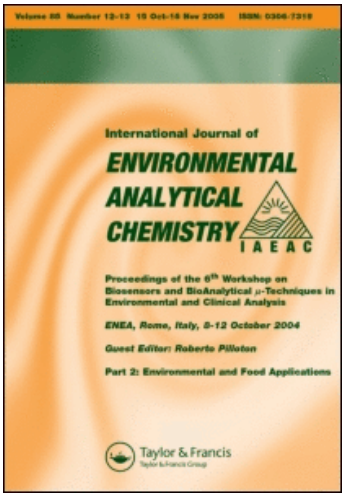

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DETERMINATION OF NATURAL AND SYNTHETIC ESTROGENIC COMPOUNDS IN COASTAL LAGOON WATERS BY HPLC-ELECTROSPRAY-MASS SPECTROMETRY

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A new analytical method was developed for the simultaneous determination of estrogenic compounds of natural (estradiol, estriol, estrone) and synthetic origin, both steroidal (ethinylestradiol, mestranol) and non-steroidal (benzophenone, bisphenol-A, diethylstilbestrol, octylphenol, nonylphenol, nonylphenol mono-ethoxylate carboxylate), in environmental aqueous samples by high-performance liquid chromatography coupled with ion trap-mass spectrometry *via* electrospray interface (HPLC-ESI-IT-MS). Quantitative MS detection was performed in the negative mode for all compounds except mestranol and benzophenone, which were detected under positive ion conditions. Very low method detection limits (MDLs), between 0.1 and 2.6 ng/L, were achieved in coastal lagoon water samples, while the developed solid-phase-extraction (SPE) procedure permitted simultaneous recovery of all analytes from spiked water samples with yields >70% (7–11 RSD%), except estriol and benzophenone, which were recovered with 60% (9 RSD%) and 50% (11 RSD%) yields, respectively. The proposed method was applied to the analysis of Venice (Italy) lagoon waters, where average concentrations of selected compounds in the 2.8–33 ng/L concentration range were found.

Keywords: Endocrine disrupting compounds; HPLC-ESI-MS; Coastal lagoon waters

INTRODUCTION

Endocrine disrupting compounds (EDCs) are receiving increasing attention owing to their wide occurrence in the aquatic environment and their potential hazard to aquatic organisms [1,2]. Estrogenic compounds are EDCs that are triggering major scientific interest, since the relatively low specificity of estrogen receptors makes not only natural hormones (such as estradiol and estrone) but also many synthetic chemicals (such as nonylphenol and bisphenol-A) capable of estrogenic activity [3]. Some substances, moreover, have been demonstrated to be active even at very low concentration levels

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(a few nanograms per liter) [2]. As a result, EDCs are being included in international conventions for the protection of the aquatic environment, especially of natural waters.

A correct evaluation of the potential impact of EDCs toward an ecosystem can be made only with a trustworthy quantitation of residual concentrations in environmental samples. Many analytical methods for the specific determination of individual compounds or classes of EDCs in environmental aqueous samples, such as river waters, municipal/industrial wastewater effluents and sea waters, were developed using both GC and HPLC separation procedures prior to detection, with claimed high selectivity and sensitivity, even in the sub-nanogram per liter range, when MS detection was employed [4–9]. GC techniques need preliminary derivatization in order to increase the volatility of analytes, thus increasing analysis time and avoiding the concurrent detection of structurally different compounds, which would require different derivatizating agents. HPLC, on the other hand, joining the absence of derivatization steps with the large variety of compounds that can be analyzed, especially the polar ones, is becoming the most suitable technique for the determination of EDCs.

Most literature reports concentrate on steroidal estrogens, both natural (estradiol, E2; estrone, E1; estriol, E3), and synthetic (ethinylestradiol, EE2; mestranol, MES), because of their high estrogenic power and potential impact. Steroidal estrogens were detected by both HPLC-MS and GC-MS with very low limits of detection (LODs: 0.01–1 ng/L) in both final effluents and surface waters [4,6–12]. Most methods adopted solid-phase extraction (SPE) for the extraction/concentration/clean-up step. Different stationary phases were used for the extraction/purification, such as C-18 or divinylbenzene-styrene copolymers [10,13]. Carbograph cartridges and solid-phase-microextraction (SPME) methods were also proposed for steroidal EDCs [6] and nonylphenol polyethoxylates and their metabolites [14–16]. In addition, extraction/purification steps with specific immunosorbents have been proposed in order to increase sensitivity and selectivity for E2 and EE2 [7].

In addition to these natural and synthetic steroidal EDCs, non-steroidal (octylphenol, OP; nonylphenol, NP; nonylphenol monoethoxylate carboxylate, NP1EC; bisphenol-A, BPA; benzophenone, BP; diethylstilbestrol, DES) estrogenic compounds were included in this work. Bisphenol-A was analyzed, by both GC and HPLC, in industrial and municipal influents and final effluents [12,17], while fewer determinations were reported in surface waters [18,19]. Diethylstilbestrol, extensively employed between 1950 and 1980 in industrialized countries for chemical castration and animal growth promotion, is nowadays prohibited, although it is illegally applied [20], while benzophenone, widely used in pesticide formulations and consumer products such as creams and drugs, has been recently reported as a potential EDC [21]. Both BP and DES were detected in final effluents from sewage treatment plants and river waters [22,23]. Alkylphenol polyethoxylates (APE), as well as their metabolites, such as OP and NP, were extensively determined in wastewaters, final effluents, river and seawaters years before the discovery of their potential effects as EDCs. Determinations were usually based on HPLC-UV and HPLC-fluorescence [24], and more recently very sensitive and selective methods based on HPLC-MS were proposed [5,15,25–27]. One method included the simultaneous extraction of NPE and their metabolites, both neutral and carboxylated, in environmental samples [14]. Only a few methods, by GC-MS, were recently proposed for the simultaneous determination of environmentally relevant natural and synthetic estrogenic compounds in aqueous samples, and they were applied to the analysis of final effluents of sewage treatment plants [12,28,29].

In this work, a method based on SPE-HPLC-ESI-MS was developed which extends the range of EDCs that can be simultaneously analyzed in environmental aqueous samples. The estrogenic compounds selected for this work were chosen to cover most potential contaminants expected to occur in an ecosystem, such as the lagoon of Venice (Italy), where treated municipal and industrial wastewaters, as well as untreated sewage, are discharged.

EXPERIMENTAL

Chemicals

The selected analytes, estriol (E3), 17β-estradiol (E2), bisphenol-A (BPA), estrone (E1), mestranol (MES), ethinylestradiol (EE2), octylphenol (OP), nonylphenol (NP), benzophenone (BP), diethylstilbestrol (DES), all >98% purity, were from Fluka (Buchs, Switzerland). Carboxylated nonylphenol monoethoxylate (NP1EC, purity approx. 90%) was purchased from CIBA (Basel, Switzerland) and further purified by semi-preparative reversed-phase HPLC on a C-18 column. Isotope-labeled estrogens used as internal standards (*n*-nonylphenol-d_4, bisphenol-A-d_{16}, ethinylestradiol-d_4 and 17β-estradiol-d_3) were obtained from Chemical Research 2000 (Rome, Italy). Ammonium acetate ($AcNH_4$), formic and acetic acid, all of analytical grade (>99% pure), HCl and NH_3 solutions (32%, v/v in water, and 37%, v/v in methanol, respectively) were from Fluka. The sorbing material employed for the solid-phase-extraction (SPE) step was C-18 (Supelclean ENVI-18) supplied by Supelco (Bellefonte, PA, USA). All organic solvents employed were HPLC ultra-gradient grade from Romil (Dublin, Ireland). Water for chromatographic purposes was purified by a MilliQ system (Millipore, Bedford, MS, USA). Standard stock solutions were prepared for all compounds except MES and E1 at 1 μg/μL by dissolving solid standards in methanol. Mestranol and E1, owing to their lower solubility, were dissolved in methanol at 0.1 μg/μL. All working solutions (100, 10, 1, 0.1 ng/μL) were prepared weekly by diluting stock solutions in 2-mL Teflon-capped glass vials from Agilent (Avondale, PA, USA), which were stored in the dark at 2°C before analysis.

Chromatographic Conditions

Analytes were injected in an Agilent 1100 HPLC system by an Agilent G1313A autosampler. Analytes were simultaneously separated by reversed-phase HPLC on a Luna (Phenomenex, Torrance, CA, USA) C8-2 5 μm, 80 Å, 250 × 4.6 mm column kept at 15°C by an Agilent G1316A thermostatted column compartment with an acetonitrile (A) / water (B) linear gradient at 0.7 mL/min. The initial mobile phase composition was 40% A, which was increased to 55% in 15 min, then to 99% in a further 25 min. The column was protected by two C8, 4 × 3 mm, guard columns by Phenomenex. Operation and settings of the HPLC system were controlled by Agilent Chemstation ver. 9.01 software.

Mass Spectrometry

LC-ion trap-MS analysis was performed, with no flow splitting, using an Agilent 1100 MSD-Trap SL detector, *via* an electrospray interface (ESI) operating under

TABLE I Monitored ions and optimal MS parameters for the detection of the examined EDCs

Compound	*MW*	*Capillary voltage (V)*	*Cone voltage (V)*	*Capillary exit voltage (V)*	*Monitored ion under MS conditions (m/z)*	*Monitored ion under MS^2 conditions (m/z)*
Estriol	288	+3500	+40	−108	287, $[M-H]^-$	257
NP1EC[a]	278	+3500	+40	−112	277, $[M-H]^-$	219
Estradiol	272	+3500	+40	−109	271, $[M-H]^-$	185
Estradiol-d_3	275	+3500	+40	−109	274, $[M-H]^-$	–[b]
Bisphenol-A	228	+3500	+40	−110	227, $[M-H]^-$	183
Bisphenol-A-d_{16}	244	+3500	+40	−110	243, $[M-H]^-$	–[b]
Ethinylestradiol	296	+3500	+40	−115	295, $[M-H]^-$	167
Ethinylestradiol-d_4	300	+3500	+40	−115	299, $[M-H]^-$	–[b]
Estrone	270	+3500	+40	−107	269, $[M-H]^-$	145
Diethylstilbestrol	268	+3500	+40	−113	267, $[M-H]^-$	223
Benzophenone	182	−3500	−40	+98	183, $[M+H]^+$	105
Mestranol	310	−3500	−40	+110	311, $[M+H]^+$	159
Octylphenol	206	+3500	+40	−107	205, $[M-H]^-$	105
Nonylphenol	220	+3500	+40	−107	219, $[M-H]^-$	105
Nonylphenol-d_4	224	+3500	+40	−107	223, $[M-H]^-$	–[b]

[a]NP1EC: nonylphenol monoethoxylate carboxylate.
[b]Internal standard, no structural confirmation in real samples.

both negative (NI) and positive ionization (PI) conditions, which were switched during the same chromatographic run. An HP (Palo Alto, CA, USA) 1050 HPLC pump connected *via* a T-union to the 1100 HPLC system between the chromatographic column and the ESI nebulizer was used for the post-column addition, at a 0.07 mL/min flow, of NH_3 (1% in methanol, v : v) and ammonium acetate (1%, w : w) solutions under NI and PI conditions, respectively. The time-scheduled conditions for the post-column addition were the following: NH_3, 0–23.5 min; ammonium acetate, 23.6–30 min; NH_3, 30–45 min. Nebulizing and drying gases were nitrogen kept at 50 psi and 350°C, 10 L/min, respectively. The mass spectrometer was controlled by Agilent 1100 series LC/MSD Trap Control Ver. 4.1 software. Capillary, cone and capillary exit voltages and monitored ions are reported for each examined compound in Table I.

Sampling

Grab water samples were collected in dark glass bottles in three stations located in the central Venice lagoon, two near the historical center of Venice (Stations 1, 2) and one near the industrial area (Station 3). Sampling sites were monitored bi-monthly in the period October 2001–July 2002. Just after collection, 10 mL of a 1% solution of $HgCl_2$ in water were added to samples (final concentration: 100 ppm) in order to prevent bacterial degradation. Particulate matter was eliminated by filtration at 0.7 μm on Whatman GF/F filters (Landspert, NJ, USA). The water samples were stored in the dark at 2°C before analysis, always performed within 96 h after sampling.

Solid-phase Extraction

The examined analytes were extracted (1000 mL, triplicate determination) from lagoon water samples by solid-phase extraction (SPE) on Envi-C18 cartridges (1 g, 6 mL) with an automated Aspec XL SPE system from Gilson (Middleton, WI, USA).

Concurrently with each lagoon water sample, 1000 mL of MilliQ water was extracted as a procedure blank. The stationary phase was conditioned at a flow of 4 mL/min with sequential elution of 10 mL of acetonitrile, 5 mL of methanol and 20 mL of water acidified to pH 2.5 with HCl (37%, v/v). Prior to extraction, proper internal standards were added to samples, which were acidified to pH 2.5 with HCl in order to eliminate carbonates and increase retention of NP1EC on the SPE cartridge. Then, water samples were passed through the SPE cartridges at a flow of 8 mL/min. Cartridges were then washed with 50 mL of MilliQ water acidified to pH 2.5, and finally dried under vacuum for 60 min. Analytes were then eluted with 12 mL (4×3 mL aliquots) of acetonitrile and concentrated under gentle nitrogen flow to 200 μL in an automated evaporator Zymark Turbovap II (Darlington, MA, USA) set at 25°C. The final extracts were diluted to 400 μL with MilliQ water in order to obtain a final solution of ACN/water 50 : 50 (v : v). Extracts were stored in 2-mL Teflon-capped screw cap glass vials from Agilent at 4°C before their injection (200 μL injected volume) into the chromatographic system.

Calibration and Quantification

Six-point calibration curves were constructed for all analytes and deuterated standards across the 1–100 ng (as injected amount) range under both MS and MS-MS modes. Limits of detection (LODs, ng, as injected amount) under MS and MS-MS modes were determined as minimum injected analyte amount giving a s/n ratio of 3. Method detection limits (MDLs) were calculated by spiking a standard mixture in 200 μL of a blank extract (1000 mL sea water collected at 5 km distance from the Venice lagoon outlet, in the Adriatic sea) and lowering the spiking level until a s/n ratio of 3, set as MDL, was recorded.

Quantification of all analytes was performed by the internal standard method using four deuterated standards: estradiol-d_3 (for the quantitation of E2, E3), ethinylestradiol-d_4 (EE2, E1, DES), bisphenol-A-d_{16} (BPA) and n-nonylphenol-d_4 (BP, MES, OP, NP). The four labeled standards were added to the water samples before the filtration step. A correction was made for analytes for which deuterated standards were not available, in order to include the matrix effect (See Results and Discussion). Quantification of NP1EC in real samples was performed under MS-MS detection mode using an external calibration curve, monitoring the ion with m/z : 219, arising from the $[\text{R-CH}_2\text{OOH}]^- \rightarrow [\text{R}]^-$ transition. In order to include the matrix suppression signal, the calibration curve for NP1EC was constructed by spiking a NP1EC standard solution in a 1000-mL seawater extract. All analyzed blanks gave <MDL values, and no blank correction was made.

RESULTS AND DISCUSSION

Mass Spectrometry Detection

Selected EDCs were analyzed with electrospray ionization interface (ESI) under both negative (NI) and positive ionization (PI) modes with formation of $[\text{M} - \text{H}]^-$ and $[\text{M} + \text{H}]^+$ quasi-molecular ions, respectively, in order to find the best s/n values. Mestranol and BP gave the best s/n ratios under PI, while the remaining analytes exhib-

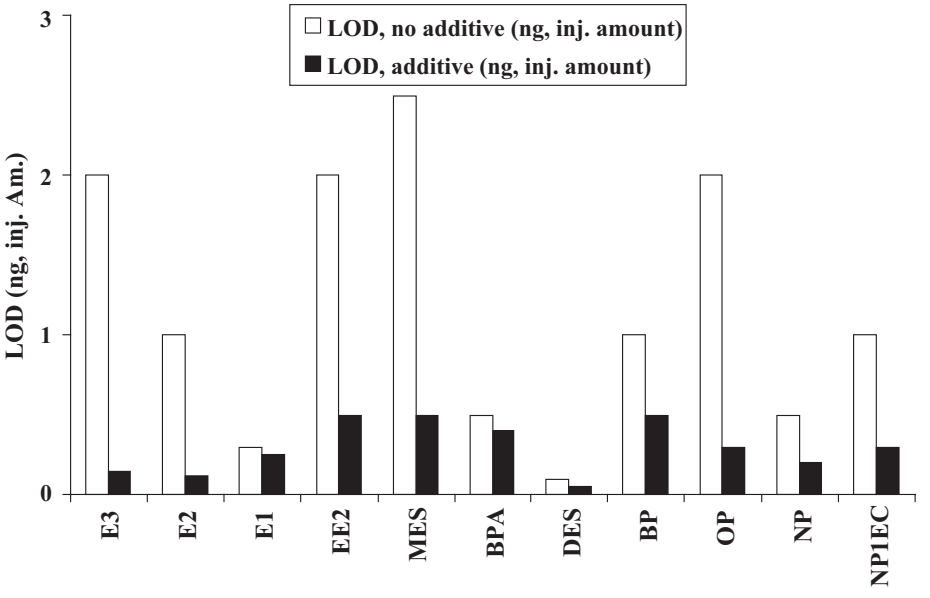

FIGURE 1 Determined LODs (ng, as injected amount) with and without additives for all examined EDCs. Additive: NH_3 (negative ionization mode) for E3, E2, E1, EE2, BPA, DES, OP, NP and NP1EC; NH_4Ac (positive ionization mode) for MES and BP.

ited higher *s*/*n* values under NI. Because of the relatively low *s*/*n* ratio exhibited by most compounds, especially steroidal EDCs, the use of additives for increasing their ionization efficiency in the ESI chamber was investigated. Such additives were added post-column in order to avoid modifications of the chromatographic conditions and for a higher versatility of the method. The best response under NI was obtained using NH_3 dissolved in methanol at 1% (w/w), while under PI detection conditions ammonium acetate dissolved in methanol at 1% (w/w) gave the best *s*/*n* ratios, with respect to formic and acetic acid, concurrently tested. Both additives were added post-column at 10% of the HPLC flow, i.e., 0.07 mL/min. In Fig. 1 the determined LODs (ng, as injected amount) with and without additives, are reported for all selected analytes. The need for using two different additives during the same chromatographic run in order to simultaneously analyze all selected analytes was overcome by using a programmable HPLC pump connected to the HPLC-MS system with a remote control cable, which synchronized the delivering of additive solutions during the chromatographic run, thus permitting the simultaneous HPLC-ESI-MS separation and detection under NI and PI conditions. The area linearity of the ion trap detector was very good for all the tested compounds under MS detection conditions, with R^2 (1–100 ng interval, as injected amount) in the 0.9939–0.9998 range (average: 0.9981), and area reproducibility (RSD%, ten consecutive injections of 10 ng standard mixture) in the 2.6–10% interval. In the MS^n detection mode, the ion trap configuration can, in principle, give structural confirmation or quantification of the selected ions, when fragmentation leads to diagnostic daughter ions. This capability is helpful for a reliable quantification of analytes in environmental samples, where matrix interferences are expected to take place. So, in addition to MS detection, the MS–MS mode was investigated for both confirmation and quantification purposes. All examined analytes, apart from NP1EC

and BP, fragment with low efficiencies and/or giving a high number of daughter ions, thus giving LODs (as injected amount) much higher (>50 ng) than those determined under MS mode. Only NP1EC exhibited a much lower LOD in MS–MS mode, compared with MS detection (0.05 vs. 0.3 ng, respectively, as injected amount), because of the selective induced elimination of the [$\cdot CH_2$–COO] radical, which permitted effective quantification of the selected compound by monitoring the $[277]^- \rightarrow [219]^-$ transition. The LOD obtained for BP (2 ng) under MS–MS detection mode was still suitable for a reliable quantification in real water samples, even if much higher than the LOD determined under MS detection (0.1 ng). The most intense daughter ions monitored under MS–MS mode are reported in Table I.

Chromatographic Separation

The need for a fully resolved chromatographic separation for all analytes to increase the overall MS sensitivity was investigated. The reversed-phase mode was chosen because of the much higher *s*/*n* ratio exhibited under MS detection, and its known high versatility in separating compounds with a large variety of molecular structures. Figure 2 shows a chromatogram of the developed separation suitable for the subsequent MS detection conditions. The best separation was obtained with a C-8 column thermostatted at 15°C. The use of this temperature was critical for obtaining

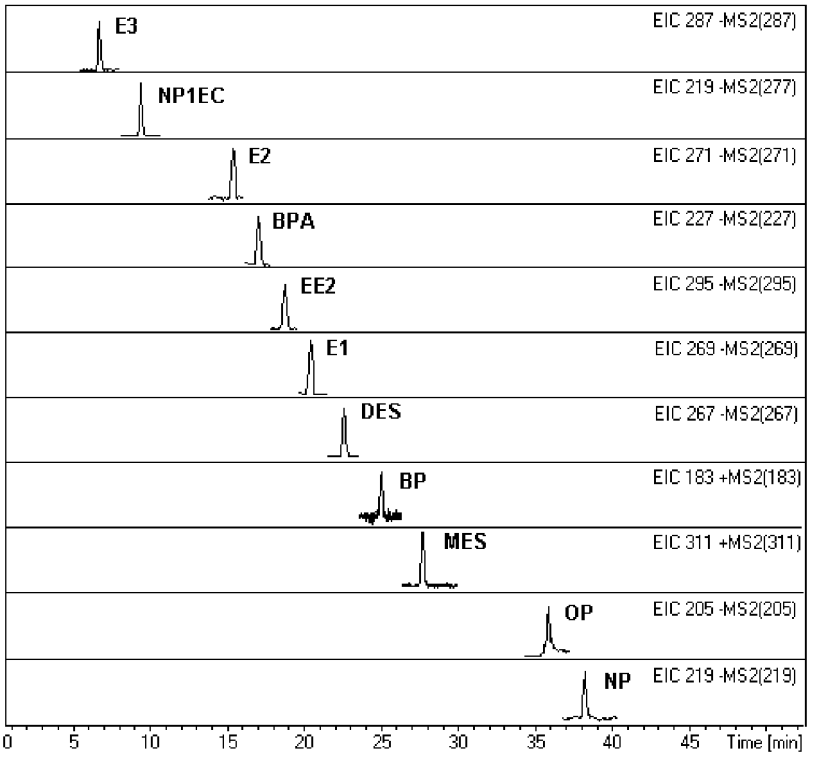

FIGURE 2 HPLC-ESI-MS chromatogram of a standard mixture under the developed separation/detection conditions. Injected amount for each analyte: 10 ng.

a complete chromatographic separation of all selected analytes, particularly of BPA and E2, which were the most difficult compounds to be separated.

Sample Extraction

Satisfactory extraction from lagoon water samples was achieved by using solid-phase extraction (SPE) on a C-18 stationary phase. Extraction was optimized to achieve acceptable recoveries for all the examined compounds, thus compromising efficiencies for some compounds, such as E3 and BP. Recoveries obtained from spiking experiments of a standard mixture added to a sea water sample (collected 5 km from the Venice lagoon outlet, in the Adriatic sea) at 100 ng/L ranged between 70 and 99%, with the exception of E3 and BP, which showed recoveries of 60 and 50%, respectively. In Fig. 3 obtained recoveries (quadruplicate determination) and relative standard deviations (RSD, %) are presented. Reported recoveries include the partial loss by evaporation during the concentration step, which proved to be approx. 10–20% for all analytes down to a final 200 μL extract volume.

Matrix-induced Suppression

A screening investigation was performed to check the potential matrix effect on the ion response of selected analytes, according to previously reported observations [5]. A strong matrix-induced suppression of ion signal was recorded for all the examined compounds when analyzing lagoon water extracts spiked with standard mixtures, with a decrease of response factor by 55–75% of their original values, with respect to those obtained with standard solutions. The observed decrease proved to be independent of the analyzed sample (RSD < 15% on ten different analyzed extracts), with no correlation with retention time. A decrease of observed linearity (R^2: 0.9721–0.9875, average: 0.9821) and reproducibility (RSD: 7–16%, ten consecutive injections

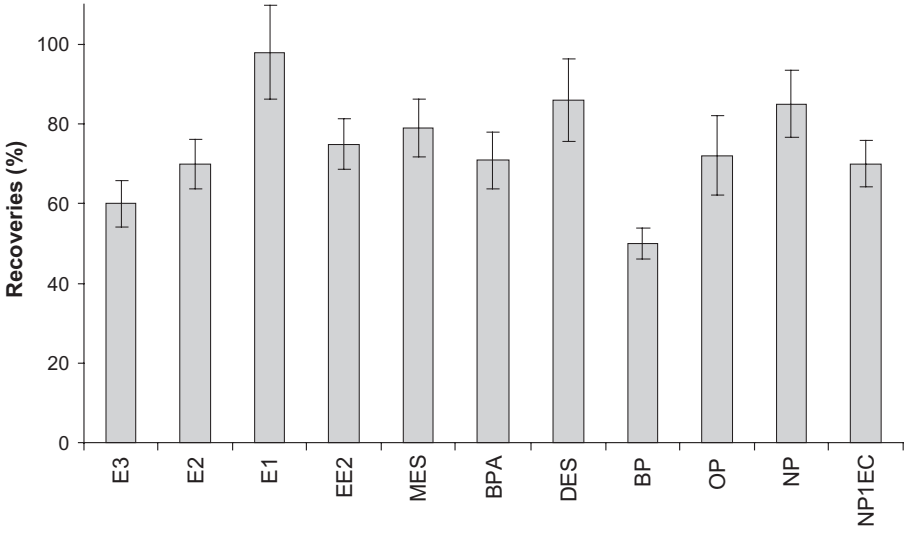

FIGURE 3 Determined recoveries and corresponding relative standard deviations (RSDs, %) obtained for the examined EDCs from 1000 mL of seawater. Spiked concentration: 100 ng/L.

of 10 ng of each analyte spiked to a sea water extract) was also observed. Since the concentrations of these analytes could be significantly underestimated if the external calibration method is used in such aqueous matrices, the use of the internal standard method, with surrogate or deuterated standards, or the standard addition method, are strongly recommended instead. The matrix effect on the internal standard method was investigated for the available deuterated standards, that is, E2, NP, EE2 and BPA. The satisfactory variation (<12%) of response factors between deuterated and non-deuterated analytes spiked to seawater extracts confirmed the suitability of the internal standard method for the quantification of EDCs in the analyzed water samples.

Environmental Applications

The developed method was applied to the determination of the selected EDCs in the central part of Venice lagoon (Italy), a highly urbanized shallow coastal lagoon with a water salt content of 28–36‰. The Venice lagoon receives both untreated sewage from the historical center of Venice (approx. 120 000 equivalent inhabitants), mechanical-biological sewage treatment plants (STPs) final effluents of municipal and industrial origin from the mainland and from the large industrial district of Porto Marghera (approx. 400 000 equivalent inhabitants), as well as contaminated fresh water from some minor rivers [30]. Despite the environmental relevance of the Venice lagoon, no data were available so far about the potential impact of EDCs on this coastal ecosystem, apart from NPE and their metabolites [31]. The developed analytical method was applied to a field survey in the Venice lagoon. Three sampling stations, located near the Porto Marghera industrial district, and near Venice historical center, were monitored for ten months, from October 2001 to July 2002. The resulting average concentrations are reported in Table II, while a typical HPLC-ESI-MS

TABLE II Concentration of estrogenic compounds analyzed in grab lagoon water samples collected over the period October 2001–July 2002

	MDL (ng/L)	*Concentration (ng/L) (min–max)*[a]		
		Station 1	*Station 2*	*Station 3*
Nonylphenol monoethoxylate carboxylate	0.1	33 (2.8–82) $n=4$	7.0 (2.3–16) $n=3$	32 (3.3–71) $n=5$
Estradiol	1.0	3.0 (2.8–3.1) $n=3$	12 (8.0–15) $n=2$	7.6 (7.2–8.0) $n=2$
Bisphenol-A	1	10 (3.4–30) $n=5$	4.5 (2.2–8.8) $n=4$	4.4 (2.3–6.4) $n=5$
Ethinylestradiol	0.8	9.0 (8.0–10) $n=2$	7.1 $n=1$	6.5 (4.6–8.4) $n=3$
Estrone	1.2	3.2 $n=1$	6.0 (5.2–6.7) $n=2$	2.8 (1.9–4.6) $n=3$
Benzophenone	2.6	15 (2.7–36) $n=4$	8.1 (3.4–16) $n=4$	15 (2.8–37) $n=5$
Nonylphenol	0.5	25 (4.0–39) $n=3$	16 (4.3–27) $n=2$	33 (5.8–69) $n=3$

Only analytes with concentrations > MDL are reported.
[a]n = number of samples with concentration > MDL.

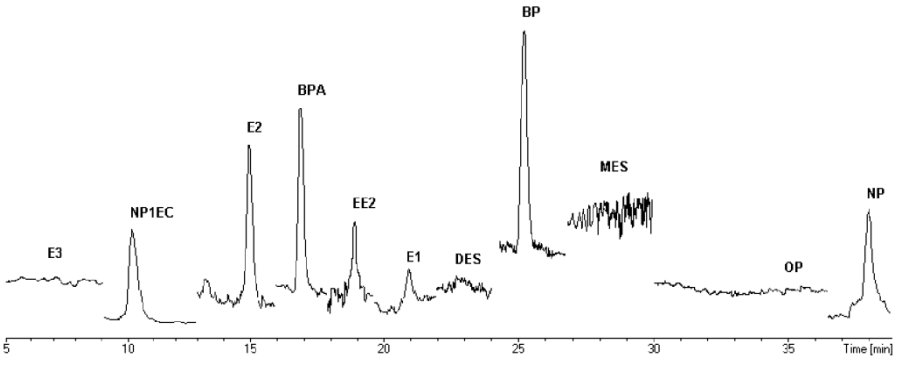

FIGURE 4 HPLC-ESI-MS chromatogram of a lagoon water extract collected at Station 1 during the February sampling session.

chromatogram of a water sample extract (February sampling campaign, station A) is presented in Fig. 4.

Only NP1EC was easily quantified under MS–MS mode, thanks to its very low MDL. All other selected analytes in the collected samples occurred at concentrations too low to apply only the MS–MS mode for quantification, which partly limited the potential of the method. The MS–MS mode was used, when possible, for the structural confirmation of analytes. All target compounds, apart from E3, DES, MES and OP (MDLs: 1.0, 0.6, 2 and 0.8 ng/L, respectively), were detected in all collected samples. Concentrations of steroidal estrogens, such as E2 and EE2 (compounds with the highest estrogenic activity), occurred in the 2.8–15 ng/L and 4.6–10 ng/L range, respectively. The remaining analyte concentrations were in the 2.3–82 ng/L, 2.2–30 ng/L, 2.7–37 ng/L and 4.0–69 ng/L range, for NP1EC, BPA, BP and NP, respectively. The highest concentrations of all examined EDCs were found in winter (November–February sampling sessions), while lower concentrations were recorded in the spring–summer period.

CONCLUSIONS

The developed method by HPLC-ESI-IT-MS proved to be suitable (i.e., sensitive, robust and rapid) for the routine determination of a wide range of natural and synthetic EDCs in coastal marine waters, thus permitting a more comprehensive evaluation of the exposure to EDCs. The reported application of the method allowed us to highlight for the first time the occurrence of natural and synthetic EDCs in a highly urbanized coastal water ecosystem, such as the lagoon of Venice.

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