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Method based on solid-phase microextraction–high-performance liquid chromatography with UV and electrochemical detection to determine estrogenic compounds in water samples

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Abstract

We determined a group of estrogenic compounds by solid-phase microextraction (SPME) coupled to high-performance liquid chromatography (HPLC) with both ultraviolet (UV) and electrochemical detection (ED). A modified liquid chromatograph was used. Polyacrylate fibers (85 μm) were used to extract the analytes from the aqueous samples. Dynamic and static modes of desorption were compared and the variables affecting both absorption and desorption processes in SPME–HPLC were optimized. Static desorption gave the best recoveries and peak shapes. The performance of the SPME–HPLC–UV–ED method was checked with river water and wastewater. The method enabled estrogenic compounds to be determined at low-μg l^{-1} levels in real water samples. Limits of detection were between 0.3 and 1.1 μg l^{-1} using UV detection and between 0.06 and 0.08 μg l^{-1} using ED. β-Estradiol was found in samples from a wastewater treatment plant at concentrations between 1.9 and 2.2 μg l^{-1}.

Keywords: Water analysis; Environmental analysis; Endocrine disruptors; Estrogenic compounds

1. Introduction

Several studies have reported that estrogenic compounds can adversely affect humans and animals [1,2]. These compounds may interact with the organism's endocrine system and disturb or inhibit the natural hormone action. As a consequence, reversible or irreversible alterations in the hormone-regulated processes may appear even at low concentrations [1,2]. A wide range of commonly used compounds are suspected of having these adverse effects on the endocrine system: some pesticides, steroid hormones and their related synthetic compounds, some plasticisers, such as bisphenol A and phthalate esters, and alkylphenols [2,3]. Urban or industrial discharges [3,4] introduce these compounds into the environment and their presence in wastewaters, municipal sewage treatment plants and river waters has recently been reported [3–7]. Nowadays, there is considerable interest in developing analytical methods for determining these compounds in environmental samples at low levels [8].

Common methods for determining such compounds in aqueous samples involve using preconcentration techniques to decrease limits of detection. Liquid–liquid extraction (LLE) or solid-phase ex-

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traction (SPE) followed by gas chromatography with mass spectrometric detection (GC–MS) are some of the most common techniques [4,9,10]. However, some of these compounds cannot be determined by GC without derivatization because they are thermally unstable, polar or ionic [5,9,10]. High-performance liquid chromatography (HPLC) combined with conventional preconcentration techniques such as LLE or SPE is a good alternative for determining these compounds in aqueous samples [3,5,7,11,12].

Solid-phase microextraction (SPME) is a preconcentration technique that has been successfully applied to determine environmental pollutants in aqueous samples [13–16] and some estrogenic compounds [4,17–19]. Desorption is usually carried out in a gas chromatograph [4,17,19], although Boyd-Boland and Pawliszyn determined alkylphenol ethoxylate surfactants in water by SPME–HPLC [18]. A SPME–HPLC interface has been developed to desorb analytes in a liquid chromatograph and some studies have been published to date [15,16,18]. Desorption in HPLC can be performed by placing an organic solvent in the desorption chamber of the SPME–HPLC interface (static desorption) during the desorption time, or by passing mobile phase through the desorption chamber (dynamic desorption).

The main objective of this study is to use SPME coupled to HPLC to determine a group of estrogens (steroid hormones and related synthetic compounds, alkylphenols and bisphenol A) by using an 85 μm polyacrylate fiber. To our knowledge, this is the first study in which SPME–HPLC coupling has been used to determine this group of endocrine disruptors. We optimised the absorption conditions and compared dynamic and static desorption modes. After optimizing the absorption and desorption procedures, we used the SPME–HPLC method to determine these compounds in real water samples by using both ultraviolet (UV) and electrochemical detection (ED).

2. Experimental

2.1. Reagents and standards

The compounds studied were a group of estrogenic compounds: bisphenol A (BA), β-estradiol, α-estradiol, 17α-ethynylestradiol, estrone, diethylstylbestrol, mestranol, 4-nonylphenol, 4-*tert*.-octylphenol and 4-*tert*.-butylphenol. Bisphenol A, 4-nonylphenol, 4-*tert*.-octylphenol and 4-*tert*.-butylphenol were purchased from Aldrich (Steinheim, Germany); β-estradiol, α-estradiol, 17α-ethynylestradiol, estrone and diethylstylbestrol were purchased from Sigma (Madrid, Spain); and mestranol was purchased from Riedel-de Häen (Seelze-Hannover, Germany). A stock standard solution of 2000 mg l^{-1} of each compound was prepared in methanol. Working standard solutions of 100 mg l^{-1} were prepared weekly in methanol. Stock and working standards were stored at 4 °C in the refrigerator. The aqueous solutions were prepared daily by diluting the working solution with water (Milli-Q and real water samples). Sodium chloride (over 99.5% pure) which was added to the aqueous samples, was obtained from Riedel-de Häen. The pH of the aqueous samples was also adjusted with hydrochloric acid from Probus (Badalona, Spain).

HPLC-grade acetonitrile from SDS (Peypin, France) and Milli-Q quality water (Millipore, Bedford, MA, USA) adjusted to pH 2.8 with acetic acid from Probus were used to prepare the mobile phase. To adjust the ionic strength of the mobile phase, we added potassium chloride, supplied by BDH (Poole, UK).

2.2. Instrumentation

The SPME device, the 85 μm polyacrylate fibers and the SPME–HPLC interface were purchased from Supelco (Bellefonte, PA, USA). The chromatographic system consisted of two LC-10AD_{VP} pumps, a DGU-14A degasser and a CTO-6AS oven from Shimadzu (Tokyo, Japan) with two detectors connected in series, a HP-1100 UV detector and a HP-1049A electrochemical detector, both from Hewlett-Packard (Palo Alto, CA, USA). This HPLC system was a modification of a conventional liquid chromatograph in which the mixing chamber was placed after the injection valve, allowing only the organic solvent of the mobile phase to pass through the injection valve. Chromatographic data were collected and recorded by an HP-3365 Series II Chemstation. The separation was performed using an 25×0.46 cm I.D. LiChrospher 100 RP-18 column with a particle size of 5 μm, supplied by Merck

(Darmstadt, Germany). Other analytical columns tested were Kromasil 100 C_{18} (25×0.46 cm I.D., 5 μm) (Technokroma, Barcelona, Spain), Spherisorb ODS-2 (25×0.4 cm I.D., 5 μm) (Technokroma) and Superspher 100 RP-C_{18} (7.5×0.4 cm I.D., 4 μm) (Merck).

2.3. Chromatographic separation

The separation conditions for the estrogens studied were optimized. The mobile phase consisted of Milli-Q water [containing 1% (v/v) acetic acid and 0.5 g l^{-1} of KCl] as solvent A, and acetonitrile as solvent B. The flow-rate of the mobile phase was 1 ml min^{-1} and the gradient profile was 35% B at 0 min, 47.5% B at 10 min (held for 8 min), 100% B at 30 min and then isocratic elution for 5 min. The mobile phase was returned to its initial composition in 5 min. The oven temperature was set at 35 °C. In the UV spectrophotometric detector, all compounds were detected at 280 nm. The potential used in the electrochemical detector was 1.0 V and the temperature 40 °C. A glassy carbon electrode and a solid state Ag/AgCl reference electrode were used. The electrochemical cleaning technique was used once a day by applying a cyclic treatment with alternate potentials to correct the electrodeposition on the surface of the electrode. The working electrode was polished in the conventional way once a week.

2.4. SPME procedure

Before they were first applied, the 85 μm polyacrylate fibers were conditioned in the desorption chamber of the SPME–HPLC interface for 30 min, according to the supplier's instructions. A fiber blank was run after the conditioning process to confirm that there were no peaks that could be assigned to compounds introduced during the manufacture of the fiber. The SPME process was carried out by introducing 3.5 ml of aqueous samples into 4-ml vials. The pH of the samples was adjusted with hydrochloric acid to pH 3.0 and the samples were half-saturated with NaCl (180 g l^{-1}). The 85 μm polyacrylate fiber was then immersed in the sample for 45 min at 65 °C. The samples were heated and stirred with a magnetic stirrer and heater unit from Selecta (Abrera, Spain) at a constant speed of 1400 rpm. To desorb the analytes from the fiber and introduce them into the chromatographic system, a commercial SPME–HPLC interface was connected to a modified HPLC system. The chromatographic system was a modification of a conventional HPLC in which the mixing chamber was placed after the desorption chamber. For this reason, the analytes were desorbed from the fiber by the organic solvent of the mobile phase. Desorption was performed in static mode: the fiber was placed in the desorption chamber filled with acetonitrile for 2 min. Then, the acetonitrile of the mobile phase (0.250 ml min^{-1}) was passed through the desorption chamber and the analytes were introduced into the analytical column by switching the valve from the load to the inject position. After 5 min, the valve was returned to its initial position. Milli-Q water was then inserted into the desorption chamber for 5 min with a syringe so as not to damage the fiber coating when the fiber was removed from the SPME–HPLC interface. Organic solvents can swell the coating and precipitate NaCl on the coating. Consequently, the fiber changes its diameter and the coating may remain inside the interface when the fiber is removed.

Real samples (from river and wastewater treatment plants) were filtered through a 0.45 μm nylon membrane filter (Whatman, Maidstone, UK) before analysis.

3. Results and discussion

3.1. Optimization of chromatographic separation and detection

We tested various analytical columns so that the resolution between the analytes was good. Firstly, we used a conventional Spherisorb ODS-2 (25×0.4 cm, 5 μm) column but we could not separate peaks corresponding to β-estradiol, α-estradiol, 17α-ethynylestradiol and estrone. Secondly, we tested a Kromasil 100 C_{18} (25×0.46 cm, 5 μm) column but resolution between the peaks did not improve. Thirdly, we used a Superspher 100 RP-C_{18} (7.5×0.4 cm, 4 μm) column, whose dimensions are quite different from the other two columns. Resolution was quite good for all the analytes in a short time when injection was direct but it decreased when we used

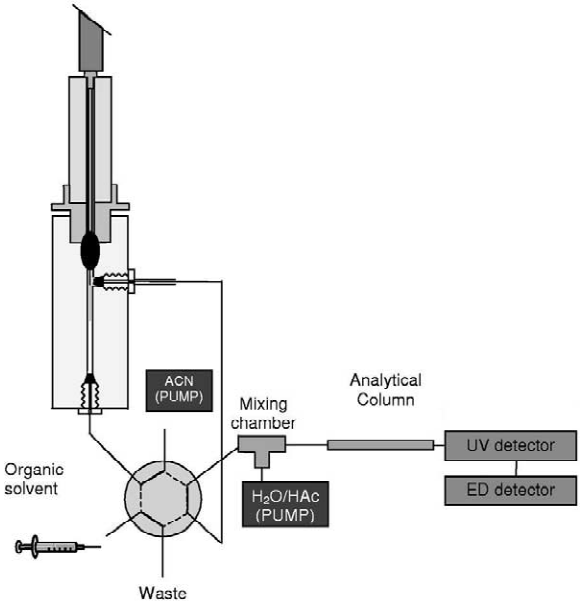

Fig. 1. Experimental set-up of the SPME–HPLC design.

the on-line SPME–HPLC system. Finally, resolution was good for all compounds, even with the on-line system, when we used the LiChrospher 100 RP-18 (25×0.46 cm, 5 μm) column. Thus, we used the LiChrospher column for further experiments.

The responses obtained with the electrochemical detector were at least five times higher than those obtained with the UV detector. However, the baseline obtained with the electrochemical detector drifted as the percentage of acetonitrile in the mobile phase increased and, consequently, the percentage of KCl decreased. The drift was more important from minute 21, when the percentage of the organic solvent in the mobile phase had to be increased from about 50 to 100% to separate the last three eluted compounds. Consequently, the responses for these compounds, mestranol, 4-*tert*.-octylphenol and 4-nonylphenol, decreased considerably and they were difficult to integrate. In order to quantify all the compounds studied in the same analysis, we used both UV and electrochemical detectors connected in

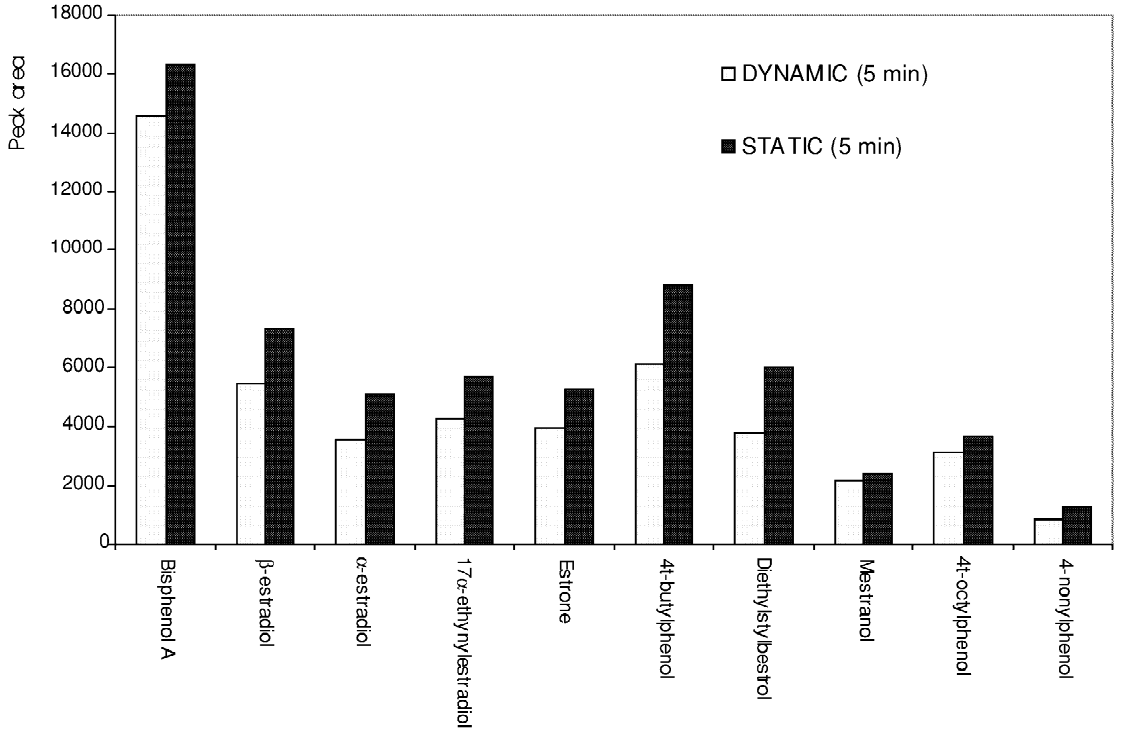

Fig. 2. Comparison of the peak areas obtained in both dynamic and static absorption for Milli-Q water samples containing the estrogenic compounds studied at 0.14 mg l^{-1}.

series. The UV detector was used to determine the three compounds eluted last and the electrochemical detector was used to determine the seven eluted first. Both signals were also used to confirm the presence of the analytes in real samples.

3.2. Optimization of the desorption process

We selected polyacrylate-coated fibers for the SPME because they had provided good results in a previous study, in which we determined phenolic compounds [16], and the compounds studied here also had a phenolic group. We also used the same HPLC design as in this previous study because it gave us better results. Fig. 1 shows the HPLC design used, which allowed only the organic solvent of the mobile phase to pass through the desorption chamber at a flow-rate of 0.250 ml min^{-1} [mobile phase flow-rate (25% acetonitrile): 1 ml min^{-1}].

To compare both the dynamic and static modes of desorption, we used aqueous samples with a concentration of 0.14 mg l^{-1} of the estrogenic compounds. Initially, all these samples were NaCl-saturated and acidified to pH 2.5. Absorption time was 30 min and the temperature was set at 45 °C for the SPME absorption process. In the dynamic mode, the fiber was placed in the desorption chamber and the valve was immediately switched from the load to the inject position. After 5 min, the valve returned to the load position and the fiber was removed from the SPME–HPLC interface. In the static mode, the fiber was placed in the desorption chamber full of acetonitrile for 5 min. Then, the valve was switched from the load to the inject position and the analytes were transferred to the chromatographic column. Fig. 2 shows the peak areas obtained with both desorption modes. Results were best in the static mode for all the compounds, so this was the mode of desorption used in further experiments. These results agree with those obtained in a previous study on phenolic compounds [16].

Then, we optimized the desorption time for the static mode by testing times between 1 and 7 min.

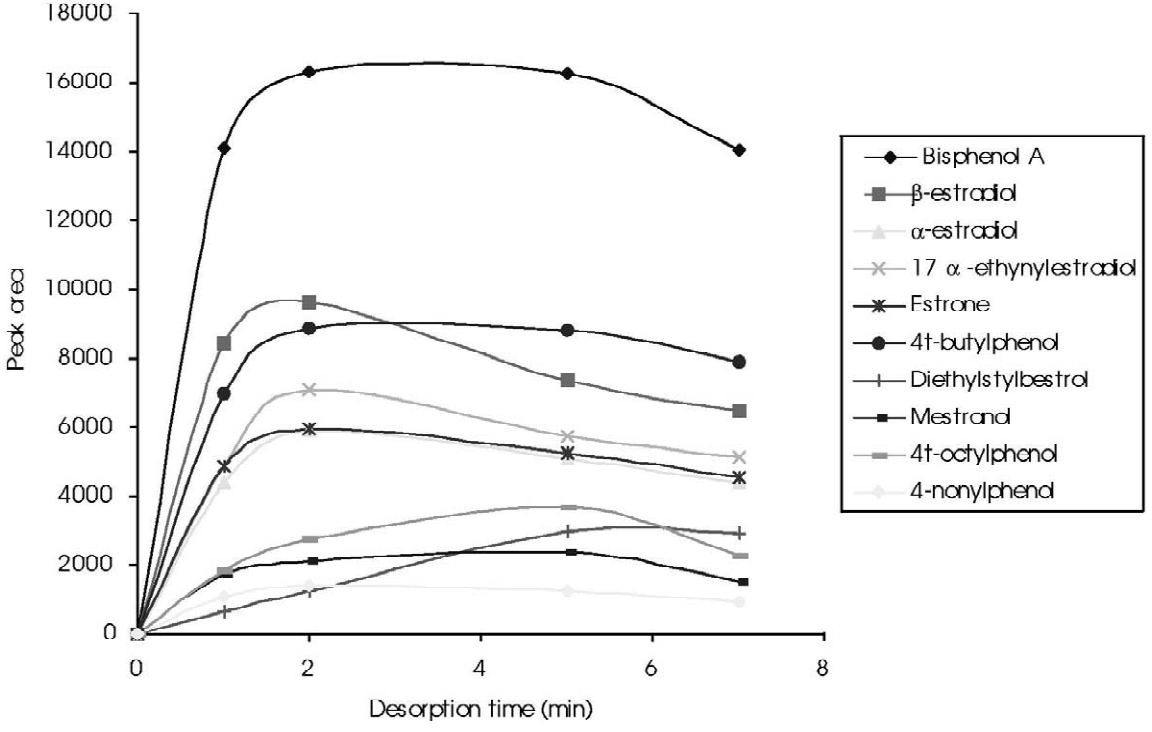

Fig. 3. Optimization of desorption time in the static mode.

Fig. 3 shows the results obtained. A desorption time of 2 min was selected because after this time the recoveries did not increase significantly and no peaks appeared at the retention time of the estrogenic compounds in subsequent analyses.

3.3. Optimization of the absorption process

The factors affecting the SPME absorption process that we optimized were: time (5–60 min) and temperature of absorption (25–75 °C), the addition of NaCl to the sample (0–360 g l^{-1}) and the pH of the sample (2–8). The concentration of estrogenic compounds in Milli-Q water samples was 0.14 mg l^{-1}. We set the desorption parameters at the previously optimized values (static desorption and 5 min of desorption time) and initially set the absorption parameters at the values used in the optimization of the desorption process.

After 60 min of absorption, some estrogens did not reach equilibrium (bisphenol A, diethylstylbestrol, estrone and β-estradiol) but we selected an absorption time of 45 min because it was a good compromise between response and time of analysis. Then, we tested various temperatures between 25 °C (room temperature) and 75 °C. Responses were higher at 65 °C for most compounds, except for mestranol, which showed an optimum temperature of 75 °C, so we selected 65 °C for further experiments. The next step optimized was NaCl concentration in the range between 0 and 360 g l^{-1} (NaCl saturated solution). The amount extracted of the most apolar compounds (diethylstylbestrol, mestranol, 4-*tert*.-octylphenol and 4-nonylphenol) decreased when the NaCl concentration in the aqueous sample increased. The most polar estrogens (bisphenol A, α-estradiol, β-estradiol, 17α-ethynylestradiol and estrone) behaved in the opposite way. We selected a concentration of 180 g l^{-1}, which corresponds to a half-saturated solution, for the next experiments because it gave good results for all the compounds studied. The amount extracted of most of the estrogens did not show a dependence on the pH except for bisphenol A, the recovery of which decreased when the pH was higher than 3.5. For this reason, the pH of the samples was adjusted to 3 in further experiments.

3.4. Performance of the SPME–HPLC method

We initially validated the SPME–HPLC–UV–ED method with Milli-Q water samples. Linear range was good, between 0.2 and 50 μg l^{-1} for all

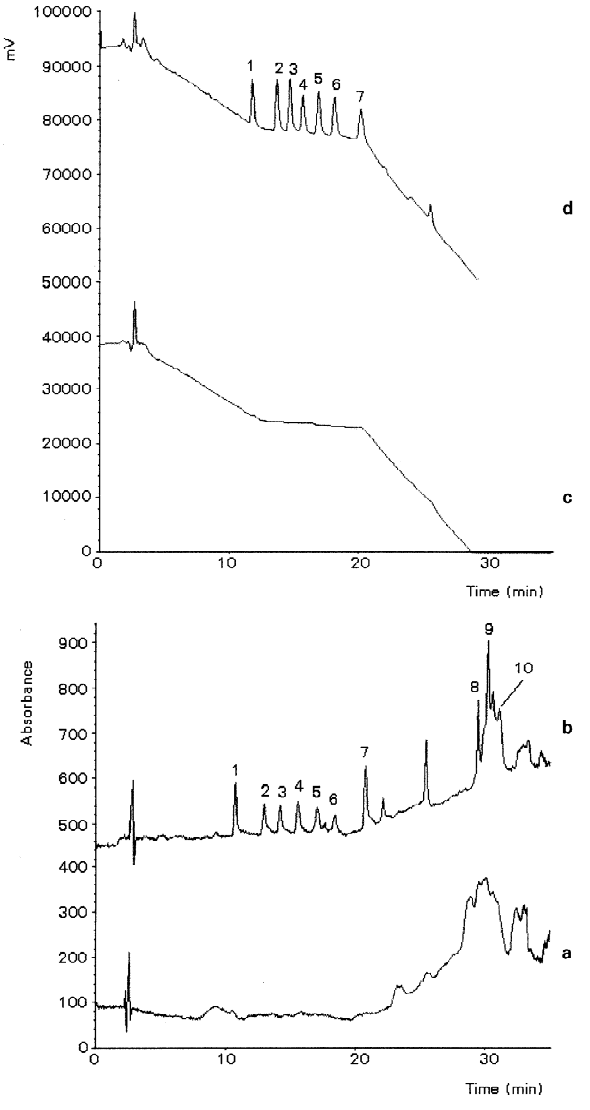

Fig. 4. Chromatograms obtained by SPME–HPLC–UV–ED of 3.5 ml of Ebro river water using (a, b) UV detection and (c, d) ED in static mode of desorption. (a, c) Unspiked Ebro river water; (b, d) Ebro river water spiked with 3 μg l^{-1} of each compound. Peaks: (1) bisphenol A; (2) β-estradiol; (3) α-estradiol; (4) 17α-ethynylestradiol; (5) estrone; (6) 4-*tert*.-butylphenol; (7) diethylstylbestrol; (8) mestranol; (9) 4-*tert*.-octylphenol; (10) 4-nonylphenol.

compounds under ED and between 1 and 50 μg l^{-1} for most compounds under UV detection. Limits of detection, repeatability and reproducibility were also determined. The limits of detection of the method, calculated by the Winefordner and Long criterion with a *K* of 6 [20], were between 0.2 and 1.1 μg l^{-1} for UV detection, and between 58 and 72 ng l^{-1} for ED. The repeatability and the reproducibility between days, calculated as the relative standard deviation (RSD, $n=3$), were determined with Milli-Q water spiked with 3 μg l^{-1}. In ED, the RSDs were under 11 and 15% for repeatability and reproducibility, respectively. In UV detection, the RSDs were under 10 and 18%, respectively.

3.5. Application to real samples

We tested the performance of the method with Ebro river water samples. First, a blank of the Ebro river water was run to verify the absence of various peaks at the retention time of compounds studied. No interfering peaks appeared in the chromatogram. Fig. 4 shows the chromatograms of the blank analysis with both ED and UV detection. The linear range, limits of detection, and the repeatability and reproducibility of the method were also determined with Ebro river water, in the same way as with Milli-Q water. Table 1 shows that the results were similar to those obtained for Milli-Q water samples. Fig. 4 shows the chromatograms for Ebro river water and Ebro river water spiked with 3 μg l^{-1} of estrogenic compounds and analyzed by the SPME–HPLC–UV–ED method.

We analyzed several samples taken from several points along the river Ebro and from a wastewater treatment plant. We found no compounds in the Ebro river water but we found one peak at the retention time of β-estradiol in a water sample from the wastewater treatment plant. This peak was identified as β-estradiol by retention time matching and comparing the signal in both UV and electrochemical detectors. Two peaks at the retention time of 4-*tert*.-octylphenol and 4-nonylphenol also appeared in this sample but they were under the quantification limit. Three replicates of this sample were performed and the mean concentration of the compound was 1.9 μg l^{-1} (RSD 14%) for β-estradiol in UV detection and 2.2 μg l^{-1} (RSD 11%) in ED. Fig. 5 shows the chromatograms of the sample from the wastewater treatment plant for both ED and UV detection.

4. Conclusions

SPME coupled to HPLC–UV–ED was successfully applied to determine a group of estrogenic com-

Table 1
Linear range, determination coefficients, limits of detection and repeatability and reproducibility for Ebro river water by SPME–HPLC–UV–ED

Compound	UV detection					ED				
	Linear range (μg l^{-1})[a]	r^2	LOD (μg l^{-1})	RSD[b] (%)	RSD[c] (%)	Linear range (μg l^{-1})[a]	r^2	LOD (μg l^{-1})	RSD[b] (%)	RSD[c] (%)
Bisphenol A	1–50	0.9949	0.3	11	17	0.2–50	0.9983	0.06	16	19
α-Estradiol	1–50	0.9951	0.4	13	21	0.2–50	0.9925	0.08	10	12
β-Estradiol	1–50	0.9928	0.3	16	24	0.2–50	0.9921	0.07	11	15
17α-Ethynylestradiol	1–50	0.9906	0.4	9	18	0.2–50	0.9906	0.07	9	12
Estrone	2–50	0.9957	0.7	6	16	0.2–50	0.9960	0.07	10	12
4-*tert*.-Butylphenol	2–50	0.9960	0.7	16	16	0.2–50	0.9998	0.07	15	18
Diethylstylbestrol	1–50	0.9917	0.3	3	17	0.2–50	0.9960	0.07	21	25
Mestranol	2–50	0.9913	0.7	18	23	–	–	–	–	–
4-*tert*.-Octylphenol	2–50	0.9961	0.8	14	24	–	–	–	–	–
4-Nonylphenol	3–50	0.9953	1.1	24	31	–	–	–	–	–

[a] Values higher than 50 μg l^{-1} were not tested, because those compounds are usually only present in real-life samples at lower concentrations.
[b] Under repeatability conditions ($n=3$).
[c] Under reproducibility between-day conditions ($n=3$).

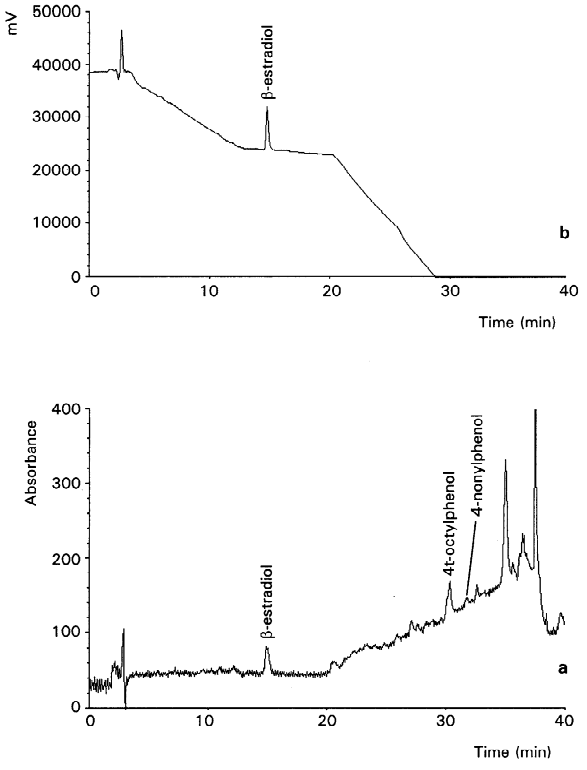

Fig. 5. Chromatograms obtained by SPME–HPLC–UV–ED of a sample from a wastewater treatment plant by: (a) ED; (b) UV detection.

pounds in water samples at low $\mu g\ l^{-1}$ levels. The various parameters that affect both absorption and desorption in SPME–HPLC were optimized.

The static desorption mode gave better results than the dynamic mode for all the compounds.

Although electrochemical detection showed a higher response than UV detection for the compounds studied, UV detection made it possible to determine the three compounds eluted last. These could not be determined by the electrochemical detector because of the baseline drift observed when the acetonitrile (mobile phase) increased.

We identified β-estradiol, 4-*tert*.-octylphenol and 4-nonylphenol by retention time matching in a sample from a wastewater treatment plant. We also quantified β-estradiol in this sample by using data from both detectors. Concentrations were 1.9 and 2.2 $\mu g\ l^{-1}$ with UV and electrochemical detection, respectively.

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