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Development and optimisation of an on-line solid phase extraction coupled to ultra-high-performance liquid chromatography–tandem mass spectrometry methodology for the simultaneous determination of endocrine disrupting compounds in wastewater samples

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ABSTRACT

A process using a fully automated on-line solid-phase extraction system combined with ultra-high-performance liquid chromatography and tandem mass spectrometry detection (SPE-UHPLC–MS/MS) has been developed and applied to the determination of 27 endocrine disrupting compounds (EDCs) in sewage samples. This technology allows separation and detection of all substances in a single chromatographic run that requires less than 4 min. In order to obtain a more sensitive method for the quantification of these compounds in sewage samples, an on-line solid-phase extraction step with Oasis HLB columns was performed prior to chromatographic determination. The complete analysis of each sample by this process requires only 9 min, provides satisfactory recoveries (72–110%) and limits of detection on the order of a few nanograms per liter (0.3–2.1 ng L^{-1}), demonstrating their potential for analyses of environmental samples. Thus, this methodology has been applied to samples collected from two wastewater treatment plants (WWTPs) located in Las Palmas de Gran Canaria (Canary Islands, Spain). One of these plants utilised conventional activated sludge treatment (CAS), while the other employed biomembrane reactor treatment (MBR).

1. Introduction

Low concentrations of endocrine disrupting chemicals (EDCs) are suspected of causing several reproductive changes reported in humans and wildlife populations [1–4]. Two different classes of endocrine disrupting substances have been identified. First are the natural steroid hormones, a group of biologically active compounds that are synthesised from cholesterol and contain a cyclopentan-o-perhydrophenanthrene ring [5], whereas the second group of EDCs consists of xenobiotic substances, including synthetic steroid hormones (norethindrone, norgestrel, 17α-ethinyloestradiol, etc.) as well as man-made chemicals and their sub-products (surfactants, pesticides, pharmaceuticals, flame retardants, etc.) [6–8].

The vast majority of existing publications regarding EDCs agree that, for both classes, the effluents of wastewater treatment plants (WWTPs) are the predominant source of EDC contamination of aquatic environments [9–11]. Furthermore, conventional activated sludge processes are reportedly unable to completely remove these EDCs, indicating that aquatic organisms may be directly exposed to a complex mixture of EDCs that continuously enters aquatic environments. Therefore, in order to assess the potential environmental impact of EDCs, it is necessary to develop sensitive and reliable methods to analyse different classes of EDCs in surface water, wastewater and sludge samples.

Toward this end, several methodologies have been developed to simultaneously identify and quantify several EDCs in different environmental matrices. Traditional analytical methods are predominantly based on gas chromatography coupled to mass spectrometry (GC–MS) due to the high degree of separation and success of identification of these combined methods [12]. However, with regard to EDC analyses, GC–MS methods exhibit some limitations, such as derivatisation requirements and conversion problems [13,14].

To overcome such limitations, high-performance liquid chromatography (LC) methods have been employed with various, predominantly spectrometric detection systems. Although optical detection systems have been successfully employed for LC analyses of these organic compounds and have exhibited reasonable detection limits, particularly for fluorescence detection (FD) systems [15], these methodologies demonstrate a lack of specificity when used to analyse complex matrices and, therefore, do not allow the unequivocal identification of EDCs [16].

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As a result, optical detectors have been "systematically" replaced during the last decade, first by single quadrupole mass spectrometer detectors (LC–MS) and more recently by triple quadrupole mass spectrometer detectors (LC–MS/MS) [14,16,17]. Moreover, novel hybrid MS systems like quadrupole-time-of flight (QqTOF) MS and quadrupole-linear ion-trap MS (QTRAP) have begun to be used for environmental analyses of EDCs [16,18]. Generally, all these analytical techniques provide further structural and quantitative information, facilitate the unambiguous identification of each analyte and increase sensitivity. Another trend that have gained great importance in the scientific community for the environmental assessment of this kind of pollutants can be found in the combination of analytical analysis and new bioanalytical tools such as bioassays, immunochemical techniques or biosensors [19].

The recent use of ultra-high-performance liquid chromatography (UHPLC) for environmental chemical analysis (first report in 2005 [20]) has enabled higher sample throughput and laboratory efficiency while maintaining or even improving the resolution obtained by conventional HPLC systems. Moreover, its considerable reduction in retention times makes UHPLC a perfect candidate for combination with on-line solid-phase extraction (SPE), which minimises manual operations and provides higher preconcentrations and recoveries than conventional SPE [21–24].

Therefore, the main objectives of this study are as follows: (1) to develop a simple and sensitive method for simultaneous extraction and determination of several EDCs that provides significant improvements in analysis/retention times, solvent(s) costs and other analytical parameters compared to other methodologies; and (2) to demonstrate the applicability of this methodology by using it to analyse real wastewater samples. Toward this end, we have employed a novel on-line solid-phase extraction (SPE) method coupled to UHPLC–MS/MS methodology, which has enabled complete separation of all analytes via a single chromatographic run that takes less than 4 min and a total analysis (including sample clean-up and extraction) that takes only 9 min. In addition to significantly reducing analysis time, this new methodology minimises manual operations as well as the automation of sample preparation steps, providing lower relative standard deviations (RSD), reducing background noise and, therefore, improving limits of detection (LODs).

2. Experimental

2.1. Chemicals and reagents

All of the IGEPAL mixtures and standards were acquired from Sigma Aldrich (Madrid, Spain). Individual compounds were used as standards (≥98% of purity) for short ethoxylated chains AP_nEOs ($n \leq 2$), testosterone, 19-norethindrone, diethylstilbestrol, norgestrel, 17β-estradiol, estriol, 17α-ethynylestradiol and bisphenol-A. The stock solutions (1000 μg mL^{-1}) of alkylphenols, steroidal hormones, and bisphenol-A were prepared by dissolving appropriate amounts of the commercial products in methanol and then storing the solutions in glass-stoppered bottles at −18 °C prior to use. Nonylphenol monoethoxylate, nonylphenol diethoxylate, octylphenol monoethoxylate and octylphenol diethoxylate were directly purchased in stock solutions (1 mL) at 10 μg mL^{-1} in acetone and were also stored at −18 °C. Work solution were prepared daily in water.

Long-chain AP_nEOs ($n \geq 3$) were only available in technical mixtures. IGEPAL CO210 and CO520 contained a range of NP_nEO oligomers between 3 and 8 ethoxy units (EO), whereas IGEPAL CA210 and CA520 contained the same EO range of OP_nEO oligomers. Stock solutions (1000 μg mL^{-1}) of long-chain alkylphenolic ethoxylated surfactants were also prepared by dissolving appropriate amounts of each mixture into methanol and were stored in glass-stoppered bottles at −18 ° C.

LC–MS-grade methanol and water, used to dissolve the standards and to prepare the mobile phases, and hexane and acetone, employed to clean up the SPE columns, were purchased from Panreac Química (Barcelona, Spain). HPLC-grade glacial acetic acid and ammonium acetate were used to prepare the mobile phase and were obtained from Scharlau Chemie S.A. (Barcelona, Spain). Ultra-high-quality water, obtained by a Milli-Q (Millipore, Bedford, MA, USA) water purification system, was used in the solid phase extraction (SPE) protocol and to dilute samples.

Glass fibre prefilters and Durapore membrane filters (0.65 μm DVPP) were purchased from Millipore (Bedford, MA, USA). The SPE On-Line columns employed in this study were Oasis HLB (20 μm, 2.1 mm × 30 mm) from Waters (Milford, MA, USA).

2.2. Sample collection

Samples were collected from two WWTPs located in north eastern Gran Canaria Island (Spain). This is the most populated region of the island and contains a population exceeding a half million people and a population density of approximately 800 persons km^{-2} (data taken from [25,26], January 2012). Moreover, although much of the limited industrial activity on the island is concentrated in this area, domestic sewage systems are the main source(s) of raw wastewater flowing into the two WWTPs.

In both treatment plants, samples were collected bimonthly from May 2011 to September 2011 on the same sampling days. The first WWTP utilised a conventional activated sludge treatment system (CAS) coupled to an electrolytic cell for tertiary treatment, whereas the second WWTP employed an advanced membrane bioreactor treatment system (MBR).

Once collected, samples were filtered through 0.65 μm membrane filters, acidified at a pH lower than 3 and stored in 20 mL amber vials at 4 °C prior to analysis, which was conducted within 48 h after sample collection.

2.3. Extraction

The on-line SPE-UHPLC–MS/MS system was obtained from Waters (Milford, MA, USA) and basically consisted of a quaternary solvent manager pump (QSM), a binary solvent manager pump (BSM), a column manager, a triple quadrupole detector (TQD) and an autosampler capable of injecting sample volumes up to 5 mL per injection. Two solid-phase extraction (SPE) columns were coupled before the column manager compartment in order to preconcentrate and "clean-up" the samples to be injected onto the UHPLC column.

Solid-phase extractions employed two Oasis HLB on-line extraction columns (20 μm; 2.1 mm × 30 mm; Waters, Milford, MA, USA) working in parallel. When a sample was injected onto the system, extraction column A concentrated all analytes, and then the BSM pump transported the eluted compounds to the UHPLC column. While the compounds were being separated chromatographically, extraction column B received the second sample (via the QSM pump) for sample preparation, while extraction column A was cleaned and re-equilibrated for the third sample. In addition, in order to avoid carryover effects, the 5 mL syringe was cleaned twice between each injection, first a weak cleaning using methanol and then a strong cleaning using a mixture of methanol:acetone:hexane 1:1:1 (v:v:v). This parallel operation significantly reduced analysis time and increased laboratory efficiency.

The BSM pump had two purposes: (1) to elute all of the compounds from the extraction columns and (2) to provide effective chromatographic separation of all analytes on the UHPLC column. For this, we used a flow rate of 0.3 mL min^{-1}, a constant

column temperature of 40 °C, and two mobile phases consisting of methanol (A1) and water (B1), both containing 0.1% ammonia and 15 mmol of ammonium acetate.

The QSM pump had three purposes: (1) to transport the samples from the injection port to the extraction columns (sample loading), (2) to carry out the sample preparation and (3) to re-equilibrate the extraction columns for the next sample injections. To achieve these goals, we used a flow rate of 2 mL min^{-1} throughout the entire extraction protocol. Mobile phases consisted of water (0.5% acetic acid) (A2), methanol (B2) and water (C), both containing 0.1% ammonia and 15 mmol of ammonium acetate and a mixture of methanol, acetone and hexane (1:1:1) (D).

The (A2) mobile phase was selected to take the sample from the injector to the extraction columns, a 90:10 (v:v) mixture of mobile phases (B2) and (C) was used for the weak sample clean-up step, and mobile phase (D) was used for the strong sample clean-up step. Details pertaining to mobile phase selections and sample preparation protocols are covered in Section 3.1.

2.4. UHPLC–MS/MS analysis

2.4.1. Chromatographic conditions

As explained in the previous section, the BSM pump was responsible for maintaining the proper chromatographic conditions ("fluidic conditions") necessary to separate all analytes in the same chromatographic run. In this regard, the gradient profile shown in Table 1 was utilised with an ACQUITY BEH C_{18} column (1.7 μm, 2.1 mm × 50 mm) from Waters (Milford, MA, USA). The injection volume was 5 mL, and the flow rate was 0.3 mL min^{-1}. The column temperature was maintained at 40 °C. Mobile phases consisted of methanol (A) and water (B), both containing 0.1% (v:v) of ammonia and 15 mmol of ammonium acetate.

Table 1 shows that the chromatographic gradient actually begins 4.1 min after sample injection, when the BSM module begins to pump mobile phase through the SPE column (elution step) and then through the UHPLC column (chromatographic separation). However, as Table 1 demonstrates, the BSM pump begins working at a lower flow rate (0.1 mL min^{-1}) at the beginning of the injection (0 min) because of the need to keep the UHPLC column conditions similar to those at the beginning of the chromatographic gradient that occurs at 4.1 min.

2.4.2. MS/MS conditions

Multiple reaction monitoring (MRM) parameters were optimised using a combined infusion of mobile phases A1 and B1 (isocratic elution 50:50 (v:v) at 0.3 mL min^{-1}) and a solution of each standard or IGEPAL mixture prepared at 1 mg L^{-1} in methanol. Mobile phases were pumped by the BSM through the entire system, whereas the standards or mixtures were infused from 2 mL vials at a flow rate of 15 μL min^{-1} by a pump module located in the TQD detector. Both solutions were mixed within a valve placed just before the electrospray interface (ESI) and injected into the triple quadrupole detector.

Thus, precursor ions included positive ions in positive ion mode ($[M+NH_4]^+$ adducts for AP_nEOs [27,13] and $[M+H]^+$ for 19-norethindrone, testosterone and norgestrel) and negative ions in negative ion mode ($[M-H]^-$ for raw alkylphenol (NP and OP), diethylstilbestrol, 17β-oestradiol, oestriol, 17α-ethynyloestradiol and bisphenol-A [28]).

Finally, ionisation within the ESI source was achieved using nitrogen as the nebuliser, cone and drying gas. The desolvation and source temperatures were maintained at 150 °C and 500 °C, respectively. The desolvation and cone gas fluxes were established at 1000 L h^{-1} and 50 L h^{-1}, respectively. The capillary voltage was fixed at 3 kV in positive mode (ESI+) and −2 kV in negative mode (ESI−). The corona voltage was set at 0.4 kV in both ESI+ and ESI− modes, whereas the cone voltage was optimised for each individual compound (Table 2). The extractor and RF lens voltages were maintained at 3 V and 0.5 V, respectively, in both ionisation modes. Collision induced dissociation (CID) was achieved using argon as the collision gas and a fixed flux of 0.15 mL min^{-1}. The fragment ions obtained for each compound and the collision potentials are displayed in Table 2.

Ions used for quantification and confirmation were monitored together with the elution time for each analyte to guarantee the presence of EDCs in the real samples.

2.5. Data acquisition and processing

Data acquisition and processing were conducted using MassLynx and TargetLynx V4.1 software from Waters. Statistical studies were performed using the SPSS 11.0 program.

3. Results and discussion

3.1. Optimisation of on-line SPE processes

3.1.1. Adsorption procedure

The capacity of the Oasis HLB sorbent to retain the analytes of interest was evaluated. Several different sample volumes and mobile phases were optimised to obtain the best results and to minimise/eliminate the background noise generated by the sample itself. Based on the results, for the experiments described below, a secondary treatment effluent sample collected from the CAS plant was spiked with all of the analytes, each at a final concentration of 500 ng L^{-1}.

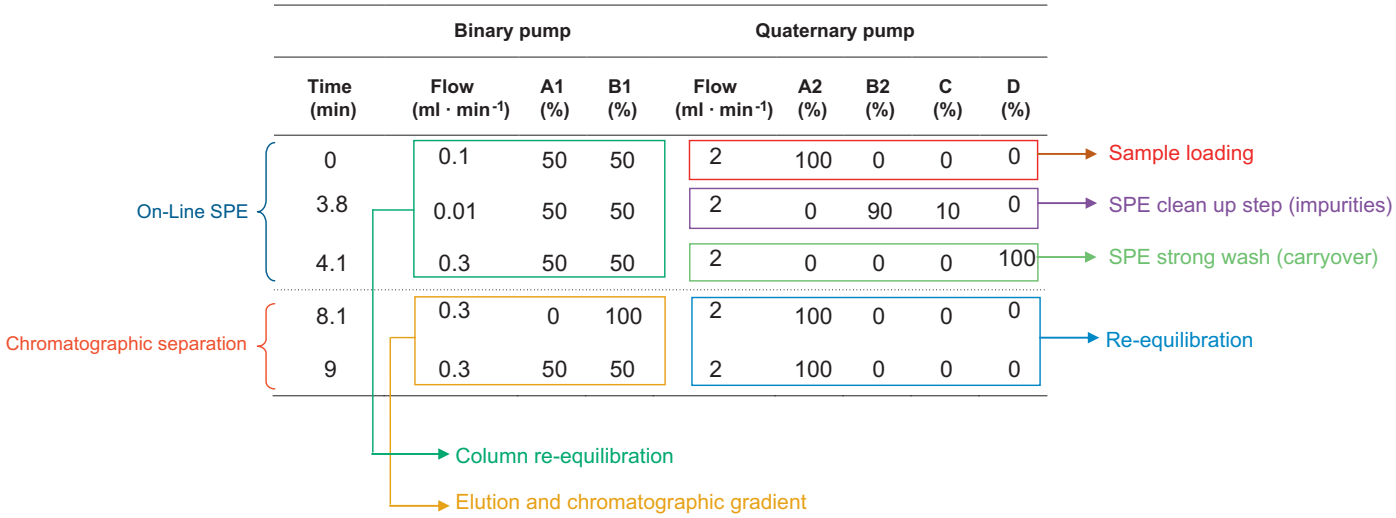
3.1.2. Sample volume

The experimental setup enables the operator to choose sample volumes ranging from 0.1 mL to 5 mL per injection cycle, with the option of injecting a number of cycles in order to increase the amount of sample passed though the SPE columns. However, this option was refused for several reasons. When the maximum sample volume (5 mL) is used, each injection cycle takes 4 min, so that using several injection cycles significantly increases the total analysis time, in contrast to one of the main objectives of this work. It is true that, when we increase the sample volume that is passed through the Oasis HLB columns, the signal detected also increases; however, due to the nature of the wastewater sample matrices, the signal-to-noise ratios do not improve significantly. Thus, the increase in time required to use multiple injection cycles in the sample loading step is not compensated by any improvements in limits of detection. Therefore, we studied the effects of different sample volumes (1, 2, 3, 4 and 5 mL) delivered by only one injection cycle and obtained the highest signal-to-noise ratios using a sample volume of 4 mL for the vast majority of target compounds.

3.1.3. Sample loading and pH

Another goal of our sample loading investigations was to minimise or even eliminate all matrix interferences. Given the neutral behaviour of the majority of analytes studied in this work [16], we investigated the affect of pH on the ability of the solvent to solubilise interfering contaminants in the sample, enabling them to pass through rather than be retained by the SPE sorbent. Thus, we tested two acidic solutions (with pH values of 3 and 5) and two basic solutions (with pH values of 8 and 10), achieving the best signal-to-noise ratios when the mobile phase pH was close to 3. For this study, an aqueous solution containing 0.5% (v:v) acetic acid (A2) was used to load the sample while eliminating many of the interfering compounds solubilised in acidic solutions.

Table 1
Gradient elution profiles employed in both binary and quaternary solvent manager pumps.



3.1.4. Wash step composition

Despite the initial clean-up that occurs during sample loading, a lot of interfering impurities remain, which can lead to signal suppression or enhancement and/or can increase the background noise, thereby impairing detection capabilities. In this regard, on-line SPE methodology provides significant advantages compared to conventional extraction procedures, including the ability to mechanise the clean-up procedure, to automatically change between different solvents, to mix different proportions of each solvent and to program various sample lists in order to optimise variables without requiring manipulation on the part of the operator (or even the presence of the operator). All these advantages lead to greater speed when optimising the system/procedure and minimise potential errors related to sample handling.

We performed several experiments for three different purposes: (1) to palliate the common detrimental matrix effects reported for LC–MS/MS using ESI mode [29,30]; (2) to improve the limits of detection (LODs) and limits of quantification (LOQs) of our method and (3) to avoid carryover effects from previous injection(s). We approached the first two goals using what we call 'weak clean-up', while the third goal was approached utilising a 'strong clean-up'. The details of weak and strong clean-up procedures are described below.

Table 2
Details of the target compounds and their multiple reaction monitoring (MRM) parameters in UHPLC–MS/MS under both positive and negative ionization modes.

Compound	Precursor ion	Product ions (collision energy)		Cone voltage (V)	Ion mode
		Quantification ion	Qualification ion		
Nonylphenol (NP)	218.7[a]	105.7 (20.5)	–	−64	ESI−
NP_1EO	282.3[b]	265.3 (6)	127.1 (8)	30	ESI+
NP_2EO	326.3[b]	183.1 (9)	121.1 (20)	30	ESI+
NP_3EO	370.3[b]	353.3 (8)	227.1 (11)	32	ESI+
NP_4EO	414.5[b]	397.4 (8)	271.2 (13.5)	32	ESI+
NP_5EO	458.6[b]	441.5 (12)	315.2 (15.5)	48	ESI+
NP_6EO	502.6[b]	485.5 (13.5)	359.3 (17)	52	ESI+
NP_7EO	546.7[b]	529.6 (14.5)	403.3 (18)	56	ESI+
NP_8EO	590.8[b]	573.6 (15.5)	447.5 (20)	64	ESI+
Octylphenol (OP)	204.7[a]	134.0 (16.5)	106.1 (19.5)	−72	ESI−
OP_1EO	268.1[b]	251.1 (6)	113.1 (7.5)	30	ESI+
OP_2EO	312.3[b]	183.1 (9.5)	121.1 (19.5)	30	ESI+
OP_3EO	356.4[b]	339.4 (8)	227.1 (14)	32	ESI+
OP_4EO	400.4[b]	383.4 (10)	271.2 (14)	32	ESI+
OP_5EO	444.5[b]	427.5 (12)	315.2 (15.5)	48	ESI+
OP_6EO	488.5[b]	471.5 (13.5)	359.3 (17)	52	ESI+
OP_7EO	532.8[b]	516.6 (15)	403.3 (18)	52	ESI+
OP_8EO	576.7[b]	559.7 (15.5)	277.2 (24)	60	ESI+
Bisphenol A (BPA)	226.7[a]	211.7 (17.5)	–	−60	ESI−
Estriol (E3)	287.2[a]	171.0 (16.5)	145.2 (19.5)	−67	ESI−
Estrone (E1)	269.2[a]	145.4 (16.5)	143.2 (19.5)	−55	ESI−
17β-estradiol (E2)	271.2[a]	183.5 (14.5)	142.2 (20.5)	−60	ESI−
17α-ethynilestradiol (EE)	295.2[a]	171.0 (16.5)	145.2 (19.5)	−71	ESI−
Testosterone (TE)	289.2[c]	187.0 (18)	205.1 (15)	33	ESI+
19-norethindrone	299.2[c]	109.0 (26)	245.1 (18)	38	ESI+
Diethylstillbestrol (DES)	267.1[a]	237.1 (19)	251.1 (17)	−60	ESI−
Norgestrel	313.2[c]	109.0 (18)	245.1 (18)	38	ESI+

[a] m/z precursor ion $[M-H]^-$.
[b] m/z precursor ion $[M+NH_4]^+$ adducts.
[c] m/z precursor ion $[M-H]^+$.

3.1.4.1. Weak clean-up. For the weak clean-up experiment, several methanol:Milli-Q water mixtures (0.5:10, 1:10, 2:10, 3:10 and 4:10 (v:v)) were tested. As shown in Fig. 1, the signal obtained for each compound remained relatively stable for methanol/Milli-Q water mixtures from 0.5:10 to 2:10 (v:v). The only significant effect observed in this range was progressive peak broadening as the percentage of methanol in the mixture was increased.

This peak broadening effect was enhanced for methanol:Milli-Q water mixtures from 3:10 to 4:10 (v:v). Significant decreases in peak signals of the most polar compounds, such as oestriol (E3) or bisphenol-A (BPA), were observed at a methanol:Milli-Q ratio of 3:10 (v:v). At a 4:10 (v:v) ratio, all compounds displayed significant broadening of their chromatographic peaks as well as decreases in signal intensities. This effect included less polar compounds and, hence, those with higher affinities for the SPE sorbent. Moreover, when these experiments employed spike levels of 500 ng L^{-1}, E3, BPA, steroidal hormones and short chained AP_nEOs ($n \leq 5$) began appearing in amounts very close to the limits of detection.

It is noteworthy that both mobile phases (methanol (B) and water (C)) contained 0.1% ammonia. Using ammonia as an additive in this step can be justified as follows:

(1) In aqueous solution, ammonia can behave as a base, forming the ammonium ion, $NH4^+$, thereby promoting the formation of ammonium adducts required for MS/MS determination of alkylphenolic polyethoxylated compounds.
(2) The addition of ammonia brings the sample to a basic pH, which favours the elimination of compounds that are soluble in basic solution. This should improve signal-to-noise ratios by reducing interferences produced by impurities. Given that the loading phase was performed in acidic solution, the basic pH caused by the addition of ammonia is of even greater significance, because it results in a much more complete cleaning of the sample.
(3) Both mobile phases are identical to those employed in the desorption step and in the chromatographic elution.

Finally, a 90:10 (v:v) mixture of mobile phases (B2) and (C) was used for the weak sample clean-up step. This mixture allows to solubilise polar impurities while minimising the peak broadening effect and the loss of analytes, so it reaches a trade off that we consider optimum.

3.1.4.2. Strong clean-up. The main objective of this step was to eliminate all carryover effects. This cleaning step is of crucial importance because we planned to reuse the extraction columns (for every even or odd injection, working in parallel). Thus, as the number of sample injections increases, a sorbent that is not completely cleaned results in cumulative interference effects on signals from the compounds of interest. To address this complication, several mixtures of methanol, acetone and hexane were tested and optimised. The results showed that a 1:1:1 (v:v:v) solution of methanol:acetone:hexane adequately prevented carryover effects.

In addition, to test the efficacy of this mixture as a strong clean-up solvent, several injections of samples with a high-concentration spike (1 μg L^{-1}), interspersed with blank Milli-Q water samples, were performed and demonstrated satisfactory results.

3.1.5. Desorption step

Elution of the analytes from the Oasis HLB SPE column was performed with the same mobile phases (A1 and B1) used for chromatographic separation (UHPLC column) and with the same chromatographic gradient. Therefore, we investigated the desorption step using gradient elution, something that cannot be done with conventional off-line SPE extraction. The main assumption supporting gradient elution is that, if the compounds are eluted from the UHPLC column by using this mobile phase, they will also elute from the SPE column, because the retention capabilities in the latter are considerably lower.

3.2. Quantification and quality control

The method developed herein was evaluated for linearity, recovery, precision, limits of detection, limits of quantification and matrix interference. Calibration curves were constructed for AP_nEOs from 5 to 1000 ng L^{-1}, for the compounds ionised in negative ESI mode from 20 to 1000 ng L^{-1} and for the rest of compounds ionised in positive ESI mode from 15 to 1000 ng L^{-1}. These curves were evaluated by analysing standard solutions prepared in Milli-Q water in triplicate at six different concentration levels. Excellent linearity was achieved in these concentration ranges, for which correlation coefficients higher than 0.991 were calculated for all validation batches.

The limits of detection (LODs) and limits of quantification (LOQs) were determined after concentration of spiked Milli-Q water samples at signal/noise ratios of three and ten, respectively. The LODs ranged between 0.3 and 2.1 ng L^{-1}, whereas the LOQs ranged from 1.0 to 7.0 ng L^{-1}. The LODs and LOQs for each compound are shown in Table 3.

Replicate analyses were performed to determine the recovery ($n = 3$) and repeatability ($n = 6$) of the proposed method. For recovery calculations, replicate samples were spiked with all of the analytes under study at two concentration levels, a high spike level (500 ng L^{-1}) and a low spike level (10 ng L^{-1}). Repeatability was expressed as the relative standard deviation (% RSD) at a concentration level of 50 ng L^{-1}, for which values lower than 10% were obtained for all EDCs. Recoveries achieved for each analyte in Milli-Q water are displayed in Table 4. Fig. 2 shows MRM chromatograms of samples spiked with all analytes (500 ng L^{-1}) after progressing through the on-line SPE process, with (a) pertaining to BPA, NP, OP and steroids, (b) pertaining to nonylphenol polyethoxylated compounds, and (c) pertaining to octylphenol polyethoxylated compounds.

3.3. Matrix effects

Matrix effects are common problems for LC–MS/MS analyses in the ESI mode [29,30], often leading to analyte signal suppression or enhancement and/or complicating clean-up procedures in complex samples, as happens in our experiments. While attempting to reduce these matrix effects as much as possible, it is very important to also consider how any such mitigation affects the specificity of the method.

Relative signal suppressions/enhancements were observed and evaluated for primary, secondary and final effluent samples of both WWTPs using the algorithm of Vieno et al. [31] (Eq. (1)). The results are expressed as percentages and presented in Table 5.

$$\frac{As - (Asp - Ausp)}{As} \times 100 \quad (1)$$

where, *As* corresponds to the peak area of the analyte in pure standard solution, *Asp* corresponds to the analyte's peak area in the spiked matrix extract, and *Ausp* corresponds to the analyte's peak area in the unspiked matrix extract.

Greater matrix effects were observed in analyses of primary treatment samples, resulting in signal suppressions from 9 to 24% for the CAS plant. More severe signal suppression was observed for hydrophobic compounds, especially NP, OP, $AP_{1,2}EO$, and EE. Ion effect suppressions were slightly lower for secondary treatment samples (between 12 and 18%) and significantly lower for final effluent samples (between 9 and 13%). Signal enhancements were only observed for testosterone and norgestrel. For the MBR plant, the matrix effects evaluated were also higher for primary

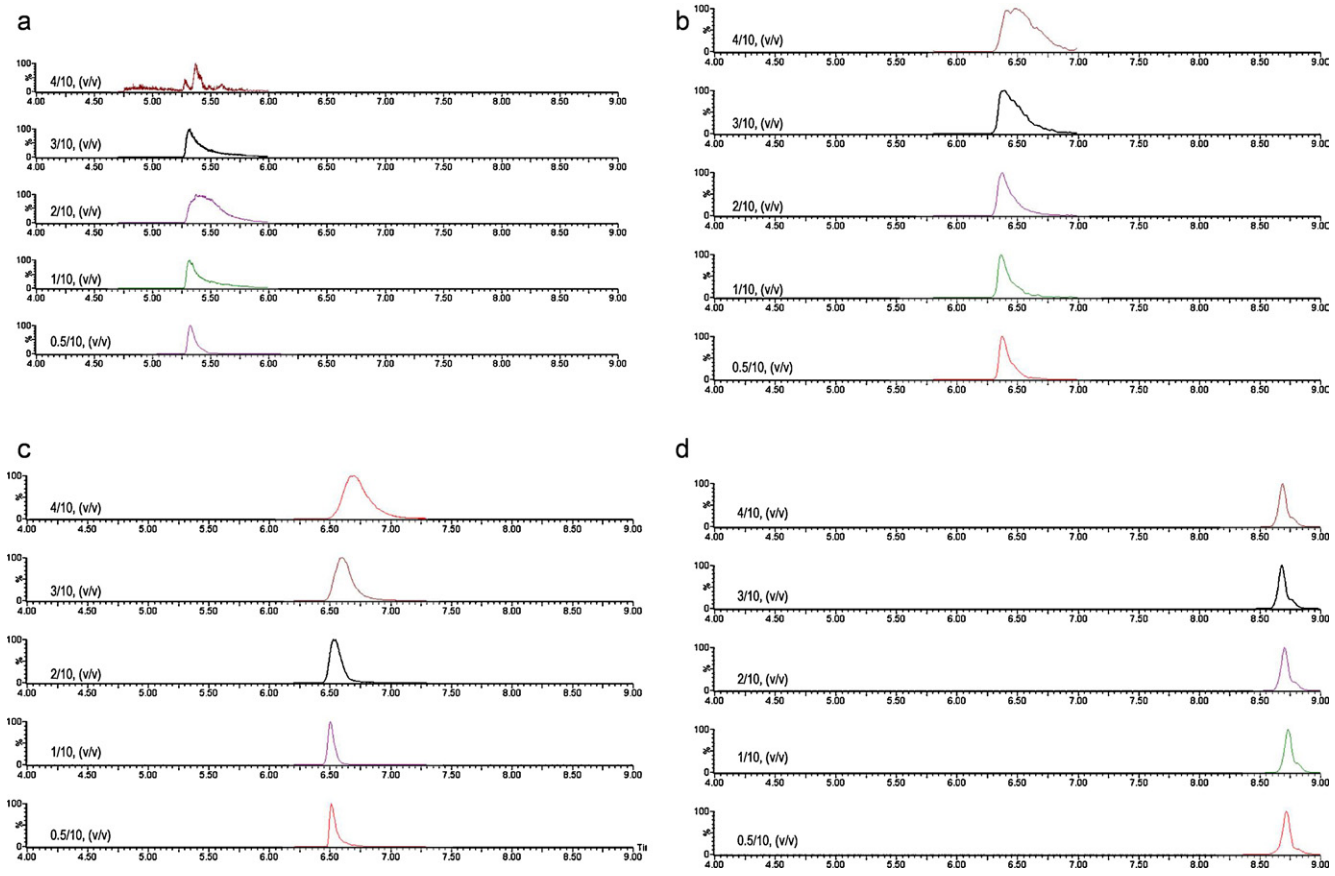


Fig. 1. Effect of the wash step composition on the analyte retention into the Oasis HLB cartridges: a) Estriol; b) Testosterone; c) 17α-ethynilestradiol; d) Nonylphenol.

Table 3
Analytical parameters of the online-SPE-UHPLC–ESI-MS/MS methodology.

Compound	Rt (min)[a]	Linear range (ng L^{-1})	RSD[b] (50 ng L^{-1})	LOD[c] (ng L^{-1})	LOQ[d] (ng L^{-1})
Nonylphenol (NP)	8.40	20–1000	5.2	1.3	4.3
NP_1EO	8.17	5–1000	3.9	1.2	4.0
NP_2EO	8.19	5–1000	3.3	0.5	1.7
NP_3EO	8.22	5–1000	5.0	0.6	2.0
NP_4EO	8.24	5–1000	5.2	0.7	2.3
NP_5EO	8.26	5–1000	2.0	1.8	6.0
NP_6EO	8.26	5–1000	4.8	0.3	1.0
NP_7EO	8.28	5–1000	8.1	1.1	3.7
NP_8EO	8.28	5–1000	6.6	1.1	3.7
Octylphenol (OP)	7.98	20–1000	9.8	1.8	6.0
OP_1EO	7.83	5–1000	3.4	2.1	7.0
OP_2EO	7.85	5–1000	4.0	1.2	4.0
OP_3EO	7.91	5–1000	7.3	0.9	3.0
OP_4EO	7.91	5–1000	2.8	1.3	4.3
OP_5EO	7.96	5–1000	1.5	1.6	5.3
OP_6EO	7.94	5–1000	4.9	1.3	4.3
OP_7EO	7.99	5–1000	4.9	0.8	2.7
OP_8EO	7.99	5–1000	3.2	0.7	2.3
Bisphenol A (BPA)	6.04	20–1000	8.5	1.9	6.3
Estriol (E3)	5.32	20–1000	10.1	1.3	4.3
Estrone (E1)	6.39	20–1000	4.9	1.3	4.3
17β-estradiol (E2)	6.36	20–1000	5.7	1.2	4.0
17α-ethynilestradiol (EE)	6.39	20–1000	9.1	0.9	3.0
Testosterone (TE)	6.51	15–1000	2.1	0.5	1.7
19-norethindrone	6.33	15–1000	1.6	1.1	3.7
Diethylstillbestrol (DES)	6.61	20–1000	6.2	0.6	2.0
Norgestrel	6.67	15–1000	3.0	0.7	2.3

[a] Retention time.
[b] Relative standard deviation ($n = 6$).
[c] Limit of detection.
[d] Limit of quantification.

Table 4
Recovery percentages and RSD (%) ($n = 3$) for different matrices spiked at two different concentration levels.

Compounds	CAS						MBR						Milli-Q	
	Primary treatment		Secondary treatment		Final effluent		Primary treatment		Secondary treatment		Final effluent		Water sample	
	10 ng L^{-1}	500 ng L^{-1}	10 ng L^{-1}	500 ng L^{-1}	10 ng L^{-1}	500 ng L^{-1}	10 ng L^{-1}	500 ng L^{-1}	10 ng L^{-1}	500 ng L^{-1}	10 ng L^{-1}	500 ng L^{-1}	10 ng L^{-1}	500 ng L^{-1}
Nonylphenol (NP)	87 ± 14	79 ± 9	84 ± 9	81 ± 11	93 ± 7	95 ± 7	77 ± 12	89 ± 6	99 ± 4	87 ± 8	92 ± 10	79 ± 10	86 ± 11	109 ± 8
NP_1EO	77 ± 7	78 ± 5	81 ± 7	90 ± 9	90 ± 11	84 ± 4	77 ± 9	81 ± 9	77 ± 7	79 ± 3	86 ± 4	85 ± 7	88 ± 8	83 ± 5
NP_2EO	82 ± 8	85 ± 9	87 ± 6	85 ± 10	83 ± 8	85 ± 2	74 ± 11	79 ± 12	80 ± 6	85 ± 8	82 ± 6	93 ± 4	87 ± 7	89 ± 7
NP_3EO	75 ± 8	75 ± 12	82 ± 9	90 ± 9	90 ± 6	90 ± 5	80 ± 9	86 ± 9	92 ± 8	94 ± 9	92 ± 2	96 ± 10	10 ± 3	103 ± 4
NP_4EO	81 ± 11	86 ± 7	99 ± 10	89 ± 7	91 ± 8	104 ± 10	88 ± 9	79 ± 8	100 ± 3	103 ± 7	107 ± 3	100 ± 7	94 ± 6	106 ± 4
NP_5EO	90 ± 13	79 ± 10	84 ± 11	90 ± 9	89 ± 3	95 ± 4	80 ± 7	79 ± 3	80 ± 7	92 ± 5	92 ± 3	99 ± 4	96 ± 6	95 ± 6
NP_6EO	76 ± 12	87 ± 9	90 ± 6	88 ± 6	98 ± 1	102 ± 7	82 ± 7	84 ± 13	89 ± 1	91 ± 5	96 ± 8	91 ± 8	102 ± 8	99 ± 6
NP_7EO	81 ± 6	83 ± 9	91 ± 3	95 ± 9	100 ± 9	96 ± 7	90 ± 15	84 ± 5	86 ± 3	92 ± 10	93 ± 1	89 ± 9	95 ± 11	101 ± 5
NP_8EO	77 ± 4	77 ± 10	88 ± 5	86 ± 6	91 ± 8	94 ± 8	81 ± 10	79 ± 5	90 ± 9	94 ± 8	92 ± 9	98 ± 4	101 ± 9	105 ± 9
Octylphenol (OP)	76 ± 10	82 ± 10	80 ± 4	81 ± 8	90 ± 6	95 ± 6	83 ± 13	79 ± 7	88 ± 6	80 ± 2	85 ± 4	88 ± 5	93 ± 9	85 ± 2
OP_1EO	80 ± 12	80 ± 11	79 ± 8	82 ± 8	86 ± 5	86 ± 11	79 ± 6	77 ± 3	86 ± 7	90 ± 6	86 ± 2	91 ± 6	89 ± 7	89 ± 3
OP_2EO	83 ± 12	80 ± 5	83 ± 10	85 ± 11	87 ± 6	91 ± 7	76 ± 9	80 ± 9	81 ± 9	79 ± 5	98 ± 6	87 ± 9	92 ± 5	83 ± 6
OP_3EO	79 ± 5	76 ± 3	76 ± 7	91 ± 10	88 ± 4	85 ± 3	81 ± 10	79 ± 11	79 ± 7	77 ± 5	85 ± 4	86 ± 3	78 ± 5	99 ± 5
OP_4EO	83 ± 8	80 ± 9	81 ± 7	87 ± 9	91 ± 6	94 ± 6	76 ± 12	77 ± 14	88 ± 4	83 ± 8	110 ± 4	85 ± 5	93 ± 5	91 ± 7
OP_5EO	81 ± 9	75 ± 7	74 ± 12	80 ± 7	104 ± 5	85 ± 3	74 ± 6	81 ± 3	79 ± 6	79 ± 3	78 ± 10	91 ± 4	82 ± 7	87 ± 10
OP_6EO	89 ± 6	82 ± 11	77 ± 8	85 ± 8	89 ± 1	90 ± 3	79 ± 7	77 ± 7	88 ± 6	90 ± 7	102 ± 4	98 ± 5	100 ± 3	92 ± 5
OP_7EO	74 ± 6	77 ± 15	88 ± 9	93 ± 8	95 ± 1	95 ± 6	83 ± 8	89 ± 7	79 ± 7	87 ± 6	93 ± 5	100 ± 11	90 ± 5	103 ± 5
OP_8EO	85 ± 8	86 ± 11	85 ± 5	82 ± 4	90 ± 9	88 ± 6	81 ± 12	80 ± 4	87 ± 1	90 ± 7	97 ± 3	94 ± 3	97 ± 8	109 ± 4
Bisphenol A (BPA)	87 ± 7	75 ± 7	79 ± 10	83 ± 11	91 ± 2	88 ± 9	81 ± 6	80 ± 6	79 ± 6	77 ± 3	86 ± 9	84 ± 13	83 ± 2	98 ± 8
Estriol (E3)	74 ± 3	75 ± 8	75 ± 8	82 ± 6	87 ± 5	85 ± 4	72 ± 8	75 ± 5	89 ± 2	80 ± 7	79 ± 4	92 ± 7	81 ± 8	80 ± 7
Estrone (E1)	80 ± 11	88 ± 8	85 ± 8	92 ± 8	85 ± 3	98 ± 1	78 ± 8	83 ± 6	91 ± 2	84 ± 4	97 ± 11	89 ± 5	90 ± 9	84 ± 3
17β-estradiol (E2)	82 ± 12	83 ± 11	82 ± 4	79 ± 8	90 ± 3	88 ± 9	76 ± 7	75 ± 6	80 ± 6	88 ± 14	90 ± 10	92 ± 5	94 ± 5	91 ± 5
17α-ethynilestradiol (EE)	76 ± 5	77 ± 3	75 ± 7	79 ± 5	88 ± 7	91 ± 5	74 ± 14	80 ± 10	87 ± 5	82 ± 12	92 ± 7	77 ± 5	79 ± 2	88 ± 3
Testosterone (TE)	100 ± 12	89 ± 4	91 ± 11	90 ± 8	105 ± 10	109 ± 5	97 ± 5	110 ± 3	90 ± 3	95 ± 10	100 ± 6	102 ± 7	102 ± 8	105 ± 9
19-norethindrone	90 ± 6	82 ± 6	89 ± 7	94 ± 4	88 ± 6	101 ± 11	76 ± 9	80 ± 6	92 ± 4	85 ± 6	91 ± 10	93 ± 10	90 ± 7	96 ± 8
Diethylstillbestrol (DES)	105 ± 6	79 ± 6	91 ± 12	90 ± 12	103 ± 6	90 ± 10	75 ± 11	75 ± 4	77 ± 4	84 ± 2	80 ± 6	82 ± 10	91 ± 5	86 ± 5
Norgestrel	75 ± 7	89 ± 9	91 ± 11	89 ± 10	87 ± 4	98 ± 6	91 ± 8	84 ± 8	90 ± 3	89 ± 1	97 ± 7	91 ± 8	93 ± 10	93 ± 8

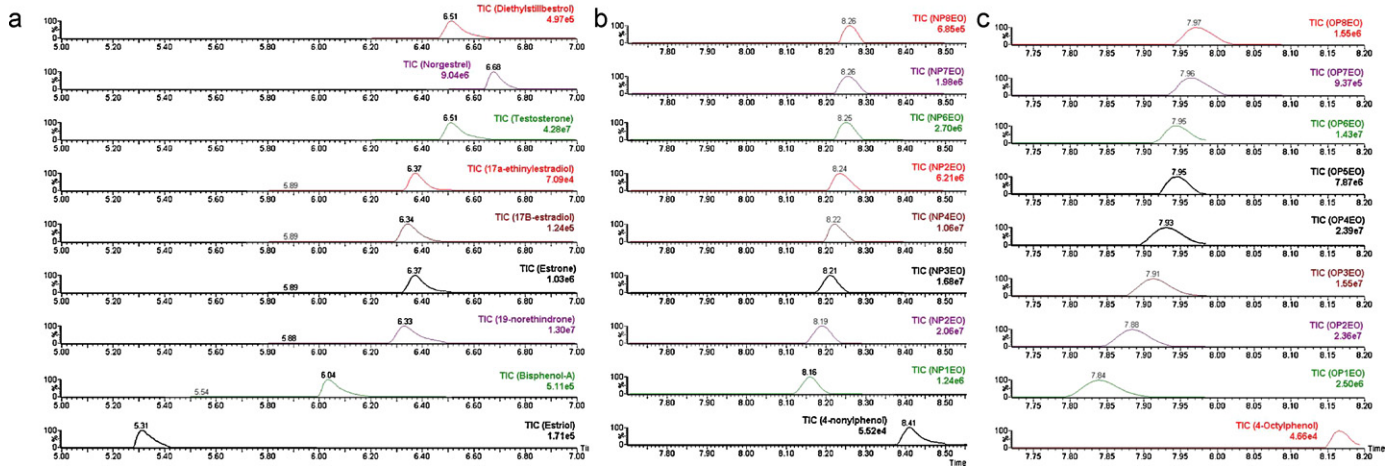


Fig. 2. MRM chromatograms of CAS final effluent sample spiked (500 ng L^{-1}) with all analytes after whole online-SPE process: a) BPA and steroids; b) Nonylphenol polyethoxylated compounds; c) Octylphenol polyethoxylated compounds.

treatment samples than for secondary treatment and final effluent samples. These results agree with previously published reports of similar studies [32].

3.4. Determination of EDCs in wastewater samples

The on-line SPE-UHPLC–MS/MS method developed herein was applied to wastewater samples obtained from two WWTPs in Las Palmas de Gran Canaria (Spain). The first WWTP was a conventional activated sludge treatment plant (CAS), whereas the second was a membrane bioreactor treatment plant (MBR). The concentrations of all target analytes in the water samples obtained during three bimonthly samplings (from May 2011 to September 2011) are given in Tables 6a, 6b and 6c. The following discussion focuses on the behaviour of contaminants through the different degradation stages and on the differences found between the different treatments in the WWTPs under study.

For the $AP_{1-8}Eos$, the total dissolved concentrations were significantly reduced from primary treatment (pt) samples to final effluent samples (fe) for both WWTPs. Although the MBR plant showed higher degradation for these compounds than the CAS plant, both purification systems demonstrated removal efficiencies above 88% and 89% for CAS and MBR, respectively.

The total dissolved concentrations of both APEO families under study, NPEOS and OPEOS, showed similar biodegradation patterns. Given that as nonylphenolic compounds represent approximately 80% of APnEOs produced worldwide, differences between NP_nEO and OP_nEO concentrations can be attributed to the global production of non-ionic surfactants, [16].

Table 5
Evaluation of the analyte matrix effects in the primary and secondary treatments and final effluent.

Compound	AST			MBR		
	Primary treatment[a]	Secondary treatment[a]	Final effluent[a]	Primary treatment[a]	Secondary treatment[a]	Final effluent[a]
Nonylphenol (NP)	24.3	18.0	12.1	19.7	14.3	8.9
NP_1EO	10.1	9.9	6.2	14.7	7.7	11.5
NP_2EO	12.3	12.1	7.2	14.7	7.9	13
NP_3EO	14.9	9.3	10.0	12.1	9.0	11.7
NP_4EO	8.6	10.0	9.5	9.5	7.5	8.2
NP_5EO	8.7	8.8	8.2	9.8	8.0	6.0
NP_6EO	10.6	8.8	6.9	10.1	6.9	4.9
NP_7EO	10.8	6.7	7.0	11.2	6.6	6.7
NP_8EO	11.8	6.9	7.1	9.0	6.6	9.0
Octylphenol (OP)	15.0	12.4	12.0	15.1	12.0	10.1
OP_1EO	16.5	10.2	7.2	10.0	10.8	11.0
OP_2EO	15.3	9.9	8.0	12.7	11.3	9.5
OP_3EO	10.3	10.7	8.9	13.0	10.5	7.4
OP_4EO	10.9	8.6	6.7	10.2	11.1	6.0
OP_5EO	8.6	8.6	6.7	9.0	6.7	6.2
OP_6EO	8.6	6.6	6.1	9.2	8.0	5.6
OP_7EO	9.3	7.0	4.7	9.2	7.5	6.3
OP_8EO	9.7	7.3	5.2	9.7	7.3	7.6
Bisphenol A (BPA)	9.0	8.2	5.5	13.0	10.1	6.8
Estriol (E3)	8.7	9.1	5.4	9.9	6.4	4.6
Estrone (E1)	12.4	7.5	7.4	8.9	8.4	5.0
17β-estradiol (E2)	13.7	6.8	6.0	8.7	9.0	7.7
17α-ethynilestradiol (EE)	18.4	12.8	13.3	14.8	10.0	8.3
Testosterone (TE)	10.0[b]	7.5[b]	4.9[b]	11.6[b]	6.8[b]	5.0[b]
19-norethindrone	13.8	10.1	8.3	14.5	9.4	6.3
Diethylstillbestrol (DES)	12.0	8.1	6.6	10.9	6.6	5.8
Norgestrel	15.9[b]	9.0[b]	8.2[b]	12.5[b]	7.3[b]	5.6[b]

[a] Mean of three determinations.
[b] Signal enhancement.

Table 6a
Concentration levels for each target compound in both WWTPs under study and at each sample conducted.

Compound	AST			MBR		
	C_{pt} (ng L^{-1})[a]	C_{st} (ng L^{-1})[a]	C_{fe} (ng L^{-1})[a]	C_{pt} (ng L^{-1})[a]	C_{st} (ng L^{-1})[a]	C_{fe} (ng L^{-1})[a]
Nonylphenol (NP)	71.0 ± 5.2	101.2 ± 3.5	89.4 ± 9.7	12.7 ± 3.3	15.5 ± 7.5	15.7 ± 3.9
NP_1EO	90.4 ± 8.2	111.7 ± 1.9	108.0 ± 10.1	30.1 ± 3.0	39.0 ± 3.5	40.7 ± 8.3
NP_2EO	95.5 ± 9.5	99.5 ± 5.5	120.6 ± 6.6	37.9 ± 0.9	35.1 ± 3.3	49.5 ± 5.5
NP_3EO	155.5 ± 6.0	72.1 ± 9.3	25.5 ± 1.8	52.3 ± 0.9	12.1 ± 0.9	3.1 ± 0.4
NP_4EO	120.6 ± 5.7	46.0 ± 8.7	39.5 ± 2.5	98.1 ± 9.0	15.1 ± 1.1	2.5 ± 0.2
NP_5EO	229.3 ± 9.3	22.5 ± 4.0	10.1 ± 0.9	80.5 ± 5.2	22.5 ± 1.6	BQL[c]
NP_6EO	505.6 ± 19.9	5.7 ± 3.5	BDL[b]	133.7 ± 9.2	10.1 ± 0.4	2.8 ± 1.9
NP_7EO	822.1 ± 15.1	12.6 ± 0.6	BDL[b]	276.2 ± 15.3	5.9 ± 2.5	5.0 ± 0.4
NP_8EO	805.3 ± 17.0	13.1 ± 0.5	1.6 ± 0.2	259.5 ± 12.5	3.0 ± 1.9	BDL[b]
Octylphenol (OP)	9.7 ± 0.7	12.5 ± 1.2	11.7 ± 0.9	18.1 ± 2.5	20.2 ± 2.0	18.5 ± 1.1
OP_1EO	21.7 ± 8.5	42.2 ± 4.1	39.9 ± 1.7	56.3 ± 0.3	61.8 ± 1.9	58.3 ± 3.8
OP_2EO	37.3 ± 6.3	55.6 ± 0.9	33.5 ± 1.7	44.5 ± 2.9	39.9 ± 0.3	37.1 ± 2.0
OP_3EO	44.2 ± 2.2	30.4 ± 2.1	10.8 ± 0.8	33.3 ± 0.3	12.2 ± 0.5	BQL[c]
OP_4EO	55.1 ± 5.0	10.1 ± 0.4	BQL[c]	58.4 ± 3.1	18.5 ± 1.5	BQL[c]
OP_5EO	76.4 ± 0.7	5.3 ± 0.1	BQL[c]	77.0 ± 6.0	3.4 ± 0.1	BDL[b]
OP_6EO	135.9 ± 12.5	6.4 ± 0.1	BDL[b]	94.4 ± 6.6	BQL[c]	BDL[b]
OP_7EO	102.1 ± 3.9	3.0 ± 0.1	BDL[b]	199.5 ± 13.3	2.9 ± 0.1	BDL[b]
OP_8EO	99.0 ± 2.5	5.2 ± 0.2	BDL[b]	203.2 ± 19.4	BQL[c]	BDL[b]
Bisphenol A (BPA)	BDL[b]	n.d.[d]	n.d.[d]	98.1 ± 2.0	44.2 ± 0.9	39.5 ± 3.1
Estriol (E3)	9.7 ± 0.3	8.1 ± 0.5	7.7 ± 0.3	18.1 ± 1.1	20.0 ± 1.3	18.0 ± 1.5
Estrone (E1)	BQL[c]	BQL[c]	BQL[c]	5.0 ± 0.3	4.3 ± 0.3	BQL[c]
17β-estradiol (E2)	39.2 ± 2.3	10.1 ± 0.4	8.3 ± 0.5	45.6 ± 3.3	14.2 ± 1.2	13.5 ± 1.3
17α-ethynilestradiol (EE)	n.d.	n.d.[d]	n.d.[d]	BQL[c]	BDL[b]	n.d.[d]
Testosterone (TE)	81.5 ± 5.9	13.5 ± 0.8	14.0 ± 1.0	100.1 ± 8.5	31.4 ± 0.6	21.6 ± 0.9
19-norethindrone	12.8 ± 0.9	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
Diethylstillbestrol (DES)	BDL[b]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
Norgestrel	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]

[a] Mean and standard deviation of three determinations.
[b] Concentration below the limit of detection.
[c] Concentration below the limit of quantification.
[d] Compound not detected.

Table 6b
Concentration levels for each target compound in both WWTPs under study and at each sample conducted.

Compound	AST			MBR		
	C_{pt} (ng L^{-1})[a]	C_{st} (ng L^{-1})[a]	C_{fe} (ng L^{-1})[a]	C_{pt} (ng L^{-1})[a]	C_{st} (ng L^{-1})[a]	C_{fe} (ng L^{-1})[a]
Nonylphenol (NP)	101.0 ± 8.3	121.4 ± 9.5	110.0 ± 9.0	55.5 ± 3.0	59.0 ± 2.0	56.6 ± 2.3
NP_1EO	123.6 ± 4.8	150.6 ± 9.4	145.0 ± 9.0	60.2 ± 5.5	75.8 ± 7.2	73.0 ± 4.9
NP_2EO	150.0 ± 3.9	109.5 ± 5.0	152.0 ± 3.7	19.1 ± 1.5	69.1 ± 3.0	65.5 ± 0.2
NP_3EO	169.2 ± 14.4	89.0 ± 5.1	50.7 ± 5.1	100.5 ± 3.8	39.9 ± 1.1	12.0 ± 0.3
NP_4EO	333.7 ± 6.2	39.5 ± 3.5	27.4 ± 2.0	103.1 ± 10.2	12.0 ± 0.4	4,5 ± 0.3
NP_5EO	302.2 ± 10.0	19.5 ± 0.9	30.2 ± 1.1	167.8 ± 10.6	12.1 ± 0.4	9.5 ± 0.7
NP_6EO	809.4 ± 41.3	78.8 ± 7.1	101.5 ± 0.6	244.4 ± 18.0	29.6 ± 2.5	2.0 ± 0.1
NP_7EO	1023.0 ± 19.2	79.5 ± 7.0	45.9 ± 1.1	250.9 ± 7.9	64.5 ± 3.9	4.8 ± 0.1
NP_8EO	1018.1 ± 56.1	90.1 ± 3.3	19.3 ± 0.5	303.6 ± 19.9	10.1 ± 0.9	7.3 ± 0.8
Octylphenol (OP)	18.5 ± 1.2	22.9 ± 2.1	23.0 ± 0.9	6.9 ± 0.3	7.1 ± 0.2	BDL[b]
OP_1EO	33.7 ± 3.0	40.0 ± 2.5	38.2 ± 2.1	18.9 ± 0.7	16.4 ± 0.8	22.2 ± 0.7
OP_2EO	25.5 ± 0.9	24.1 ± 1.1	35.6 ± 3.0	40.5 ± 2.6	56.3 ± 5.1	51.0 ± 4.4
OP_3EO	56.9 ± 0.8	10.5 ± 0.5	14.0 ± 0.8	69.6 ± 3.8	19.7 ± 0.5	BDL[b]
OP_4EO	156.5 ± 4.9	7.7 ± 0.5	6.1 ± 0.1	101.1 ± 10.2	BDL[b]	BDL[b]
OP_5EO	138.7 ± 10.7	9.5 ± 0.8	11. 0 ± 1.0	99.5 ± 5.8	BDL[b]	BDL[b]
OP_6EO	140.5 ± 1.5	BQL[c]	BDL[b]	121.0 ± 9.5	BDL[b]	BDL[b]
OP_7EO	187.3 ± 12.0	BDL[b]	BDL[b]	150.0 ± 9.2	n.d.[d]	n.d.[d]
OP_8EO	245.1 ± 8.5	BDL[b]	BDL[b]	120.5 ± 12.0	n.d.[d]	n.d.[d]
Bisphenol A (BPA)	51.4 ± 2.1	22.6 ± 2.0	25.1 ± 3.2	18.2 ± 1.0	7.8 ± 0.4	8.0 ± 0.8
Estriol (E3)	14.3 ± 1.1	15.0 ± 1.2	12.8 ± 0.9	6.5 ± 0.4	5.0 ± 0.2	BQL[c]
Estrone (E1)	5.5 ± 0.4	BQL[c]	BDL[b]	BQL[c]	BDL[b]	BDL[b]
17β-estradiol (E2)	51.9 ± 2.8	15.1 ± 1.0	14.7 ± 1.3	24.8 ± 1.5	7.6 ± 0.6	7.1 ± 0.6
17α-ethynilestradiol (EE)	5.9 ± 0.6	n.d.[d]	n.d.[d]	3.1 ± 0.2	n.d.[d]	n.d.[d]
Testosterone (TE)	258.6 ± 24.9	69.2 ± 5.3	48.0 ± 3.7	19.5 ± 1.1	1.7 ± 0.3	BDL[b]
19-norethindrone	18.2 ± 1.5	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
Diethylstillbestrol (DES)	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
Norgestrel	9.0 ± 0.6	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]

[a] Mean and standard deviation of three determinations.
[b] Concentration below the limit of detection.
[c] Concentration below the limit of quantification.
[d] Compound not detected.

Table 6c
Concentration levels for each target compound in both WWTPs under study and at each sample conducted.

Compound	AST			MBR		
	C_{pt} (ng L^{-1})[a]	C_{st} (ng L^{-1})[a]	C_{fe} (ng L^{-1})[a]	C_{pt} (ng L^{-1})[a]	C_{st} (ng L^{-1})[a]	C_{fe} (ng L^{-1})[a]
Nonylphenol (NP)	73.9 ± 1.0	82.5 ± 7.5	77.0 ± 0.9	15.3 ± 0.9	17.0 ± 3.3	17.5 ± 1.1
NP_1EO	154.9 ± 14.9	145.8 ± 10.0	160.0 ± 7.9	19.6 ± 1.1	22.5 ± 0.7	22.6 ± 2.2
NP_2EO	60.0 ± 13.3	74.1 ± 1.9	63.5 ± 6.3	14.7 ± 1.1	19.0 ± 1.5	17.6 ± 1.1
NP_3EO	129.5 ± 7.4	49.1 ± 3.3	12.6 ± 0.6	74.3 ± .3.9	10.7 ± 0.1	8.0 ± 0.7
NP_4EO	126.7 ± 10.0	22.0 ± 0.9	29.2 ± 1.5	195.3 ± 10.6	9.0 ± 0.1	12.7 ± 0.3
NP_5EO	301.9 ± 21.0	39.7 ± 2.1	20.0 ± 1.8	282.5 ± 10.9	18.3 ± 1.2	22.8 ± 1.0
NP_6EO	369.2 ± 30.1	9.9 ± 0.4	6.0 ± 0.3	218.7 ± 20.1	29.6 ± 0.9	10.3 ± 0.3
NP_7EO	800.4 ± 7.5	26.0 ± 2.0	12.2 ± 0.7	390.1 ± 20.0	10.0 ± 0.6	BDL[b]
NP_8EO	905.6 ± 19.3	17.2 ± 1.1	9.5 ± 0.3	508.5 ± 34.9	19.6 ± 0.9	12.8 ± 0.6
Octylphenol (OP)	12.3 ± 0.7	13.5 ± 1.1	17.8 ± 1.1	9.0 ± 0.6	12.0 ± 0.6	11.4 ± 0.4
OP_1EO	6.7 ± 0.3	16.4 ± 0.9	13.5 ± 0.4	21.1 ± 0.8	30.9 ± 2.2	31.6 ± 3.0
OP_2EO	19.0 ± 0.2	22.9 ± 1.8	23.0 ± 2.2	19.0 ± 1.0	5.0 ± 0.3	BQL[c]
OP_3EO	49.9 ± 0.3	19.6 ± 1.5	12.4 ± 0.9	29.9 ± 1.5	5.8 ± 0.6	14.5 ± 0.7
OP_4EO	90.4 ± 0.7	20.5 ± 2.1	18.0 ± 0.8	47.5 ± 3.3	12.2 ± 1.0	10.5 ± 0.7
OP_5EO	79.5 ± 0.7	5.3 ± 0.3	BQL[c]	60.6 ± 3.3	BQL[c]	BDL[b]
OP_6EO	234.8 ± 2.1	BQL[c]	BDL[b]	75.2 ± 0.9	BQL[c]	BDL[b]
OP_7EO	153.9 ± 0.9	n.d.[d]	n.d.[d]	120.2 ± 4.7	4.0 ± 0.4	BDL[b]
OP_8EO	301.8 ± 2.9	n.d.[d]	n.d.[d]	99.0 ± 8.0	n.d.[d]	n.d.[d]
Bisphenol A (BPA)	12.1 ± 0.5	BQL[c]	BDL[b]	BQL[c]	BDL[b]	BQL[c]
Estriol (E3)	18.0 ± 1.1	22.5 ± 0.6	19.2 ± 1.9	11.9 ± 0.3	10.5 ± 1.0	8.9 ± 0.2
Estrone (E1)	10.0 ± 0.8	8.1 ± 0.5	8.2 ± 0.5	7.5 ± 0.6	8.0 ± 0.5	5.4 ± 0.4
17β-estradiol (E2)	60.0 ± 3.1	18.9 ± 2.2	n.d.[d]	52.5 ± 2.7	14.9 ± 0.9	14.5 ± 1.5
17α-ethynilestradiol (EE)	15.1 ± 0.5	BDL[b]	n.d.[d]	3.9 ± 0.2	BDL[b]	n.d.[d]
Testosterone (TE)	209.1 ± 18.6	48.9 ± 0.2	55.2 ± 0.5	93.5 ± 3.9	20.1 ± 1.7	24.4 ± 2.4
19-norethindrone	6.5 ± 0.5	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
Diethylstillbestrol (DES)	3.3 ± 0.2	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
Norgestrel	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]

[a] Mean and standard deviation of three determinations.
[b] Concentration below the limit of detection.
[c] Concentration below the limit of quantification.
[d] Compound not detected.

Steroidal hormones and BPA were present in almost all samples at low ng L^{-1} levels. Compared to APEOs, both WWTPs exhibited lower biodegradation efficiencies for steroidal hormones and BPA, with the MBR treatment plant, once again, achieving higher removal rates than the CAS plant. As shown in Tables 6a, 6b and 6c, the concentration determined for BPA was lower than the method's detection limits or simply was not detected in some instances. Furthermore, reductions of approximately 60% and 58% were observed for BPA detection in CAS and MBR samples, respectively.

Oestrogenic steroidal hormones displayed behaviour that was intermediate between that described for APEOs and BPA. During the biological treatment, both WWTPs showed efficient degradation of 17 β-oestradiol (E2), with slightly higher removal rates than those observed for BPA (64% in CAS and 71% in MBR). As in the case of APEOs, the biodegradation of E2 leads to the formation of oestrogenic metabolites. In the present case we determined and evaluated the presence of two of these, oestrone (E1) and oestriol (E3).

Unlike the results obtained thus far for all other compounds, concentrations of E1 and E2 remained relatively stable throughout treatment at both CAS and MBR treatment plants, showing biodegradations that exceeded 20% efficiency (19.5% in July 2011 sampling). This lead us to hypothesise that, although both wastewater treatment plants may be eliminating both metabolites more efficiently, the continual addition of E1 and E3 resulting from the biodegradation of E2 could be masking the true elimination of these analytes. Norgestrel and 19-norethindrone were exclusively detected in a timely manner in CAS primary-treatment samples. Thus, it was impossible to evaluate the related biodegradation in both WWTPs. Testosterone ($\log K_{ow}$ 3.27) showed a similar biodegradation pattern to that observed for BPA. Taking all analysed samples into account, an average degradation of 77% and 82% was estimated for this analyte in CAS and MBR, respectively.

4. Conclusions

A sensitive and selective on-line-SPE-UHPLC–MS/MS method has been developed for the determination of endocrine disrupting compounds in sewage samples at low nanogram-per-liter levels. The entire analytical procedure, including the extraction of all analytes, requires only 9 min per sample. The advantages of this methodology can be summarised as follows: (1) with regard to EDCs, the total analysis time is considerably reduced compared with another methodologies; (2) the minimisation of manual operation helps to lower RSDs; (3) the on-line solid-phase extraction process minimises and can even eliminate manual sample preparation steps; (4) automating the process helps reduce background noise, thus improving LODs and (5) the parallel processing of samples coupled to ultra-high-performance liquid chromatography increases laboratory throughput and efficiency.

In addition to enabling rapid extraction and analysis of all of the analytes under study, our methodology also offers low limits of detection (ranging from 0.3 to 2.1 ng L^{-1} in MRM mode) and high selectivity, which are required to detect these analytes in complex environmental matrices at ultra trace levels. Moreover, recoveries between 74% and 110% with RSDs lower than 15% were obtained. This newly developed method detected the target analytes at low ng L^{-1} levels in real liquid wastewater samples obtained from two WWTPs. In addition, analysis of these EDCs along throughout all treatment stages in both treatment plants revealed that these compounds are not completely eliminated but are ultimately released into the environment through their respective submarine emissaries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.01.077.

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