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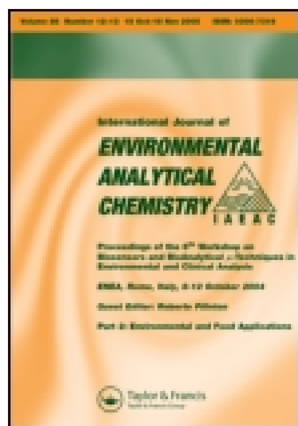

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Development and optimisation of a GC-MS method for the evaluation of oestrogens and persistent pollutants in river and seawater samples

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Development and optimisation of a GC-MS method for the evaluation of oestrogens and persistent pollutants in river and seawater samples

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This paper describes the development and validation of a GC-MS method which allows the simultaneous quantification of 11 endocrine disrupting compounds (EDCs) in surface water samples from both estuary and sea. The analysed EDCs are oestrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2), 4-tert-octylphenol, 4-n-octylphenol, 4-nonylphenol, bisphenol A and finally, mono and diethoxylates of 4-nonylphenol and 4-octylphenol. The method includes the pre-concentration of water samples, 1000-fold factor, in OASIS HLB cartridges by solid phase extraction, the derivatisation of all EDCs by *N,O*-bis(trimethylsilyl)trifluoroacetamide added with 1% trimethylchlorosilane and pyridine (at 65°C for 30 min) and, finally the stabilisation of the EDCs-silylated derivatives, in hexane, for 72 h. The validation parameters revealed that this method was highly specific for all target compounds using real samples. The linearity of the calibration curves (r^2) showed correlation factors higher than 0.990. The detection limits ranged from 0.10 to 1.45 ng L^{-1}, depending on each analysed compound, and recoveries were satisfactory for most of the assayed EDCs (>60%). Analysis of samples from four polluted areas of Douro River estuary and from two points of the Atlantic Ocean (Portugal) showed high amounts of E1 (up to 1.96 ng L^{-1}), E2 (up to 14.36 ng L^{-1}) and EE2 (up to 2.76 ng L^{-1}).

Keywords: oestrogens; alkylphenols; alkylphenol ethoxylates; bisphenol-A; endocrine disrupting compounds (EDCs); estuarine and sea water

1. Introduction

Endocrine disrupting compounds (EDCs) are exogenous substances able to interfere with the normal functioning of the endocrine system of both aquatic and terrestrial animals [1]. Exposure to sex hormones, either of natural (oestrone, E1 and, 17β-estradiol, E2) or synthetic origins (17α-ethynylestradiol, EE2) induce, even at low ng L^{-1} levels, vitellogenin [1,2], intersex and feminisation of male fish [3,4]. Lately, these compounds have been linked to diverse reproductive and immune system disorders in humans making them

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important targets in environmental monitoring programmes [5,6]. Nonetheless, beyond hormones, other anthropogenic compounds, the alkylphenol polyethoxylates (APEOs) and the bisphenol A (BPA), arrive continuously into the environment sometimes in $\mu g\,L^{-1}$ levels [7,8]. The APEOs, especially the branched-chain nonylphenol (NPEOs) and octylphenol (OPEOs), constitute environmental persistent pollutants due to their slow, and frequently incomplete, biodegradation [9]. This occurrence leads to the formation of completely deetoxylated nonylphenol and octylphenol, which are more lipophilic and toxic than their parents [9]. Besides, they also have the ability to remain in the aquatic compartments for a long time [8–10]. These observations, together with the APEOs ability to mimic or antagonise the synthesis, hormonal metabolism and/or modify receptor levels of aquatic animals [11] conducted to their inclusion in the group of '*priority substances in the field of water policy* (Decision No. 2455/2001/EC)' [12]. Presently, it is well known that the simultaneous measurement of oestrogens, bisphenol A, APEOs and their metabolites are difficult due to their distinct physicochemical properties [13]. In addition, environmental water matrices, as those coming from polluted estuaries and sea, may well increase the difficulty of analysing the above referred compounds [13]. Furthermore, the measurement of all target compounds involves the usage of derivatisation reagents to generate volatile EDCs-trimethylsylil derivatives (EDCs-TMS) [14]. In general, phenols and APEOs are usually derivatised with *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane (BSTFA, 1% TMCS) whereas oestrogens are preferentially derivatised with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) [15,16]. Consequently, the majority of the methods are generally committed to analyse only one, maximum two families, of the present compounds [15–18].

Taking into account the above concerns, the main purpose of this study was to develop and validate a gas chromatography-mass spectrometry (GC-MS) technique able to quantify simultaneously E1, E2, EE2, 4-tert-octylphenol (4-t-OP), 4-n-octylphenol (4-OP), 4-nonylphenol (4-NP), BPA and finally, mono and diethoxylates of 4-nonylphenol (NP1EO and NP2EO) and 4-octylphenol (OP1EO and OP2EO) in estuarine and sea water samples. The selection of the target compounds is supported by their worldwide occurrence and persistence in aquatic environment [1,7,8], on the knowledge that some grey mullets, *Mugil cephalus*, caught from Douro River estuary show ovotestis [19] and on the fact that this area is considerably polluted [20]. To test the effectiveness of the developed GC-MS method, for the quantification of the selected EDCs several surface water samples, taken either from Douro River estuary or the coastal area located nearby (Atlantic Ocean), were analysed. This data is the first of this kind ever done in the vast Portuguese coast.

2. Experimental

2.1 *Chemicals and materials*

Analytical grade solvents including hexane, ethyl acetate, methanol and, pyridine were purchased by Sigma-Aldrich (Steinhein, Germany). Ultrapure water was supplied by a Milli-Q water system. The cartridges used for solid-phase extraction were 200 mg Oasis HLB (Hydrophilic-Lipophilic Balance), 6 cc, from Waters Corporation (Milford, MA, USA) and 0.45 μm glass fibre filters were purchased from Millipore (Ireland).

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2.2 *Reference standards*

Oestrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2), 17β-estradiol-d_2 (E2-d_2), 4-t-octylphenol (4-t-OP), 4-n-octylphenol (4-OP), bisphenol A (BPA), bisphenol A-d_{16} (BPA-d_{16}), Igepal CA-210 (4-octylphenol monoethoxylate, OP1EO and, 4-octylphenol diethoxylate, OP2EO) and Igepal CO-210 (4-nonylphenol monoethoxylate, NP1EO and 4-nonylphenol diethoxylate, NP2EO) were obtained from Sigma-Aldrich (Steinhein, Germany), whereas 4-n-nonylphenol (4-NP) was supplied from Riedel-de-Haën (Seelze-Hannover, Germany). Stock solutions of individual standards ($1\,g\,L^{-1}$) were prepared in methanol, transferred to amber bottles and stored in the dark at $-20°C$ to minimise their potential decay. All standard solutions were stable and evidence of decomposing was never observed. Working solutions were prepared diluting the stock solution with methanol. From the stock solutions six nominal calibration standard mixtures were prepared and spiked in both estuary and sea water matrices [21]. Fortified matrices were used as calibration standards and to demonstrate the applicability of the method. The range of concentrations added to water matrices were: 10–500 $\mu g\,L^{-1}$ for 4-t-OP, 4-OP, NP1EO, NP2EO, E1, E2, EE2, 10 – 100 $\mu g\,L^{-1}$ for 4-NP, OP1EO, BPA, OP2EO, 90 $\mu g\,L^{-1}$ for E2-d_2 and 75 $\mu g\,L^{-1}$ for BPA-d_{16} (surrogate internal standards, IS). Similarly, for precision, accuracy and recovery assays three quality control (QC) standard solutions containing each EDC and IS were prepared: 10, 100, 500 $\mu g\,L^{-1}$ for 4-t-OP, 4-OP, NP1EO, NP2EO, E1, E2, EE2 and 10, 50, 100 $\mu g\,L^{-1}$ for 4-NP, OP1EO, BPA, OP2EO. The calibration curves were produced using standard/IS ratios *vs.* the above referred standard concentrations ($\mu g\,L^{-1}$).

2.3 *Sample collection and preparation*

Water samples were taken from Douro River estuary (Samples 1 to 4) and Atlantic Ocean (Samples 5 and 6) (Figure 1). These were collected in low tide during March 2009 into 2.5 L amber glass bottles, which were rinsed in the laboratory with ultrapure water and later, on site, with water sample. Surface waters were sampled from a depth of approximately 1 m using a peristaltic pump (Global Water, Model: WS 3000,

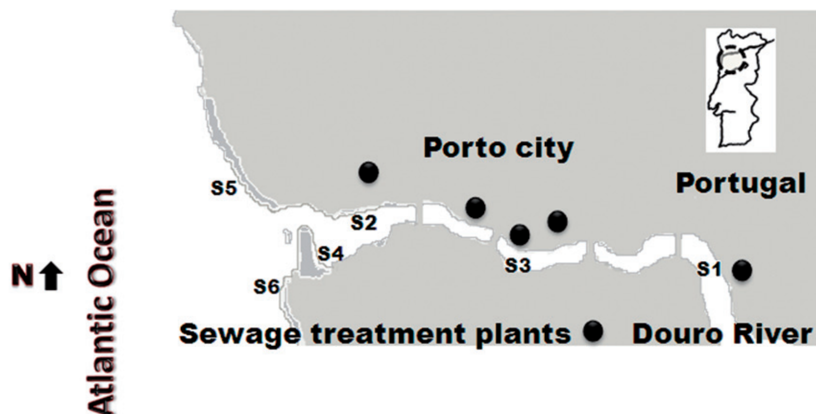

Figure 1. Map of Douro River estuary and Atlantic Ocean (Portugal) with the location of each sampling areas (Sites 1 to 6) and of the sewage treatment plants.

California, USA). Temperature and pH were measured immediately after collection using a Consort C868 electrochemical apparatus. After sampling, all water was kept refrigerated (±4°C), transported in the dark to the laboratory and immediately vacuum filtered through 0.45 μm glass fibre filters to remove suspended particles. Each filter was washed with approximately 2 mL of methanol and this volume was added to the filtrate. All samples were adjusted to pH 5 with few drops of H_2SO_4 (conc.) and maintained at ±4°C in dark until extraction. The last step occurred, always, within a maximum of 48 h after sampling.

2.4 *Solid-phase extraction*

All target EDCs were extracted, from both fortified water matrices and real water samples, by solid phase extraction (SPE) using OASIS HLB cartridges adapted in an off-line SPE vacuum extraction device (Waters). The breakthrough volume, pH adjustment, washes and elution conditions followed a method initially developed to extract phenolic compounds and steroids in water [16]. In this study, the last method was broadened for the analysis of 4-OP and alkylphenol ethoxylates (NP1EO, NP2EO, OP1EO, OP2EO). Briefly, the condition step was carried out with 5 mL of ethyl acetate, to remove residual bonding agents, followed by 5 mL of methanol and 3 × 5 mL of ultrapure water, at a flow rate of 1mL min^{-1}. Spiked water samples and surface water matrices (500 mL) added with the above referred IS, were loaded onto SPE cartridges at a constant flow rate of 5 mL min^{-1} followed by a washing step with 10 mL of ultrapure water and methanol (9:1). Cartridges were dried under vacuum for 30 min and then eluted with 10 mL of ethyl acetate, at 1 mL min^{-1}. The resulting extracts were evaporated to dryness in a heating block at 40°C under a gentle stream of nitrogen and reconstituted in 500 μL of anhydrous methanol.

2.5 *Derivatisation procedure*

Due to the low volatility of the majority of the present compounds, which gave rise to poor chromatographic peaks, derivatisation was essential [14–16]. In this step 50 μL of each SPE extracted fractions, containing the studied compounds in mixture, were transferred into GC vials and evaporated at 40°C to dryness under a gentle nitrogen stream. Fifty μL of pyridine were added to the dry residues which were derivatised by the addition of 50 μL of BSTFA (1% TMCS) or MSTFA. In all conditions, the vials were mixed using a vortex system and heated, in a heating block, for 15 min, 30 min and 45 min at 60°C, 65°C and 70°C to test the best derivatisation conditions. TMS- derivatives were further evaporated to dryness under a gentle nitrogen stream, reconstituted with 100 μL of hexane and subjected to GC-MS analysis; initially the same procedure was applied to individual compounds. The stability of the TMS-derivatives was evaluated by analysing aliquots of the same samples immediately after derivatisation and then after 12, 24, 48 and 72 h at room temperature (*c*. 20°C).

2.6 *GC-MS analysis*

GC-MS analysis was performed using a gas chromatograph (Trace GC ultra, Thermo Finnigan Electron Corporation) coupled with an ion trap mass spectrometer

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(Thermo Scientific ITQ™ 1100 GC-MS^n), an autosampler (Thermo Scientific TriPlus™) and a TR5MS capillary column ($30\,m \times 0.25\,mm$ i.d., $0.25\,\mu m$ film thickness). Helium carrier gas (99.99% purity) was maintained at a constant flow rate of $1.0\,mL\,min^{-1}$. Column oven temperatures were programmed using several ramps: (a) from 100°C (initial equilibrium time 1 min) to 260°C at $10°C\,min^{-1}$; (b) from 260°C to 270°C at $1°C\,min^{-1}$; and, finally (c) from 270°C to 280°C at $10°C\,min^{-1}$, at this point the GC oven was maintained at 280°C for 2 min. The mass spectrum (MS) was achieved by electron impact ionisation and operated in full-scan mode from 40 to 650 (total ion chromatogram, TIC, Figure 2). For quantitative analysis the selected-ion monitoring mode (SIM) was preferred (Figure 3). A solvent delay time of 5 min was used to protect the ion multiplier of the MS instrument from saturation. Temperatures of PTV liner ranged from 35°C to 250°C via a ramp of $10°C\,s^{-1}$. Both MS transfer line and ion source were at 280°C. Sample injection ($3\,\mu L$) was programmed in splitless mode using an 80 mm injection needle.

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2.7 *Matrix effect*

The matrix effect was evaluated fortifying real water samples from estuary and sea with QC standards added with both IS at three different levels and injected in triplicate [22,23]. The ratio areas and MS spectra of standards spiked in real samples were compared with those of artificial fortified matrices and those acquired using methanol standards.

2.8 *Validation studies*

The current GC-MS method was validated following the analytical performance parameters established by international validation guidelines [24,25]. According to that, the validation process includes the evaluation of linearity, accuracy, precision, limits of detection and quantification. Artificial estuarine and sea waters were used as blank matrices (free of all target EDCs). Accuracy, intra- and inter-batch precision were evaluated analysing three replicates of each QC samples. Precision was expressed in terms of relative standard deviation (% RSD) of the replicate measurements. Accuracy was estimated as the percentage of agreement between the method results and the nominal amount of added compound [22–24]. Blank matrices, of estuarine and sea water, fortified at three QC concentrations allowed the calculation of recovery and the effectiveness of the extraction step. These values were obtained comparing the QCs concentrations, calculated after the SPE procedure, with those of equivalent QCs prepared in methanol. Limits of detection, LOD (1) and, quantification, LOQ (2), were calculated based on the standard deviation of the response and the slope of three calibration curves ($n = 3$), each one done in triplicate, using the mathematic formulas:

$$\text{LOD} = 3.3 \times (s/S) \tag{1}$$

$$\text{LOQ} = 10 \times (s/S) \tag{2}$$

Here, s is the standard deviation of y-intercepts, and S the slope of the calibration curves [22–24]. The confirmation of the target compounds identity was performed in conformity with the European regulations (EU Commission Decision 2002/657/EC) [25].

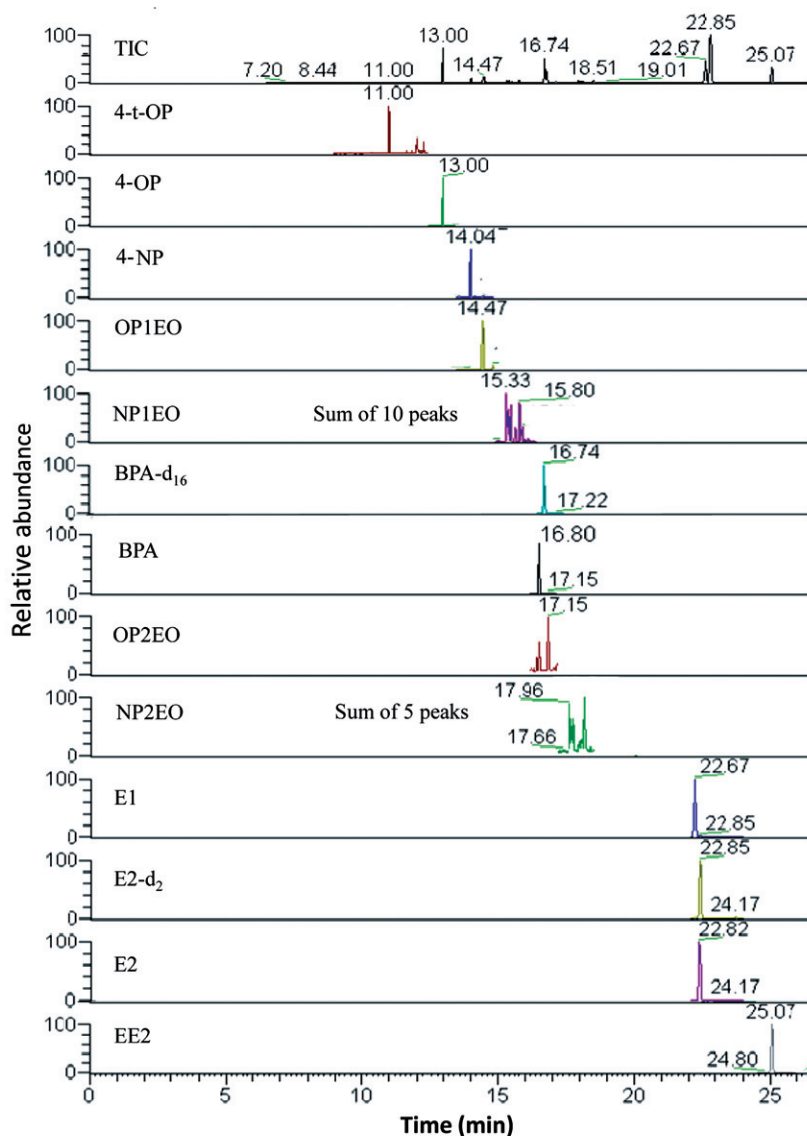

Figure 2. Chromatograms of a standard mixture of the target EDCs ($100\,\mu g\,L^{-1}$ for 4-t-OP, 4-OP, NP1EO, NP2EO, E1, E2, EE2 and $50\,\mu g\,L^{-1}$ for 4-NP, OP1EO, BPA, OP2EO) and their internal standards E2-d_2 ($90\,\mu g\,L^{-1}$) and BPA-d_{16} ($75\,\mu g\,L^{-1}$) in full-scan mode (TIC).

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3. Results and discussion

3.1 *Derivatisation procedure and GC-MS analysis*

Two derivatisation reagents, BSTFA (1% TMCS) and MSTFA were assayed in this study since there was in literature some controversy about this issue [15–17] and because the present method deals with 11 EDCs with different physicochemical properties. In this sense many different combinations of time and temperature were assayed. Finally, our

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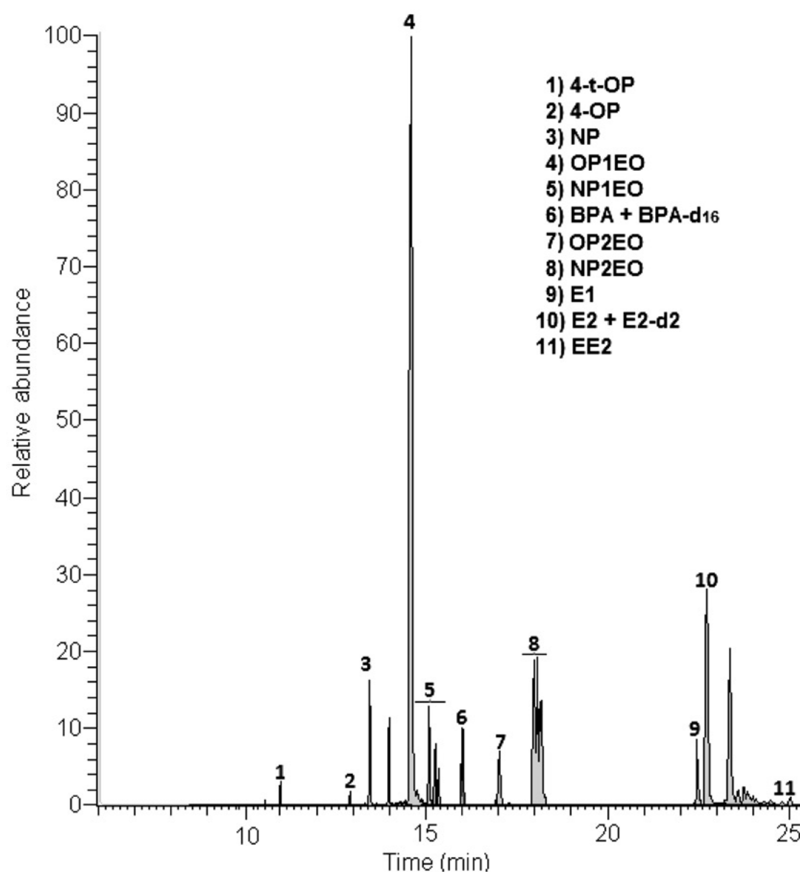

Figure 3. Chromatogram in selected-ion monitoring mode (SIM) of a blind seawater sample from S5 (1 L) spiked with the QC standard containing $25\,\mu g\,L^{-1}$ for 4-t-OP, 4-OP, NP1EO, NP2EO, E1, E2, EE2 and $15\,\mu g\,L^{-1}$ for 4-NP, OP1EO, OP2EO, BPA and their internal standards E2-d_2 ($90\,\mu g\,L^{-1}$) and BPA-d_{16} ($75\,\mu g\,L^{-1}$).

data demonstrated that the best derivatisation time and temperature were respectively, 30 min and 65°C. Using the last combination of time/temperature both BSTFA (1% TMCS) and MSTFA produced high amounts of all 11 EDCs-TMS derivatives. However, comparing the chromatograms produced by the last two derivatisation reagents BSTFA (1% TMCS) showed to be more efficient in terms of peak areas and, mainly, in peak symmetries than MSTFA. Room temperature stability assays demonstrated that the addition of hexane to dry EDCs-TMS derivatives allowed their permanence in the auto-sampler tray for 72 h at room temperature (approximately 20°C) without significant effect on the quantitative determination of the target TMS derivatives (RSD $< 11.0\%$).

GC separation was achieved evaluating different ranges of temperatures. Initially full-scan mass spectra of individual silylated EDCs (EDCs-TMS) were analysed. Then, all standards were injected in separated groups (oestrogens, phenols, and APEOs) and finally all EDCs-TMS in mixture. Finally, the SIM segments were established containing for each compound the specific ion mass-to-charge ratio (m/z) (Table 1). The selection of high mass

Table 1. Quantification and diagnostic ions used in GC-MS analysis. Text inside parentheses refers to the relative abundance of ions (*m*/*z*) for each target EDC.

Compound	t_R (min)	Molecular mass	Quantification ions (base ions, *m*/*z*)	Diagnostic ions (*m*/*z*)	Segment [time (min)]
4-t-OP	11.00	206.3	207	–	8.50–12.50
4-OP	13.00	206.3	278	–	12.50–13.50
4-NP	14.04	220.4	179	292 (38.0)	13.50–14.80
OP1EO	14.47	250.4	251	206 (67.4), 135 (49.7)	13.50–14.80
NP1EO	15.33–15.90	264.4	251	265 (3.5), 335 (1.0)	14.80–16.20
BPA-d_{16}	16.74	244.3	386	368 (38.0)	16.20–16.95
BPA	16.80	228.3	357	372 (1.0)	16.20–16.95
OP2EO	17.15	294.4	207	295 (67.4)	16.95–17.50
NP2EO	17.96–18.20	308.5	207	295 (64.0)	17.50–20.00
E1	22.67	270.4	342	257 (60.0)	20.00–23.50
E2	22.82	272.4	285	416 (32.8)	20.00–23.50
E2-d_2	22.85	274.4	287	418 (57.0)	20.00–23.50
EE2	24.60	296.4	425	285 (53.5)	23.50–26.00

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fragments associated to high percentages of *m*/*z* quantification ions is considered of great interest when complex matrices are analysed [26]. In the present study the TMS^+ ion (*m*/*z*, 73.0) was always present in all target compounds. Besides, for 4-t-OP the base peak had a *m*/*z*, 207 (abundance 100%) corresponding to $[(CH_3)_3Si\text{-}O\text{-}C_6H_4\text{-}C(CH_3)_2]^+$ [17]. 4-OP major ion was the one with *m*/*z*, 278 (abundance 100%) matching to the molecular ion chemical structure [26]. The 4-NP-TMS ion *m*/*z*, 179 (abundance 100%) was produced by the loss of $–C_8H_{17}$ group from the molecular ion m/z, 292 (abundance 38.0%) (Table 1). For BPA, the ion at *m*/*z*, 357 (abundance 100%) was attributed to the fragment $\{[(CH_3)_3Si\text{-}O\text{-}C_6H_4\text{-}C(CH_3)_2\text{-}C_6H_4\text{-}O\text{-}Si\text{-}(CH_3)_2]^+\}$, pointing to the synthesis of bis-TMS ethers at both hydroxyl groups [17]. For oestrogens, complete derivatisation of all free hydroxyl groups was accomplished as identical data was obtained by others [17]. Therefore, the mono-TMS derivative was formed for E1 as shown by the molecular ion at *m*/*z*, 342 (abundance 100%) and the ion fragment *m*/*z* 257 (abundance 60.0%) of the $[M\text{-}85]^+$ ion. For E2, in addition to the base peak of *m*/*z*, 285, the molecular ion at *m*/*z*, 416 (abundance 32.8%) was also seen (Table 1). EE2 produced di-TMS derivatives of the $[M\text{-}15]^+$ ion at *m*/*z*, 425 (abundance 100%) and ion fragment at *m*/*z*, 285 (abundance 53.5%) (Table 1). OP1EO and NP1EO produced identical base peak fragments at *m*/*z*, 251 (abundance 100%) but different ion fragments at *m*/*z*, 206 (abundance 67.4%) and 265 (abundance 3.5%), respectively. In contrast, both OP2EO and NP2EO, produced identical ion fragments at *m*/*z*, 207(abundance 100%) and identical molecular ion fragments at *m*/*z*, 295 (similar abundance rates 64.0 for NP2EO and 67.4% for OP2EO). For these two compounds the analysis of other chromatographic parameters such as the tr and the peak shapes (one peak for OP2EO and several peaks for NP2EO) were extremely important.

3.2 *Solid-phase extraction*

The sample pre-treatment was based in a previous study carried out for extraction of phenols and oestrogens [17]. Herein, the last SPE method was the optimised for the

extraction of a larger number of environmental pollutants that include other phenols and APEOs not only in river water but also in other, more complex matrices such as the estuarine and the sea water samples. All compounds showed satisfactory recovery rates (>60%), with the exception of 4-NP (approx. 50%), supporting the idea that this method can be used for SPE extraction of the present EDCs in future monitoring assays. This observation was confirmed by the analysis of two blind samples, prepared in triplicate for both matrices, containing the following concentrations which were not known by the analyst: 25, 150, 250 ng L^{-1} for 4-t-OP, 4-OP, NP1EO, NP2EO, E1, E2, EE2 and 15, 25, 50 ng L^{-1} for 4-NP, OP1EO, BPA, OP2EO. These results produced accuracies ranging from 92.3–109.0% and precision RSD values from 1.9 to 7.5%. Similar values were obtained for all assayed levels within the dynamic range of the calibration curve. One previous study report lower recovery levels for almost all compounds referred herein [13]. Those values, also lower than those referred by others measuring steroids and phenolic compounds [17], was probably due to the usage of BSTFA, in absence of TMCS and pyridine, and the absence of stabilisation of the TMS derivatives, which is fundamental when environmental matrices are analysed [17].

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3.3 *Matrix effects*

Humic substances (humic and fulvic acids) constitute the greatest part of dissolved organic matter in surface waters that generally impair the efficiency of sample extraction and the detection of the target compounds in aquatic environments [16,17]. Since, it was impossible to find water samples from estuary and sea without the presence of the current target compounds, the authors considered the usage of artificial matrices of those aquatic environments [21]. The SPE method, previously developed by others referred that the above interferences, as well as different salinities, did not affect the SPE extraction step [17]. To confirm that our matrix did not affect the last process the prepared QC standard solutions, spiked in real water samples, were analysed (Figure 3). These data confirmed that both tr (RSD < 5.0%) and ion fragmentation were not affected. Relatively to the last item all fragments were within the ranges proposed by the 2002/657/EC European Commission Decision [25], i.e. the tolerances were ±10% for ions with a relative intensity >50% of the base peak, ±15% for ions with a relative intensity of 20–50%, ±20% for ions with a relative intensity of 10–20% and ±50% for ions with a relative intensity of <10%. Also peak areas were similar when comparing QC standards spiked in water samples or in water artificial matrices. Thus, it was concluded that external calibration using artificial water matrices was possible and unaffected by innate matrix components. Consequently, EDCs present in real water samples were quantified using the SIM mode and following the equations reported in Table 2.

3.4 *Method validation parameters*

3.4.1 *Linearity*

The linearity and range of application were established by the calibration curves in ranges given at Table 2 with coefficients of correlation (r^2) values ranging from 0.990 and 0.998.

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Table 2. Analytical characteristics of the optimised GC-MS method: calibration equations, coefficients of correlation (r^2), limits of detection (LOD) and quantification (LOQ) for all EDCs spiked in both estuarine and sea water.

Compound	Linearity parameters estuarine water		$LOD_{estuary}$ ($ng\,L^{-1}$)	$LOQ_{estuary}$ ($ng\,L^{-1}$)	Linearity parameters seawater		LOD_{sea} ($ng\,L^{-1}$)	LOQ_{sea} ($ng\,L^{-1}$)
	Calibration equation	r^2			Calibration equation	r^2		
4-t-OP	$y = 0.129x - 0.060$	0.995	1.40	4.62	$y = 0.093x + 0.003$	0.995	1.20	3.99
4-OP	$y = 0.168x - 0.022$	0.992	1.40	4.62	$y = 0.157x + 0.005$	0.990	1.20	3.99
4-NP	$y = 0.277x - 0.006$	0.997	0.50	1.67	$y = 0.221x + 0.008$	0.990	0.50	1.67
OP1EO	$y = 0.249x - 0.008$	0.990	0.20	0.67	$y = 0.210x + 0.002$	0.998	0.20	0.67
NP1EO	$y = 0.072x - 0.002$	0.991	1.30	4.29	$y = 0.078x - 0.024$	0.993	1.00	3.33
BPA	$y = 0.866x + 0.011$	0.992	0.10	0.33	$y = 0.825x + 0.037$	0.997	0.10	0.33
OP2EO	$y = 0.183x - 0.002$	0.994	0.50	1.67	$y = 0.133x - 0.013$	0.993	0.50	1.67
NP2EO	$y = 0.024x - 0.002$	0.996	1.45	4.83	$y = 0.018x + 0.005$	0.996	1.00	3.33
E1	$y = 0.151x - 0.004$	0.995	0.50	1.67	$y = 0.114x - 0.021$	0.991	0.60	1.99
E2	$y = 0.136x - 0.065$	0.990	0.60	1.99	$y = 0.116x + 0.024$	0.990	0.80	2.66
EE2	$y = 0.105x - 0.002$	0.997	0.58	1.93	$y = 0.103x - 0.028$	0.994	0.61	2.03

It is important to stress that these data are in conformity with all the validation requisites used in this work [23–25]. These results are within the range of other works [17,18] with the advantage of evaluating, in the same chromatographic process, 11 important EDCs within a short analysis time.

3.4.2 *Precision and accuracy*

The precision of this method was based on the determination of the repeatability (intra-day assays) and the intermediary precision (inter-day assays) (Tables 3 and 4). In this method precision was lower than 11.0% and accuracy values ranged from 71.0% and 120.0% for all calibration concentrations in the established ranges. These data were considered as acceptable results since the values obtained encompassed the complete sample preparation and not only a consecutive sequence of injections of the same sample [23,27]. Instrumental precision (%RSD), calculated automatically by the GC-MS software, was 7.5% for E2-d_2 and 13.3% for BPA-d_{16} ($n = 50$ injections).

3.4.3 *Limits of detection and quantification*

After applying the pre-concentration factors, that were a result of the SPE process, the LODs ranged from 0.10 to 1.45 $ng\,L^{-1}$ and from 0.10 to 1.20 $ng\,L^{-1}$ for estuarine and seawater samples, respectively (Table 2). These values were considered suitable for environmental analysis when comparing the usual amounts of these EDCs in several field works with those obtained herein [18,28–31].

3.4.4 *Selectivity*

EDCs-TMS showed well resolved peaks when QC was spiked in real samples. The identity of each chromatographic peak was confirmed not only by its retention time but also by its mass spectrum. Similar results are found in other environmental validation methods [28].

3.5 *EDCs in water samples from Douro River estuary and Atlantic Ocean*

To evaluate the applicability of the current method to monitor the presence of EDCs in both estuarine and sea water, water samples from four areas in Douro River estuary (Portugal) and from two sites in Atlantic Ocean were analysed. Temperatures and pH of all sampling sites ranged from 12 to 14°C and 7.5 to 8.5, respectively. GC-MS results are summarised in Table 5. Here, almost all compounds were measured above the detection limits demonstrating the feasibility of the current method for future monitoring assays in both estuarine and sea water matrices. The oestrogenic load found in this first sampling assessment was extremely high [1] and compatible with the previous information, biologic [19] and chemical [20], previously reported in Douro River estuary. The EDCs found in the Atlantic Sea are the first of this kind ever studied in Portuguese coast.

4. Conclusions

A SPE-GC-MS method has been developed and validated for the simultaneous evaluation of 11 relevant endocrine disrupters in environmental water matrices – oestrogens

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Table 3. Intra- and inter-day precision, accuracy and recovery data for EDCs spiked in estuarine water.

EDCs spiked in estuarine matrix ($ng\,L^{-1}$)	1st day[a] Accuracy (%)	1st day[a] RSD (%)	2nd day[a] Accuracy (%)	2nd day[a] RSD (%)	3rd day[a] Accuracy (%)	3rd day[a] RSD (%)	Recovery (%)[a] (RSD %)
4-t-OP							
10	87.1	10.7	99.5	1.5	113.4	4.3	76.6 (9.4)
100	92.0	1.4	99.4	8.4	108.6	4.6	78.9 (8.3)
500	107.1	1.0	102.7	4.6	106.0	4.6	79.1 (4.6)
4-OP							
10	92.3	0.3	99.5	2.3	108.2	1.0	93.7 (8.0)
100	100.0	0.6	101.0	4.6	106.5	2.7	92.0 (3.4)
500	82.2	1.5	108.0	3.4	105.9	3.4	91.8 (10.5)
4-NP							
10	99.3	0.6	100.6	0.8	100.2	1.0	52.4 (0.7)
50	83.6	3.0	84.5	3.0	84.1	3.9	52.5 (2.4)
100	107.5	5.6	104.0	5.8	105.2	6.7	52.5 (5.6)
OP1EO							
10	108.8	2.3	119.5	4.1	96.5	3.0	68.1 (8.5)
50	107.9	0.6	96.9	1.0	95.4	3.2	68.2 (2.0)
100	86.0	1.4	86.3	1.3	85.3	1.7	68.2 (2.4)
NP1EO							
10	100.3	2.3	98.3	0.5	101.5	1.7	64.1 (1.6)
100	97.2	3.2	100.2	1.8	102.6	1.4	63.3 (2.7)
500	99.0	3.3	100.1	3.7	99.8	4.2	63.2 (4.4)
BPA							
10	70.8	0.7	84.0	7.4	77.0	4.0	72.8 (3.4)
50	104.4	5.0	99.8	0.8	99.6	0.6	72.7 (6.7)
100	80.0	3.0	94.1	5.3	89.5	3.4	72.7 (3.2)
OP2EO							
10	93.3	7.2	99.3	0.3	107.4	1.0	91.9 (7.4)
50	107.1	1.0	92.8	0.7	98.0	6.7	92.0 (1.5)
100	80.2	2.8	89.9	4.1	90.3	3.4	92.0 (1.6)
NP2EO							
10	101.3	6.0	102.0	1.9	92.8	8.7	80.4 (2.8)
100	103.3	2.3	93.5	8.4	103.2	1.9	79.8 (5.7)
500	92.6	2.0	97.8	1.9	112.2	3.4	79.8 (2.4)
E1							
10	100.0	6.0	99.8	4.4	100.2	8.9	96.7 (0.2)
100	102.6	6.8	100.7	8.4	96.7	9.0	96.1 (3.0)
500	104.6	0.6	97.1	2.3	98.4	4.3	96.1 (1.6)
E2							
10	102.0	0.8	98.2	6.4	99.8	2.7	79.7 (1.9)
100	106.9	4.7	97.5	1.5	95.7	1.5	82.2 (6.0)
500	103.0	3.4	105.5	1.8	104.5	2.4	82.4 (1.6)
EE2							
10	100.1	0.5	100.9	0.1	99.0	2.7	101.5 (0.9)
100	99.6	1.3	100.3	2.1	100.1	0.3	101.9 (0.3)
500	98.1	0.2	102.5	2.2	101.7	2.4	101.9 (8.7)

[a] $n = 3$.

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Table 4. Intra- and inter-day precision, accuracy and recovery data for EDCs spiked in sea water.

	1st day[a]		2nd day[a]		3rd day[a]		
EDCs in seawater matrix (ng L^{-1})	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Recovery (%)[a] (RSD %)
4-t-OP							
10	89.0	9.4	78.0	7.2	93.4	8.8	58.1 (4.8)
100	111.4	9.0	90.9	8.0	97.7	2.1	57.2 (5.1)
500	113.6	1.8	100.7	1.6	109.3	4.2	57.1 (1.5)
4-OP							
10	97.8	1.2	100.0	3.0	102.2	1.8	89.0 (2.1)
100	98.1	4.4	101.0	4.1	100.9	4.0	86.1 (1.6)
500	100.0	0.2	108.0	3.4	105.9	3.4	85.9 (1.9)
4-NP							
10	99.2	1.9	102.2	3.1	98.6	3.5	42.1 (1.9)
50	92.4	4.1	92.4	4.1	94.6	0.7	41.9 (9.4)
100	116.6	0.2	96.1	3.6	117.0	0.7	41.9 (5.9)
OP1EO							
10	85.1	2.1	87.3	1.6	120.0	1.4	58.1 (1.8)
50	98.6	4.1	108.9	4.6	108.9	4.6	57.7 (9.3)
100	101.0	1.0	100.7	1.2	98.6	3.2	57.6 (0.2)
NP1EO							
10	100.2	3.2	98.2	9.6	101.6	0.6	67.4 (1.8)
100	92.4	8.3	103.6	0.6	104.1	1.9	68.3 (8.1)
500	95.7	1.5	106.7	3.2	104.8	2.9	68.4 (4.1)
BPA							
10	100.0	0.8	99.4	3.5	100.6	1.7	72.0 (0.6)
50	98.3	1.8	98.8	1.0	99.4	0.8	71.7 (1.9)
100	99.5	1.0	98.4	1.8	99.3	1.3	71.6 (1.0)
OP2EO							
10	92.3	8.0	107.7	9.5	100.0	11.0	66.2 (10.9)
50	94.5	1.4	95.1	1.0	100.0	5.0	66.7 (4.1)
100	99.0	4.9	96.7	3.6	99.1	5.0	66.8 (5.5)
NP2EO							
10	95.3	0.9	100.5	7.6	104.2	6.4	62.5 (4.5)
100	98.5	2.2	98.3	4.6	103.3	0.8	60.1 (2.8)
500	105.0	1.5	100.6	4.1	102.0	4.4	59.9 (6.7)
E1							
10	94.5	2.7	103.5	3.4	102.1	7.8	100.0 (4.8)
100	79.7	0.6	107.1	5.4	113.2	10.2	93.4 (4.6)
500	110.6	1.2	101.8	2.1	102.9	1.0	89.7 (8.9)
E2							
10	99.5	3.9	100.5	4.2	100.0	8.5	72.9 (0.8)
100	95.2	0.7	99.3	2.1	105.5	3.2	70.6 (5.2)
500	100.9	4.9	104.4	4.9	114.0	0.2	70.4 (0.2)
EE2							
10	110.9	0.8	93.7	6.1	95.5	6.7	97.0 (9.5)
100	95.3	6.9	102.1	0.1	102.6	1.8	99.7 (4.1)
500	102.8	2.3	99.0	1.0	100.0	1.5	99.9 (1.6)

[a] $n = 3$.

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Table 5. Environmental levels of the EDCs measured in both the Douro River estuary and the Atlantic Ocean.

Environmental levels (ng L^{-1})	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
4-t-OP	23.11	26.80	25.59	22.49	18.90	20.31
4-OP	12.10	11.40	13.70	11.80	8.50	9.50
4-NP	11.60	11.80	13.20	11.50	10.90	11.60
OP1EO	18.03	21.23	16.52	21.13	13.94	11.83
NP1EO	332.32	478.39	445.65	554.27	263.67	259.77
BPA	42.80	57.06	43.92	47.43	46.80	46.76
OP2EO	175.23	214.40	156.68	118.25	199.46	151.26
NP2EO	1451.85	1950.26	2145.19	1987.54	1755.96	1377.31
E1	0.52	0.51	0.52	0.52	1.96	1.87
E2	14.08	13.39	14.36	14.13	6.25	8.39
EE2	<0.58	<0.58	1.43	1.62	2.76	2.74

and persistent pollutants. International validation guidelines were strictly followed to guarantee the quality of results, even when trace levels were found (ng L^{-1}). The SPE procedure, used for preconcentrate all EDCs and remove matrix interferences, was used in conjunction with a silyl derivatisation protocol for the analysis of the target compounds by GC–MS. Since the GC-MS chromatographic analysis is very fast (<30 min) and all EDCs-TMS derivatives are stable during a reasonable period of time (72 h) the current method is suitable for continuous monitoring assays. To confirm the efficacy of this method to real environmental samples, several estuarine (Douro River estuary) and sea (Atlantic Ocean) water samples were successfully assessed.

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