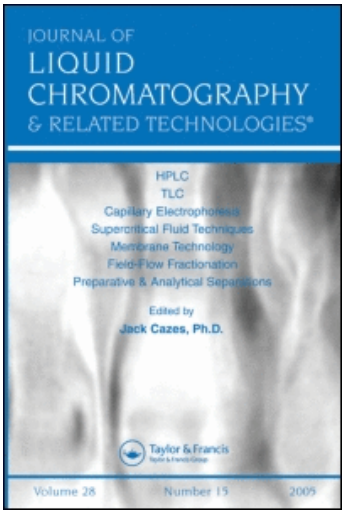

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Development and Validation of a HPLC-DAD Method for Determination of Several Endocrine Disrupting Compounds in Estuarine Water

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Development and Validation of a HPLC-DAD Method for Determination of Several Endocrine Disrupting Compounds in Estuarine Water

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Abstract: This paper presents the development and validation of an analytical procedure, which allows the simultaneous quantification of nine endocrine disrupters (EDCs) in polluted surface waters. The compounds selected for this study were

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the natural hormones (17β-estradiol and estrone), the synthetic hormone (17α-ethynylestradiol), the bisphenol A, the alkylphenols (4-octylphenol and 4-nonylphenol), and the phytoestrogens (daidzein, genistein, and biochanin A). Briefly, this method consisted of the preconcentration of water samples (2 L) in 500 mg Oasis HLB cartridges, followed by a cleanup step in 1 g silica cartridges, and analysis of all EDCs by High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD). The chromatographic separation occurred in a RP-18 analytical column in a gradient mode of CH_3CN:H_2O (pH 2) at a flow rate of 1 mL/min. The validation parameters revealed that this method was highly specific for all assayed EDCs (>99%), the linearity of the calibration curves, obtained either by external patronization or by fortified matrix calibration techniques, always showed a correlation higher than 0.99 and their intermediate precision and repeatability, evaluated in terms of intra and inter assays, were optimal for all EDCs (RSD < 3%). The precision and accuracy, estimated as the recovery rates that occurred during the preconcentration and the cleanup processes showed, for all EDCs, excellent results (RSD < 3% and recoveries up to 116%), and preconcentration factors (enrichment) up to 10000. The limits of detection (LOD) and quantification (LOQ) were mathematically estimated from the calibration curves. As expected, these values were different for each of the nine assayed EDCs and were higher when calculated from the addition standard calibration curves. Here, 4-octylphenol was the compound with the lowest LOD and LOQ (3.8 ng/L and 12.0 ng/L) and 17α-ethynylestradiol the one with the highest levels for both cited parameters (18.0 ng/L and 54.5 ng/L). Finally, the performance of the method was checked with water samples from a highly polluted area in the Douro river estuary (Portugal). The field results revealed high levels of estrogens (up to 176 ng/L), bisphenol A (up to 5.1 μg/L), alkylphenols (up to 449 ng/L), and phytoestrogens (up to 74 ng/L), in accordance with the high local pollution load, and suggested that this method can be easily and successfully used in water monitoring surveys of, at least, highly polluted estuarine areas.

Keywords: Method validation, HPLC-DAD, Water analysis, Environmental estrogenic compounds

INTRODUCTION

Currently, there is a wide variety of compounds able to act as endocrine disruptor compounds (EDCs) in both fish and mammals, due to their ability to either mimic or counteract endogenous hormones.[1,2] The presence of those compounds, even in treated effluents, demonstrates the difficulty in eradicating these kinds of pollutants by sewage treatment plants.[3] As a consequence, recent studies confirmed that several EDCs are extensively distributed in numerous ecosystems where they persist for more time than was initially expected.[4] This condition is so worrying that it attracted the attention of several international regulatory agencies, including the US Environmental Protection Agency (EPA), which are highly concerned with the impact of these compounds in the aquatic environment.[5] This occurrence has been demonstrated for natural estrogens and estrogen mimics, such as

alkylphenols or phytoestrogens, which can actually negatively interfere with the endocrine system of wild animals.[6] Amongst the most active EDCs, those that are usually present in higher amounts in polluted rivers are several kinds of estrogens, such as estrone (E1), 17β-estradiol (E2), and ethynylestradiol (EE2), the phenols, as bisphenol A (BPA), the alkylphenols, as 4-octylphenol (4-OP) and 4-nonylphenol (4-NP), and the phytoestrogens, as daidzein (DAID), genistein (GEN), and biochanin A (BIO-A). Although those compounds have different origins and physical chemical properties, in polluted environments they all cause endocrine disruption in fish.[7,8]

Concerning the physical chemical properties of these substances, the variety found in their structures (Figure 1) are linked to diverse action mechanisms *in vivo* and to the concentration that each compound needs to attain to be considered an environmental risk.[9] In this sense, recent studies revealed that whereas estrogens are harmful for the aquatic environments at ng/L levels, the alkylphenols and phytoestrogens seem to need higher concentrations, μg/L levels, to be hazardous.[9] Besides, there is evidence that

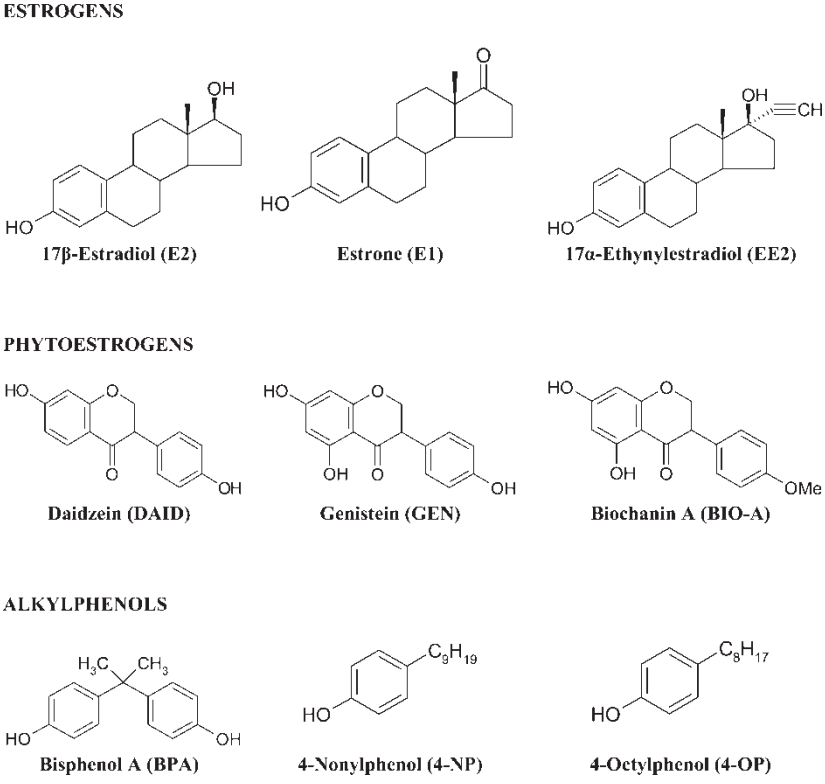


Figure 1. Chemical structures and trivial names of all compounds analysed in this study.

mixture of the above referred EDCs act as "estrogen cocktails", which are responsible for the appearance of ovotestis in fish.[9] Since recent studies in grey mullet, *Mugil cephalus*, caught at the Douro river estuary reported the presence of ovotestis in this species,[10] it is suspected that there is significant pollution by the above referred EDCs in this estuary in levels that can be evaluated by High Performance Liquid Chromatography with Diode-Array Detector (HPLC-DAD). Thus, because valid interpretation of environmental data needs a validated method,[11,12] this study describes the development and the validation of an analytical method, which followed the validation parameters established by the International Conference of Harmonization (ICH).[11,12] Finally, to evaluate the efficacy of this technique to monitoring proposes, water samples were collected from a highly polluted zone in the Douro river estuary, Portugal.

EXPERIMENTAL

Standard Preparation

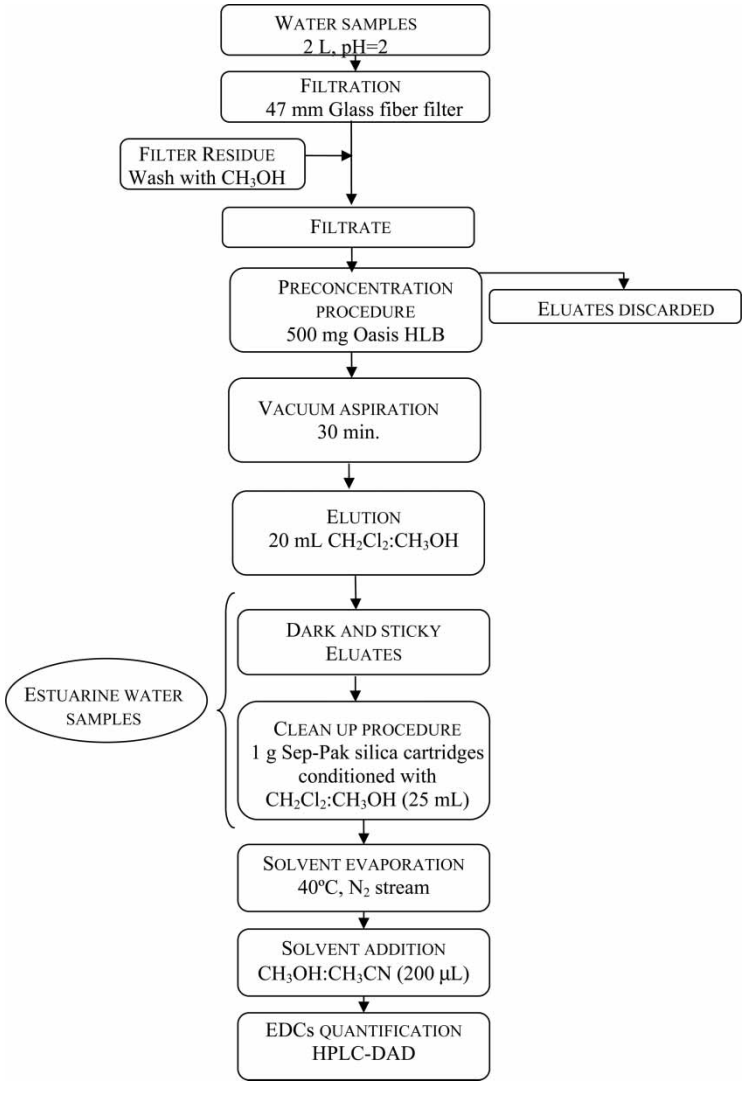
Estrone (E1), 17β-estradiol (E2), ethynylestradiol (EE2), bisphenol A (BPA), 4-octylphenol (4-OP), daidzein (DAID), genistein (GEN), and biochanin A (BIO-A) were obtained from Sigma-Aldrich (Steinhein, Germany), 4-nonylphenol (4-NP) was obtained from Riedel-de-Haën (Seelze-Hannover, Germany). Stock solutions of individual standards were prepared by dissolving known amounts of each compound in CH_3OH:CH_3CN (50:50, v/v) HPLC-grade, acquired from Sigma-Aldrich (Steinhein, Germany), to obtain final concentrations of 500 mg/L. Working standard solutions were obtained by further diluting stock solutions with CH_3OH:CH_3CN (50:50, v/v) and the nominal concentrations used for each EDCs calibration curve is referred to in Table 1. Other solvents were of analytical grade and were supplied by Sigma-Aldrich (Steinhein, Germany). Ultrapure water was supplied by a Milli-Q water system.

Sample Collection and Preparation

For the fortified matrix calibration method, estuarine water samples were randomly collected from a low polluted reference station of the Douro river estuary, located about 20 km away from the point were the river joins with the Atlantic sea. Also, for the final evaluation of the suitability of this method, estuarine water samples were collected from a highly polluted area located about 1–2 km away from the point where the river joins with the Atlantic sea. In both situations, 2 L of estuarine water samples were collected by a peristaltic sampler pump (Global Water, Model: WS300) at a depth of 1 m. Before sampling, sample bottles were rinsed two or three

Table 1. Chromatographic data and calibration results obtained by external patronization (**1**) and by the standard-addition technique (**2**)

Chemicals (EDCs)	Wavelength (nm)	Retention times (t_M, min)	Nominal concentrations of all EDCs in the calibration curves (μg/mL)	Intercept (**1**)	Slope (**1**)	R^2 (**1**)	Intercept (**2**)	Slope (**2**)	R^2 (**2**)
DAID	246	8.10	0.40–0.60–0.80–1.00–1.20	−1452.8	61866	0.998	2462.6	60865	0.994
GEN	260	10.60	0.40–0.60–0.80–1.00–1.20	−4605.7	67852	0.993	−5742.5	72165	0.994
BPA	278	12.60	0.90–1.35–1.80–2.25–2.70	−140.4	8276.5	0.998	−136.5	8540.4	0.995
E2	280	13.60	0.80–1.20–1.60–2.00–2.40	227.6	4230.7	0.994	250.0	4026.8	0.997
EE2	280	14.40	1.00–1.50–2.00–2.50–3.00	−462.5	4216.7	0.997	−453.0	4337.9	0.996
E1	280	15.20	0.80–1.20–1.60–2.00–2.40	−477.0	4662.1	0.999	181.2	4624.1	0.992
BIO-A	260	15.70	0.12–0.18–0.24–0.30–0.36	−227.9	39919	0.996	626.3	41352	0.990
4-OP	278	25.90	2.00–3.00–4.00–5.00–6.00	−1030.3	5952.4	0.992	−528.1	5939.8	0.997
4-NP	278	27.80	2.00–3.00–4.00–5.00–6.00	−596.4	4527.8	0.996	−528.13	5939.8	0.997



Scheme 1. Flow diagram of the analytical method for the preconcentration and quantification of the EDCs proposed in this study.

times using the collected waters and, during transport to the laboratory all flasks were stored at $<5^{\circ}C$. To eliminate particulate matter and other suspended solid, all the samples were filtered through a 47 mm GF/C glass fiber filter, acquired from Millipore (Ireland). Then, each filter was washed with a small amount of CH_3OH and the acquired solution added to the filtrate before extraction. All samples were acidified with H_2SO_4 to pH 2, and treated as shown in Scheme 1.

Sample Extraction Method

The preconcentration method was done according to Scheme 1. The cartridge employed for solid phase extraction (SPE) was a 500 mg Oasis HLB Cartridge, purchased from Waters Corporation (Milford, MA, USA). This cartridge, prior to use, was sequentially washed with 25 mL of CH_2Cl_2:CH_3OH (50:50, v/v), 12 mL of CH_3OH, and 25 mL of ultrapure Milli-Q water. Estuarine water samples (2 L) or ultrapure water samples (2 L), fortified with all EDCs assayed in this study, were vacuum forced through these cartridges and the flow rate was kept constant in 5–7 mL/min range. After passing the water samples, the cartridges were washed with 25 mL of ultrapure Milli-Q water and 1 mL of CH_3OH and all eluates were discarded. Afterwards, the cartridges were kept under vacuum aspiration for 30 min to dry out residual water. The elution process, was performed with 20 mL of CH_2Cl_2:CH_3OH (50:50, v/v); since the extracts from the estuarine water samples had a dark and sticky appearance, a clean up step was implemented for all samples, using 1 g Sep-Pak silica cartridges, acquired from Waters Corporation (Milford, MA, USA). Briefly, the SPE extracts were quantitatively transferred to the silica cartridges previously washed with CH_2Cl_2:CH_3OH (50:50, v/v), and eluted with 7 mL of CH_2Cl_2:CH_3OH (50:50, v/v).[13,14] Then, these eluates were collected in a round bottomed tube and evaporated to dryness in a thermostatic bath at 40°C under a nitrogen stream and further dissolved in 200 μL of CH_3OH:CH_3CN (50:50, v/v). Finally, 20 μL was injected into the HPLC-DAD system for analysis.

Instrumental Conditions

The chromatographic system consisted of a LiChroCART C_{18} reversed-phase analytical column 250 × 4 mm i.d., 5 μm particle size (Merck, Darmstadt, Germany) and a Merck Hitachi HPLC apparatus, equipped with a LaChrom pump L-7100, a programmable autosampler L-7200 with the volume injection set to 20 μL, an interface D-7000, and a LaChrom diode array detector L-7455. Data acquisition was performed by a HPLC System Manager HSM D-7000, Version 3.0 (Merck-Hitachi).

The chromatographic analysis was performed at room temperature using a gradient solvent program with a flow rate of 1 mL/min. The initial composition of the mobile phase was CH_3CN:H_2O (25:75, v/v) acidified with CF_3CO_2H, pH 2, in order to suppress the ionic moieties of all assayed compounds. The gradient was programmed to linearly increase the amount of organic solvent as follows: 0–5 min (25–40%), 5-14 min (40–55%), 14–17 min (55–57%), and 17–30 min (57–90%). After the chromatographic run, the amount of CH_3CN was increased to 100% and maintained isocratically during 5 min before a new injection. The wavelengths used for detection of the nine EDCs proposed in this study are referred in Table 1. Peak areas were used for quantitative analysis.

Validation Conditions

The method was validated following the analytical performance parameters established by the International Conference of Harmonization (ICH).[11,12] According to that, the validation process includes the evaluation of selectivity, linearity, range of application, accuracy, precision, and evaluation of the limits of detection and quantitation.

In this work, the assessment of selectivity was evaluated using two different strategies. First, the retention times, UV spectra, and peak purity tests (by diode array analysis) of all EDCs in standard solutions or in fortified matrix were compared.[11,12] In order to evaluate possible matrix interferences in this study, the last step was conducted in low polluted and in highly polluted matrixes. Secondly, the parallelism among the calibration curves obtained by external calibration and those achieved by the fortified matrix calibration method was observed. A selective method show a parallelism between the last calibration curves.[11,12]

The linearity of response between concentrations ($\mu g/L$) and the analytical responses (mAU) was assessed for each assayed compound by linear regression. For this propose, five different concentrations were prepared from the standard stock solutions. A similar procedure was accomplished using the fortified matrix (Table 1). During the evaluation of linearity, the range of application for the present method was established for each compound considering an interval between the quantification limit and, at least, 150% of the expected value found in highly polluted environments.[11,12] According to several validation guidelines, all analytical curves must show a correlation level, R^2, higher than 0.99.[11,12]

The instrumental precision accuracy was evaluated by the measurement of the peak areas of ten injections in both standard mixture and fortified matrix. For validation, RSD must be inferior to 1% ($n > 5$).[11,12,15]

The method precision was assessed using three concentration levels of the standard mixture (low, medium, and high), prepared both in CH_3OH:CH_3CN and in the fortified matrix (Table 2), injected in triplicate.[11,12] In validated methods, these results are expressed as the relative standard deviation (RSD) and have to be inferior to 20%.[11,12]

The intermediate precision (intra-day assays) was estimated in parallel with accuracy. This parameter was evaluated by the injection, in triplicate, of the extractive solutions in three concentration levels of the standard mixture (Table 3). The repeatability (inter-day assays) was assessed repeating the last procedure during three consecutive days (Table 3). The estimation of accuracy was expressed in terms of recovery percentages. In literature, it is referred that recoveries must range from 50% to 120%, with a precision inferior to 15%.[11,12]

Both limits of detection (LOD) and quantification (LOQ) can be evaluated using different approaches.[12] Here, both parameters were calculated based on the standard deviation of the response and the slope of the

Table 2. Intra and inter-day precision and accuracy ($n = 3$) for the HPLC-DAD analysis.

Chemicals (EDCs)	Nominal concentration (μg/mL)	1st day		2nd day		3rd day	
		RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
DAID	0.40	1.20	101	1.10	98	0.20	98
	0.80	1.00	101	0.70	104	0.20	105
	1.00	0.40	98	0.50	99	0.40	98
GEN	0.40	0.50	103	1.30	104	0.50	105
	0.80	1.10	97	0.60	108	0.30	96
	1.00	0.40	99	0.03	100	0.60	98
BPA	0.90	0.30	103	1.30	104	0.70	105
	1.80	1.30	98	0.30	98	1.00	106
	2.25	1.60	98	1.80	98	0.60	100
E2	0.80	2.10	97	2.90	97	1.00	105
	1.60	1.00	103	0.60	103	0.60	104
	2.00	1.90	102	0.40	102	0.60	105
EE2	1.00	1.70	106	0.80	107	0.60	108
	2.00	1.70	100	0.30	100	0.20	101
	2.50	1.50	98	0.70	97	0.03	97
E1	0.80	1.40	96	0.50	98	0.50	97
	1.60	1.40	102	0.20	98	0.50	98
	2.00	2.70	101	0.70	101	0.20	102
BIO-A	0.12	1.80	95	1.10	95	1.90	94
	0.24	0.40	103	0.60	102	0.07	102
	0.30	0.50	99	0.30	101	0.90	101

(*continued*)

Table 2. Continued

Chemicals (EDCs)	Nominal concentration (μg/mL)	1st day		2nd day		3rd day	
		RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
4-OP	2.00	2.40	103	0.80	95	2.50	94
	4.00	0.90	99	1.50	100	0.50	103
	5.00	1.20	96	1.20	97	0.30	96
4-NP	2.00	2.00	102	0.50	100	1.00	100
	4.00	2.50	100	0.80	101	0.20	103
	5.00	1.30	98	0.60	99	0.20	98

calibration curves using the mathematic formulas:[12]

$$LOD = 3.3 \times \frac{s}{S} \text{ and } LOQ = 10 \times \frac{s}{S}$$

s is the standard deviation of y-intercepts, and S the slope of the calibration curves (n = 3).[11,12]

RESULTS AND DISCUSSION

Chromatography

A typical chromatogram obtained using a standard mixture of the nine EDCs is illustrated in Figure 2. In Figure 3 shows a typical chromatogram of a estuarine water spiked with the nine EDCs referred to in this study. Both retention times (t_R) and wavelengths used for the evaluation of each EDC are reported in Table 1 and agree with the requisites required by the United States Food and Drug Administration (US-FDA).[15] So, all compounds had selectivity factors (α) superior to 1.5, capacity factors (k) superior to 2, and resolution parameters (Rs) higher than 2. Besides, the number of plates (N) of this chromatographic column was higher than 2000, and the instrumental precision for all assayed EDCs showed a repeatability (RSD $<$ 1%) and a t_R precision (RSD $<$ 5%) for all compounds. Thus, since in this chromatographic system all the nine compounds had demonstrated optimal resolution parameters and were processed in less than 30 minutes, it was concluded that this method guarantees that all chromatographic parameters agree with the requisites required by the ICH guidelines.[11,12]

Method Validation

Selectivity

In this study, it was observed that when standard solutions of all EDCs were spiked in ultrapure water, low polluted or highly polluted estuarine water samples, both the retention times (t_R) and the UV spectra was maintained between standards and fortified matrixes (RSD $<$ 5%). The peak purity tests performed by the HPLC-DAD software also revealed that all peaks maintain their purity at levels higher than 99%, independently of the matrix. Besides, in Table 1 it is demonstrated that there are, for all compounds, a parallelism among the slopes of the calibration curves, calculated either by external patronization or by the fortified standard mixture method. Therefore, it was concluded that this chromatographic procedure is a selective method for the quantification of the nine EDCs referred to in Table 1.[11,12] Furthermore, the absence of matrix interferences, even in highly polluted matrixes, guarantees the suitability of this method for monitoring proposes in polluted estuarine water samples.

Table 3. Precision (RSD, %), recoveries (%), preconcentration factors, limits of detection (LOD) and quantification (LOQ) in solvent (**1**) or matrix (**2**)

		Precision and recovery rates of each EDC spiked in 2 L of ultra pure water		Precision and recovery rates of each EDC spiked in 2 L of estuarine water sample						
Chemicals (EDCs)	Nominal concentrations (μg/mL)	Recovery (%) (n = 3)	RSD (%) (n = 3)	Recovery (%) (n = 3)	RSD (%) (n = 3)	Preconcentration factors	LOD (1) ng/L	LOQ (1) ng/L	LOD (2) ng/L	LOQ (2) ng/L
DAID	0.40	98	0.70	104	3.40	9800	3.0	8.0	10.0	31.7
	0.80	99	0.04							
	1.00	97	0.60							
GEN	0.40	95	2.20	100	3.50	9500	2.6	7.9	3.2	9.8
	0.80	95	3.50							
	1.00	96	0.80							
BPA	0.90	99	2.30	99	2.60	9900	6.6	20.0	8.0	24.5
	1.80	99	0.60							
	2.25	100	1.30							
E2	0.80	100	0.80	87	2.60	10000	3.0	9.2	7.0	21.3
	1.60	100	0.60							
	2.00	98	1.30							

EE2	1.00	100	2.30	108	1.80	10000	12.0	36.0	18.0	54.5
	2.00	99	0.80							
	2.50	99	1.90							
E1	0.80	94	4.60	116	1.90	9400	5.0	16.0	15.0	44.0
	1.60	98	1.90							
	2.00	98	0.40							
BIO-A	0.12	92	2.10	92	4.30	9200	8.4	25.7	12.4	37.5
	0.24	95	4.30							
	0.30	85	1.90							
4-OP	2.00	87	2.40	69	3.60	8700	2.0	7.0	3.8	12.0
	4.00	66	0.60							
	5.00	58	0.60							
4-NP	2.00	71	2.60	53	4.00	7100	5.8	17.6	7.0	21.8
	4.00	59	4.00							
	5.00	53	1.80							

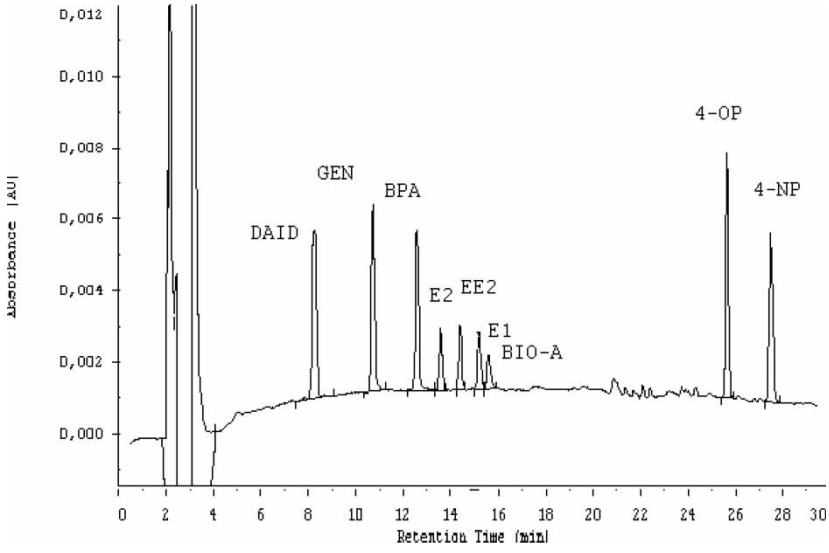


Figure 2. Chromatogram of a standard mixture containing all EDCs assayed in this study.

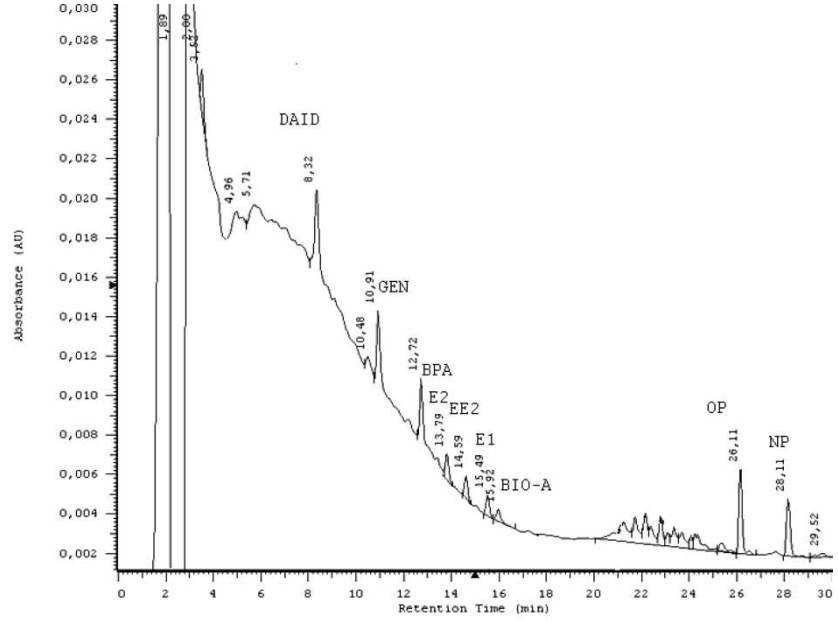


Figure 3. Chromatogram of a water sample collected from a highly polluted area of Douro river estuary spiked with all nine EDCs referred in this study.

Linearity and Range

Calibration curves, prepared as external patronization or standard addition method, were linear for all compounds in the ranges indicated in Table 1. The range, slope, interception points, and correlation levels ($R^2 > 0.99$) of each analytical curve are shown in Table 1. It is important to note that these data are in conformity with the ICH validation requisites.[11,12]

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) (Table 2). Descriptive statistical evaluation of intra and inter-day precision demonstrated that this process has the adequate precision (RSD $< 3\%$) and accuracy (RSD $< 3\%$) recommended in literature.[11,12]

Accuracy was evaluated in parallel with precision. Results in Table 3 refer to the calculation of the recovery rates of the preconcentration and cleanup steps referred in Scheme 1. These results demonstrate that all compounds have high recovery rates, which are within the levels accepted as suitable by the ICH validation procedures.[11]

Limits of Detection and Quantification

The LOD and LOQ concentrations were calculated mathematically using calibration data of both external patronization and the calibration curve obtained by fortified matrix. These results, summarized in Table 3, are within the range of other works using similar procedures.[16] Presently, other methods such as LC-MS,[17,18] and GC-MS[17–19] are referred as excellent methods to determine the levels of estrogens, drugs, alkylphenols, and phytoestrogens in waste waters, because, comparatively to the present method both LOD and LOQ levels are lower. However, LC-MS is highly expensive equipment, and the GC-MS needs for the type of compounds analysed in this study a derivatization procedure, which implies an additional step. Therefore, since these techniques involve higher costs and because we are interested in monitoring the nine EDCs by a low cost and fast procedure (all nine EDCs are analysed in less than 30 minutes), and targeting proven environmentally relevant amounts, we think that this method is an excellent option for the monitoring purposes.

Evaluation of the Efficacy of the Present Method to Water Samples Collected from the Douro River Estuary (Portugal)

To evaluate the applicability of the validated method to monitor relatively high levels of EDCs, several water samples were collected in one highly

polluted area of the Douro river estuary. This location, visibly polluted and receiving both treated and untreated sewage waste waters, was situated near the spot where Ferreira *et al.* described the appearance of fish, grey mullet, with gonadal disruption (ovo-testis).[10] Our data, which is to our knowledge the first of this kind published for these compounds, reveal the existence in this estuary of high levels of estrogens, such as EE2 (up to 56 ng/L) that is the active principle of the contraceptive pills, and E1 (up to 176 ng/L), which is a biologically produced oestrogen from female ovaries.[8,20] These values, compared to others referred to in the literature are very high, but within the range reported in other works in highly polluted aquatic environments.[6,21] Also, the bisphenol (BPA) and the alkylphenol (4-OP), which are industrial products still widely used in household and industrial processes, were found in biological environmental relevant amounts in this estuary, BPA (up to 5.1 μg/L) and 4-OP (up to 449 ng/L). Other works also reported high levels of these compounds in highly polluted rivers and their tributaries.[22,23] Although, high amounts of phytoestrogens that are compounds released into the aquatic environment as byproducts of paper and pulp mills and sewage wastewaters, were found in these estuarine samples, DAID (up to 29 ng/L), GEN (up to 74 ng/L), and BIO-A (up to 47 ng/L) in levels supported by literature.[24] Thus, the present data suggest that this estuary receives urban, industrial, and agricultural pollution, which are consistent with the emergence of endocrine disruption in fish.[25,26] Moreover, the data is in agreement with the above cited endocrine disruption effects in grey mullet.[10] Besides, our results show the importance of monitoring programs in Portuguese estuaries located near highly urbanized and industrialized areas. Finally, since the identities of all measured compounds were recently confirmed by GC-MS,[27] we believe that the present method is excellent for monitoring proposes in highly polluted estuarine waters or suspected hot spots of water pollution.

CONCLUSIONS

This paper describes the development and validation of a simple and fast HPLC-DAD method, which can be easily adjusted to monitoring programs of water assays. The main advantage of this chromatographic method is that nine well known EDCs, with different origins and physical chemical properties, can be simultaneously quantified by HPLC-DAD at room temperature in less than 30 minutes in one single chromatographic run. This optimized method also possesses the specificity, linearity, precision, accuracy, and sensitivity required for monitoring environmental risky levels of all proposed pollutants assayed in this study. After all, taking into account economic aspects, since this method is relatively fast and allows the simultaneous analysis of a high number of pollutants, it proves to be cost effective and efficient when used to monitor a large number of samples. Finally, this study also reports

the first time data about the levels of seven well known endocrine disruptors (E1, EE2, BPA, 4-OP, BIO-A, GEN, and DAID) in the Douro river estuary.

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