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Determination of alkylphenol polyethoxylates, bisphenol-A, 17α-ethynylestradiol and 17β-estradiol and its metabolites in sewage samples by SPE and LC/MS/MS

T. Vega-Morales, Z. Sosa-Ferrera, J.J. Santana-Rodríguez*

Department of Chemistry, Faculty of Marine Sciences, University of Las Palmas de Gran Canaria, 35017 Las Palmas de Gran Canaria, Spain

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ABSTRACT

Recently, many chemicals released into the environment have been shown to mimic endogenous hormones such as estradiol. It has been demonstrated that these compounds cause several adverse effects on wildlife and humans, such as the feminization of animal species, development of physical abnormalities and birth defects, and reproductive failure. In an effort to model the behaviour of some endocrine-disrupting chemicals (EDCs) and to establish the level of contamination in sewage samples, a quantitative method for the simultaneous determination of nonylphenol, octylphenol and corresponding ethoxylates (1–12), 17α-ethynylestradiol, bisphenol-A, and 17β-estradiol and two of its metabolites have been developed. Identification and quantification were achieved by high performance liquid chromatography–tandem mass spectrometry (LC–ESI–MS/MS). Satisfactory detection limits (between 0.5–6 ng L^{-1} in the dissolved phase and 1.4–12.7 ng g^{-1} in the particulate phase) and analyte recoveries (between 60% and 108%) were achieved for target compounds. The optimised method was applied to the determination of EDCs in liquid sewage samples collected from a wastewater treatment plant (WWTP) in Las Palmas de Gran Canaria (Spain). Concentrations of EDCs ranged from <10 ng L^{-1} to nearly 1200 ng L^{-1} in the dissolved phase, and from 0.005 μg g^{-1} to 2.8 μg g^{-1} in the suspended particulate matter.

1. Introduction

A large amount of anthropogenic and natural chemicals are able to disrupt the endocrine system of wildlife and humans [1]. Although these endocrine-disrupting chemicals (EDCs) can alter the endocrine system in various ways, one of the most widely demonstrated modes of hormonal disruption corresponds to the mimicry of endogenous estradiol by binding to and activating oestrogen receptors (ER agonist) [2]. As a result, the principal functions of this hormone in the organism are severely compromised, directly affecting reproduction and development.

In particular, alkylphenolic ethoxylated surfactants (APEOs), bisphenol-A (BPA), steroidal hormones, such as 17β-estradiol (E2) and its metabolites including estrone (E1), estriol (E3) and 16α-hydroxyestrone, and synthetic steroids, such as 17α-ethynylestradiol (EE), have aroused an enormous interest in the scientific community due to worldwide production, extensive use in industrial, agricultural and household applications, and chronic toxicity [3–7]. Due to their physicochemical properties including high octanol–water partition coefficients and low water solubilities (Table 1), these compounds tend to bind tightly to sediments and bioaccumulate in aquatic organisms, which contributes to the persistence of these compounds in the environment and requires their determination from several matrices [8–12].

The presence of EDCs in different environments and matrices from various input activities has generated the development of analytical methods for the determination of these substances in various liquid and solid matrices [13].

Extraction methodologies for solid samples include conventional methods such as the Soxhlet extractor and advanced extraction techniques such as supercritical fluid extraction (SFE), ultrasonic assisted extraction (UAE) or microwave assisted extraction (MAE) [14,15]. In liquid samples, extraction methodologies range from classical liquid–liquid extraction (LLE) to advanced extraction and preconcentration techniques such as solid phase extraction (SPE), solid phase microextraction (SPME) or stir bar sorptive extraction (SBSE) [16–18].

Although liquid chromatography (LC) with optical detection systems have been successfully employed for the analysis of these organic compounds and reasonable detection limits have been reported using fluorescence detection systems (FD) [19]. However, the lack of specificity of these methodologies in complex matrices does not allow the unequivocal identification of EDCs [20]. Mass spectrometry (MS) may provide further structural and

* Corresponding author. Tel.: +34 928 45 44 25; fax: +34 928 45 29 22.
E-mail address: jsantana@dqui.ulpgc.es (J.J. Santana-Rodríguez).

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Table 1
Physiochemical properties of the compounds studied [4,6].

Chemical name	Molecular weight	Water solubility (mg L^{-1} at 20 °C)	log K_{ow}
Bisphenol-A (BPA)	228.0	120	3.32
17β-Estradiol (E2)	272.4	13	3.94
Estrone (E1)	270.4	13	3.43
Estriol (E3)	288.4	13	2.81
17α-Ethynylestradiol (EE)	296.4	4.8	4.15
4-Octylphenol (OP)	206.0	12.6	4.12
Octylphenol monoethoxylate (OP_1EO)	250.0	8.0	4.10
Octylphenol diethoxylate (OP_2EO)	294.0	13.2	4.00
Octylphenol triethoxylate (OP_3EO)	338.0	18.4	3.90
Octylphenol tetraethoxylate (OP_4EO)	384.0	24.5	3.90
4-Nonylphenol (NP)	220.0	1.57	4.48
Nonylphenol monoethoxylate (NP_1EO)	264.0	3.02	4.17
Nonylphenol diethoxylate (NP_2EO)	308.0	3.38	4.21
Nonylphenol triethoxylate (NP_3EO)	352.0	5.88	4.20
Nonylphenol tetraethoxylate (NP_4EO)	396.0	7.65	4.30

quantitative information, facilitating the correct and unambiguous identification of each analyte and an increase in sensitivity. The major weakness of "traditional" methodologies for EDCs analysis can be solved by coupling LC to MS, particularly to a tandem mass spectrometer (LC/MS/MS) [13].

The objective of this study was to develop a simple and rapid analytical procedure for the simultaneous extraction and determination of nonylphenol, octylphenol and corresponding ethoxylates (1–12), 17α-ethynylestradiol, 17β-estradiol and its two metabolites estrone (E1) and estriol (E3), and bisphenol-A (BPA) in sewage samples and to apply the optimised method to the quantification of EDCs in wastewater samples obtained from a wastewater treatment plant (WWTP) in Las Palmas of Gran Canaria (Spain). Compounds were determined in both the dissolved and particulate phases at different stages of the WWTP process, including its final effluent located on the city's coast.

2. Experimental

2.1. Chemicals and reagents

Methanol and water used to dissolve standards or Igepal mixtures and to prepare mobile phases were LC–MS grade and obtained from Panreac Química (Barcelona, Spain). Methanol and water were filtered through a 0.22 μm acetate membrane filter. Glacial acetic acid used to adjust the pH of the mobile phase was high performance liquid chromatography (LC) grade and was purchased 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 solid phase extraction.

All standards and Igepal technical mixtures were purchased from Sigma Aldrich (Madrid, Spain). Short ethoxylated chains AP_nEOs ($n \leq 2$), 17β-estradiol, estrone, estriol, 17α-ethynylestradiol and bisphenol-A were greater than 98% pure and were used as standards. Stock solutions (1000 μg mL^{-1}) of alkylphenols, steroidal hormones, and bisphenol-A were prepared by dissolving the standards into methanol. Stock solutions were stored in glass-stoppered bottles at −20 °C prior to use. 10 μg mL^{-1} of nonylphenol monoethoxylate, nonylphenol diethoxylate, octylphenol monoethoxylate and octylphenol diethoxylate were obtained as stock solutions (1 mL) in acetone and were stored at −20 °C.

Long-chain AP_nEOs ($n \geq 3$) were only available in technical mixtures. Igepal CO210, CO520 and CO720 contained a range of NP_nEO oligomers with 3–12 ethoxy units (EO) while Igepal CA210, CA520 and CA720 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 20 °C.

The cartridges (6 mL) employed in this study included Sep-Pak Vac C_{18} (500 mg) and Oasis HLB (200 mg) from Waters (Madrid, Spain), Bond Elut-ENV (500 mg) and Bond Elut Plexa (500 mg) from Varian (Madrid, Spain), and LiChrolut EN (500 mg) from Merck (Darmstadt, Germany). A Varian Vac Elut 20 SPE Manifold coupled to a Sartorius vacuum pump was used for extractions.

2.2. Sample collection

To test the applicability of the method, three samples from primary, secondary and final effluent were collected from one of the major wastewater treatment plants in Las Palmas de Gran Canaria (Spain). Treatment in the WWTP relies on settling and flotation (primary treatment) and biological treatment with activated sludge (secondary treatment). A tertiary treatment was not conducted in the WWTP process. Samples were collected in July 2009 and acidified to a pH < 3 to prevent the loss of analytes by abiotic reactions (such as hydrolysis) and by biological degradation [21]. The samples were stored at 4 °C in 2.5 L glass bottles and extracted within 48 h. Primary and secondary treatment samples were taken from the effluent of each stage.

Prior to extraction, wastewater samples were filtered through 0.65 μm filter paper and 0.45 μm membrane filters (Millipore, Bedford, MA, USA). A pore size of 0.45 μm was used to separate dissolved and particulate phases [22].

2.3. Extraction

Compounds were isolated from the dissolved phase using solid phase extraction (SPE). The cartridge was conditioned with 3× 5 mL of methanol and 3× 5 mL of Milli-Q water at a flow-rate of 5 mL min^{-1} before each extraction. The sample (250 mL) percolated through the cartridge at a flow-rate of 10 mL min^{-1}. A wash step was conducted using 2× 5 mL of Milli-Q water to remove impurities retained in the cartridge. Subsequently, the cartridge was dried under vacuum for 10 minutes, and the retained analytes were eluted at a low flow-rate (approximately 1 mL min^{-1}) with 2 mL of methanol. Blanks were run to confirm the absence of carryover.

0.45 μm membrane filters with retained particulate matter (between 0.1 and 0.2 g for all samples) were immersed in an ultrasonic bath with 10 mL of methanol for 10 min. The methanol extract was collected in a flask, evaporated to dryness under a gentle stream of nitrogen and reconstituted in 100 μL of methanol. The final extracts were analysed separately and concentrations of dissolved and particulate phases are reported separately for each sample.

Table 2
Characteristic of ESI/MS/MS parameters for each compound studied.

Compound	*m*/*z*Precursor $[M+NH_4]^+$	*m*/*z*Precursor $[M-H]^-$	Cone (V)	Fragment ions (collision potential)	Ion mode
NP_1EO	282.3	–	30	265.3 (6)[a], 127.1 (8)	ESI+
NP_2EO	326.3	–	30	183.1 (9)[a], 121 (20)	ESI+
NP_3EO	370.3	–	32	353.3 (8)[a], 227.1 (11)	ESI+
NP_4EO	414.5	–	32	397.4 (8)[a], 271.2 (13.5)	ESI+
NP_5EO	458.6	–	48	441.5 (12)[a], 315.2 (15.5)	ESI+
NP_6EO	502.6	–	52	485.5 (13.5)[a], 359.3 (17)	ESI+
NP_7EO	546.7	–	56	529.6 (14.5)[a], 403 (18)	ESI+
NP_8EO	590.8	–	64	573.6 (15.5)[a], 447.5 (20)	ESI+
NP_9EO	634.8	–	64	617.8 (16)[a], 335 (21)	ESI+
$NP_{10}EO$	678.9	–	68	661.8 (16.5)[a], 132.7 (24.5)	ESI+
$NP_{11}EO$	722.9	–	72	704.9 (17.5)[a], 291 (26.5)	ESI+
$NP_{12}EO$	766.9	–	88	749.9 (18)[a], 291 (28.5)	ESI+
$NP_{13}EO$	811.1	–	80	794 (18)[a], 291.1 (29)	ESI+
$NP_{14}EO$	855.2	–	88	838 (18)[a], 291 (32.5)	ESI+
OP_1EO	268.1	–	30	251.1 (6)[a], 113 (7.5)	ESI+
OP_2EO	312.3	–	30	183.0 (9.5)[a], 121 (19.5)	ESI+
OP_3EO	356.4	–	32	339.4 (8)[a], 227.1 (14)	ESI+
OP_4EO	400.4	–	32	383.4 (10)[a], 271.2 (14)	ESI+
OP_5EO	444.5	–	48	427.5 (12)[a], 315.2 (15.5)	ESI+
OP_6EO	488.5	–	52	471.5 (13.5)[a], 359.3 (17)	ESI+
OP_7EO	532.8	–	52	516.6 (15)[a], 403.3 (18)	ESI+
OP_8EO	576.7	–	60	559.7 (15.5)[a], 277.2 (24)	ESI+
OP_9EO	620.8	–	64	603.7 (16.5)[a], 277.2 (25.5)	ESI+
$OP_{10}EO$	664.9	–	68	647.8 (17.5)[a], 277.2 (27.5)	ESI+
$OP_{11}EO$	708.9	–	68	691.8 (18)[a], 277.2 (29)	ESI+
$OP_{12}EO$	752.9	–	68	735.9 (19)[a], 277.2 (29.5)	ESI+
$OP_{13}EO$	797.1	–	72	780 (18.5)[a], 277.2 (30.5)	ESI+
$OP_{14}EO$	841.2	–	84	823.2 (20)[a], 132.6 (27)	ESI+
NP	–	218.7	−64	105.7 (20.5)[a]	ESI−
OP	–	204.7	−72	134 (16.5)[a], 106 (19.5)	ESI−
BPA	–	226.7	−60	211.7 (17.5)[a]	ESI−
E2	–	271.1	−60	183.5 (14.5)[a], 145.2 (20.5)	ESI−
E1	–	269.3	−55	145.4 (16.5)[a], 143.2 (19.5)	ESI−
E3	–	287.2	−67	171.0 (16.5)[a], 145.2 (19.5)	ESI−
EE	–	295.3	−71	159.5 (17.5)[a], 145.2 (21.5)	ESI−

[a] Fragment ion used for quantitation (MRM).

2.4. Instrumentation and chromatographic conditions

2.4.1. LC/MS/MS analysis

Analysis was performed by reversed phase liquid chromatography coupled to a triple quadrupole (TQ) mass spectrometer equipped with an electrospray interface (LC/ESI/MS/MS). The apparatus was composed of a Varian 320-MS TQ Mass Spectrometer (Varian Inc., CA, USA) equipped with a Varian LC system consisting of a binary pump, autosampler and temperature controlled column compartment.

Chromatographic separation was performed on a Pursuit XRs Ultra-C18 reversed phase column (2.8 μm particle size, 50 mm × 2 mm i.d.) from Varian Inc. (CA, USA). The mobile phase consisted of water (solvent A) and methanol (solvent B) with 0.1% (v/v) glacial acetic acid and 15 mM ammonium acetate. Gradient elution consisted of a solution of 30:70 (v/v) methanol: water for 9 min, followed by an increase in methanol to 100% over 10 min. The injection volume was 10 μL and the flow-rate was 200 $\mu L\,min^{-1}$ for 10 min. The temperature in the column compartment was set to 40 °C.

Multiple reaction monitoring (MRM) parameters were optimised for subsequent quantitative analysis. Precursor ions included $[M+NH_4]^+$ for AP_nEOs in positive ion mode and $[M-H]^-$ for APs, steroidal hormones, and BFA in negative ion mode. This procedure was conducted using a 1 mL syringe pump (Hamilton Company, Reno, NV, USA), employing a continuous flow-rate of 20 $\mu L\,min^{-1}$. Each standard or mixture was prepared as 10 $mg\,L^{-1}$ in methanol. 0.1 mL of each solution was taken up by the Hamilton syringe and the remaining 0.9 mL of syringe volume were filled with mobile phase. The composition of the mobile phase depended on the ionisation of each analyte in ESI.

Ionisation in the ESI source was achieved using nitrogen as a nebuliser and drying gas. Housing and desolvation temperature were set to 60 °C and 250 °C, respectively, for the optimisation of syringe pump injections for MS/MS. However, to obtain a strong signal for each analyte, the desolvation temperature was set to 200 °C during the first 4 min and was increased linearly to 350 °C until the end of the chromatographic run. The drying and nebulising gas pressures were fixed at 30 psi and 65 psi, respectively. The capillary voltage was set to 4.5 kV in positive mode (ESI+) and −3 kV in negative mode (ESI−). The shield voltage was maintained at −600/600 V (ESI+/ESI−) and the cone voltage was optimised for each individual compound (Table 2). Collision induced dissociation (CID) was conducted with argon as the collision gas at a fixed pressure of 2 mTorr. The fragment ions obtained for each compound and the collision potential are displayed in Table 2.

3. Results and discussion

3.1. Optimisation of MS/MS conditions

The optimisation of MS/MS conditions for each compound was performed by direct infusion of pure standards or Igepal mixtures. Under positive ion mode (ESI+), AP_nEOs possess a high affinity for alkali metal ions in unmodified mobile phases and often lead to the formation of sodium adducts $[M-Na]^+$ rather than protonated molecules $[M-H]^+$ [23]. These adducts are reluctant to fragment in the collision cell and cannot be used in MRM detection [19]. For this reason, an aqueous mobile phase of 0.1% (v/v) glacial acetic acid and 20 mM ammonium formiate was used to force the formation of ammonium adducts $[M+NH_4]^+$, which have a relevant and reproducible fragmentation in the collision cell [19,20].

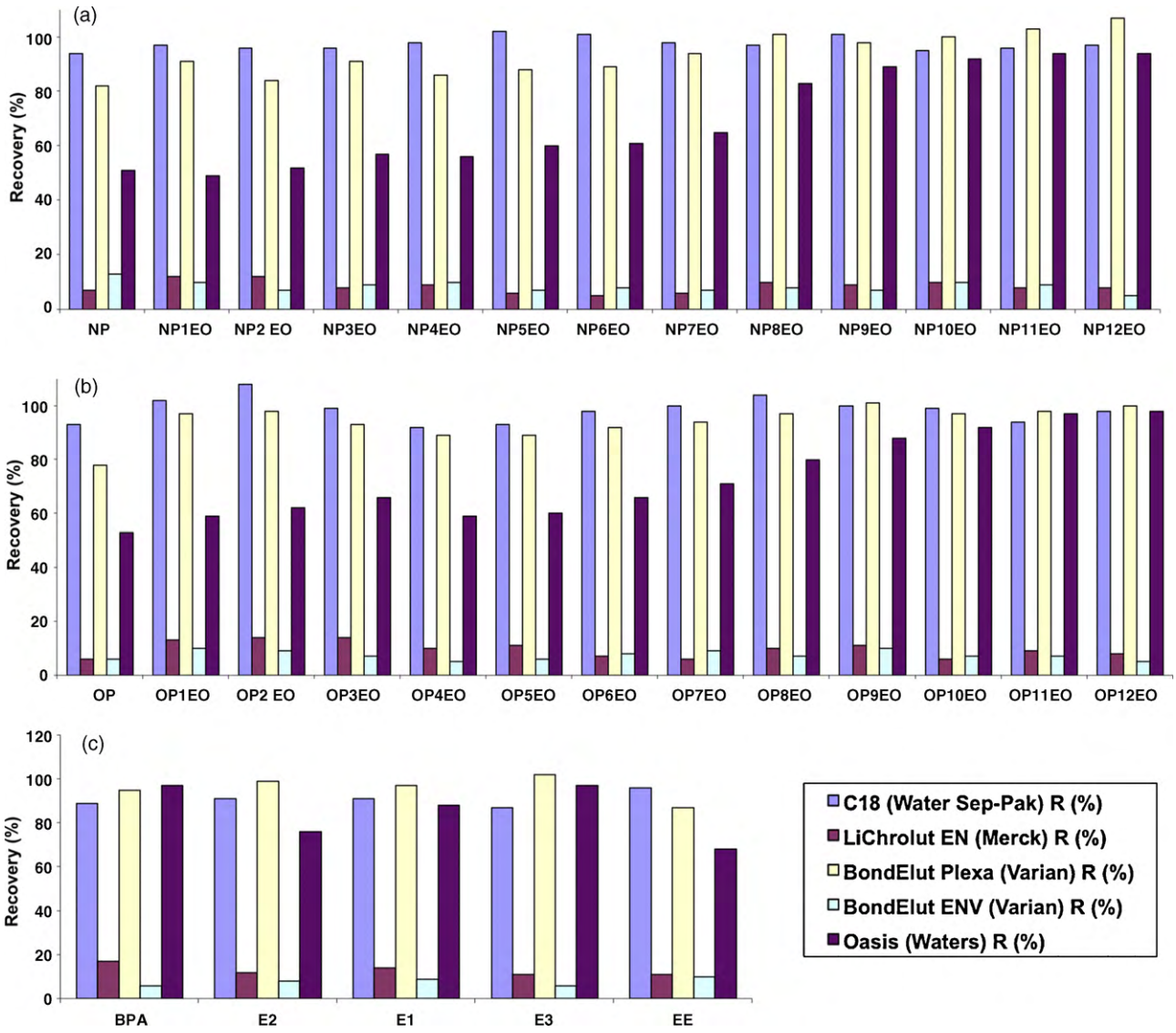

Fig. 1. Recoveries obtained by different types of SPE cartridges. (a) Recoveries obtained for NPEOs oligomers; (b) recoveries obtained for OPEOs oligomers; (c) recoveries obtained for BPA and steroidal hormones.

In negative ion mode (ESI−), transitions from the $[M-H]^-$ ion for APs, steroidal hormones and bisphenol-A were monitored. For these compounds, mobile phases with different percentages of methanol were used for direct flow injections. The optimal response for these transitions was observed with a 70% (v/v) methanol, 30% (v/v) aqueous solution, which is consistent with previous studies [24].

3.2. Optimisation of SPE process

The optimisation of SPE for wastewater samples included the evaluation of several experimental variables to achieve maximum extraction efficiency for each compound. Hence, cartridge type, pH, ionic strength, sample volume, wash step and desorption volume were optimised. A sample volume of 100 mL of Milli-Q water containing 500 ng L^{-1} of each analyte and a desorption volume of 2 mL of methanol were used initially. Samples were passed through cartridges under the conditions described in Section 2.3.

3.2.1. Type of cartridge

Five solid phase materials with different characteristics were tested to obtain an optimal extraction for the analytes, including a Sep-Pak Vac C_{18} (500 mg), Oasis HLB (200 mg), Bond Elut-ENV (500 mg), Bond Elut Plexa (500 mg) and LiChrolut EN (500 mg). Fig. 1 shows the results obtained with the aforementioned cartridges.

Among the cartridges tested, Sep-Pak Vac C_{18} showed superior performance under the initial conditions, even for long-chained AP_nEO oligomers (recoveries >90%). Despite this result, it was expected that an increase in sample volume passed through the cartridges would decrease the recoveries of polar compounds considerably [25]. However, due to the nature of wastewater samples, especially those taken from primary treatment, a few hundred millilitres (~300 mL) of sample were enough to saturate the cartridges [19], even when the samples had been properly filtered.

The Oasis HLB cartridge showed poor recoveries for APs and short-chain alkylphenolic compounds (from 1 to 5 ethoxylate units). However, due to its hydrophilic nature [26,27], a satisfactory extraction was obtained for "water soluble" AP_nEO ($n > 5$ units) oligomers (recoveries between 78% and 92%).

The Bond Elut Plexa cartridge showed the best recoveries for polar compounds ($n > 9$), but the results obtained for hydrophobic species were clearly inferior to those achieved with the C_{18} cartridge, especially for nonylphenol (NP) and its ethoxylates. Due to the highly cross-linked materials of LiChrolut EN and Bond Elut-

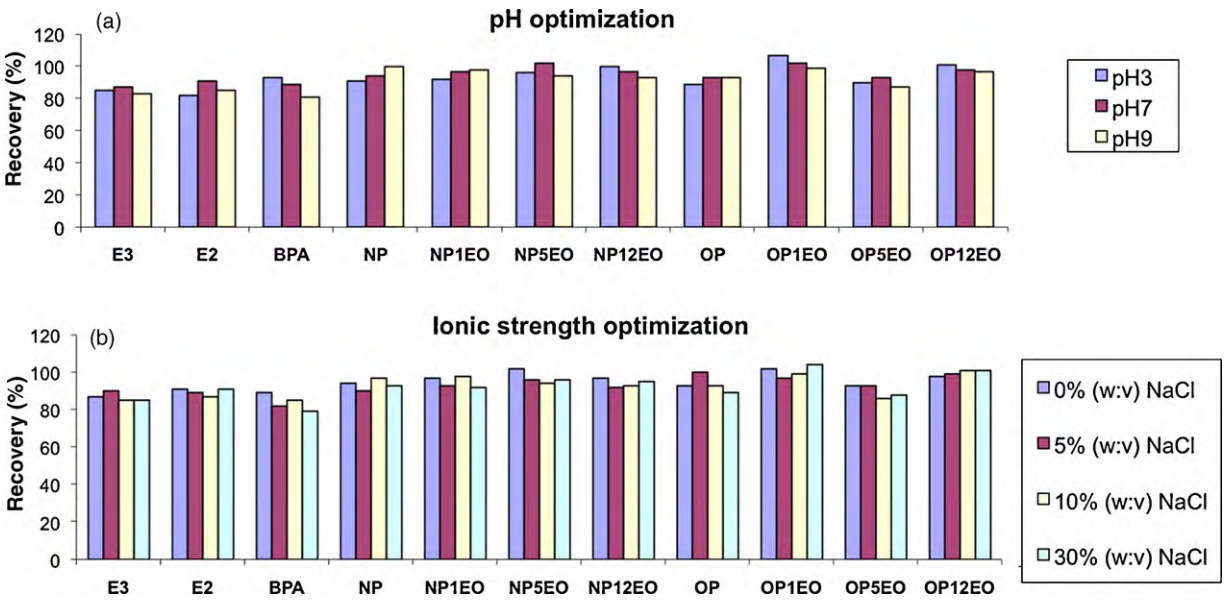

Fig. 2. Relative extraction efficiencies at several pH (a) and ionic strength (b) values for estriol, 17β-estradiol, bisphenol-A, nonylphenol, NP_1EO, NP_5EO, $NP_{12}EO$, octylphenol, OP_1EO, OP_5EO and $OP_{12}EO$.

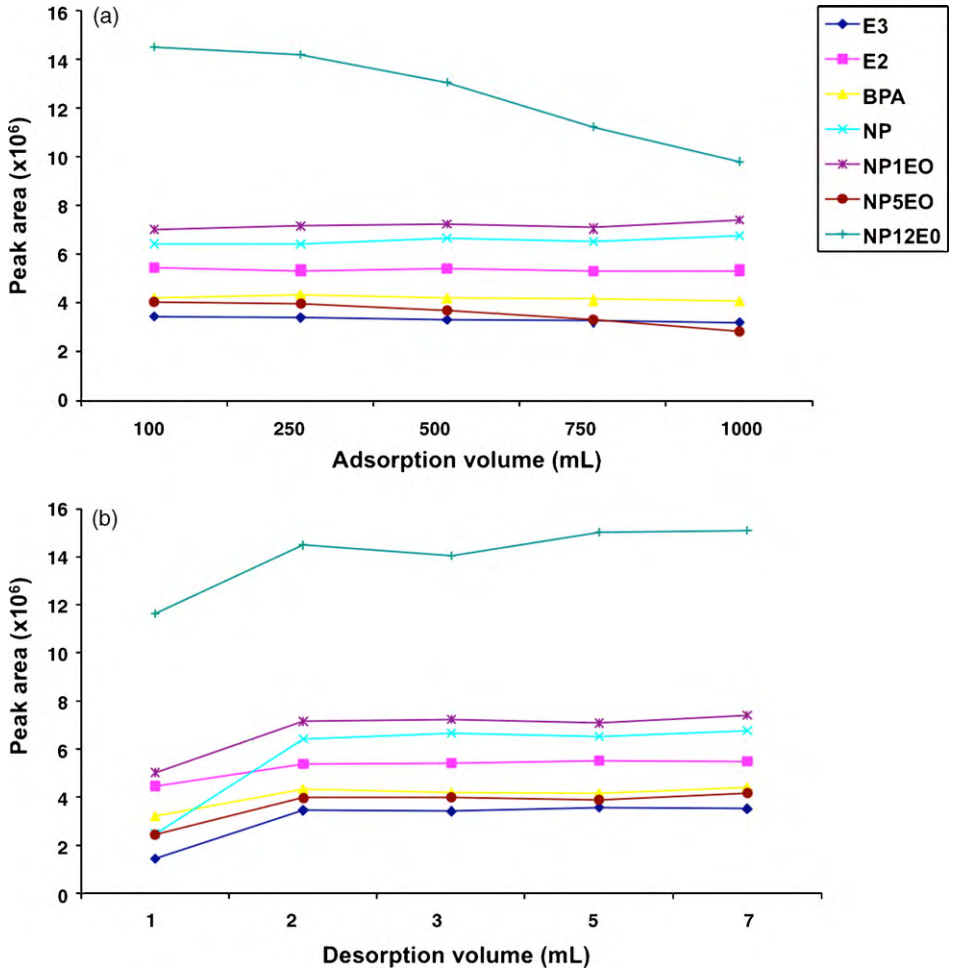

Fig. 3. (a) Normalized peaks areas obtained for different volumes of Milli-Q water samples. A volume of 2 mL of methanol was employed for desorption of analytes. (b) Normalized peak areas obtained for extraction of 250 mL of Milli-Q water samples spiked with 500 ng L^{-1} of each analyte using different desorption volumes.

ENV, recoveries obtained under the initial conditions were inferior for all analytes studied. Typically, large volumes of organic solvents such as dichloromethane, trichloromethane, hexane, ethyl acetate or acetone are required for the elution of adsorbed compounds from this kind of adsorbents [28]. The use of large volumes of these highly toxic organic solvents generally leads to the inclusion of an evaporation step to allow the final extract to be compatible with the mobile phase and analytical instrument (e.g., LC/MS^n interfaces) or to reduce the injection volume and increase the preconcentration factor [28]. This additional step lengthens analysis time, increases the final cost and leads to the loss of volatile analytes, which affects the quality of the results.

On the basis of the results presented in this section, the Sep-Pak Vac C_{18} (500 mg) cartridge was selected for the analysis of the selected compounds.

3.2.2. pH and ionic strength

Fig. 2 shows the effects of pH (a) and ionic strength (b) on extraction recoveries of EDCs using Sep-Pak Vac C_{18} (500 mg) cartridges. The results revealed that a pH between 3 and 9 did not significantly affect extraction recovery [29,30]. Thus, a pH between 6 and 7.5 was selected as the sample pH for SPE.

The effect of ionic strength on the recovery of EDCs was performed by the addition of 0% to 30% (w/v) sodium chloride to the aqueous medium. The results show that an increase in ionic strength did not produce an increase in the signals of EDCs. Thus, a sample solution was used without salt additions for subsequent experiments.

3.2.3. Sample volume

The effect of sample volume between 100 and 1000 mL on signal intensity was evaluated. To compare the signals with the same concentration (500 ng L^{-1}) of analyte, the data was normalised and preconcentration was applied in each case. Two millilitres of methanol was employed for the desorption of analytes. Fig. 3a shows the normalised signals for selected volumes of aqueous samples.

Similar responses between 100 and 250 mL were observed for all analytes. However, a significant decrease in the AP$_n$EO water soluble fraction ($n > 5$) was obtained with 500 mL of sample. For the other compound, equal signal intensities were recorded for sample volumes ranging from 100 to 1000 mL.

Even though samples had been thoroughly filtered (0.45 μm), cartridges were completely saturated when 300 mL of sample was used in identical experiments on wastewater matrices (primary and secondary treatment). Based on these results, a sample volume of 250 mL was used to obtain the best analytical results.

3.2.4. Wash step and desorption volume

The effect of the wash solution composition on extraction recovery was also evaluated. The proper elution of analytes is significant because a selective extraction and thorough sample cleaning allows for minimal ion suppression effects in the ESI interface [31]. The percentage of methanol was varied (0% (v/v), 1% (v/v), 5% (v/v), 10% (v/v), and 20% (v/v)) in 5 mL of Milli-Q water and the resultant solutions were used to elute the analyte. Equal signal intensities were obtained with the use of 0% (v/v) to 5% (v/v) methanol for all compounds; however, a decrease in signal intensity of the most polar compounds was observed with 10% (v/v) methanol and was most significant with estriol and the water soluble fraction of AP$_n$EOs ($n > 5$). Thus, 5 mL of a Milli-Q water/methanol (5% (v/v)) solution was selected for the wash step.

Desorption volume was optimised to ensure the complete extraction of analytes. Volumes of methanol between 1 and 7 mL were tested. Fig. 3b shows normalised peaks obtained for EDCs with different volumes of wash solution. Similar responses were observed for desorption volumes of 2–7 mL; however, 1 mL of methanol was clearly insufficient for proper elution of the analytes. Thus, a desorption volume of 2 mL of methanol was chosen to obtain the highest preconcentration possible.

In summary, the optimal conditions for the extraction of the target compounds included a Sep-Pak Vac C_{18} (500 mg) cartridge with a sample volume of 250 mL at a pH between 6 and 7.5, a wash step with 5 mL of Milli-Q water/methanol (5%, v/v) and a desorption volume of 2 mL of methanol. Under these conditions, a preconcentration factor of 125 was obtained.

Table 3
Evaluation of the analyte signal suppression in the primary and secondary treatments and final effluent.

Compound	Primary treatment[a] (%)	Secondary treatment[a] (%)	Final effluent[a] (%)
NP$_1$EO	14.3	15.2	10.6
NP$_2$EO	12.1	14.5	10.8
NP$_3$EO	14.3	14.9	11.8
NP$_4$EO	14.9	15.2	10.8
NP$_5$EO	16.6	15.5	10.5
NP$_6$EO	16.6	16.7	10.4
NP$_7$EO	17.6	15.9	10.8
NP$_8$EO	17.8	15.2	11.5
NP$_9$EO	18.4	14.2	10.5
NP$_{10}$EO	18.0	14.9	11.8
NP$_{11}$EO	21.0	14.5	12.3
NP$_{12}$EO	19.6	15.2	11.8
OP$_1$EO	14.3	12.7	9.1
OP$_2$EO	13.9	12.4	10.3
OP$_3$EO	17.6	13.3	10.6
OP$_4$EO	17.0	12.5	10.5
OP$_5$EO	16.8	12.3	11.0
OP$_6$EO	17.8	12.7	11.4
OP$_7$EO	20.8	12.7	11.6
OP$_8$EO	18.6	13.6	11.1
OP$_9$EO	18.0	14.0	11.9
OP$_{10}$EO	19.4	12.5	12.6
OP$_{11}$EO	19.4	12.8	11.4
OP$_{12}$EO	20.4	13.6	11.4
NP	23.8	18.6	13.4
OP	19.7	18.1	12.9
BPA	19.0	14.9	10.3
E1	17.8	13.7	10.1
E2	16.4	14.5	11.4
E3	18.7	13.6	11.0
EE	18.4	13.9	9.9

[a] Mean of three determinations.

3.3. Matrix effects

Despite the high sensitivity and low chemical noise in LC/MS/MS systems, the sample composition has a great influence on the analyte signal [31]. Thus, a low analyte signal may be the result of co-eluting compounds that impair ionisation [13].

To evaluate the relative signal suppression in the dissolved phase, the algorithm published by Vieno et al. [32] was used and is shown in Eq. (1). The results are expressed as a percentage and presented in Table 3.

$$\frac{A_s - (A_{sp} - A_{usp})}{A_s} \times 100 \quad (1)$$

where A_s corresponds to the peak area of the analyte in pure standard solution, A_{sp} corresponds to the peak area in the spiked matrix extract, and A_{usp} corresponds to the matrix extract.

This procedure was applied to primary and secondary wastewaters, as well as to final effluent matrices. Greater matrix effects were observed during the analysis of primary treatment samples, resulting in signal suppressions from 14% to 24%. More severe signal suppression was observed for hydrophobic compounds, especially for NP, OP, AP$_{1,2}$EO, and EE. Ion effect suppressions were signifi-

cantly lower for secondary treatment samples (between 12% and 18%) and final effluent samples (between 9% and 3%). The results obtained are in agreement with those reported in similar studies [33].

3.4. Quantification and quality control

The developed method was evaluated for recovery, precision and detection limit. Calibration curves were evaluated by analysing external standard solutions in triplicate at six different concentration levels. Linearity was established from 50 ng L^{-1} to 3000 ng L^{-1}. Satisfactory linearity was obtained with correlation coefficients over 0.991 for all analytes studied. The instrumental detection limits (IDLs) were defined and determined to equal the concentration of analyte that yielded a signal to noise ratio of 3. The limit of detection for the entire method (MDLs) in different matrices was calculated with the following equation:

$$\mathrm{MDL} = \frac{\mathrm{IDL} \times 100}{R(\%) \times c} \quad (2)$$

where IDL is the instrumental detection limit, R (%) is the recovery of each analyte in the corresponding matrix and c is the preconcentration factor (125 for all matrices in the dissolved phase and 10 in the particulate phase). The results obtained are shown in Table 4.

Recovery and reproducibility were evaluated from replicate analysis in Milli-Q water, wastewaters from primary and secondary treatment, and particulate matter (n = 3). Replicate samples were spiked with 500 ng L^{-1} of the standard mixture in each matrix (dissolved phases) or 10 μg g^{-1} in approximately 0.2 g of particulate dry weight (particulate phase). The replicate samples were extracted and analysed according to the respective methodology. Reproducibility was expressed as the relative standard deviation (%

Table 4
Method detection limits obtained in the different matrices: MDL_w in Milli-Q water, MDL_{pt} in primary treatment, MDL_{st} in secondary treatment, and MDL_{pm} in particulate matter (primary treatment).

Compound	MDL_w (ng L^{-1})	MDL_{pt} (ng L^{-1})	MDL_{st} (ng L^{-1})	MDL_{pm} (ng g^{-1})
NP_1EO	1.2	3.5	3.2	6.2
NP_2EO	0.5	1.5	1.4	3.1
NP_3EO	0.6	1.6	1.6	2.8
NP_4EO	0.7	2.0	1.9	3.4
NP_5EO	1.8	5.4	4.7	8.2
NP_6EO	0.3	0.9	0.7	1.4
NP_7EO	1.1	3.2	2.8	4.6
NP_8EO	1.1	3.0	3.0	4.2
NP_9EO	0.9	2.7	2.6	3.7
$NP_{10}EO$	1.6	4.3	4.4	6.0
$NP_{11}EO$	0.5	1.3	1.4	1.5
$NP_{12}EO$	0.5	1.3	1.3	1.7
OP_1EO	2.1	7.2	6.0	12.7
OP_2EO	1.2	3.7	3.5	6.6
OP_3EO	0.9	2.6	2.4	3.8
OP_4EO	1.3	3.9	3.7	5.8
OP_5EO	1.6	4.5	4.7	6.8
OP_6EO	1.3	3.6	3.7	5.1
OP_7EO	0.8	2.2	2.3	2.6
OP_8EO	0.7	2.0	1.9	2.7
OP_9EO	1.1	2.9	2.8	3.8
$OP_{10}EO$	1.2	2.9	3.4	3.8
$OP_{11}EO$	1.4	3.8	3.9	5.0
$OP_{12}EO$	1.0	2.7	2.7	3.4
NP	1.3	2.7	2.7	5.1
OP	1.8	5.7	5.5	9.4
BPA	1.9	5.7	5.2	9.8
E1	1.3	4.0	3.9	5.7
E2	1.2	3.3	2.8	5.1
E3	1.3	3.7	3.3	6.9
EE	0.9	2.8	2.6	5.0

Table 5
Recovery percentages and RSD (%) (n = 3) for different matrices spiked with 500 ng mL^{-1} (dissolved phase) or 10 μg g^{-1} (particulate phase from primary treatment samples) of each analyte.

Compound	Milli-Q waterRecovery (%)	Primary treatmentRecovery (%)	Secondary treatmentRecovery (%)	Particulate matterRecovery (%)
NP_1EO	97 ± 10	85 ± 5	93 ± 7	71 ± 9
NP_2EO	96 ± 11	86 ± 2	89 ± 5	60 ± 9
NP_3EO	96 ± 12	94 ± 6	91 ± 6	71 ± 8
NP_4EO	98 ± 8	86 ± 11	93 ± 6	74 ± 7
NP_5EO	102 ± 8	84 ± 10	95 ± 7	82 ± 6
NP_6EO	101 ± 7	83 ± 6	102 ± 5	82 ± 7
NP_7EO	98 ± 9	86 ± 1	97 ± 6	87 ± 9
NP_8EO	97 ± 6	92 ± 2	93 ± 10	88 ± 5
NP_9EO	101 ± 6	84 ± 10	87 ± 8	91 ± 6
$NP_{10}EO$	95 ± 7	94 ± 7	91 ± 3	89 ± 8
$NP_{11}EO$	96 ± 8	98 ± 8	89 ± 7	104 ± 9
$NP_{12}EO$	97 ± 7	94 ± 9	93 ± 7	97 ± 6
OP_1EO	102 ± 13	73 ± 2	88 ± 8	71 ± 10
OP_2EO	108 ± 11	82 ± 5	86 ± 11	69 ± 12
OP_3EO	99 ± 12	85 ± 6	92 ± 9	87 ± 9
OP_4EO	92 ± 9	84 ± 6	87 ± 5	84 ± 5
OP_5EO	93 ± 9	88 ± 7	85 ± 8	83 ± 7
OP_6EO	98 ± 8	91 ± 7	88 ± 6	88 ± 6
OP_7EO	100 ± 7	93 ± 7	88 ± 9	103 ± 14
OP_8EO	104 ± 8	89 ± 8	94 ± 3	92 ± 6
OP_9EO	100 ± 7	95 ± 11	97 ± 4	95 ± 8
$OP_{10}EO$	99 ± 6	101 ± 5	87 ± 4	96 ± 9
$OP_{11}EO$	94 ± 7	91 ± 7	89 ± 5	96 ± 8
$OP_{12}EO$	98 ± 8	91 ± 8	94 ± 11	101 ± 8
NP	94 ± 12	82 ± 14	84 ± 12	67 ± 7
OP	97 ± 9	79 ± 12	82 ± 13	76 ± 7
BPA	93 ± 13	82 ± 10	91 ± 7	73 ± 7
E1	91 ± 7	81 ± 7	84 ± 9	88 ± 6
E2	91 ± 8	91 ± 6	89 ± 5	81 ± 8
E3	87 ± 7	88 ± 6	83 ± 7	67 ± 9
EE	96 ± 11	79 ± 7	85 ± 7	71 ± 8

Table 6
Concentrations of each analyte in sewage from WWTP: C_{pt} in primary treatment, C_{st} in secondary treatment, C_{fe} in final effluent, and C_{pm} in particulate matter (primary treatment).

Compound	C_{pt}[a] ($ng\,L^{-1}$)	C_{st}[a] ($ng\,L^{-1}$)	C_{fe}[a] ($ng\,L^{-1}$)	C_{pm}[a] ($\mu g\,g^{-1}$)
NP_1EO	170.9	278.8	274.3	2.6
NP_2EO	188.0	154.4	161.7	2.9
NP_3EO	305.2	140.9	135.2	2.8
NP_4EO	413.1	51.8	42.8	0.9
NP_5EO	485.2	42.6	41.3	1.1
NP_6EO	615.4	47.8	41.3	0.3
NP_7EO	927.2	34.0	29.8	0.5
NP_8EO	1097	33.0	27.3	0.5
NP_9EO	981.1	25.7	19.4	0.5
$NP_{10}EO$	890.0	27.0	21.5	0.4
$NP_{11}EO$	759.2	27.5	20.5	0.4
$NP_{12}EO$	589.0	24.7	16.1	0.3
OP_1EO	41.92	58.6	51.8	0.2
OP_2EO	205.7	10.3	9.2	0.3
OP_3EO	216.9	9.2	9.1	0.4
OP_4EO	113.8	10.9	9.9	0.2
OP_5EO	68.3	8.6	8.5	32.8[c]
OP_6EO	39.2	8.0	7.0	18.8[c]
OP_7EO	28.0	7.2	6.3	12.3[c]
OP_8EO	29.7	7.6	7.2	10.7[c]
OP_9EO	33.8	7.0	7.0	16.2[c]
$OP_{10}EO$	23.6	5.1	5.0	11.3[c]
$OP_{11}EO$	17.0	4.9	4.9	8.1[c]
$OP_{12}EO$	9.8	4.3	4.2	4.7[c]
NP	9.7	18.7	17.5	0.8
OP	6.1	9.3	8.6	0.2
BPA	13.4	7.8	6.4	0.4
E2	16.3	5.0	3.5	0.4
E1	15.5	13.4	11.6	0.5
E3	22.2	18.7	16.1	0.4
EE	9.3	n.d.[b]	n.d.[b]	0.1

[a] Mean of three determinations.
[b] Not detected.
[c] $\times 10^{-3}$.

RSD) and RSDs lower than 14% were achieved for all EDCs. Recoveries into different matrices were tested for each analyte and are displayed in Table 5.

3.5. Determination of EDCs in sewage samples

3.5.1. Dissolved phase concentrations

To demonstrate that the proposed method can be used to adequately quantify EDCs in actual environmental samples, the methodology was applied to sewage samples from a wastewater treatment plant of Las Palmas of Gran Canaria (Spain). Dissolved phase concentrations are presented in Fig. 4. Fig. 4a shows the results of NP and 1–12 ethoxylate concentrations in different treatment stages.

The total dissolved concentration of NP and $NP_{1-12}EO$ was reduced from 7.60 $\mu g\,L^{-1}$ in the primary treatment to 0.84 $\mu g\,L^{-1}$ in the final effluent (Table 6 shows the concentration of single compounds). Thus, an 89% decrease in total NP was observed. However, the relative composition of the homologous mixture was enriched in short-chained $NP_{1-2}EO$ and NP as the treatment progressed, which is in agreement with the results of other publications [29,33]. This phenomenon is a direct consequence of AP_nEOs breakdown in WWTPs, especially during biological treatments. These compounds progressively lose ethoxylate units, which results in the formation of APs (a raw material for microorganisms), short-chain APEOs, and other biotransformation products such as carboxylated and halogenated derivatives [19].

The total dissolved OP and $OP_{1-12}EO$ concentration showed a similar biotransformation pattern to nonylphenol ethoxylates (NPEOs) family. A reduction of approximately 84% (from 0.83 $\mu g\,L^{-1}$ to 0.13 $\mu g\,L^{-1}$) was observed in the waste water treatment process (Fig. 4b). Nonetheless, only OP and OP_1EO showed a slight increase in concentration as the treatment progressed. Differences between NP_nEO and OP_nEO concentrations can be attributed to the global production of non-ionic surfactants, as nonylphenolic compounds represent approximately 80% of AP_nEOs worldwide production [3].

The absence of a mass balance between $AP_{0-12}EO$ concentrations in the primary treatment and the biotransformation products in the final effluent could be explained by two distinct processes. Logarithmic values of octanol/water partition coefficients ($\log K_{ow}$) for $AP_{1-4}EO$ metabolites are between 3.90 and 4.48, suggesting that these substances might become associated with organic matter in particulate matter and sediment [4]. Additionally, AP_nEOs under aerobic conditions are susceptible to oxidation to more polar short-chain and long-chain AP ethoxycarboxylate (APEC) and carboxylated AP ether carboxylate (CAPEC) derivatives [19]. It has been reported that short-chain $AP_{1-2}ECs$ account for the majority of AP_nEO-related compounds, especially in secondary and final effluents, where 80% of AP_nEOs exist as $AP_{1-2}ECs$ in the dissolved phase [26,34].

Fig. 4c shows the concentrations of BPA and steroidal hormones during the treatment process. Bisphenol-A concentration was reduced by approximately 47%, from 13.4 $ng\,L^{-1}$ in the primary treatment to 6.36 $ng\,L^{-1}$ in the final effluent.

Throughout all stages of treatment, steroidal hormones were present at low $ng\,L^{-1}$ levels. 17β-estradiol (E2) showed an efficient biodegradation during the activated sludge treatment, reflected in the rapid decline in concentration from the primary treatment (16.3 $ng\,L^{-1}$) to the final effluent (3.45 $ng\,L^{-1}$). This result is in agreement with those of previous publications [35,36]. Concentrations of E1 (from 15.54 $ng\,L^{-1}$ in the primary treatment to 11.57 $ng\,L^{-1}$ in the final effluent) and E3 (from 22.25 $ng\,L^{-1}$ to 16.12 $ng\,L^{-1}$) remained relatively constant throughout the treatment, which can be explained by the continuous enrichment of these metabolites by E2 biotransformation. A small decline in the concentration of E2 and E3 may in part be due to the affinity of these metabolites to organic matter present in sediments and particulate matter (E3 has a $\log K_{ow}$ of 2.81 and E1 has a $\log K_{ow}$ of 3.43). This affinity is particularly plausible for 17α-ethynylestradiol (EE), which was found in primary treatment samples (9.34 $ng\,L^{-1}$) but was not observed in the secondary treatment and final effluent. However, EE concentrations were determined throughout all stages of treatment for particulate phase samples.

3.5.2. Particulate phase concentrations

Concentrations of EDCs in the particulate phase (Table 6) demonstrated the tendency of hydrophobic compounds to bind tightly to particulate matter and sediments. In Fig. 5, particulate phase concentrations in different stages of wastewater treatment are highlighted. The concentration profiles of AP_nEOs homologues in the particulate phase were similar to the profiles of dissolved compounds, except that short-chain AP_nEOs were present in higher proportions (Fig. 5a and b). In primary treatment samples, more than 60% of short-chain AP_nEOs ($n < 3$) were associated with particulate matter. Moreover, more than 80% of total NP and more than 60% of total OP were found in the particulate phase. The partitioning of target compounds between the dissolved and particulate fraction of the sample was similar for each treatment stage and only the relative concentrations of the analytes varied between stages.

BPA and steroidal hormones were also found in the particulate phase (Fig. 5c). The partitioning of these compounds between the particulate and dissolved phase was significant, especially for hydrophobic compounds. Up to 60% of total BPA and steroidal hormones was present in the particulate phase.

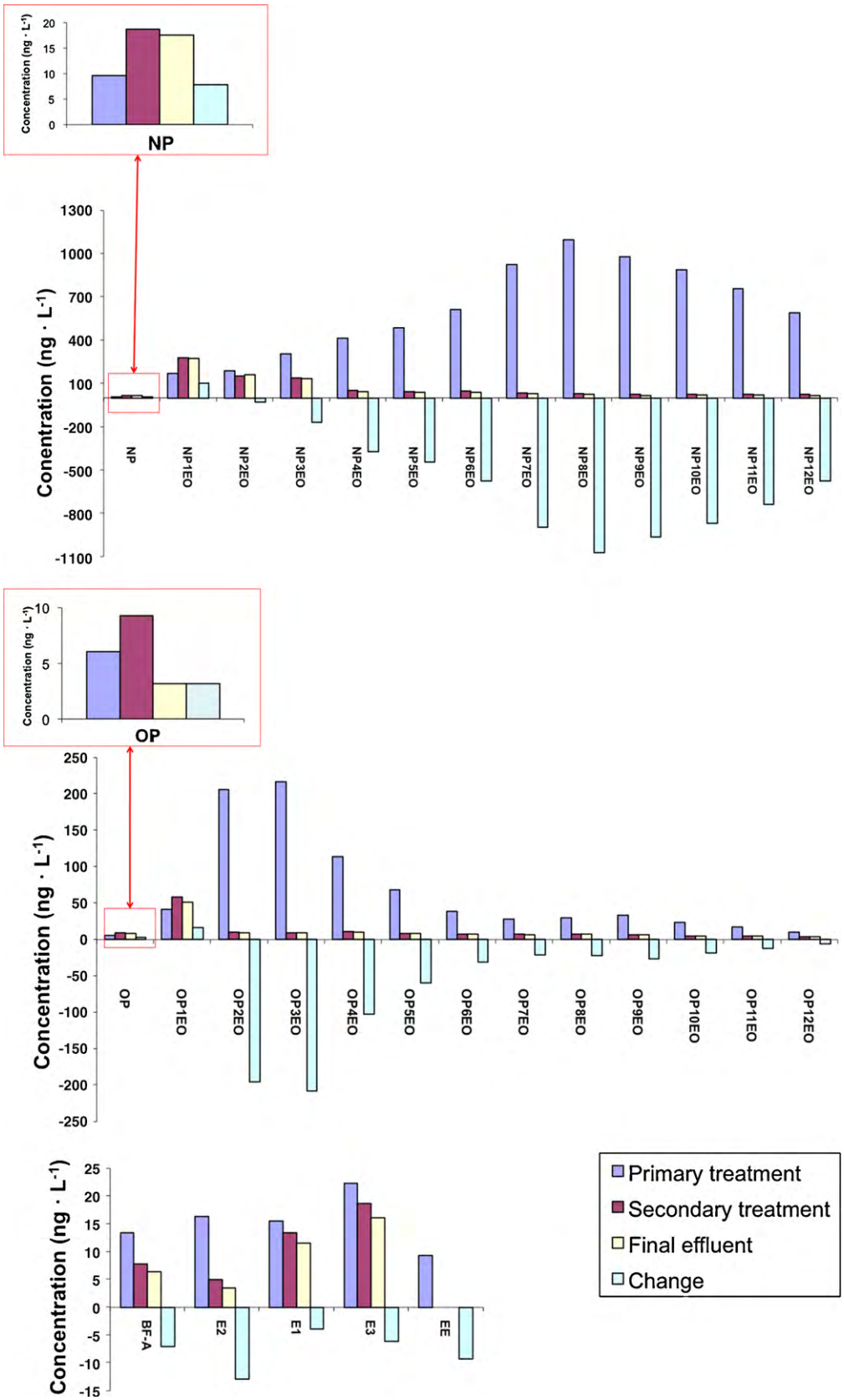

Fig. 4. Dissolved phase concentrations of NPEOs (a), OPEOs (b), BPA, and steroidal hormones (c) in primary treatment, secondary treatment, and final effluent with the change in concentrations during the process highlighted.

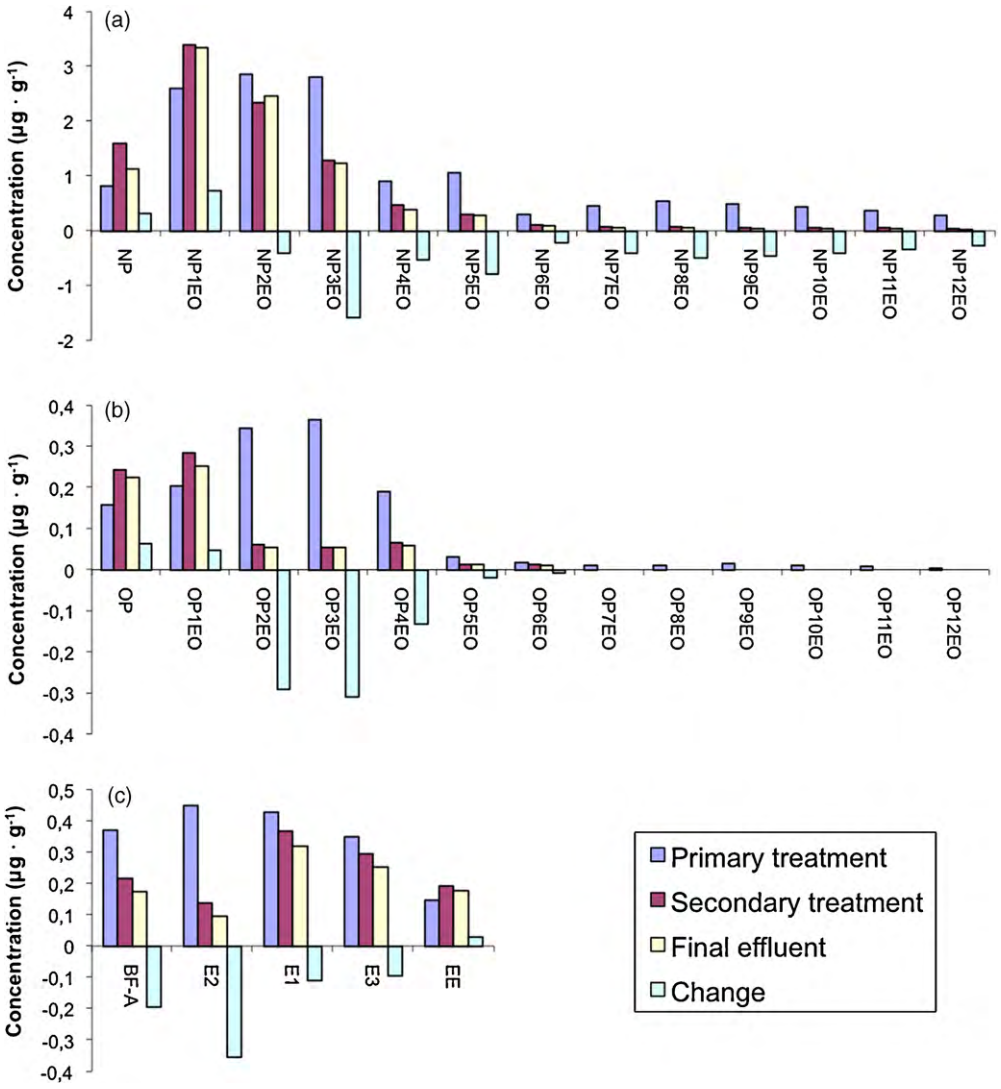

Fig. 5. Particulate phase concentrations of NPEOs (a), OPEOs (b), BPA, and steroidal hormones (c) in primary treatment, secondary treatment, and final effluent with the change in concentrations during the process highlighted.

4. Conclusions

In this work, an analytical method for the simultaneous extraction, identification, and quantification of nonylphenol, octylphenol and corresponding ethoxylates (1–12), bisphenol-A, 17α-ethynylestradiol, and 17β-estradiol and two of its metabolites (estriol and estrone) from wastewater matrices has been developed and applied to sewage samples. An SPE method (dissolved phase) coupled with LC/MS/MS allows for rapid extraction and analysis, offering the low detection limits (from 0.5 ng L^{-1} to 6 ng L^{-1} in MRM mode) and high selectivity required to detect these analytes in complex environmental matrices. Ultrasonic extraction of the particulate phase with methanol has several advantages including low desorption volumes and fast extraction times. Moreover, recoveries between 60% and 104% with a RSD lower than 14% and detection limits from 1.4 ng g^{-1} to 12.7 ng g^{-1} were obtained.

The application of the methodology to samples from a WWTP revealed that almost all of the target analytes were present in every stage of wastewater treatment, with concentrations on the order of ng L^{-1} or µg g^{-1} in the dissolved and particulate phase, respectively. Moreover, the results show that a complex mixture of endocrine-disrupting compounds occurs in the effluent of the waste water treatment plant under study, and that this mixture will eventually enter the environment where aquatic organisms are exposed to these pollutants.

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