detailed discussion of optimization parameters impacting overall performance of the method has been presented (sample collection, filtration and storage). All optimization and validation experiments for the on-line SPE method and chromatographic separation were performed in environmentally-relevant wastewater matrices. This method represents a compromise between analysis time, higher sample throughput capabilities, sample volume and simplicity for the analysis of both progestagenic and estrogenic steroid hormones in a single run, with LODs and LOQs sufficiently low to detect and quantify them in environmental wastewater matrices. Thus, the applicability of the method was tested on affluent and effluent wastewaters from two wastewater treatment facilities using different processes (biological and physico-chemical) to evaluate their removal efficiency for the detected steroid hormones.

1. Introduction

The monitoring of endocrine disrupting compounds (EDCs), such as steroid hormones, in the aquatic environment is progressively becoming a priority for government agencies, regulatory agencies as well as the general public. They originate from naturally-occurring (e.g. normal urine excretion from mammals) and synthetic sources (e.g. oral contraceptives and hormone replacement therapy). Given their strong endocrine-disrupting potency and their occurrence, selected estrogens, progestagens and androgens have been targeted and detected in wastewater, surface water and drinking water [1–6]. With growing populations and increased discharges from wastewater treatment plants (WWTPs), the presence of steroid hormones in surface waters could be cause for concern since conventional treatment methods have proven to be inadequate to sufficiently eliminate them. There is strong evidence that impacts on the reproductive physiology of wildlife populations occur at very low concentrations, i.e. from 0.1 to 1.0 ng L^{-1} [7–10]. Several studies conducted in numerous countries [2,11], have shown that WWTP effluents and receiving water bodies contain sufficient amounts of estrogenic and progestagenic compounds to induce harmful effects on fish, with their concentrations varying from sub-ng L^{-1} levels to hundreds of ng L^{-1} in wastewater samples [11–13]. Therefore, the development of analytical methods able to detect and quantify these steroid hormones is of critical importance.

To date, numerous analytical procedures have been developed to identify and quantitate steroid estrogenic hormones in water matrixes and often include the use of chromatography (liquid or gas) coupled to tandem mass spectrometry (MS/MS) [5,14]. However, gas chromatographic (GC) methods often require labor and time-consuming steps that improve sensitivity, given the low-molecular weight and low volatility of steroid hormones, but these manipulations could induce some loss of analyte. The necessary sample preparation could include complex hydrolysis as well as derivatization reactions [3,15]. This has led to the development of

* Corresponding author. Tel.: +1 514 343 6749; fax: +1 514 343 7586.
E-mail address: sebastien.sauve@umontreal.ca (S. Sauvé).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.05.038

liquid chromatographic (LC) methods that do not necessitate the use of such sample chemical pre-treatment methods for steroid hormones before sample detection, since analyte volatility and thermolability are not limiting factors. Indeed, a review of the literature (from 1981 to the present, i.e. 64 articles) on steroid hormones [3,5,14,16] shows that of the methods developed, 70% used LC, 25% use GC while the remaining 5% used an immunoassay analysis procedure. Pre-concentration and purifying processes, such as solid-phase extraction (SPE) or liquid–liquid extraction (LLE) are necessary because of matrix complexity and the low-nanogram per liter levels at which steroid hormones have been reported in the aquatic environment [3]. More than half (56%) of all LC methods developed used an off-line SPE sample enrichment process that necessitates the use of 100 to 2000 mL of sample volume, making it a slow, tedious and labor-intensive practice. As a result, the identification and quantification of steroid hormones can be time consuming, costly and often result in slow turnover and parsimonious environmental sampling strategies. The concept of on-line SPE where all the steps (conditioning, sample enrichment, wash and elution) involved in the off-line method are integrated into an automated procedure was first introduced in the mid 1990s [17]. This on-line SPE approach, is quicker, allows for reduced sample size, handling and preparation, improved reproducibility, higher sample throughput as well as less waste and solvent consumption.

Only two articles [18,19] have developed an on-line method by LC-MS/MS that include both estrogens and progestagens in the same analytical run, with the use of a 1 to 5 mL injection method with limits of detection (LODs) ranging from 0.3 to 50 ng L^{-1}. The others [16,20–24] have focused on estrogens (including androgens and other analytes of interests) using injection volumes between 1 and 500 mL with LODs between 0.01 and 6.8 ng L^{-1}. Analysis time varies significantly for the proposed methods, with the longest between 25 and 65 min and the others ranging from 10 to 17 min. Also, method validation parameters have not always been clearly defined. Indeed in one study [19], the LODs and limits of quantifications (LOQs) were determined in Milli-*Q* water with the measured concentrations in wastewater samples for estrogens being lower than the reported linearity range. In another work [18], the LODs were determined in river water while the calculated concentrations were reported in wastewater or surface matrices and the LOQ values were not reported for the steroid hormones analyzed. Finally, sample collection, storage and pre-treatment are not discussed in the majority of the methods and could have a significant impact on the reported results.

Our objectives in this study is to optimize and apply an on-line tandem SPE–LC–MS/MS method for the determination of eight selected hormones in water, i.e. estrogens (estriol, estradiol, estrone and 17-α-ethinylestradiol) and progestagens (progesterone, levonorgestrel, medroxyprogesterone and norethindrone). We aim to validate the method starting with sample collection, filtration and storage, up to the on-line pre-concentration followed by LC–MS/MS detection. A proposed method for the determination of LODs and LOQs according to the product ions will be used in order to consider both SRM transitions for the quantification and detection of selected steroid hormones. Their determination at low-nanogram per liter levels in affluent and effluent wastewater was done to confirm the applicability of the method in real environmental samples.

2. Experimental

2.1. Chemicals, reagents and stock solutions

All selected steroid hormone standards (purity≥97%); estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol (EE2), levonorgestrel (LEVO), medroxyprogesterone (MEDRO), norethindrone (NORE) and progesterone (PROG) were purchased form Sigma Aldrich (St. Louis, MO) and are illustrated in Fig. S1. Isotopically-labeled estradiol, [$^{13}C_2$]-E2 was obtained from Cambridge Isotope Laboratories (Andover, MA) and used as internal standard (IS). Individual stock solutions were prepared in methanol (MeOH) at a concentration of 1000 mg L^{-1} and kept at −20 °C for a maximum of six months. A primary mix of steroid hormone working solution was prepared weekly at a concentration of 50 mg L^{-1} by dilution in MeOH of individual stock solutions aliquots. Subsequent working solutions were prepared daily in water to give solutions of desired concentration. All organic solvents and water used for dilutions were of HPLC grade purity from Fisher Scientific (Whitby, ON, Canada).

Analyte-free effluent wastewater samples were generated by maintaining previously collected samples in the laboratory under conditions to favorable to degradation (exposed to light and keep at room temperature) for long periods of time, until the targeted analytes were no longer detectable.

2.2. Instrumental conditions

The pre-concentration of selected steroid hormone water samples was performed using the Equan™ (Thermo Fisher Scientific, Waltham, MA) system. It consists of a sample delivery system, a dual switching-column array and an LC–MS/MS system. The delivery system comprised an HTC thermopal autosampler manufactured by CTC analytics AG (Zwingen, Switzeraland) used for in-loop sample injection and a quaternary pump Accela 600 (Thermo Finnigan, San Jose, CA) used to load the SPE column with the contents of the sample loop. The column switching system was composed of two-position six-port and ten-port valves (VICI® Valco Instruments Co. Inc., Houston, TX) and a quaternary pump Accela 1200 (Thermo Finnigan, San Jose, CA) used for sample elution from the SPE column and separation on the analytical column. The on-line SPE was achieved using two Hypersil Gold aQ (20 mm × 2 mm, 12 μm particle size) columns in tandem and chromatographic separation was done with a Hypersil Gold (100 mm × 2.1 mm, 1.9 μm particle size) column kept at 55 °C. All columns were manufactured by Thermo Fisher Scientific (Thermo Finnigan, San Jose, CA). Ionization of steroid hormones was achieved using the Ion Max API Source mounted on a Quantum Ultra AM triple quadrupole mass spectrometer by Thermo Fisher Scientific (Waltham, MA) operated in selected reaction monitoring (SRM) mode for quantification and detection.

2.2.1. On-line solid phase extraction and chromatographic conditions

In order to improve signal intensities and method detection limits (MDLs) we tested multiple injection volumes of a 150 ng L^{-1} mix steroid hormone solution in HPLC water and affluent wastewater, using a 20-mL injection loop. This allowed us to establish the maximum injectable volume without loss of analyte. According to the optimized procedure, a sample loading volume (using a 5-mL syringe) ranging between 1 and 10 mL is possible and adjustable depending on the expected steroid hormone concentrations in the sample matrix. The sample transfer rate (loading speed) from the injection loop to the SPE column was tested between 1.0 and 5.0 mL min^{-1}, for a concentration of spiked steroid hormones (500 ng L^{-1}) in analyte-free affluent wastewater, in order to reduce total analysis time. The maximum sample loading flow rate from the sample loop (10 mL) to the SPE columns was 1.5 mL min^{-1} from the loading pump using water with 0.1% formic acid (FA). Following the sample loading step, the pre-concentration columns were back-flushed and the eluting analytes were transferred using the analytical pump gradient directly

Table 1
Valve program, on-line SPE (loading pump) and LC (analytical pump) gradient elution conditions used for the pre-concentration and separation of selected steroid hormones.

Loading pump (to six port valve)					Analytical pump (to ten port valve)				
	Time (min)	A (%)	B (%)	Flow rate (μL min^{-1})	Time (min)	A (%)	B (%)	Flow rate (μL min^{-1})	
On-line SPE loading step	0.00	0	100	1500	0.00	40	60	550	Column re-equilibration
	6.96	0	100	1500	6.96	40	60	550	
Loop wash	6.97	100	0	3000	8.45	55	45	550	Elution and chromatographic separation
	10.00	100	0	3000	10.00	75	25	550	
SPE column and loop conditioning	10.01	0	100	3000	11.54	85	15	550	
	14.00	0	100	3000	11.55	40	60	550	Column re-equilibration
					12.00	40	60	550	

Table 2
MS/MS optimized parameters for the analysis of selected steroid hormone analytes in positive (PI) ionization mode.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Intensity ratio (%)	TL (V)	CE (eV)
E1	271	157	100	91	22
	$[M+H]^+$	133	93 ± 6	91	22
E2	255	159	100	100	16
	$[M-H_2O+H]^+$	133	29 ± 2	100	17
E3	271	157	100	91	22
	$[M-H_2O+H]^+$	133	91 ± 9	91	22
EE2	279	133	100	87	15
	$[M-H_2O+H]^+$	159	80 ± 7	87	18
LEVO	313	245	100	103	17
	$[M+H]^+$	91	87 ± 7	103	26
MEDRO	345	123	100	127	22
	$[M+H]^+$	97	24 ± 2	127	18
NORE	299	109	100	114	22
	$[M+H]^+$	91	90 ± 10	114	37
PROG	315	109	100	100	26
	$[M+H]^+$	97	98 ± 5	100	17

through the analytical column using the solvent (MeOH and water with the addition of 0.1% FA—3.1 min). The on-line SPE and elution gradients are shown in Table 1. To eliminate carryover effects, the 5-mL syringe and the six-port sample loop injection valve were cleaned twice between each injection, during the chromatographic separation step. Initially with a strong cleaning mixture of solvents, i.e. acetonitrile:methanol:isopropanol (1:1:1, v/v/v), and then with HPLC grade water with 0.1% FA. The sampling step, the SPE column conditioning, loading and elution steps as well as the LC-MS/MS analysis are all automated. This configuration allowed for a short total analysis time, i.e. 15 min per sample, using a 10-mL injection loop.

2.2.2. Mass spectrometry

Ionization of steroid hormones was achieved with an APCI source in positive (PI) mode. The initial compound-dependent (tube lens and collision energy) parameters for MS and MS/MS optimization conditions in PI mode were performed by infusion of the standard steroid hormones and the IS at a concentration of 10 mg L^{-1} with a mobile phase of MeOH (A) and water (B) (50:50, v/v with 0.1% FA) and are presented in Table 2. Once the chromatographic separation was optimized, source-dependent parameters were re-evaluated for the steroid hormones as a function of mobile phase composition at their respective retention times on column. The resulting APCI parameters used to maximize signal intensity were as follows: capillary temperature (350 °C), vaporizer temperature (490 °C), sheath gas pressure (50 arbitrary units), aux gas pressure (15 arbitrary units), ion sweep gas pressure (0 arbitrary units) and discharge current (5 μA). The scan time was adjusted to 0.015 s, giving a minimum of 20 points per peak, the first and third quadrupole (Q1 and Q3) were operated at unit resolution (0.7 Da FWHM), with the second quadrupole (Q2) collision gas pressure set at 1.5 mTorr.

2.3. Data analysis and method validation

All optimization and validation experiments for the on-line SPE method and chromatographic separation were performed in HPLC grade water and analyte-free composite wastewater affluent collected from several wastewater treatment plants (WWTPs) to mimic environmentally-relevant conditions. The positive identification of target analytes was confirmed by matching chromatographic retention times within ± 2% of those from standard spiked in analyte-free matrix and using a minimum of two selected reaction monitoring transitions (SRM) as well as the relative intensities of their ratios. The most abundant product ion was used for quantification whereas the second most abundant was used for confirmation. In accordance with the European Commission [25], the SRM ratios were acceptable if for relative intensities greater than 50%, the error was within ± 20% and within ± 50% for relative intensities inferior to 10%. Data acquisition was performed in the selected reaction monitoring (SRM) mode. Resulting MS/MS peaks were integrated using the ICIS algorithm of the Xcalibur 1.2 software from Thermo Fisher Scientific. The instrument response was determined as the ratio of the analyte area to that of the isotopically-labeled IS.

The stability of sampled wastewater was evaluated to determine the maximum acceptable period from sample collection to sample analysis. Analyte-free affluent, effluent and HPLC grade waters were spiked with selected steroid hormones (2 μg L^{-1}) and kept in amber glass bottles. The bottles were repeatedly shaken and left to equilibrate for 1-h at room temperature, as it was previously illustrated that sorption was maximal within that time for steroid hormones [26]. The compared working conditions were temperature (25 °C versus 4 °C), filtration (non-filtered versus filtered) and time (from 0 to 48 h). The IS was added to the sampled aliquots from each bottle prior to analysis ($n=2$) and their peak area ratios were calculated to evaluate the impact of sample storage conditions and maximum time allowed before sample degradation.

To evaluate the impact of filter material on the retention of the selected steroid hormones in the initial filtration step, several types of filters were tested (all 0.22 μm with a 25 mm diameter except for glass fiber at 0.3 μm with a 47 mm diameter), i.e. cellulose acetate (CA), mixed cellulose ester (MCE), nylon (NYL), polycarbonate (PC), polyethersulfone (PES) and glass fiber (GF) obtained from Sterlitech (Sterlitech Corporation, Kent, WA). The solutions were spiked with the selected steroid hormones (5 μg L^{-1}) in HPLC grade water and analyte-free filtered affluent wastewater samples that were left to equilibrate overnight at 4 °C

prior to filtration. The resulting filtrate aliquots, with added IS, were analyzed ($n=4$) and their peak area ratios were compared to those of the non-filtered solutions (centrifugation was used for the non-filtered affluent) of equal concentration to determine recovery values. Recovery values were reported as percentages.

The recovery values and matrix effects for the on-line SPE method were evaluated at two concentration levels, i.e. 100 and 500 ng L^{-1}, in spiked HPLC water and analyte-free affluent wastewater. Extraction recoveries were determined in two ways. The mean peak areas ($n=5$, 100 ng L^{-1}) of the selected steroid hormones of, (i) a direct chromatographic injection (25 μL) and, (ii) an on-line small injection (100 μL, with a 100-μL loop) were compared with those of the on-line high volume injection (10 mL) used for the standard sample analysis. The same mass of analyte was injected in all cases. Matrix effects were calculated by comparing peak areas of spiked affluent wastewater to the peak areas found in HPLC water according to a 10-mL injection volume. Recoveries and matrix effect values were reported as percentages.

The limits of detection (LOD), limits of quantification (LOQ), linearity, precision and accuracy were determined using a six to eight point calibration curve, analyzed in duplicate, in HPLC water as well as analyte-free effluent and affluent wastewater. These values were evaluated using the complete, optimized, on-line SPE–LC–MS/MS method and therefore represent the validation parameters for the entire analytical process. Method blank samples for each matrix were also added following the highest calibration point to evaluate carryover. For the calibration curve, the back-calculated concentrations (Table S2) of calibrant standards were acceptable if within 15% of the nominal value, except for the determined LOQ for which a 20% error was deemed satisfactory. The linear calibration range used was tested for homoscedasticity (Table S2, Breusch–Pagan test, P value of 0.05) to confirm the application of the linear least-squares method (constant variance).

The LOQ was measured using the calibration curve corresponding to the most abundant product ion; whereas the LOD was determined using the calibration curve associated with the second most abundant product ion. Both LOD and LOQ were calculated by multiplying by 3.3 and 10 the error on the y-intercept and dividing by the slope of the regression line equations, respectively. Once calculated, a test sample was prepared at the LOQ concentration and injected to confirm the expected linearity range.

Precision and accuracy were determined at two concentration levels (QC1 and QC2, $n=5$) of steroid hormones spiked in HPLC water and in analyte-free affluent wastewater. They were calculated as the relative standard deviation (RSD) in percentage of the steroid hormone to IS peak area ratio from the replicates.

The validation process was performed using the criteria's form the International Conferences of Harmonization (ICH), more specifically the Q2(R1) guidelines [27]. We used the Statistical Package for Social Science (SPSS 16.0, Chicago, IL), for a Tukey's b post hoc ANOVA test to compare the optimization parameters of the method. Statistical significance was reported for P value of <0.05 or <0.01 according to number of samples tested.

2.4. Sample collection, preparation and quantification

Affluent and effluent wastewater samples were collected, in pre-cleaned 1-L amber bottles, from two WWTP facilities in the Greater Montreal Area (Quebec, Canada) using different treatment processes, i.e. WWTP-A and WWTP-B. The WWTP-A uses a physico-chemical treatment approach, whereas the WWTP-B applies a bio-filtration and ultraviolet disinfection process for contaminant removal. A time-proportional sampling mode was chosen, with wastewater samples collected hourly or every half hour between 7:30 am and 11:30 am for each treatment plant. All samples, prior to analysis, were passed through a 0.3-μm pore size glass fiber filter (Sterlitech Corporation, Kent, WA) to eliminate particulate material and stored at 4 °C to avoid microbial growth. Once filtered, the samples could be stored for 48 h before analysis without affecting sample integrity according to our results. No prior treatment of sample matrix was necessary before applying the on-line SPE procedure.

In order to correct for matrix effect, the selected steroid hormones were quantified using four to five point standard addition calibration curves (Fig. S9), with each calibration point injected twice and the unknown analyzed in triplicate ($n=3$). A least-square linear regression model was applied, with coefficients of determination (R^2) required to be greater than 0.9950 for all analytes, with IS added to all calibration levels, blanks and unknowns prior to analysis.

A summary of the analytical steps, described here in this section and used during method development and validation is presented in Fig. S10.

3. Results and discussion

3.1. Sample storage and filtration conditions

The stability of the selected steroid hormones in three different water matrices (HPLC grade, effluent and affluent wastewater) was evaluated according to temperature (22 versus 4 °C) and filtration conditions (Figs. 1 and S2–S4). These tests are necessary to guaranty both sample integrity and accuracy of reported concentrations and to provide important information on the permissible delay from the collection of a sample to its analysis. In earlier studies [28–31], the integrity of steroid hormones in the collected wastewater and river samples relied on the addition of preservation agents, i.e. formaldehyde (1%, v/v), acidic preservatives (sulfuric acid, sodium azide or hydrochloric acid) and solvent addition (MeOH, 2.5% v/v). The addition of preservatives can, however, lead to unwanted secondary reactions, such as the cleavage of conjugated steroid hormones by the addition of acids or desorption form the particulate phase when solvents are used. This could lead to the overestimation of free steroids hormones. Thus, it was necessary to evaluate the recovery values for the selected steroid hormones without the addition of any conservation agents to avoid modifying the nature of the collected samples. The steroid hormones EE2 and MEDRO were chosen to represent both estrogens and progestagens, respectively, as a function of their stability during short term storage (Fig. 1), while the results for the remaining steroid hormones are shown in Figs. S2–S4. It was shown that for E3, EE2, LEVO and NORE, there was no statistically-significant differences between the initial (0-h) and the remaining time scheduled samples analyzed in a period of 48 h as a function of: temperature, filtration or water type ($n=2$; $P<0.01$, with 98% of all RSD≤20%). At a temperature of 25 °C, only filtered effluent wastewater samples were unchanged as a function of time, for all tested analytes.

The selected steroid hormones were stable for a period of 48 h at 4 °C with no significant differences between filtered and unfiltered samples or the nature of the water, with the exception of PROG at the 8-h mark in unfiltered effluent wastewater at 4 °C (Fig. S3). Poor recoveries from unfiltered and filtered affluent and effluent water samples were seen for PROG, MEDRO and E2 at 25 °C. These results are in agreement with a previous study [32] for the stability of progesterone in surface and drinking matrices under similar conditions. These losses are attributed to their strong hydrophobic character, with a log K_{ow} ranging from 3.50 (PROG) to 4.01 (E2) (Table S1), making them susceptible to interact with suspended and dissolved natural organic matter as well as particulates in solution, with these exchanges being favored at

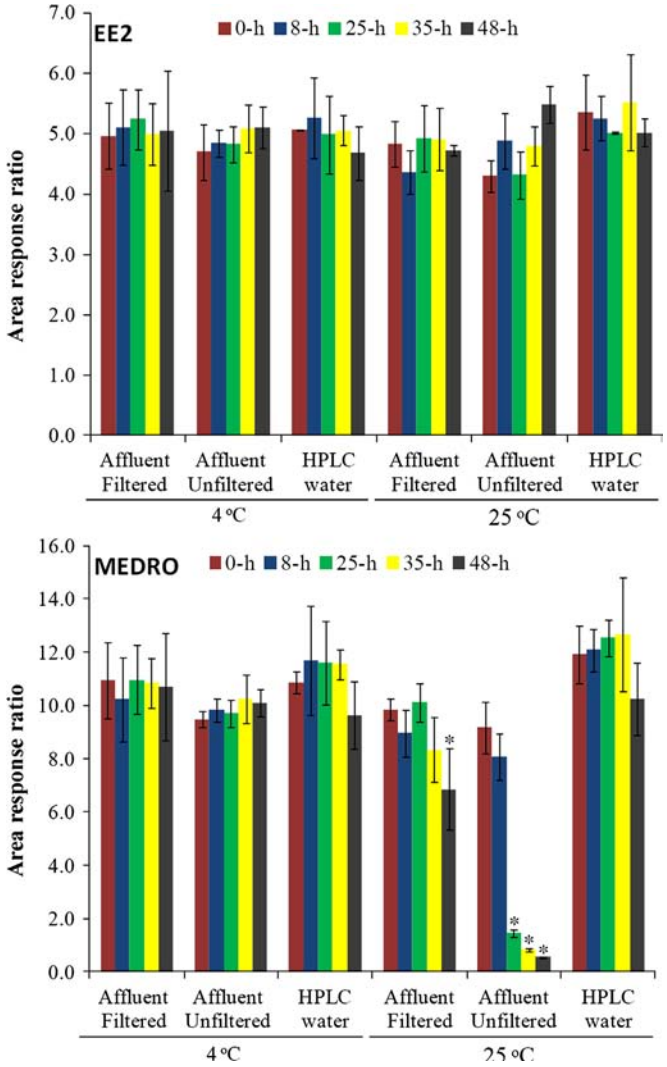

Fig. 1. Influence of temperature (4 °C and 25 °C), suspended material (filtered versus unfiltered), water type (HPLC grade water and affluent wastewater) and time (0-h to 48-h) on the degradation of spiked steroid hormone solutions (mean ± SD, $n=2$, 2 μg L^{-1}) during storage. As examples, EE2 and MEDRO were chosen to represent estrogens and progestagens, respectively. The symbols (*) represent statistically significant ($P<0.01$) differences between the initial 0-h and the subsequent area response ratios as a function of time within a sample.

room temperature. Therefore, with these results, sample integrity was not compromised with the addition of preservation agents and it was decided to filter the samples and keep them refrigerated prior to analysis within 48 h of collection.

Although sometimes overlooked, the filtration step involved prior to sample pre-treatment for analysis can have an impact on adsorption and loss of analytes due to undesirable interactions between the filter material and the analytes of interest. This has been discussed in detail for membrane technology applied to estrogenic steroid hormone removal in wastewater treatment processes [32,33] and should be investigated when filtration is applied in the analysis of steroid hormones in water matrices (or any trace contaminant for that matter). One study has evaluated the impact of several microfiltration membrane filters on estrone solution following photocatalytic degradation treatment processes in water [33]. Several filter materials have been used to isolate the unbound water-soluble fraction of steroid hormones from the particular phase in water analysis, such as nylon [16,34,35], mixed cellulose ester [18,36], cellulose acetate [37,38], polytetrafluoroethylene (PTFE) [39], and glass fiber [22,28,40], while others do not mention the nature of the filter material or the use of a filtration step [19,21,24]. The retention of steroid hormones on the filter material needs to be evaluated since it could result in the underestimation of reported concentration or generate false negative results.

The impact of filter material on the retention of the selected steroid hormones in the initial filtration step was tested on several filter types in spiked HPLC grade water as well as analyte-free filtered affluent wastewater and is presented in Figs. 2 and S5. In both cases, three (EE2, MEDRO and PROG) of the eight selected steroid hormones gave statistically significant higher recovery values with GF filters ($n=4$; $P<0.05$). Mean recovery values ranged from 92 to 103% when GF was used (with SD < 8% in all cases), 57 to 106% for CA, 6 to 91% for MCE, 1 to 96% for NYL, 37 to 113% for PC and 14 to 104% for PES, for all compounds in both water matrices. This made GF the most reliable filter material for the elimination of suspended material for the selected steroid hormones while minimizing losses of dissolved analyte due to sorption onto the filter. Progesterone (PROG) was the only compound (Fig. S5) for which the recovery values were significantly ($n=4$; $P<0.05$) lower in affluent wastewater than in HPLC grade water. This result could be attributed to its interaction with the natural dissolved organic matter present in the affluent matrix, given the hydrophobicity of PROG (log $K_{ow}=3.87$, Table S1). The nature of the sorption and interaction mechanisms involved between the steroid hormones and the selected filter material are complex and are not completely understood. These mechanisms include hydrophobic interactions, hydrogen bonding and π–π stacking, all of which are dependent on the nature of the analytes and the physico-chemical characteristics of the filter material. The emphasis of this paper was to evaluate the impact of filter material on the retention of the selected steroid hormones prior to chromatographic analysis, a more detailed discussion on the sorption mechanisms can be found elsewhere [32,33,38].

A recent review [41] demonstrates the importance and the need for such studies (storage conditions and filter material) to measure the depletion levels of several analytes, such as pharmaceutical compounds as well as certain hormones, as a function of storage conditions. They mention the lack of standardized protocols for the collection and storage of environmental waters which would undoubtedly help in improving the results (accuracy) and confidence for the reported concentration levels in environmental samples.

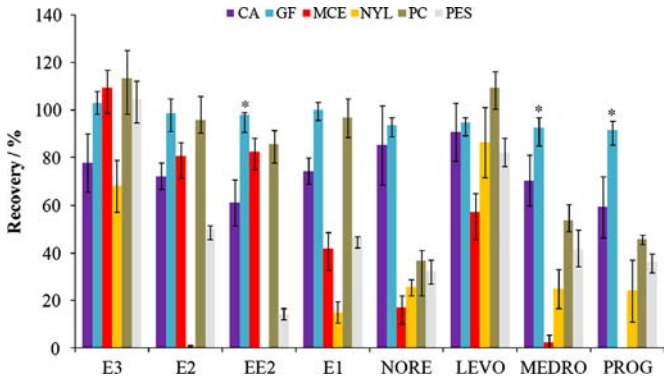

Fig. 2. Comparison of recovery values (mean ± SD, $n=4$) on different filter materials, cellulose acetate (CA), glass fiber (GF), mixed cellulose ester (MCE), nylon (NYL), polycarbonate (PC) and polyethersulfone (PES), for selected steroid hormones. Tests were done in spiked (5 μg L^{-1}) analyte-free filtered affluent wastewater. The GF filters gave significantly higher recovery values for three out of the eight steroid hormones (*, $P<0.05$).

3.2. Optimization of the on-line SPE procedure

3.2.1. SPE loading speed and breakthrough volume

The time limiting factor in on-line SPE is the sample transfer time from the injection loop to the SPE column in the sample enrichment step. The more rapidly the loading speed from the injection loop to the SPE column, the less time is spent on each sample, increasing sample throughput. This parameter must be optimized so as not to generate sample loss from diminished sample contact time with sorbent phase at higher loading flow rates or, inversely, increased retention behavior from the sorbent phase due to lower flow rates which subsequently affects elution strength. Also, since the breakthrough volume could be a flow rate dependent step [42], the loading speed should be tested in the conditions used for sample analysis. The optimum loading speed values, in terms of both extraction efficiency and speed, were evaluated on a spiked steroid hormone solution ($n=3$, 500 ng L^{-1}) in analyte-free affluent wastewater, using a 10-mL injection sample loop (Fig. 3). As shown in Fig. 3, for the loading speeds of the selected steroid hormones, with the exception of MEDRO and PROG, there is no significant differences ($n=3$; $P<0.05$) between 1000 and 2000 μL min^{-1}, such flow rates yielding the highest analyte peak area responses. Therefore, a flow rate of 1500 μL min^{-1} was selected as a compromise in order to decrease sample loading time while not affecting analyte response for six of the eight analyzed compounds. The negative impact of peak area response for MEDRO and PROG was considered acceptable ($<25\%$), since they are the two most sensitive compounds and have the lowest LODs and LOQs.

The overloading of the SPE columns is considered to be a function of the retention capacity of the sorbent material towards analyte concentration or interfering matrix compounds. The impact of these two factors has been considered negligible when working with trace level contaminants and with the nature of the matrix in environmental water samples [42–44]. Previous on-line SPE breakthrough volume experiments for steroid hormones were performed in HPLC grade or Milli-Q water in the absence of wastewater of surface water matrix, according to analyte peak area or recovery [16,36,45]. The breakthrough volume is important in that it is directly related to the pre-concentration factor, thus influencing the LOD and the LOQ which are both related to the signal-to-noise ratio (*S/N*), as well as the sensitivity. This would suggest that looking for a peak area increase or recovery value to determine breakthrough volume should be accompanied by an evaluation of the *S/N* response, since a larger volume of sample could also cause an increase in unwanted interfering compounds. The breakthrough volume for the selected steroid hormones was established by injecting variable volumes (1 to 20-mL) of a sample (constant concentration) spiked with steroid hormones ($n=3$, 150 ng L^{-1}) in analyte-free affluent wastewater, using a 20-mL injection loop (Figs. 4 and S6). Both peak areas and *S/N* were monitored as a function of injection volumes. Linear regression lines were plotted against the experimental data points to

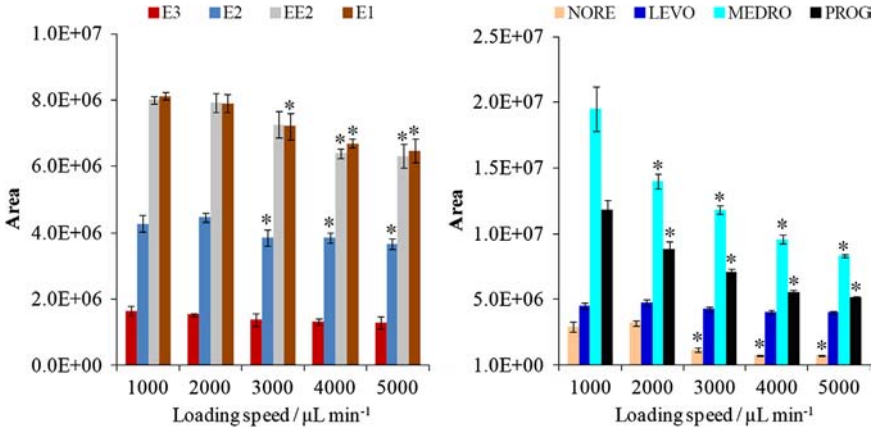

Fig. 3. Effect of loading speed (1000 to 5000 μL min^{-1}) for spiked steroid hormones (mean ± SD, $n=3$, 500 ng L^{-1}) peak areas from sample loop (10-mL) to on-line SPE column in analyte-free affluent wastewater. The symbols (*) represent significantly ($P<0.05$, $n=3$ with RSD≤15 in all cases) smaller peak areas than observed at the lowest loading speed (1000 μL min^{-1}).

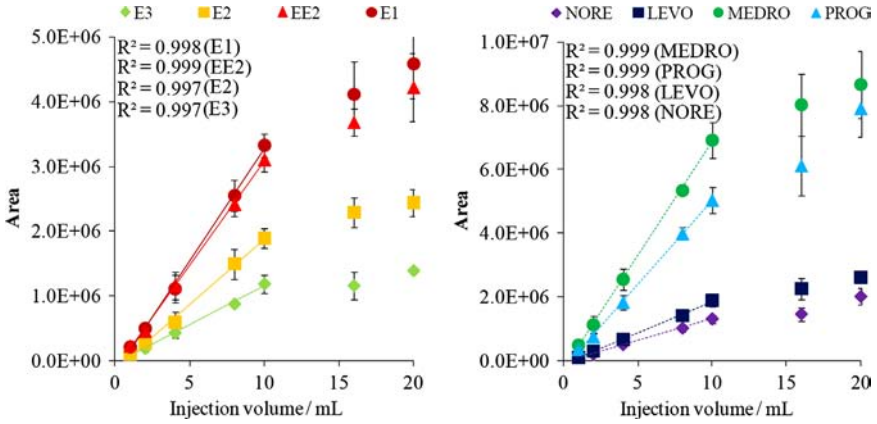

Fig. 4. Determination of breakthrough volumes (1 to 20-mL) of a mix steroid hormone solution ($n=3$, 150 ng L^{-1}) in analyte-free affluent wastewater, using a 20-mL injection loop. The maximum injectable volume is represented by the last point of the linear regression line of analyte peak area. A sample loading volume between 1 and 10 mL, without loss of analyte, is possible in affluent wastewater (R^2≥0.997).

determine the maximum injection volume. Linearity (R^2≥0.997) was observed for all selected steroid hormones between 1 and 10-mL (Fig. 4) when peak areas were used, whereas five of the compounds showed an acceptable linear increase (R^2≥0.983) between 1 and 10-mL when looking at *S/N* (Fig. S6). Indeed, for LEVO, MEDRO and NORE a linear increase was achieved between 1 and 4-mL and then a plateau was observed for the following injection volumes (Fig. S6). A maximum injection volume of 10-mL was chosen, even if *S/N* was not improved for three progestagenic hormones, since it did have a positive impact for the remaining compounds and because the progestagens show higher *S/N* ratios than the estrogens.

3.2.2. SPE matrix effect and recovery

The nature of the composition of the sample matrix can have a negative (ion suppression) or positive (ion enhancement) impact on analyte signal and will influence the reproducibility, linearity, accuracy and ion-ratio of a method as well as produce false positives. Although APCI is less susceptible to matrix effect than ESI [46,47], it should still be evaluated, since it is matrix dependent, unpredictable and can affect the reliability of a method [48,49]. The matrix effects for the on-line SPE method were evaluated at two concentration levels, i.e. 100 and 500 ng L^{-1}, in spiked HPLC water and a mixture of several analyte-free affluent wastewaters (Fig. S7). Matrix effect was calculated by comparing peak areas of spiked affluent wastewater to the peak areas found in HPLC water according to a 10-mL injection volume. Multiple affluent wastewater samples were pooled prior to being spiked, to evaluate a modified relative matrix effect [47] in a more representative matrix. This will affect both precision and accuracy measurements of the method, whereas the majority of methods employ an absolute matrix effect calculation method [16,18], that only affects accuracy. The results in Fig. S7 show that for all the analytes of interest there was a signal suppression, with matrix effect varying from 63 to 86% for both concentrations (100 and 500 ng L^{-1}). To compensate for these matrix effects, a standard addition calibration method using a single IS was chosen for the quantification and detection of the selected steroid hormones in wastewater samples. This alternative was previously investigated and compared with external and matrix-matched calibration methods, using one or multiple IS, to overcome matrix effects in the analysis of phytoestrogens by LC–MS/MS [50]. While traditional off-line standard addition methods remain laborious and time consuming, the automation of the SPE procedure using on-line SPE–LC–APCI–MS/MS method makes this approach feasible and not so labour-intensive, while giving the most reliable results.

Extraction recoveries have an impact on the LODs and LOQs and were determined in two ways, (i) a direct chromatographic injection (25 μL) and (ii) an on-line small volume injection (100 μL, with a 100-μL loop) and compared with those of the on-line high volume injection (10-mL) used for sample analysis in affluent wastewater (Fig. S8). The on-line small injection volume method [18] has the advantage of using the same instrumental setup and is based on the complete retention of analytes on the SPE column due to small injection loop volume (100-μL versus 10-mL in this case) and reduced sample transfer times. As illustrated in Fig. S8, the two methods are not statistically ($n=5$; $P<0.05$) equivalent. Indeed, for only two of the selected steroid hormones (EE2 and NORE) both methods were statistically similar. In addition, when the signal intensities for the off-line small injection volume and the on-line small injection were compared (results not shown) the averages were also significantly different for six of the eight selected hormones. The recoveries for the on-line small injection method ranged from 64 to 101%, while for the off-line small injection method, they ranged from 71 to 95%. This would imply that the differences in recovery values are not simply due to matrix effect as would be expected, but rather a combination of matrix effect and other factors, such as the instrumental setup or retention mechanism on the sorbent mass for smaller volumes. As expected, E3 gave the lowest recovery values, being the least hydrophobic of the studied steroid hormones (log K_{ow}=2.45, Table S1), whereas the more hydrophobic progestagenic steroid hormones (NORE, PROG, MEDRO and LEVO) had higher recovery values. Given that the recovery values could not be determined with matrix-matched solutions, the process efficiency [47,51] was also calculated to take into account both matrix affects and recovery values (Table 3). Process efficiencies ranged from 54 to 79%, which represents satisfactory values since the least hydrophobic steroid hormones, i.e. E1, E3 and NORE (Table S1), did not permit an effective wash step without loss of analyte.

Table 3
Calculated process efficiency (PE) for the selected steroid hormones for the on-line SPE-LC-APCI-MS/MS method.

Compound	Process efficiency[a] (%)
E1	54
E2	72
E3	67
EE2	55
NOR	60
LEVO	79
MPROG	65
PROG	73

[a] Calculated using matrix effect (ME) and recovery values (RE) according to the off-line small injection method (25 μL): PE=(ME × RE)/100. Equation taken from Ref. [46].

3.3. Chromatographic and MS/MS conditions

The complete method was validated following the previously described on-line SPE optimization results for the selected steroid hormones. The chromatography was the first challenge, with the adjustment of the elution gradient conditions. A minimum of 40% MeOH in the initial elution step (Table 1) was needed to simultaneously elute all the steroid hormones from the SPE column prior to reaching the analytical column, thus significantly reducing peak tailing and broadening. With this amount of organic modifier, the un-retained polar and less hydrophobic compounds are quickly eluted in the solvent front and could cause matrix effects. The gradient was adjusted in real matrix, affluent wastewater, so as to shift the retention times of the selected steroid hormones away from the time windows of interfering compounds to avoid their co-elution (Fig. 5). Given the short analysis time and their structural similarities, the steroid hormones E1, E2, EE2 and NORE (Fig. 5) were poorly resolved with the applied gradient. This was also the case in other studies [16,18,19], where these compounds were investigated in short run times using reversed phase analytical columns. A limited number of papers [24,45] show proper separation for these steroid hormones and require long analysis times, between 20 and 55 min and the use of dansyl chloride derivatization in one case. Although it can reduce matrix effect and improves detectability, complete separation is not required for the selective MS/MS detection, especially since E1, E2, EE2 and NORE do not have the same precursor ions (Table 2). The chromatographic run was 2.28 min and column re-equilibration was performed during the sample transfer step from the sample loop to the SPE column. The positive identification of target steroid hormones in real samples was confirmed by

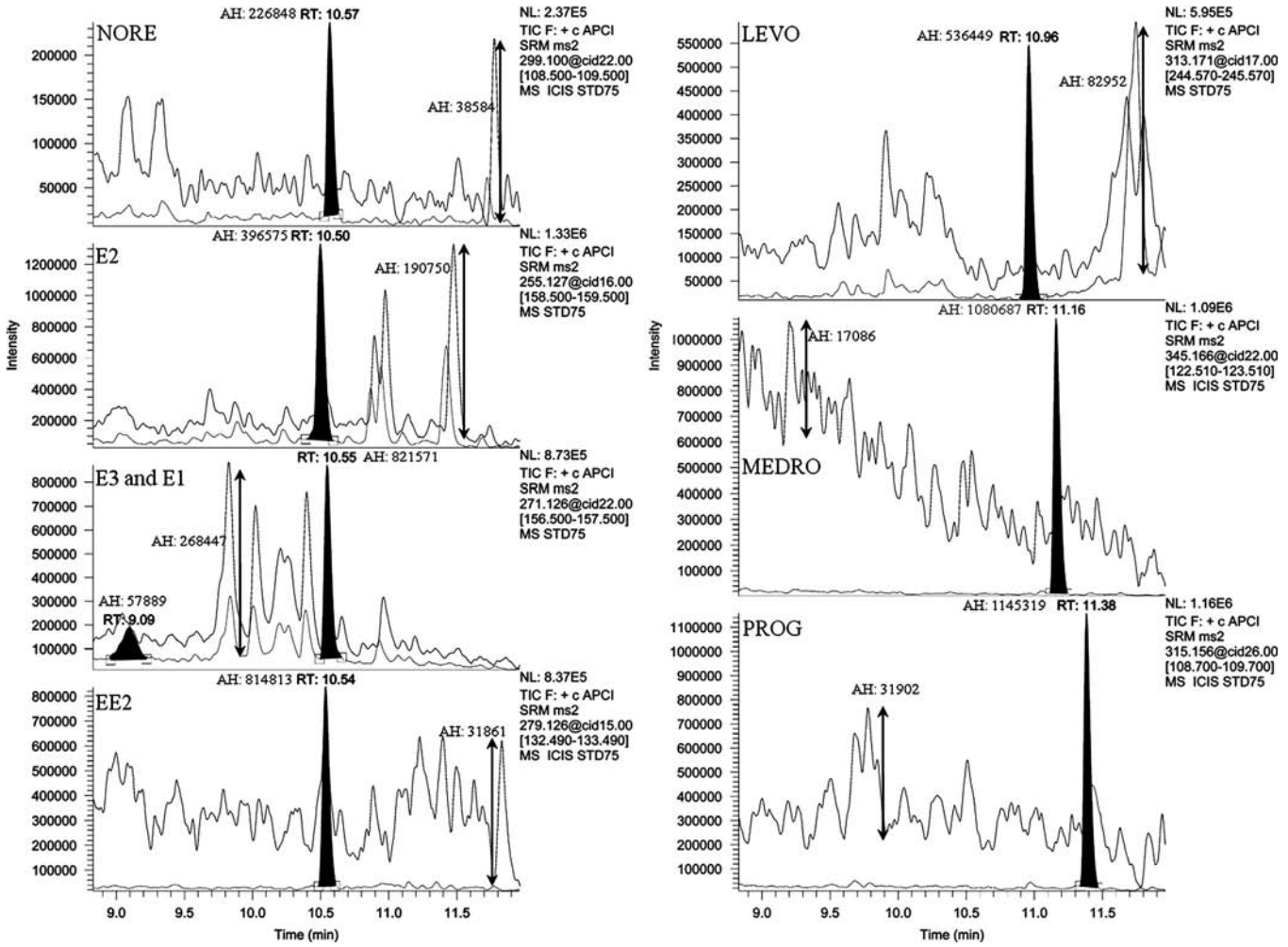

Fig. 5. Representative superimposed chromatograms of a blank and spiked (75 ng L^{-1}) wastewater affluent sample. The blank is represented by the non-integrated chromatograms, whereas the spiked sample is represented by the integrated peaks with corresponding retention times (RT) for each steroid hormone. The ordinates are given in intensities for the spiked sample only, therefore absolute peak height (AH) for both spiked samples and maximum peak blank values (black arrows) are also reported.

matching chromatographic retention times within ±2% of those observed in standards spiked in analyte-free matrix.

The internal standard was an isotopically-labeled E2 steroid hormone and was mainly used to compensate for the signal irreproducibility and variations within runs more than for matrix effect, since a standard addition calibration method was applied for quantification. As previously reported [52], the impact of cross-contribution from the isotopic pattern of steroid hormones with a two mass unit difference, i.e. E2 and its isotopically-labeled counterpart (IS, [$^{13}C_2$]-E2), was considered insignificant (less than 5%) and did not affect accuracy (% bias, Table 3). A minimum of two selected reaction monitoring transitions (SRM) as well as the relative intensities of their ratios (Table 2) were used for positive analyte identification in real samples.

3.4. On-line SPE–LC–APCI–MS/MS method validation

Method validation was done by evaluating linearity, precision (inter-day), accuracy, limits of detection (LOD) and limits of quantification (LOQ) for HPLC grade water as well as in analyte-free effluent and affluent wastewater (Tables 4 and 5).

The calibration curves of the spiked selected steroid hormones showed good linearity in all water matrices, with R^2≥0.9962 (Table 4). The precision and accuracy (% bias) of the method was determined at two concentration levels (QC 1 and QC 2) by analyzing replicates of spiked HPLC water and affluent wastewater samples (n=5, Table 5). The precision for all eight analytes in both waters for both QCs ranged between 6 and 23% while the accuracy, as % bias from the expected concentration, was between 0.8 and 21%, with the exception of NORE at 25% for the lowest QC level (QC 1, Table 5). The LODs and LOQs were matrix dependent with values ranging from 5 to 22 ng L^{-1} and 11 to 36 ng L^{-1} in HPLC grade water, from 8 to 34 ng L^{-1} and 21 to 60 ng L^{-1} in effluent wastewater and 20 to 60 ng L^{-1} and 24 to 98 ng L^{-1} in affluent wastewater, respectively (Table 5).

In all the reported methods using MS/MS, none rely on the use of both SRM transitions to determine the LODs and LOQs (Table 6). The use of the most abundant ion product will lead to lower values of LODs, but the obtained concentration could result in the absence of analytical signal from the background noise for the second, less abundant, monitored product ion. This is why the second most abundant SRM (Table 6) was chosen to measure the reported LODs in the herein method, since its presence is mandatory for proper compound confirmation in real samples. This approach, referred to as limits of determination or limits of confirmation, has been applied in previous studies [53,54] and will generally result in superior, although more reliable, values.

Table 6 summarizes the LODs and LOQs found in the other on-line SPE-LC-MS/MS methods developed for measuring steroid hormones in water matrices. In some cases, LODs and limits of

Table 4
Method validation results for linearity (R^2), limits of detection (LOD) and quantification (LOQ) for HPLC grade water, effluent (Effl) wastewater and affluent (Affl) wasterwater.

Steroids	R^2[a]			LOD[b] (ng L^{-1})			LOQ[c](ng L^{-1})			Highest calibration point[d] (ng L^{-1})		
	HPLC water	Effl	Affl	HPLC water	Effl	Affl	HPLC water	Effl	Affl	HPLC water	Effl	Affl
E3	0.9998	0.9967	0.9981	17	30	60	35	49	98	1525	697	3041
E2	0.9998	0.9993	0.9996	5	21	24	13	36	44	1014	689	3029
E1	0.9996	0.9976	0.9987	9	16	23	27	30	44	1030	686	3077
EE2	0.9998	0.9964	0.9989	13	18	21	18	33	24	1030	680	3077
NORE	0.9990	0.9962	0.9986	22	34	53	23	35	61	1542	704	3121
LEVO	0.9991	0.9977	0.9962	13	18	30	36	50	58	1531	689	3053
MEDRO	0.9989	0.9973	0.9987	20	32	42	21	38	53	1543	680	3122
PROG	0.9994	0.9990	0.9994	6	8	20	11	21	24	1579	697	3184

[a] R^2 determined by internal standard calibration for spiked solution of analyte free wastewater ($n=2$, with six to eight point calibration standards).
[b] The LOD, $(3.3 \times SD_{y\text{-intercept}})/m$), was determined using the calibration curve of the second most abundant product ion.
[c] The LOQ, $(10 \times SD_{y\text{-intercept}})/m$), was determined using the calibration curve of the most abundant product ion.
[d] This value was not determined until saturation of signal (ULOQ), but was evaluated according to the maximum expected concentration in the environment or to confirm linearity for the optimization parameters of the method.

Table 5
Retention times and method validation for precision (inter-day) and accuracy (bias) for two concentration levels (QC 1 and QC 2) for the selected steroid hormones[a].

Steroids	RT (min)	QC 1 (ng L^{-1}) amount		QC 2 (ng L^{-1}) amount		Bias (%)			
						QC #1		QC #2	
		HPLC water	Affl	HPLC water	Affl	HPLC water	Affl	HPLC water	Affl
E3	9.09 (0.03)	112 (5)	342 (20)	852 (3)	817 (12)	0.8	20	4	4.3
E2	10.50 (0.03)	109 (6)	317 (12)	862 (4)	855 (13)	1.6	21	6	6
E1	10.55 (0.02)	104 (5)	346 (6)	877 (5)	874 (10)	6	14	1.8	8
EE2	10.54 (0.02)	105 (6)	342 (12)	876 (7)	887 (8)	6	15	2.3	5
NORE	10.57 (0.02)	106 (10)	312 (7)	906 (4)	832 (10)	3	25	5	0.2
LEVO	10.96 (0.03)	99 (9)	338 (8)	858 (3)	864 (9)	9	16	0.2	3.2
MEDRO	11.16 (0.02)	112 (6)	316 (23)	826 (4)	852 (8)	17	20	5	1.5
PROG	11.38 (0.02)	108 (7)	330 (22)	905 (4)	697 (16)	2.7	13	1.5	7

[a] Values in parenthesis represent standard deviations from the mean ($n=5$).

quantifications (LOQs) were determined in Milli-Q water, when calculated concentrations were given in wastewater or surface water matrices while one study did not report any LOQ values for analyzed steroid hormones (Table 6). In others, the measured concentrations in wastewater samples for estrogens were lower than the determined linearity range [19,24]. Given the number of different methods used to determine the LODs and LOQs as well as the nature of the matrix in which they are determined (Table 6), a direct comparison among them is not necessarily justified [55,56]. The LODs values in this study are higher than the previous studies, with a relatively low sample volume (10-mL), but could be improved by using higher injection volumes for the pre-concentration (loading) step given the use of a higher bed mass for the SPE columns, permitting larger breakthrough volumes. Of the methods reported, four have used lower injection volume (1, 2.5 and 5-mL) and obtained better LODs (Table 6). In two of them, an ESI method in NI mode was used [16,19] which will produce better *S/N* values and lower matrix effect than in PI mode, but is not suitable for the analysis of progestagens. In another, an atmospheric pressure ionization (APPI) interface was used and showed better signal intensities and sensitivity for the steroid hormones when compared to ESI and APCI [18]. The last one, used a derivatization step (dansyl chloride) followed by an ESI method in PI mode [21], which is more time consuming and necessitates more complex instrumentation. The remaining methods (Table 6) used very large sample volumes (50 to 500-mL) to achieve low LODs, causing longer analysis times (25 to 60 min) and reducing sample throughput. The herein method represents a compromise between analysis time, higher sample throughput capabilities, sample volume and simplicity for the analysis of both progestagenic and estrogenic steroid hormones in a single run, with LODs and LOQs sufficiently low to detect and quantify them in environmental wastewater matrices, as shown in Section 3.5.

3.5. Method application to environmental samples

The optimized and validated on-line SPE–LC–APCI–MS/MS method was applied to analyze the selected steroid hormones in affluent and effluent wastewaters. Two wastewater treatment plants (WWTPs) were chosen, WWTP-A using a physico-chemical treatment approach and WWTP-B using a bio-filtration as well as an ultraviolet disinfection process for contaminant removal. In order to evaluate the removal efficiency of each WWTP in the most representative fashion, a time-proportional sampling mode was chosen. Wastewater affluent samples were collected hourly or every half hour between 7:30 am and 11:30 am for each treatment plant. Effluent samples were collected as a function of the retention times (hours) and flow rates ($m^3\ h^{-1}$) of the WWTPs studied so that the outflow samples corresponded to the inflow (affluent) samples. This sampling method allowed us to evaluate the removal efficiency performance of the WWTPs as a function of incoming wastewater peak flow hours (morning) and non-peak flow hours (early morning and noon) [57]. The results for the selected steroid hormones found in wastewater matrices are presented in Table 7, with the four natural steroid hormones (E1, E2, E3 and PROG) detectable and quantifiable. The synthetic steroid hormones (EE2, LEVO, NORE and MEDRO) were not detected in any of the analyzed samples. Removal efficiencies

Table 6
Analytical performance of the described herein method compared to previously reported on-line SPE–LC–MS/MS methods for the detection of steroid hormones.

Compounds studied[a]	Injection volume (mL)	Analysis time (min)	Water matrix[b]		LOD (ng L^{-1})	LOQ[c] (ng L^{-1})	Method	Ref.
			Sample	Validation				
E1, E2, E3, EE2, DES, BLD, NDL, ADD, TTR	50	30	Affluent Effluent River	Affluent Effluent River Milli-*Q*	0.1–5.0 SRM 1	1.0–10.0	LOD: *S/N*=3 LOQ: *S/N*=10	[7]
E1, E2, E3, EE2, LEVO, MEDRO, NORE, PROG	1	15	Affluent Effluent River	River	3–50 SRM 1	n.a.	LOD: 3.70 × S.D.[d]	[3]
E1, E2, EE2 (Dansylated)	1	17	Affluent Effluent	Pure water	0.4–0.7 SRM 1	1	LOD: *S/N*=3 LOQ: n.a.	[6]
E1, E2, E3, EE2, TTR, NORE, DES, LEVO	5	9	Wastewater	Milli-*Q*	0.3–2.1 SRM 1	1.0–7.0	LOD: *S/N*=3 LOQ: *S/N*=10	[5]
E1, (a-β)E2, E3, EE2	2.5	10	Wastewater Freshwater	Milli-*Q*	0.15–0.95 SRM 1	0.25–2.0	LOD: *S/N* > 3 LOQ: *S/N* > 5	[1]
E1, E2, E3, E3-16G, E2-3S, E1-3G, E2-3G, E3-3S, E3-3G, E1-3S (free estrogens dansylated)	500	25	River	River	0.04–6.8 SRM 1	0.2–2	LOD: 2.821 × S.D.[e] LOQ: n.a.	[4]
E1. E2, E3, EE2, E2-17G, E1-3S, E2-17A, DES	250	60	River Drinking	Ground	0.01–0.38 SRM 1	0.02–1.02	LOD: *S/N*=3 LOQ: *S/N*=8	[2]
E1, E2, E3, EE2, LEVO, MEDRO, NORE, PROG	10	15	Affluent Effluent	Affluent Effluent Milli-*Q*	5–60 SRM 1	11–98 SRM 2	$(3.3 \times S.D._{y\text{-int}})/m$ $(10 \times S.D._{y\text{-int}})/m$	Our study

[a] DES: diethylstilbestrol, NDL: nandrolone, ADD: androstenedione, TTR: testosterone, BLD: boldenone, E3-16G: estriol-16-glucuronide, E1-3S: estrone-3-sulfate, E2-3S: estradiol-3-sulfate, E1-3G: estrone-3-glucuronide, E2-3G: estradiol-3-glucuronide, E3-3S: estriol-3-sulfate, E3-3G: estriol-3-glucuronide, E2-17A: estradiol-17-acetate, E2-17G: estradiol-17-glucuronide.
[b] The water matrix sample corresponds to the analysis of real samples for the detection and quantification of the compounds, whereas the validation water matrix is the one used to calculate the respective LODs and LOQs.
[c] n.a., not available.
[d] S.D., standard deviation of seven replicates of spiked river samples at concentrations of 2 to 5 times the estimated LOD.
[e] S.D., standard deviation of calculated concentrations from 10 spiked river samples.

Table 7
Concentration of detected selected steroid hormones in WWTP-A and WWTP-B according to sampling times[a,b].

	Time 1				Time 2				Time 3			
WWTP-A	E3	E2	E1	PROG	E3	E2	E1	PROG	E3	E2	E1	PROG
Affl (ng L^{-1})	92 (7)	< LOQ	n.d.	< LOQ	117 (10)	31 (7)	< LOQ	< LOQ	74 (6)	32 (5)	< LOQ	< LOQ
Effl (ng L^{-1})	46 (8)	< LOQ	25 (6)	< LOQ	50 (10)	< LOQ	< LOQ	< LOQ	82 (8)	< LOQ	33 (3)	< LOQ
Removal (%)	50				57				n.a.			
WWTP-B												
Affl (ng L^{-1})	155 (30)	70 (1)	284 (14)	25 (2)	148 (5)	66 (5)	275 (6)	< LOQ	234 (7)	68 (4)	376 (36)	30 (8)
Effl (ng L^{-1})	120 (12)	33 (5)	28 (2)	n.d.	88 (13)	36 (4)	29 (2)	n.d.	144 (19)	37 (6)	30 (2)	n.d.
Removal (%)	23	53	90	n.a.	40	40	89		38	46	92	n.a.
	Time 4				Time 5				Time 6			
WWTP-A	E3	E2	E1	PROG	E3	E2	E1	PROG	E3	E2	E1	PROG
Affl (ng L^{-1})	143 (9)	47 (3)	< LOQ	< LOQ	111 (4)	55 (11)	47 (9)	29 (1)	Not sampled			
Effl (ng L^{-1})	117 (2)	51 (2)	< LOQ	< LOQ	104 (20)	39 (7)	35 (6)	30 (7)				
Removal (%)	18	n.a.			6	30	26	n.a.				
WWTP-B												
Affl (ng L^{-1})	206 (6)	74 (2)	336 (11)	< LOQ	176 (10)	70 (1)	313 (25)	< LOQ	156 (37)	68 (5)	313 (11)	44 (9)
Effl (ng L^{-1})	175 (10)	34 (2)	25 (3)	n.d.	138 (11)	38 (4)	31 (3)	n.d.	82 (8)	40 (8)	42 (5)	n.d.
Removal (%)	15	54	93		22	51	90		47	41	87	n.a.

[a] WWTP-A time schedule ranged from 7:30 am (Time 1) to 11:30 am (Time 5) with sampling done hourly. The corresponding effluent samples were collected from 10:00 am (Time 1) to 1:55 pm (Time 5); WWTP-B time schedule ranged from 9:00 am (Time 1) to 11:30 am (Time 6) for the affluent with sampling done every half-hour. The corresponding effluent samples were collected from 11:15 am (Time 1) to 1:45 pm (Time 6).
[b] n.a.: Not available; n.d.: not detected (< LOD). Values in parentheses represents standard deviations (SD) from de mean.

from both WWTPs were calculated (Table 7) and the percent removals for WWTP-B were stable as a function of time, with an average of 31 ± 8 for E3, 48 ± 6 for E2 and 90 ± 2 for E1. This suggests that the WWTP-B consistently removed these estrogens with the same efficiency independently of peak flow times. This could not be determined for the WWTP-A, since insufficient data

was obtained for both affluent and effluent at the different times for this facility. Of the estrogens (E1, E2 and E3) quantified in WWTP-A, the removal efficiencies were low with values ranging from 6 to 57% (Table 7). This could result from there less hydrophobic character among the steroid hormones which does not favor their elimination by sorption mechanisms to suspended solids by the physico-chemical treatment. The bio-filtration process of WWTP-B effectively removed ($90 \pm 2\%$) of E1, while having less impact on E3 ($31 \pm 8\%$) and E2 ($48 \pm 6\%$). Overall, the bio-filtration process does remove the observed steroid hormones more successfully than the physico-chemical approach, but the resulting effluent concentrations (ranging from 25 to 175 ng L^{-1}, Table 7) remain high. Indeed, although the dilution factor will be significant, these levels are more than sufficient to induce severe estrogenic activity in the receiving waters [7–10]. The concentrations found in this work for the natural hormones are similar to those previously reported for wastewater samples [18,58,59]. The synthetic hormones have not been detected for the present samples, but the presence of steroid hormone in environmental waters is associated with consumption rates as well as transformation rates from their conjugated to the un-conjugated compounds.

4. Conclusion

An analytical method based on on-line SPE–LC–APCI-MS/MS has been developed for the detection and quantification of eight selected steroid hormones in wastewater matrices in a single analytical run. A detailed discussion on optimization parameters often overlooked and that have an impact on the overall performance of the method (sample collection, filtration and storage) has been presented as a function of water matrix. Indeed, only glass fiber was the most reliable filter material for the elimination of suspended material for the selected steroid hormones while minimizing losses of dissolved analytes due to sorption (92 to 103% recovery). Real samples, free of conservation agents, once filtered and kept at 4 °C could be analyzed within a 48 h period without affecting the integrity of the analytes. All optimization and validation experiments for the on-line SPE method and chromatographic separation were performed in environmentally relevant water matrices. The sensitivity and limits of detection in this study could be improved by using higher injection volumes than the proposed 1 to 10-mL, although this would lead to increased analysis time and should be adjusted depending on the expected steroid hormone concentrations in the matrix of interest. The proposed method is simple and provides a high throughput approach (15 min per injection) for the determination, at low-nanogram per liter levels, of steroid hormones in affluent and effluent wastewaters.

The applicability of the method was demonstrated on affluent and effluent samples from two wastewater treatment plants using different treatment processes. The results revealed that the treatment plants were not successful in removing the detected steroid hormones and ultimately released them into their receiving waters with concentrations sufficiently high to induce estrogenic activity in aquatic species.

Novelty statement

An on-line SPE–LC–MS/MS method for the analysis of both estrogens and progestagens in wastewater in a single run is proposed. We evaluated sample storage over time at different temperature and filtration protocol including various different filtration materials. The method was validated in real environmental matrices including in wastewater effluents.

Acknowledgments

This work was made possible through the financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC), the NSERC Industrial Chair on Drinking Water at the École Polytechnique de Montréal and the Canadian Foundation for Innovation (equipment).

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.2013.05.038.

References

[1] M.J. López de Alda, S. Diaz-Cruz, M. Petrovic, D. Barceló, J. Chromatogr. A 1000 (2003) 503.
[2] M. Auriol, Y. Filali-Meknassi, R.D. Tyagi, C.D. Adams, R.Y. Surampalli, Process Biochem. 41 (2006) 525.
[3] M.J. López de Alda, D. Barceló, Fresenius J. Anal. Chem. 371 (2001) 437.
[4] R.D. Briciu, A. Kot-Wasik, J. Namiesnik, J. Chromatogr. Sci. 47 (2009) 127.
[5] H. Tomšíková, J. Aufartová, P. Solich, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, L. Nováková, TRAC—Trend Anal. Chem. 34 (2012) 35.
[6] H. Chang, Y. Wan, S. Wu, Z. Fan, J. Hu, Water Res. 45 (2011) 732.
[7] E.J. Routledge, D. Sheahan, C. Desbrow, G.C. Brighty, M. Waldock, J.P. Sumpter, Environ. Sci. Technol. 32 (1998) 1559.
[8] P.D. Hansen, H. Dizer, B. Hock, A. Marx, J. Sherry, M. McMaster, C. Blaise, TRAC—Trend Anal. Chem. 17 (1998) 448.
[9] C.E. Purdom, P.A. Hardiman, V.V.J. Bye, N.C. Eno, C.R. Tyler, J.P. Sumpter, Chem. Ecol. 8 (1994) 275.
[10] V.J. Kramer, S. Miles-Richardson, S.L. Pierens, J.P. Giesy, Aquat. Toxicol. 40 (1998) 335.
[11] J.P. Besse, J. Garric, Environ. Pollut. 157 (2009) 3485.
[12] D. Koubovec, K. Ronacher, E. Stubsrud, A. Louw, J.P. Hapgood, Mol. Cell Endocrinol. 242 (2005) 23.
[13] R. López-Roldán, M.J. López de Alda, M. Gros, M. Petrovic, J. Martín-Alonso, D. Barceló, Chemosphere 80 (2010) 1337.
[14] G. Streck, TRAC—Trend Anal. Chem. 28 (2009) 635.
[15] M. Petrovic, E. Eljarrat, M.J. López de Alda, D. Barceló, J. Chromatogr. A 974 (2002) 23.
[16] L. Ciofi, D. Fibbi, U. Chiuminatto, E. Coppini, L. Checchini, B.M. Del J. Chromatogr. A 1283 (2013) 53.
[17] E.R. Brouwer, S. Kofman, U.A.T. Brinkman, J. Chromatogr. A 703 (1995) 167.
[18] L. Viglino, K. Aboulfadl, M. Prévost, S. Sauvé, Talanta 76 (2008) 1088.
[19] T. Vega-Morales, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, J. Chromatogr. A 1230 (2012) 66.
[20] S. Rodriguez-Mozaz, M.J. López de Alda, D. Barceló, Anal. Chem. 76 (2004) 6998.
[21] A. Salvador, C. Moretton, A. Piram, R. Faure, J. Chromatogr. A 1145 (2007) 102.
[22] F. Guo, Q. Liu, G.B. Qu, S.J. Song, J.T. Sun, J.B. Shi, G.B. Jiang, J. Chromatogr. A 1281 (2013) 9.
[23] S. Wang, W. Huang, G. Fang, J. He, Y. Zhang, Anal. Chim. Acta 606 (2008) 194.
[24] F. Qin, Y.Y. Zhao, M.B. Sawyer, X.F. Li, Anal. Chim. Acta 627 (2008) 91.
[25] Council Regulation (EEC), OJEC L224 (1990) 1, Annexe 1.
[26] K.M. Lai, K.L. Johnson, M.D. Scrimshaw, J.N. Lester, Environ. Sci. Technol. 34 (2000) 3890.
[27] Anon, 1995. CPMP/ICH/381/95. European Medicines Agency (adopted by the CPMP in November 1994.
[28] C. Baronti, R. Curini, G. D'Ascenzo, A. Di Corcia, A. Gentili, R. Samperi, Environ. Sci. Technol. 34 (2000) 5059.
[29] K. Aboulfadl, C. De Potter, M. Prévost, S. Sauvé, Chem. Cent. J. 4 (2010) 10.
[30] B.J. Vanderford, R.A. Pearson, D.J. Rexing, S.A. Snyder, Anal. Chem. 75 (2003) 6265.
[31] S.M. Havens, C.J. Hedman, J.D. Hemming, M.G. Mieritz, M.M. Shafer J.J. Schauer, Environ. Toxicol. Chem. 29 (2010) 2481.
[32] A.I. Schafer, I. Akanyeti, A.J. Semiao, Adv. Colloid Interface Sci. 164 (2011) 100.
[33] J. Han, S. Meng, Y. Dong, J. Hu, W. Gao, Water Res. 47 (2013) 197.
[34] M.L. Hedgespeth, Y. Sapozhnikova, P. Pennington, A. Clum, A. Fairey, E. Wirth, Sci. Total Environ. 437 (2012) 1.
[35] M. Pedrouzo, F. Borrull, E. Pocurull, R. Marcé, Water Air Soil Pollute 217 (2011) 267.
[36] A. Azzouz, B. Souhail, E. Ballesteros, J. Chromatogr. A 1217 (2010) 2956.
[37] A. Krein, J.Y. Pailler, C. Guignard, A.C. Gutleb, L. Hoffmann, B. Meyer, S. Kefler, P. Berckmans, H.E. Witters, Environ. Pollut. 1 (2012) 86.
[38] P.A. Neale, B.I. Escher, A.I. Schafer, Sci. Total Environ. 407 (2009) 1164.
[39] Y. Morishima, Y. Irata, K. Inno, C. Ujimoto, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 3217.

[40] A. Lagana, A. Bacaloni, G. Fago, A. Marino, Rapid Commun. Mass Spectrom. 14 (2000) 401.
[41] S. Mompelat, A. Jaffrezic, E. Jardé ,B. Le Bot, *Talanta*, 109, 2013, 31.
[42] C.F. Poole, A.D. Gunatilleka, R. Sethuraman, J. Chromatogr. A 885 (2000) 17.
[43] M.L. Larrivee, C.F. Poole, Anal. Chem. 66 (1994) 139.
[44] M.C. Hennion, V. Pichon, Environ. Sci. Technol 28 (1994) 576A.
[45] M.J. López de Alda, D. Barceló, J. Chromatogr. A 911 (2001) 203.
[46] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom 11 (2000) 942.
[47] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[48] J.P. Antignac, K. De Wasch, F. Monteau, H. de Brabander, F. Andre, B. Le Bizec, Anal. Chim. Acta 529 (2005) 129.
[49] H. Trufelli, P. Palma, G. Famiglini, A. Cappiello, Mass Spectrom. Rev. 30 (2011) 491.
[50] J. Kang, L.A. Hick, W.E. Price, Rapid Commun. Mass Spectrom. 21 (2007) 4065.
[51] D.L. Buhrman, P.I. Price, P.J. Rudewicz, J. Am. Soc. Mass Spectrom 7 (1996) 1099.
[52] P.B. Fayad, M. Prévost, S. Sauvé, Anal. Chem. 82 (2009) 639.
[53] P.A. Segura, C. Gagnon, S. Sauvé, Chromatographia 70 (2009) 239.
[54] N. Negreira, M.J. López de Alda, D. Barceló, J. Chromatogr. A 1280 (2013) 64.
[55] G.L. Long, J.D. Winefordner, Anal. Chem 55 (1983) 712A.
[56] J.A. Glaser, D.L. Foerst, G.D. McKee, S.A. Quave, W.L. Budde, Environ. Sci. Technol. 15 (1981) 1426.
[57] K.P. Singh, N. Basant, A. Malik, G. Jain, Anal. Chim. Acta 658 (2010) 1.
[58] M.P. Fernandez, M.G. Ikonomou, I. Buchanan, Sci. Total Environ. 373 (2007) 250.
[59] M.R. Servos, D.T. Bennie, B.K. Burnison, A. Jurkovic, R. McInnis, T. Neheli, A. Schnell, P. Seto, S.A. Smyth, T.A. Ternes, Sci. Total Environ. 336 (2005) 155.