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A simple and sensitive UFLC-fluorescence method for endocrine disrupters determination in marine waters

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ABSTRACT

The present study proposes a fast and simple analytical methodology employing C18 SPE cartridges (for preconcentration and clean-up), and a ultra-fast liquid chromatography coupled to fluorescence detector (UFLC-FLD) for determination of the following endocrine disrupters (ED): bisphenol A (BPA), 4-n-nonylphenol (4NNP), 4-n-octylphenol (4NOP), 4-t-octylphenol (4TOP), estriol (E3), estrone (E1), 17β-estradiol (E2) and 17α-ethynylestradiol (EE2) in seawater. The proposed method was developed, optimized and validated. Separation was done by a total running time of 10 min in a Shim-pack XR-ODS C-18 (2.0 mm ID × 50 mm) chromatographic column, mobile phases were acetonitrile/ultra-pure water under gradient programming; eluent flow rate at 0.120 mL min^{-1}; column temperature set at 60 °C; emission wavelength of 306 nm and excitation wavelength of 280 nm. The method was validated through assessment of the following parameters: linear range, linearity, selectiveness, precision, recovery test, limit of detection (LOD), and limit of quantification (LOQ). Recoveries ranged from 91% (for EE2) to 104% (for 4NNP) and also was found a suitable repeatability (RSD < 4.5%) for all considered compounds. LOD and LOQ ranged from 2.0 ng L^{-1} (EE2) to 23 ng L^{-1} (E1) and 9.3 ng L^{-1} (EE2) to 96 ng L^{-1} (E1), respectively. The analytical method using SPE UFLC-FLD was applied to seawater samples collected from Todos os Santos Bay (BTS), Brazil to determine the concentration of eight ED.

1. Introduction

The endocrine disrupting chemicals (EDC) are a group of several classes of substances defined not only by its chemical nature but also by its probable biological effect, which may affect the health of a given population and/or its descendants [1]. These substances, including organochlorine pesticides, alkylphenols, phthalates, polychlorinated biphenyls and dioxins, and bisphenols, among others, disturb the hormonal equilibrium of organisms, which is particularly dangerous at developmental age, when changes are in most cases irreversible [2,3].

Moreover, there are thousands of substances currently being used in the pharmaceutical industry to produce drugs such as contraceptives, antibiotics, painkillers, among others. Various substances have also a veterinary use and are employed to prevent and control diseases and to enhance productivity in livestock, poultry, fish and shellfish farming. Considering the diverse use and applicability of pharmaceuticals, it is expected that these substances to be transported from wastewater and/or point sources to river, estuarine and finally coastal and open waters, where they can cause contamination. Whether the low concentration of many of these substances in the environment causes adverse effects in humans and biota is still a question to be answered.

Among the pharmaceuticals, hormones have become important emerging contaminants, due to their presence in various environmental compartments [4–7] and concerns about possible estrogenic and other effects both to wildlife and humans have been risen lately [8–10]. The hormones 17 α-ethynylestradiol (EE2), 17 α-estradiol, 17 β-estradiol (E2), estriol (E3) and estrone (E1) have already been included as priority drinking water contaminants on the basis of health effects and occurrence in environmental waters [11,12].

Bisphenol A (BPA), 4-tert-octhylphenol (4TOP), 4-n-octhylphenol (4NOP), 4-n-nonylphenol (4NNP) are man-made alkylphenols (AP) known as xenoestrogen compounds. In the literature, there is good support from both in vitro and in vivo that many APs have hormone-disrupting effects in a large number of organisms, including humans [13,14]. These substances bind to and affect the estrogen receptors in a similar way as E2 and act competing

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towards natural hormones, although with much weaker responses [15,16]. The BPA is a high production chemical used in the industry as an intermediate in the production of resins and polymers. As such, it can be found in many different materials and products from bottles and pipes to flame retardant materials. The European Union has banned the use of BPA in plastic infant bottles. The endocrine disrupting properties of BPA are controversial, and conflicting data can be found in the literature [17].

The determination of these compounds in environmental samples still is a challenge, especially if the compartment of interest is seawater. Firstly, due to physical (dilution, advection, and dispersion), chemical (sorption, volatilization, and photolysis) and biological (transformations and uptake) processes may promote attenuation on the concentration of the substances from sources (waste treatment plants or other point sources) to the marine environment [18]. Secondly, seawater is a complex matrix with high concentrations of major ions that can interfere in the analysis. It is, therefore, essential to extract and separate the substances of interest from the original matrix. It is expected that the BPA, APs and hormones occur in very low concentrations in seawater, so a concentration step, prior to detection, is usually also necessary.

A number of techniques have been used for both the extraction and analysis of endocrine disrupters (EDs) in environmental matrices. For water analysis, sample preparation techniques mostly used are solid phase extraction (SPE) [19,20], stir bar sorptive extraction (SBSE) [21–23], ultrasonic solid–liquid extraction [2], solid-phase microextraction (SPME) [24,25], and liquid–liquid microextraction (LPME) [26], among others. The most frequently used procedure to isolate and concentrate endocrine disrupters is the SPE, which is based on the partition equilibrium of analytes between sorbent and samples [27] and it allows high enrichment and cleaning up [28]. Several SPE sorbents are available, including octadecylsiloxane [29], graphitized black carbon [30], a polymeric sorbent [28,31], the Oasis MCX, a mixed polymeric and cation-exchange sorbent [32] and hypercrosslinked polymer resin – HXLPP [33].

The main analytical techniques for determination of EDC are based either on gas chromatography (GC) or on liquid chromatography (LC) coupled to Mass Spectrometry (MS) [20] but other detectors coupled to LC, such as photodiode array (PDA) or fluorescence (FLD) are also reported [34]. The GC/MS and LC/MS methods include different practical aspects of their use with different ionization and monitoring modes, where GC/MS/MS or LC/MS/MS techniques are indicated for identification of unknowns in environmental samples [20,22].

There are both advantages and disadvantages for choosing GC and LC methods. For GC analysis it becomes essential a derivatization step during sample preparation. Derivatization is done in order to obtain more volatile derivatives, making them more suitable for GC analysis [35–38]. However, the derivatization step may augment the risk of contamination of the samples and it is also time consuming and cannot be overcome. Due to its simpler sample preparation than GC methods, LC methods are in many cases a good tool for routine environmental sample analysis, since derivatization is not necessary [39]. But LC/MS or LC/MS/MS require a highly specialized analyst.

Most of analytical techniques used are reaching the highest accuracy with low limit of detections, but are expensive, time-consuming, and require the use of highly trained personnel. In this way, the development and employment of reliable, simple and affordable analytical methods is highly suggested for the current demand for field monitoring. Furthermore, suitable methods employing adequate techniques could be then used both by regulatory authorities and by industry to provide enough information for routine testing and screening of samples [1].

The objective of this study was to develop sensitive and simple analytical procedures (extraction and clean-up) for the routine detection of eight hormone-disrupting substances (bisphenol A (BPA), estriol (E3), estrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2), 4-n-nonylphenol (4NNP), 4-n-octhylphenol (4NOP), 4-tert-octhylphenol (4TOP)) in seawater by UFLC-Fluorescence. The developed method was validated and then applied to seawater samples from Todos os Santos Bay (BTS), Brazil.

2. Experimental

2.1. Sample collection

Todos os Santos Bay (BTS), Northeastern Brazil, is the second largest Brazilian bay (1233 km^2). A large urban area (3 million inhabitants), and an important industrial complex (petrochemical, chemical, textile, fertilizers, paper mill, etc.) are located in the BTS basin. In addition, a large amount of untreated and/or poorly treated sewage is discharged daily all around the bay. Details of the environmental characteristics and contamination status of the BTS can be found elsewhere [40]. Fig. 1 shows the sites sampled in the BTS.

In each site, three independent replicates were collected in 4 L amber bottles. Each bottle was rinsed three times with local seawater prior to sample collection. Samples were kept at dark and under refrigeration during transport to the laboratory.

2.2. Chemicals, analytical standards, and instrumentation

All chemical solutions, standard solutions, and eluents used in this work were prepared using ultrapure water from MILLI-Q-PLUS (Millipore Corporation, Bedford, USA) with resistivity higher than 18.2 $M\Omega\ cm^{-1}$ and conductivity of 0.054 $\mu S\ cm^{-1}$ at 25 °C, and organic solvents (acetonitrile, methanol and dichloromethane – JTBaker, Santana, USA) of chromatographic and/or spectroscopic grade.

Field and laboratory materials were soaked in 10% Extran (Merck, Germany) solution under sonication for 20 min (ultrasound bath, 25 °C, 25 Hz, Elma, Singen, Germany). Subsequently, materials were rinsed three times with ultrapure water, one time with hexane (JTBaker, Santana, USA), and dried in a dust free environment at room temperature. After the decontamination procedure, bottles were capped and wrapped with tin foils. Dust free nitrile gloves were used at all times in the preparation of materials and sample handling to minimize potential contamination.

Standards of BPA (99% degree of purity, Sigma-Aldrich, USA), 4NNP (99% purity, Sigma-Aldrich, USA), 4NOP (99% chromatographic grade, Sigma-Aldrich, USA), 4TOP (99% purity, Sigma-Aldrich, USA), E1 (99% purity, Sigma-Aldrich, USA), E2 (99% purity, Sigma-Aldrich, USA), EE2 (99% HPLC grade, Sigma-Aldrich, USA), and E3 (98% purity, Sigma-Aldrich, USA) were used. Stock solutions of 1000 $mg\ L^{-1}$ for each analyte were prepared in methanol and kept at 4 °C, at dark, for up to 3 months. Fresh analytical multistandards were made by successive dilutions from stock solutions. For the external calibration analytical curve, 10–12 points were used in the concentration range of 1 $\mu g\ L^{-1}$ to 200 $\mu g\ L^{-1}$. Each standard concentration was injected three times in the chromatographic system. All standard solutions and eluents were filtered through regenerated cellulose Millex filtration units (0.22 μm pore size, 15 mm diameter, Millipore, USA) immediately prior to injection into the liquid chromatographic system.

Chemical determinations were performed in an ultra-fast liquid chromatograph (model LC-20AD Prominence, Shimadzu, Japan) coupled to a fluorescence detector (model RF-20A, Shimadzu, Japan), equipped with high pressure dual pump (model LC-6AD, Shimadzu, Japan), degasser (model DGU-20A3, Shimadzu, Japan), and autosampler (model SIL-20A HT, Shimadzu, Japan). Separation

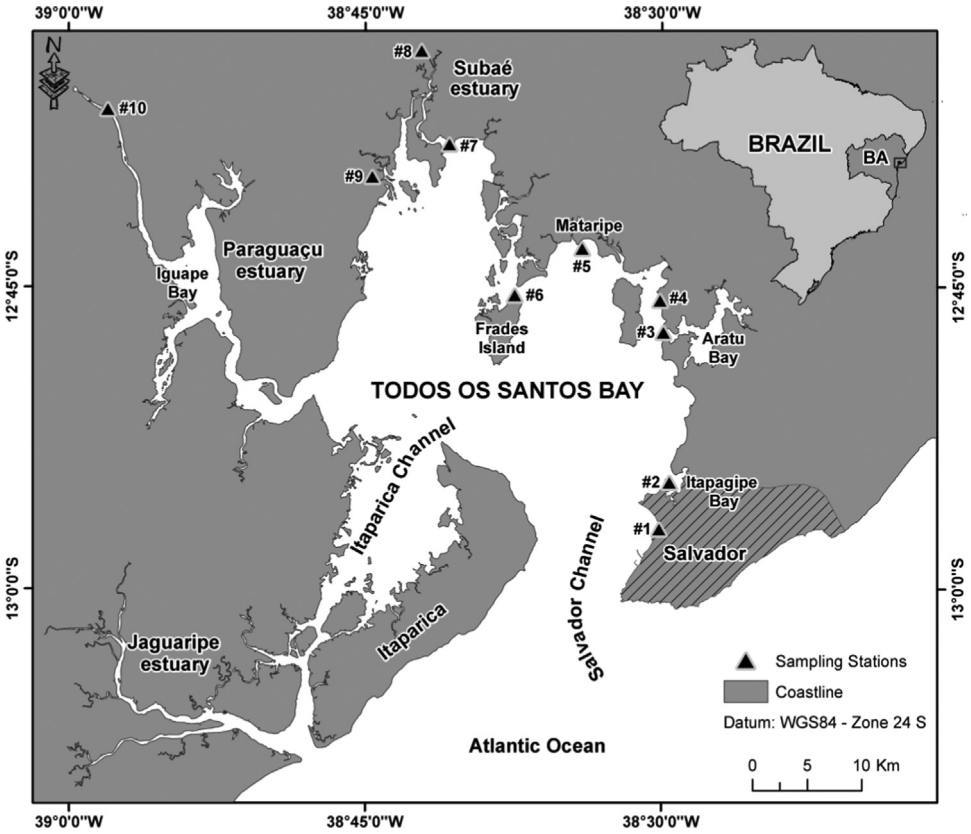

Fig. 1. Sampling sites in Todos os Santos Bay, Bahia, Brazil. Sites are: #1 São Joaquim, #2 Ribeira, #3 Porto de Aratu, #4 Caboto, #5 Mataripe, #6 Temadre, #7 São Francisco do Conde, #8 Santo Amaro, #9 Acupe, and #10 Cachoeira.

was done by using a Shimadzu column Zorbax XDB-C18 column (50 × 2 mm, 2.2 μm particle size and 12 nm pore size).

2.3. Analytical method development, optimization and validation

Method development and optimization was performed by univariate procedures. Validation was done following recommendations from IUPAC, i.e. determination of analytical figures such as linearity, calibration curve, evaluation of sample matrix effect, repeatability, selectivity, precision, accuracy, limit of detection, limit of quantification, and method application to real samples [41–44].

2.4. Sample preparation

The preconcentration apparatus employed was a modified version of the system proposed by Sodré et al. [45]. The system was designed to handle large volume samples (up to 4 L) coupled to commercial SPE cartridges. Four sets containing PTFE-made connectors, brass adapters and ball valves were used to fit SPE cartridges and sample bottles to a 4-port manifold attached to a 20 L carboy connected to a vacuum pump (Millipore, USA).

Immediately upon arrival in the laboratory, seawater samples were filtered through regenerated cellulose (0.45 μm pore size, 47 mm diameter, Millipore, EUA) for separating suspended particulate matter. Endocrine disrupters presented in each seawater sample (4 L each) were concentrated and extracted through C18 solid phase extraction (SPE) cartridges (6 mL polypropylene tube, 200 mg of C18 resin, Waters, Milford, EUA). After finishing the preconcentration step, we rinsed the C18 cartridge three times with 2 mL deionized water by fitting a 5 mL syringe plunger in it, in order to remove the high salt content of the sample. EDs were eluted from cartridges with 2 mL methanol into a vial then they were injected in the UFLC-FLD.

3. Results and discussion

3.1. Sample preparation

The preconcentration apparatus (modified from Sodré et al. [45]) was properly set and worked well for our study. It is robust and much less expensive than the typical commercially available manifold system. Moreover, it also provides less experimental handling, minimizing cross contamination and sample losses.

The preconcentration procedure was adjusted well in order to separate suspended particulate matter (SPM) from seawater before preconcentration step. SPM samples were not considered in this study. The C18 cartridges were set in the constructed manifold and 4.0 L of seawater was pushed into the cartridges. After preconcentration step, both ED and interfering species were trapped and concentrated in the cartridges. The high sea salt content present in this kind of sample causes relevant interference in the ED determination. If salts were not quantitatively removed prior ED elution from cartridges, they will be present during analysis what could potentially clog and damage the liquid chromatograph system. Due to that, salt elimination becomes a crucial step and should be done before ED elution from cartridges. In this way, salts were excluded by passing deionized water into cartridges and then the ED elution was done by using 2 mL methanol. The complete extraction procedure provided a preconcentration factor of 2000 times.

One interesting point to be addressed in this discussion is the role of the salt content during sample preparation. If, on one hand, the salts present in the samples could interfere the analysis of ED and therefore they should be eliminated, on the other hand, while each 4 L of seawater was passing through C18 resin, the salts enabled an inherent salting out effect and facilitated retention of the ED. This happened since ED considered in this study possess

low polarity and should have more affinity to apolar C18 resins. In turn, when passing deionized water through C18 cartridges, the amount of salts retained there would prefer to be dissolved in the water and therefore be eliminated from apolar resin matrix. In this point of view, this proposed sample preparation is very suitable for ED determination in seawaters.

3.2. Chromatographic method optimization and validation

The following tests were performed: (a) evaluation of different eluents, (b) eluent gradient programming, (c) evaluation of eluent flowrate, and (d) column temperature variation. In the evaluation of eluents, two different binary sets were assessed, methanol and ultrapure water and acetonitrile and ultrapure water. The best results were obtained when injecting 200 μg L^{-1} ED standard solution and using acetonitrile and ultrapure water as eluents. In a second experiment, the evaluation of the gradient of eluent was realized in order to obtain the best separation of the analytes from a 200 μg L^{-1} ED multi-standard solution. In the next step, 200 μg L^{-1} individual standard solution of each ED was injected, and the retention time of each analyte was determined. Finally, a known amount of each individual standard solution was added to a 200 μg L^{-1} multi-standard solution with all EDs. A match between the identity of each ED and the chromatographic peak could be observed, therefore the elution order and the retention time of all EDs could be determined.

The eluent program that produced the best separation in the shortest run time was: (i) run starting at 45% eluent B (acetonitrile); (ii) increasing from 45% to 90% eluent B during 3.5 min; (iii) keeping eluent B at 90% for 0.5 min; (iv) rising from 90% to 100% eluent B in 0.2 min; (v) decreasing from 100% to 45% eluent B during 0.3 min; (vi) finally keeping eluent B at 45% for 6.5 min. The total run time was 10 min.

In the third test, the influence of the eluent flowrate variation was evaluated. The flowrate tested varied from 0.100 to 0.300 mL min^{-1}, with 0.020 mL min^{-1} increments. The optimum conditions were reached at 0.120 mL min^{-1} flowrate. In the last test the influence of the column temperature was tested. Experimental conditions varied from 25 to 60 °C, while temperature increments of 5 °C were evaluated. Based on the results, 60 °C was defined as the best column temperature. Additionally, excitation wavelength (λ_{exc}) and emission wavelength (λ_{em}) were set at 208 nm and 306 nm, respectively, as proposed by Yu et al. [46]. A 200 μg L^{-1} standard solution chromatogram was acquired following the optimized conditions set in this study (Fig. 2). EDs elution order was: E3 (Rt=1.45 min), BPA (Rt=2.58 min), E2 (Rt=2.99 min), EE2 (Rt=3.57 min), E1 (Rt=3.89 min), 4TOP (Rt=7.50 min), 4NOP (Rt=8.10 min), and 4NNP (Rt=8.50 min). Method validation was carried out by the analytical figures, as described below.

3.2.1. Selectivity

A simple way of verifying selectivity of a given chromatographic method is observing if there is any interfering peak around analyte peak when comparing chromatograms of a real sample blank (a seawater sample proved to be absent of analytes), and a seawater sample enriched with analyte standard solution [47,48]. Fig. 2 shows that there are no other interfering peaks eluting around analyte peaks which indicates that the developed method is selective for the eight endocrine disrupters evaluated.

3.2.2. Response function, linearity and linear range

The performance of the analytical curves (assessed by the equation $y=ax+b$, where "a" is the angular coefficient and "b" is the linear coefficient of the curve for a given compound), linearity (assessed by R^2 of the curve) and linear range are presented

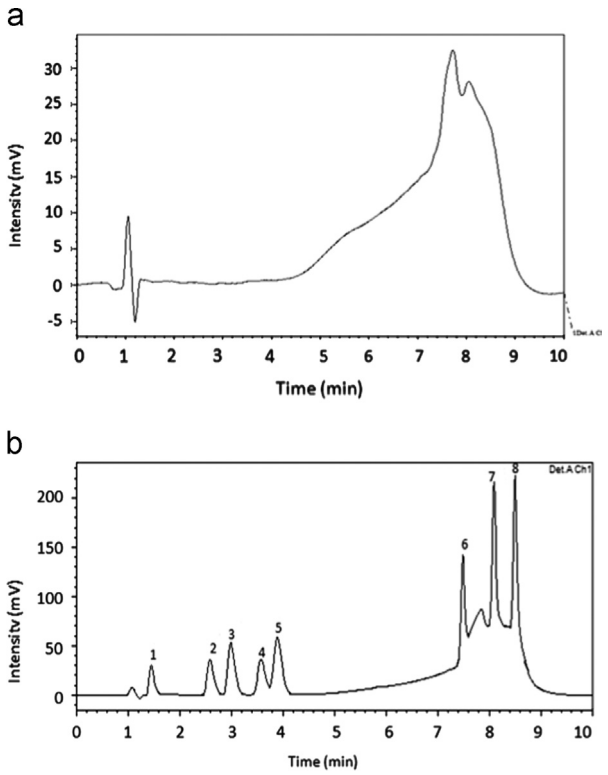

Fig. 2. A comparison between real seawater sample free of endocrine disrupter chromatogram (A) and a real seawater sample fortified with endocrine disrupter standards (B). Chromatographic conditions: 45% acetonitrile (ACN), 45–90% (ACN) for 3.5 min, 90% ACN for 0.5 min, 90–100% ACN for 0.2 min, 100–45% ACN for 0.3 min, 45% ACN for 6.5 min. Total run time was 10 min. Flowrate was 0.120 mL min^{-1}, column temperature 60 °C as well as λ_{exc} 208 nm and λ_{em} 306 nm.

Table 1
Analytical figures obtained from optimized method.

Analytes	Rt (min)	Linear range (μg L^{-1})	Analytical curve ($y=ax+b$)	Linearity (R^2)	Conc. levels (n)
E3	1.45	5–200	$y=916x-525$	0.9938	10
BPA	2.58	5–200	$y=1473x+136$	0.9944	10
E2	2.99	5–200	$y=2285x-1470$	0.9936	10
EE2	3.57	5–200	$y=1673x-644$	0.9952	10
E1	3.89	10–2000	$y=296x-477$	0.9965	12
4TOP	7.50	5–200	$y=2082x+14097$	0.9916	10
4NOP	8.10	5–200	$y=2914x+103975$	0.9906	10
4NNP	8.50	5–200	$y=4135x+68226$	0.9936	10

in Table 1. It was considered that the ideal data fit was achieved whenever R^2 was equal or above 0.99. For all endocrine disrupters, R^2 were above 0.99, which indicated good linearity. Linear range was between 1 and 200 μg L^{-1}, except for E1 that linear range was 10–2000 μg L^{-1} (Table 1). For all EDs, RSD ranged from 1.5% (E1) to 4.5% (both BPA and 4TOP), due to random fluctuations in detector response.

3.2.3. Test for matrix sample effect

In the present study the matrix sample effect was evaluated by comparing angular coefficient (a) of two linear regression curves constructed with and without standard additions. The first curve was prepared with six different concentrations of standards added to a seawater sample ($a_{standard+sample}$) while the second curve was prepared with a standard solution ($a_{standard}$). When inclination of both curves is equal or very close one to another,

Table 2
Comparison between angular coefficient (a) in methanol and seawater.

Analytes	$a_{standard}$[a]	$a_{standard+sample}$[b]	$a_{standard}/a_{standard+sample}$	RSD (%)
E3	1379	1370	1.01	0.007
BPA	1315	1497	0.88	0.4
E2	2082	2143	0.97	0.1
EE2	1656	1642	1.01	0.05
E1	190	172	1.11	0.6
4TOP	2077	2056	1.01	0.1
4NOP	3821	4170	0.92	0.3
4NNP	3746	4060	0.92	0.3

[a] Angular coefficient from external analytical curve (standards dissolved in methanol).
[b] Angular coefficient from standard addition analytical curve (standards added to real seawater samples).

Table 3
Limit of detection (LOD) and limit of quantification (LOQ) for endocrine disrupters in seawater.

Analytes	LOD[a] (μg L^{-1})	LOQ[a] (μg L^{-1})	LOD[b] (ng L^{-1})	LOQ[b] (ng L^{-1})
E3	5.6	19	2.8	9.0
BPA	6.5	33	3.3	16
E2	6.0	19	3.0	9.7
EE2	4.0	74	2.0	37
E1	56	185	23	96
4TOP	27	90	13	45
4NOP	9.0	31	4.5	15
4NNP	9.0	29	4.0	15

[a] LOD and LOQ calculated from analytical curve.
[b] LOD and LOQ calculated considering a nominal sampled seawater volume of 4.0 L and the concentration step to 2 mL during sample preparation.

the $a_{standard+sample}/a_{standard}$ ratio tends to approach to 1, meaning that there is not matrix effect. The results obtained (Table 2) indicated that there is no matrix effect. Additionally there is no need to use standard addition method (which is time-consuming) for quantifying ED in seawater samples.

3.2.4. Limit of detection and limit of quantification

The LOD of an analyte may be described as that concentration which gives an instrumental signal significantly different from "blank" or "background" signal. LOQ is the smallest value that an analyte can be determined quantitatively, with a certain limit of confidence. Below the value determined for LOQ, measurements do not represent sufficient confidence for quantification [48]. According to Ribani et al. [48], an alternative to determine the LOD and LOQ of a method is to calculate them based on response SD-to-inclination of the analytical curve rate, $LOD = 3\,s/a$ and $LOQ = 10\,s/a$, where s is the standard deviation (SD) of linear coefficient and a is the angular coefficient (inclination) from analytical curve.

In the present study, LOD calculated from analytical curve varied from 4.0 μg L^{-1} to 56 μg L^{-1} and LOQ ranged 74–185 μg L^{-1} for EE2 and E1, respectively (Table 3). Considering the original seawater sample volume of 4.0 L and the final volume of 2.0 mL obtained after sample preparation, the LOD for EE2 and E1 varied between 2.0 ng L^{-1} and 23 ng L^{-1}, and 37 ng L^{-1} and 96 ng L^{-1}, respectively. LOD and LOQ found in the present study were, in general, lower than those reported for EDs in freshwaters [34,49–52] and within those found in [21] for seawater. Seawater is a much more complex matrix, due to high concentration of major ion and complex dissolved organic matter, than freshwater used in the above cited literature. The LOD and LOQ obtained in this study can be considered adequate for the determination of endocrine disrupter in seawater samples.

3.2.5. Precision

Intra-day or within day precision expresses the repeatability, under the same operating conditions (i.e. same procedure, analyst, instrumental conditions, laboratory), of different experiments realized in the same day. In this study, repeatability was evaluated for retention time and detector response (as peak area) with standard solution of 200 μg L^{-1}, injected in 10 replicates during the same day, and expressed as RSD, as can be observed in Table 4.

Intermediate precision or inter-day precision expresses variability in results obtained in different days. In this test, standard solutions, at three concentration levels (5, 100 and 200 μg L^{-1}), were injected in triplicate during five consecutive days. Results were evaluated in terms of the variability (% RSD) of the retention time and peak area (Table 5). Results for all EDs considered in this study presented RSD below 5.5% (for both repeatability and intermediate precision; Tables 4 and 5). Considering that is acceptable RSD values up to 20% for trace analysis of complex matrices, the proposed method can be considered precise.

3.2.6. Recovery tests

Accuracy or trueness of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted value and the value found. Since there is no certified reference material (CRM) for EDs in seawater samples, accuracy was measured by a recovery test. In this test a known amount (25, 100, and 150 μg L^{-1}) of standard was added to a real seawater samples. Then, the enriched sample followed the developed sample clean-up, extraction and chromatographic analysis procedure. Recoveries are presented in Table 6. Recovery ranged from 91% (EE2) to 103% (4TOP) at 25 μg L^{-1}; from 85% (4TOP) to 99% (E3) at 100 μg L^{-1}; and from 88% (E1) to 104% (4NNP) at 150 μg L^{-1}. Considering these recovery values were around 90–100%, we could consider this method accurate.

Table 4
Repeatability (intra-day precision) for retention time (Rt) and peak area (Area), expressed as RSD (%).

Analyte	Rt	Area
E3	0.28	1.6
BPA	0.12	4.5
E2	0.30	2.0
EE2	0.22	3.0
E1	0.13	1.5
4TOP	0.08	4.5
4NOP	0.07	2.1
4NNP	0.38	1.8

Table 5
Intermediate precision (inter-day precision) for retention time (Rt) and peak area (Area), expressed as RSD (%).

Analyte	5(μg L^{-1})		100 (μg L^{-1})		200 (μg L^{-1})	
	Rt	Area	Rt	Area	Rt	Area
E3	0.035	2.3	0.069	0.6	0.26	3.4
BPA	0.11	2.0	0.19	2.8	0.62	6.1
E2	1.75	5.0	1.63	2.7	2.5	3.9
EE2	0.025	4.5	0.59	5.5	0.14	2.5
E1	0.028	2.3	0.96	2.3	0.13	3.1
4TOP	0.020	5.9	0.21	3.8	0.40	3.2
4NOP	0.058	5.4	0.11	2.5	0.13	2.0
4NNP	0.034	5.4	0.18	2.5	0.036	2.0

3.2.7. Application to real seawater samples

In order to demonstrate the feasibility and applicability of the proposed method, real seawater samples obtained from sites of Todos os Santos Bay (BTS; Fig. 1), Bahia, were analyzed. The studied compounds were not found at all studied stations above LOQ (Table 7). In fact, EE2, E1, 4TOP and 4NNP concentrations at all studied sites were below LOQ, whereas E3 was found only at Ribeira Bay (Fig. 1; Table 7). These results were unexpected once all studied sites are subject to anthropogenic activities [40]. On the other hand, the 4NOP was detected in all samples, showing the ubiquity of this compound in BTS. Similar results were also found for BPA. The highest BPA and 4NOP levels were observed in Santo Amaro, at the Subaé River (Fig. 1), which has a long history of contamination by trace metals and untreated sewage inputs [53]. Downstream of the Subaé, at the estuary mouth, at the São Francisco do Conde and Acupe (Fig. 1), the concentrations of BPA and 4NOP were still relatively high, despite the attenuation processes due to the seawater mixture and other physical-chemical processes (e.g. sorption, desorption, degradation) that could occur during water transport along the estuary. The chromatogram of the Santo Amaro site was shown in Fig. 3. Concentrations of 4NOP were also relatively high at Temadre and Mataripe (Fig. 1). These values could be explained considering the proximity of these sites to a large petrochemical and port complex. Alkylphenols and BPA are the largest group of surfactants used as detergents, emulsifiers, wetting agents, dispersants, solubilizers or plasticizers. On the contrary what is happening in Europe [53], Australia and USA, until the present date there has been no regulation on the sale and use of these products in Brazil.

The E2 was the hormone that presented both the highest occurrence and concentrations (Table 7). The former compound, together with the synthetic hormone EE2, E1 and E3, contribute to the estrogenic activity of domestic sewage and water and sewage treatment plants [54,55]. The regions around Santo Amaro, Acupe, Cachoeira and Ribeira present a disordered occupation, many of which occurs over mangrove areas where untreated sewage is directly discharged in coastal waters.

All results presented at Table 7 are the average of three independent replicate samples collected at each site. The relative standard deviation (RSD) for the independent replicate samples was lower than expected, and varied from 0.1% to 12%. These RSD values can be considered good, once environmental conditions are naturally highly variable. The only two exceptions were the BPA concentrations at Mataripe (RSD=24%) and 4NOP levels at São Joaquim site (RSD=33%). The São Joaquim is a particular site, once it is a low circulation, small embayment which is subject to several point sources of untreated domestic effluents. Based on these results, and the laborious and time consuming process to filter, concentrate and extract each of the 4 L replicates, it is not necessary to work with independent environmental water replicates.

Not many data on hormones, alkylphenols and BPA for coastal and seawater have been reported in the literature. The concentrations of the EDs in the waters collected from BTS are lower than the values reported for impacted coastal waters (Table 7). The maximum BPA concentrations found in waters from Florida [56], Mondego estuary [57], Venice Lagoon [51] are twice as high the values obtained for BTS. Both 4TOP and 4NNP concentrations were lower than values reported for the seawater from Liaohe, Northeast China [58].

4NNP, 4NOP and 4TOP are considered priority pollutants in the aquatic environment and an EQS-MAC (Environmental Quality Standard-Maximum Allowable Concentration) have been proposed as water quality standards [59]. The EQS criteria refer to the total (dissolved and particulate) concentration of these compounds. The maximum allowable concentration for 4NNP is 2.0 $\mu g\,L^{-1}$. For OPs, the EQS-AA (Environmental Quality Standard-Annual Average) concentration is 0.01 $\mu g\,L^{-1}$. Regarding BPA, which was mentioned as a possible priority hazardous substance

Table 6
Recoveries values (%) for enriched seawater samples (standard addition of 25, 100 and 150 $\mu g\,L^{-1}$).

Analyte	25 ($\mu g\,L^{-1}$)	100 ($\mu g\,L^{-1}$)	150 ($\mu g\,L^{-1}$)
E3	94	99	95
BPA	100	96	102
E2	93	95	103
EE2	91	88	94
E1	89	89	88
4TOP	103	97	96
4NOP	97	85	103
4NNP	99	97	104

Table 7
Average concentration ($ng\,L^{-1}$) and standard deviation of independent replicates ($n=3$) collected at Todosos Santos Bay, Bahia and data published in the literature.

Site ($n=3$)	BPA	E3	E2	EE2	E1	4TOP	4NOP	4NNP	References
Ribeira	< 3.3	37.9 ± 0.52	< 3.0	< 2.0	< 23	< 13	19.5 ± 0.21	< 4.0	*This study*
São Joaquim	18.6 ± 2.20	< 2.8	< 3.0	< 2.0	< 23	< 13	35.4 ± 11.8	< 4.0	*This study*
Acupe	28.8 ± 0.09	< 2.8	6.20 ± 0.06	< 2.0	< 23	< 13	28.7 ± 0.20	< 4.0	*This study*
São Francisco do Conde	48.2 ± 0.43	< 2.8	< 3.0	< 2.0	< 23	< 13	39.0 ± 0.99	< 4.0	*This study*
Santo Amaro	76.8 ± 0.43	< 2.8	4.90 ± 0.04	< 2.0	< 23	< 13	134 ± 1.81	< 4.0	*This study*
Cachoeira	13.3 ± 1.33	< 2.8	18.2 ± 0.03	< 2.0	< 23	< 13	74.5 ± 0.10	< 4.0	*This study*
Aratu	5.42 ± 00.9	< 2.8	< 3.0	< 2.0	< 23	< 13	16.4 ± 0.10	< 4.0	*This study*
Caboto	< 3.3	< 2.8	< 3.0	< 2.0	< 23	< 13	126 ± 4.40	< 4.0	*This study*
Mataripe	8.86 ± 2.16	< 2.8	< 3.0	< 2.0	< 23	< 13	77.1 ± 1.10	< 4.0	*This study*
Temadre	3.78 ± 0.10	< 2.8	< 3.0	< 2.0	< 23	< 13	80.7 ± 0.10	< 4.0	*This study*
A Coruña, Spain	< LOD–35	–	–	–	–	< LOD–110	< LOD–65	< LD–59	[61]
Baltic Sea	0.11–5.7	< LOD	< LOD	< LOD–17.9	0.08–0.54	0.04–1.1	–	1.3–21.3	[62]
Scheldt, France	–	< LOD	< LOD	< LOD	0.37–10	–	–	–	[63]
Mondego, Portugal	< LOD–880	–	< LOD	< LOD	< LOD	–	< LOD	–	[57]
Venice, Italy	< LOD–145	–	< LOD–175	< LOD–34	< LOD–10	–	–	< LD–211	[50]
Florida, USA	< LOD–190		< LOD–1.8	–	< LOD–5.2	–	–	–	[56]
Liaohe, China	69.7–100	–	–	–	–	5.75–37.2	–	44.9–146	[58]
Singapore	40–190	–	–	–	–	–	< LOD–190	320–2760	[64]
ThermaikosGulf, Greece	10.6–52.3	< LOD	< LOD	< LOD	< LOD	1.7–18.2	< LOD	–	[65]

LOD = limit of detection.

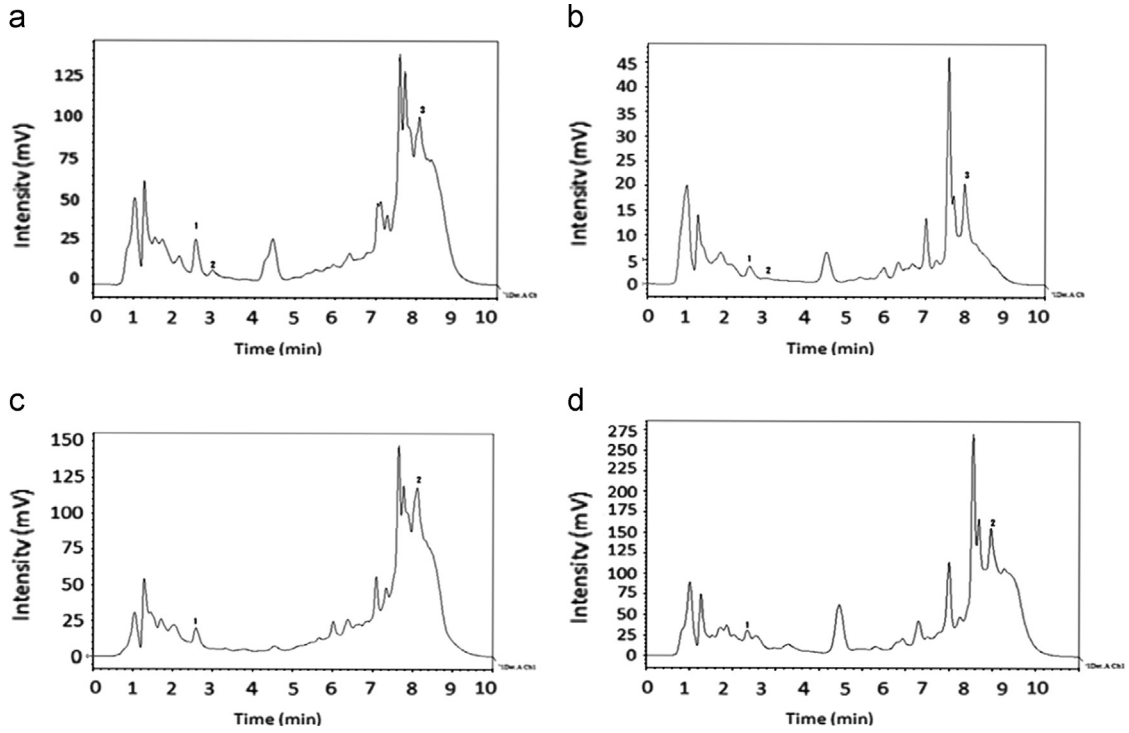

Fig. 3. Chromatograms of seawater samples collected at BTS. Chromatographic conditions: 45% acetonitrile (ACN), 45–90% (ACN) for 3.5 min, 90% ACN for 0.5 min, 90–100% ACN for 0.2 min, 100–45% ACN for 0.3 min, 45% ACN for 6.5 min. Total run time was 10 min. Flowrate was 0.120 mL min^{-1}, column temperature 60 °C as well as λ_{exc} 208 nm and λ_{em} 306 nm. Peaks: (*a*) *Acupe* [#1: BPA (2.58 min), #2: E2 (2.99 min), #3: 4NOP (8.10 min)]; (*b*) *Santo Amaro* [#1: BPA (2.58 min), #2: E2 (2.99 min), #3: 4NOP (8.10 min)]; (*c*) *São Joaquim* [#1: BPA (2.58 min), #2: 4NOP (8.09 min)]; (*d*) *São Francisco do Conde* [#1: BPA (2.58 min), #2: 4NOP (8.09 min)].

in water, a predicted, no effect concentration of 0.15 μg L^{-1} was recently proposed for marine water [60]. The measured concentrations of BPA, 4NNP and 4NOP were below the environmental quality standards.

4. Conclusions

This is a low cost, fast, simple and sensitive method for determination of ED at low ng L^{-1} range in seawater samples. The UFLC-FLD technique does not require a highly specialized analyst (as should be necessary when using LC-MS or LC-MS/MS techniques). Furthermore, it is adequate for ED analyses of a large number of samples, as desired by regulatory/monitoring agencies and industries.

The applicability of the proposed method was demonstrated analyzing seawater samples from Todos os Santos Bay. These analyses showed the presence of BPA, 4NOP, E3 and E2 in collected samples. The ubiquitous occurrence of BPA and 4NOP requires the monitoring of these compounds not only in seawater but also in sediments and biota. Moreover, the sources and the effects of these components in biota require further investigation.

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