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Low cost methodology for estrogens monitoring in water samples using dispersive liquid–liquid microextraction and HPLC with fluorescence detection

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ABSTRACT

A new low cost methodology for estrogens' analysis in water samples was developed in this work. Based on dispersive liquid–liquid microextraction followed by high-performance liquid chromatography with fluorescence detection, the developed method is fast, cheap, easy-to-use, uses low volumes of organic solvents and has the possibility of a large number of samples to be extracted in parallel. Under optimum conditions (sample volume: 8 mL; extraction solvent: 200 μL of chlorobenzene; dispersive solvent: 2000 μL of acetone), the enrichment factor and extraction recoveries were 145 and 72% for 17β-estradiol (E2) and 178 and 89% for 17α-ethinylestradiol (EE2), respectively. Limits of detection of 2.0 ng L^{-1} for E2 and 6.5 ng L^{-1} for EE2 were achieved, allowing the detection and quantification of these compounds in surface and waste water samples with concentrations from 12 to 32 ng L^{-1} for E2 and from 11 to 18 ng L^{-1} for EE2. Also, recovery tests were performed to evaluate possible matrix effects. Recoveries between 98% and 106% were obtained using humic acids (HA) to simulate the effect of organic matter, and between 86% and 120% in real water samples.

1. Introduction

It was only after 1990 that the presence of hormones in the aquatic environment started to draw some attention, due to the established relation between synthetic birth-control pharmaceutical and its impact on aquatic living organisms [1]. Since then, and due to their endocrine disrupting effects, there has been a growing concern about the presence of steroidal hormones in natural waters. Recently, the Water Framework Directive, that foresees a list of priority substances that present a significant risk to or via the aquatic environment, was reviewed and on the 31st of January 2012 the Legislative Commission proposed 15 additional priority substances, which include 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) [2].

Steroid hormones can enter the environment through sewage discharge and animal waste disposal, since all humans and animals can excrete them from their bodies [3,4] in different amounts, depending on gender, age, state of health, diet or pregnancy [4,5]. Released in urine as conjugated glucuronides or sulfate complexes [6], they can rapidly be converted to fully potent hormones by cleavage and/or metabolization during transport and treatment of sewage [7]. E2 and EE2 are amongst the estrogens most commonly found in waste water [8,9]. Also, E2 and EE2 have been detected in effluents of different sewage treatment plants (STPs) (up to 94 ng L^{-1} and 64 ng L^{-1}, respectively) [3,6,10,11] and in surface waters (up to 7 ng L^{-1} and 27 ng L^{-1}, respectively) [3,12]. Even at such low concentrations, these compounds may represent serious risks, particularly to the aquatic population [8,9].

Due to their extremely low environmental concentrations, E2 and EE2 direct quantification in water samples usually requires instruments with high sensitivity, such as gas chromatography coupled with mass spectrometry (GC–MS) [13] and GC–MS/MS [14], as well as liquid chromatography coupled with mass spectrometry (LC–MS) [15] and LC–MS/MS [16]. Among these analytic techniques, LC–MS and LC–MS/MS have experienced an impressive progress in last decades and have been indicated as techniques of choice for environmental analysis of steroid hormones because of their high sensitivity and selectivity [17]. However, their use accounts with several drawbacks, such as the expensive instrumentation, high maintenance costs and the requirement of specifically trained analysts. As a consequence, considering current financial restrictions, the elevated related costs may be unaffordable for many analytical laboratories.

Compared with LC–MS and LC–MS/MS, high-performance liquid chromatography (HPLC) coupled either to UV or fluorescence detector

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is a simpler, faster, easy-to-use and widely available technique. Still, detection limits are not as good as with MS detectors so a pre-concentration step is required. Among pre-concentration methodologies that can be applied are solid-phase extraction (SPE) and liquid–liquid extraction (LLE). An SPE coupled online with liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) has been used for determination of hormones in water samples with limits of quantification between 0.02 and 1.02 ng L^{-1} [18]. However, SPE implies a high consumption of organic solvents. In this sense, solid phase microextraction (SPME) has an advantage over SPE, which is a minor solvent consumption. However, for SPME, the fragile, expensive, limited lifetime and sample carryover of the fiber is also an issue [19]. Regarding classical LLE, main weaknesses are that it is time-consuming and requires large volumes of toxic organic solvents. Nonetheless, liquid-phase microextraction (LPME) overcomes many disadvantages of LLE as well as some of those of SPME [20]. Among LPME techniques, several have been applied successfully to steroid hormones pre-concentration. Hollow-fiber LPME (HF-LPME) combined with GC–MS was optimized for the determination of steroid hormones with LODs of 1.6–10 ng L^{-1} [21]. Chang and Huang [22] applied liquid–liquid microextraction with solidification of a floating organic drop (DLLME–SFO) followed by HPLC, obtaining LODs ranging from 0.8 to 3.1 μg L^{-1}.

DLLME, which was first introduced in 2006 by Rezaee and co-workers [23], is a simple and fast microextraction technique based on a ternary component solvent system that has been applied to the extraction and pre-concentration of several compounds e.g. [24–28]. Succinctly, a few microliters of an appropriated organic solvent —extracting solvent— together with a dispersive solvent (with high miscibility in both extractant and aqueous phase) are rapidly injected into the sample, producing high turbulence. Such turbulence originates the formation of small droplets, which are dispersed throughout the aqueous sample. After the formation of a cloudy solution, the equilibrium state is achieved, the mixture is centrifuged and the sedimented phase containing the analyte is collected. The main advantages of this extraction procedure are the short extraction time, the possibility of a large number of samples to be extracted in parallel, simplicity, low quantity of organic solvents, low cost and high recovery and enrichment factors [20]. Thus, DLLME may be an interesting pre-concentration option for the HPLC analysis of hormones at a moment when it is crucial to develop new and low cost methodologies able to concentrate and determine, at environmental levels, these “emerging” contaminants that have raised great concern in the last years.

The main purpose of this work was the development of a low cost methodology for the analysis of steroid hormones in environmental samples, focusing in the sample preparation and on lowering the limit of detection. DLLME–HPLC with fluorescence detection (FLD) was optimized for simultaneous determination of E2 and EE2 in tap, surface and waste water samples. Also, matrix effects, such as presence of organic matter, which can decrease the extraction efficiency, were evaluated.

2. Materials and methods

2.1. Reagents and standards

Steroid hormones 17β-estradiol (≥97%) and 17α-ethinylestradiol (≥98%) and chlorobenzene (C_6H_5Cl) (99.9%) were supplied by Sigma. Trichloroethylene (C_2HCl_3) (99%) and carbon tetrachloride (CCl_4) (99.9%) were obtained from Panreac. Methanol, acetone and acetonitrile, all for HPLC 99.9%, were from Fischer Chemical, Carlo Erba and HiPerSolv CHROMANORM, respectively. Chloroform ($CHCl_3$) (99%) was from Scharlau and dichloromethane (CH_2Cl_2) (99.8%) was purchased from Riedel-de Haën. Commercial humic acids (HA) (technical) were also obtained from Sigma. Ultrapure water was obtained from a Milli-Q water purification system from Millipore.

Individual standard stock solutions of E2 and EE2 were prepared in methanol at a concentration of 100 mg L^{-1}. Each solution was further diluted to the appropriate concentration using ultrapure water. A stock HA solution of 1 g L^{-1} at pH 9 (in 1 mol L^{-1} ammonium hydroxide) was also prepared.

2.2. Instrumentation

E2 and EE2 analysis was performed on a Shimadzu High-Performance Liquid Chromatograph Prominence system equipped with a fluorescence detector. This device consists of a degasser DGU-20A5, a bomb LC-20AD, and a column oven CTO-10ASVP. A new ACE® C18 column-PFP (5 μm, 150 mm × 4.6 mm) connected to an ACE® 5 C18 4.6 mm i.d. guard column was used for the separation. The mobile phase consisted of a water:acetonitrile mixture (50:50, v/v), at a flow rate of 0.8 mL min^{-1} with an injection volume of 20 μL. Detection was performed using a Shimadzu Prominence RF-20A XS fluorescence detector at an excitation wavelength of 280 nm and an emission wavelength of 310 nm [29]. Both the column and cell temperatures were maintained at 25 °C.

Water and acetonitrile used in the mobile phase were pre-treated by filtering through a 0.2 μm polyamide membrane filters from Whatman. A Lab Dancer Mini Vortex from VWR (Portugal) was used to perform the agitation during the extraction procedure.

2.3. DLLME procedure

Aliquots of 8 mL of samples or ultrapure water spiked with E2 or EE2 were added to 12 mL glass centrifuge tubes with conical bottom. Then, a mixture containing 2000 μL of acetone and 200 μL of chlorobenzene was injected into each tube, which was immediately shaken for 30 s using a vortex. After the formation of the cloudy solution, as a result of the dispersion of fine droplets of C_6H_5Cl in aqueous sample, the tubes were centrifuged at 4000 rpm for 5 min. Chlorobenzene organic phase sedimented at the bottom of the conical centrifuge tube was transferred to a 2 mL vial, dried under a nitrogen stream and redissolved using 40 μL of acetonitrile. The redissolved fraction was then analyzed by HPLC–FLD.

2.4. Optimization of extraction conditions

2.4.1. Selection of extracting solvent

In DLLME there are several factors that affect the extraction efficiency. One of these factors is the choice of the suitable extracting solvent. Organic solvents with higher density than water, good chromatographic behavior and high extraction capability of the analyte of interest are generally used in these extractions [20]. Among them, halogenated hydrocarbons, such as CCl_4, $CHCl_3$ and C_6H_5Cl, are usually selected as extracting solvents due to their high density.

From all organic solvents available, CCl_4, $CHCl_3$, C_6H_5Cl, C_2HCl_3 and CH_2Cl_2 were tested to extract E2 and EE2 from water samples. The procedure consisted in injecting into an 8 mL sample, spiked with 1 μg L^{-1} of E2 or EE2, a mixture containing 500 μL of methanol and 50 μL of one of the following solvents: CCl_4, C_6H_5Cl, C_2HCl_3, or 75 μL $CHCl_3$ (a higher volume was used in order to obtain a similar volume of sedimented phase). For CH_2Cl_2, 8 mL sample spiked with 1 μg L^{-1} of E2 or EE2 was injected with only 200 μL of CH_2Cl_2, since using methanol, as dispersive solvent, no two-phase system was formed.

2.4.2. Selection of dispersive solvent

Miscibility of dispersive solvent in both aqueous phase and extracting solvent is an essential factor to select a dispersive solvent. Acetone, methanol and acetonitrile are usually the solvents of choice. In this work, those three dispersive solvents were tested along with the three extracting solvents with the best results in the previous section. Also, an extraction without the use of a dispersive solvent was performed for comparison.

2.4.3. Selection of volume of extracting and dispersive solvents

The optimal ratio between extracting and dispersive solvent volumes should ensure high extraction efficiency. This ratio affects directly the formation of the cloudy solution, the dispersion degree of the extracting solvent in aqueous phase and also the extraction efficiency [20]. The procedure consisted in injecting to an 8 mL sample spiked with 1 $\mu g\,L^{-1}$ of E2 or EE2, 500 μL of acetone containing different volumes of chlorobenzene. The different volumes of chlorobenzene will result in a different extracting solvent:dispersive solvent ratio. The volumes tested were 50, 70, 85 and 100 μL resulting in the ratios 1:10, 1:7.1, 1:5.9 and 1:5, respectively.

2.4.4. Salt and agitation time effect

It is well known that, generally, the increase in the ionic strength of sample solution results in a decrease of analyte solubility and enhances extraction recovery (ER), due to the salting-out effect [22,30]. The addition of salt on the extraction efficiency was tested by adding NaCl 5% (w/w). Also the effect of agitation time was evaluated between 30 s and 5 min. The agitation time is defined as the time interval between the mixture injection of extracting and dispersive solvents and the starting time of centrifugation.

2.5. Matrix effect

Since the DLLME method is intended to be applied directly to environmental samples, it is necessary to evaluate possible interferences of water matrix. The influence of organic matter was first simulated using HA with concentrations ranging from 0 to 30 $mg\,L^{-1}$. Also, and in order to evaluate real matrix effects, the extraction recovery rates for E2 and EE2 were determined by spiking 15, 30 and 60 $ng\,L^{-1}$ of E2 and EE2 into three different types of water samples—tap, surface and waste water.

2.6. Determination of E2 and EE2 in environmental water samples

Finally, and in order to evaluate the applicability of the DLLME proposed, tap, surface and waste water samples from a STP were collected in glass containers and subjected to the optimized method described previously. Waste water samples were collected at three different points of the treatment: after primary decantation, after secondary biological treatment and after secondary decantation (which corresponds to the final treated effluent). Immediately after collection, all samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore) and stored at 4 °C prior to extraction.

3. Results and discussion

3.1. Optimization of extraction conditions

3.1.1. Selection of extracting solvent

Results obtained on the selection of the extracting solvent are presented in Fig. 1.

From all the extracting solvents tested, chloroform and chlorobenzene presented similar results for EE2; however, for E2, the extraction capacity of chlorobenzene was higher than that of chloroform. For both estrogens, carbon tetrachloride and dichloromethane, as extracting solvent, presented lower peak areas, thus suggesting a lower extraction capacity. In fact, the formation of the cloudy solution was not evident.

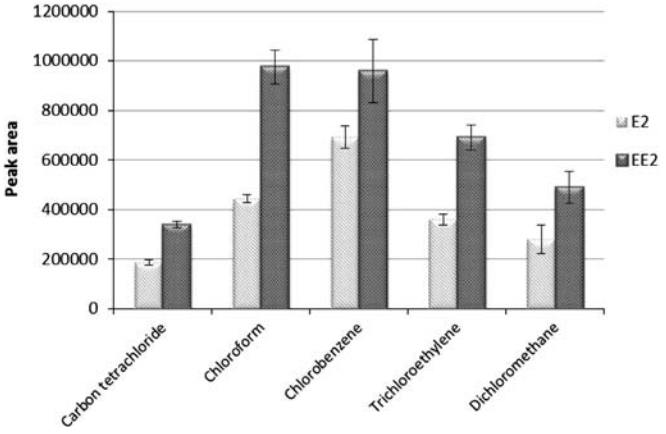

Fig. 1. Effect of extracting solvent ($n=3$). Extraction conditions: 8 mL of sample spiked with 1 $\mu g\,L^{-1}$ E2 or EE2; extracting solvent: 50 μL carbon tetrachloride, chlorobenzene, trichloroethylene, 75 μL chloroform and 200 μL dichloromethane; dispersive solvent: 500 μL of methanol, except with dichloromethane where no dispersive solvent was used; extraction time: 30 s.

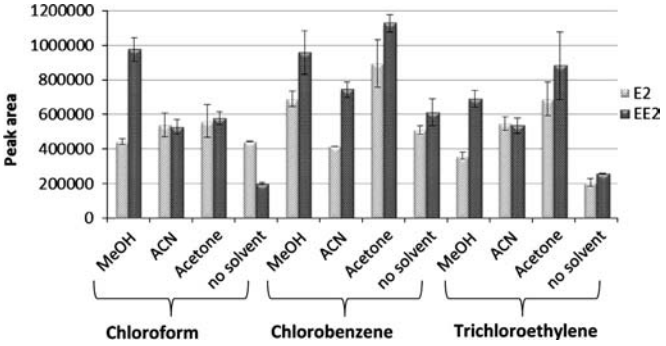

Fig. 2. Effect of dispersive solvent ($n=3$). Extraction conditions: 8 mL of sample spiked with 1 $\mu g\,L^{-1}$ E2 or EE2; extracting solvent: 75 μL chloroform, 50 μL chlorobenzene and trichloroethylene; 500 μL of dispersive solvent; extraction time: 30 s.

3.1.2. Selection of dispersive solvent

Since the extraction capacity of the organic solvent is also influenced by the dispersive solvent, the three extracting solvents with the best results (chloroform, chlorobenzene and trichloroethylene) were chosen to be tested with different dispersive solvents (acetone, methanol and acetonitrile). For all extracting solvents tested, acetone seems to be the more suitable dispersive solvent for the extraction of both compounds (Fig. 2). The only exception is chloroform, for which methanol seems to be a more efficient dispersive solvent for EE2 extraction. The combination chlorobenzene–acetone was the one chosen between all possibilities tested, due to the higher peak areas obtained. It is also possible to observe that extraction without dispersive solvent is generally poorer than using a dispersive solvent.

3.1.3. Selection of volume for extracting and dispersive solvents

The volumetric ratio between extracting and dispersive solvents was also tested and results are shown in Fig. 3. It is possible to observe a different behavior for E2 and EE2. While for EE2 the peak area tends to decrease when increasing the ratio between the extracting and the dispersive solvents, for E2 there is not such an obvious trend. The ratio of extracting solvent:dispersive solvent giving the best results is not the same for both compounds. For E2,

85 μL of extracting solvent (1:5.9 ratio) is the best choice; however, for EE2, 50 μL of extracting solvent (1:10 ratio) is a better solution. These results require a compromise in order to choose a common suitable ratio. Since the EE2 concentration expected in water samples is generally lower than for E2, it is necessary to choose the experimental procedure that allows a higher enrichment factor (EF) for EE2. For this reason, the ratio extracting solvent: dispersive solvent chosen was 1:10 (50 μL of extracting solvent and 500 μL of dispersive solvent).

The extracting solvent:dispersive solvent total volume has a direct influence on the EF. Increasing the volume will directly increase the volume of sedimented phase, thus decreasing the EF. However, the halogenated hydrocarbons are not compatible with the reverse-phase-HPLC mobile phase and thus, an extra step is needed to evaporate them before final analysis. Previously to HPLC analysis, the fraction is redissolved in acetonitrile. Considering the volume of acetonitrile as the sedimented volume to obtain the EF, the volume of extracting mixture can be increased to improve the extraction efficiency without a direct influence on the EF.

A mixture with 1:10 ratio between extracting solvent:dispersive solvent was prepared and different volumes of this mixture were used to perform the DLLME (Fig. 4).

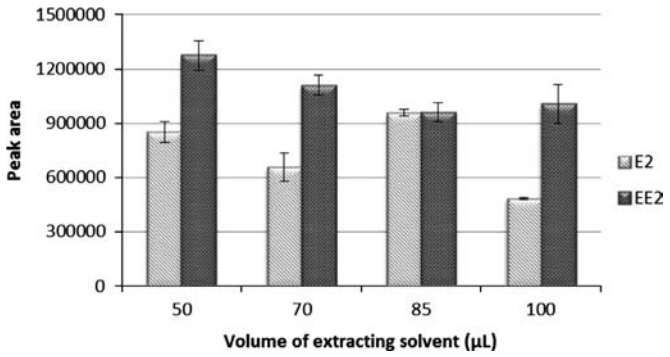

Fig. 3. Effect of extracting solvent volume ($n=3$). Extraction conditions: 8 mL of sample spiked with 1 μg L^{-1} E2 or EE2; extracting solvent: chlorobenzene; 500 μL of acetone as dispersive solvent; extraction time: 30 s. The volumes tested 50, 70, 85 and 100 μL correspond to the ratios 1:10, 1:7.1, 1:5.9 and 1:5, respectively.

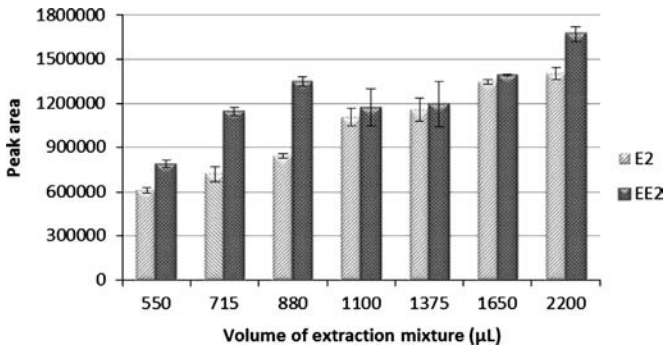

Fig. 4. Effect of extracting mixture volume ($n=3$). Extraction conditions: 8 mL of sample spiked with 1 μg L^{-1} E2 or EE2; extracting solvent:dispersive solvent ratio 1:10; extraction time: 30 s.

The results show a predictable increase in the peak areas with the increase of the extraction mixture volume. However, this increase will result in a higher organic solvent volume for evaporation, prior to analysis. For EE2, with 2200 μL, the recovery rates reach around 90%, a value considered very good for this pre-concentration procedures. For E2, the recovery is around 70%, a value that should be improved. However, when the volume of extraction mixture increases from 1650 to 2200 μL, the E2 peak area, and thus the associated recovery, does not change significantly, suggesting that a volume higher than 2200 μL will not improve the extraction. For this reason, the extraction mixture volume of 2200 μL was selected.

3.1.4. Salt and agitation time effect

Results obtained showed that the addition of NaCl 5% (w/w) had no influence in the recovery of E2 and EE2. Also, no difference in the recovery was observed for agitation times between 30 s and 5 min, concluding that 30 s are enough to reach the maximum extraction yield.

3.2. Analytical performance

Under optimized conditions, linear range, correlation coefficient (r), linearity, limit of detection (LOD), ER and EF of the DLLME method, for both estrogens, were calculated and are presented in Table 1. The performance of the method was evaluated with the r, LOD and linearity (Lin (%) $=100-RSD_b$, where RSD_b is the relative standard deviation of the slope). LOD was calculated from each calibration curve as $a+3s_{y/x}$, where a is the intercept of the regression line and $s_{y/x}$ is the statistical parameter which estimates the random errors in the y-axis (signal). Linear range was 10–300 ng L^{-1} for E2 and 10–500 ng L^{-1} for EE2, while LOD was 2.0 ng L^{-1} for E2 and 6.5 ng L^{-1} for EE2. Both estrogens presented good correlation coefficients and good linearity values in the linear range used in this study. EF is defined as the ratio between the analyte concentration determined by HPLC and the initial analyte concentration in the sample. The ER is given by ER (%) $=EF\times V_{ACN}/V_{sample}$, where V_{ACN} is the volume of acetonitrile used to redissolve the dried sedimented phase and V_{sample} is the sample volume used in the extraction. EF and ER (%) were calculated and are presented in Table 1. Extraction recoveries obtained were 72% for E2 and 89% for EE2, leading to an enrichment factor of 145 and 178, respectively.

3.3. Matrix effect

Results obtained on the matrix effects of organic matter, simulated by HA are shown in Fig. 5, which represents the peak area for E2 and EE2 standards subjected to the previously optimized DLLME procedure in absence and in presence of different HA concentrations. On applying the one way ANOVA to compare the peak area means obtained with different HA concentrations the null hypothesis was established: the peak area means do not differ significantly. By the obtained results for $P=0.05$ it was possible to conclude that peak areas obtained do

Table 1
Quantitative parameters for typical analytical curves obtained by DLLME–HPLC–FLD.

Analyte	Linear range (ng L^{-1})	Correlation coefficient (r)	Linearity (%)	Limit of detection (ng L^{-1})	Extraction recovery (%)[a]	Enrichment factor[a]
E2	10–300	0.99967	98.182	2.0	72 ± 4[b]	145 ± 8[b]
EE2	10–500	0.99996	99.466	6.5	89 ± 3[b]	178 ± 7[b]

[a] Values obtained for a 0.1 μg L^{-1} concentration of each compound extracted simultaneously ($n=3$). Extraction conditions: 8 mL of E2 or EE2 standard; extracting solvent: 200 μL of chlorobenzene; 2000 μL of acetone as dispersive solvent; extraction time: 30 s.
[b] Mean value ± standard deviation ($n=3$).

not differ significantly, indicating that HA present in sample solution upon the extraction procedure do not interfere in any way on the extraction efficiency.

Also, E2 and EE2 extraction recoveries were determined and are presented in Table 2. Results obtained, between 98% and 107% for E2 and from 100% to 106% for EE2, confirm that HA do not influence in any way the extraction yield.

Although results obtained using HA to simulate organic matter reflect the absence of matrix effects in the proposed procedure, recovery tests were performed in real water samples in order to evaluate a real matrix influence. The chromatogram of a surface sample spiked with 0.1 μg L^{-1} of E2 and EE2 subjected to DLLME–HPLC–FLD is presented in Fig. 6(a). Also, the chromatogram of the surface sample (without spiking) subjected to the same procedure is presented in Fig. 6(b). Several peaks, other than the ones attributed to E2 and EE2, can be observed in both chromatograms. These peaks are also present when ultrapure water (control sample) is subjected to DLLME–HPLC–FLD, ensuring that its provenance is not from the water sample, but due to the extraction procedure itself. It is important to notice that, as we can see in Fig. 6, these peaks do not interfere with the E2 and EE2 determination.

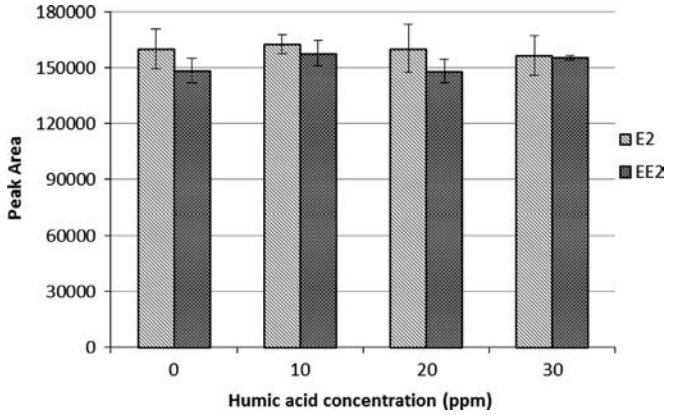

Fig. 5. Effect of humic acid concentration on the extraction efficiency ($n=3$). Extraction conditions: 8 mL of 0.1 μg L^{-1} E2 or EE2 standard with HA concentrations ranging from 0 to 30 mg L^{-1}; extracting solvent: 200 μL of chlorobenzene; 2000 μL of acetone as dispersive solvent; extraction time: 30 s.

Table 2
Effect of HA concentration on the extraction recovery of E2 and EE2 ($n=3$).

[HA] (mg L^{-1})	Recovery (%)	
	E2	EE2
0	119 ± 6	102 ± 5
10	102 ± 7	106 ± 7
20	100 ± 10	100 ± 6
30	98 ± 9	105 ± 5

Extraction conditions: 8 mL of 0.1 μg L^{-1} E2 or EE2 standard with HA concentrations ranging from 0 to 30 mg L^{-1}; extracting solvent: 200 μL of chlorobenzene; 2000 μL of acetone as dispersive solvent; extraction time: 30 s.

The extraction recovery rates were determined by spiking 15, 30 and 60 ng L^{-1} of E2 and EE2 into three different types of water samples—tap, surface and waste water. The obtained extraction recoveries are presented in Table 3. As it may be seen, recovery rates obtained in tap water ranged between 89.9% and 96.3% for E2 and between 104% and 119.7% for EE2, while in surface water results ranged between 96.4% and 104.1% for E2 and between 93.2% and 117.3% for EE2. When using waste water samples, that comprise a much more complicated matrix, results ranged between 86% and 106% for E2 and between 114% and 119% for EE2. Comparing results in Table 3, it is possible to observe an increase in standard deviation for recoveries obtained in the waste water sample, especially for lower concentrations. This can be explained by the complex matrix expected in such type of water, which can interfere in the extraction process. However, results obtained can be considered acceptable considering the very low concentrations used.

3.4. Analysis of environmental water samples

From all river and tap water samples analyzed, only one river water presented E2 and EE2 in a quantifiable level. The collected

Table 3
Effect of water sample matrix on the extraction recovery of E2 and EE2 ($n=3$).

Water samples	Recovery (%) Spiking level = 15 ng L^{-1}		Recovery (%) Spiking level = 30 ng L^{-1}		Recovery (%) Spiking level = 60 ng L^{-1}	
	E2	EE2	E2	EE2	E2	EE2
Tap water	95 ± 13	119.7 ± 0.1	90 ± 15	110 ± 7	96 ± 10	104 ± 6
Surface water	104 ± 3	115 ± 3	96 ± 6	93 ± 12	99 ± 5	117 ± 6
Waste water	94 ± 5	114 ± 23	86 ± 15	119 ± 20	106 ± 8	117 ± 2

Extraction conditions: 8 mL of spiked water sample; extracting solvent: 200 μL of chlorobenzene; 2000 μL of acetone as dispersive solvent; extraction time: 30 s.

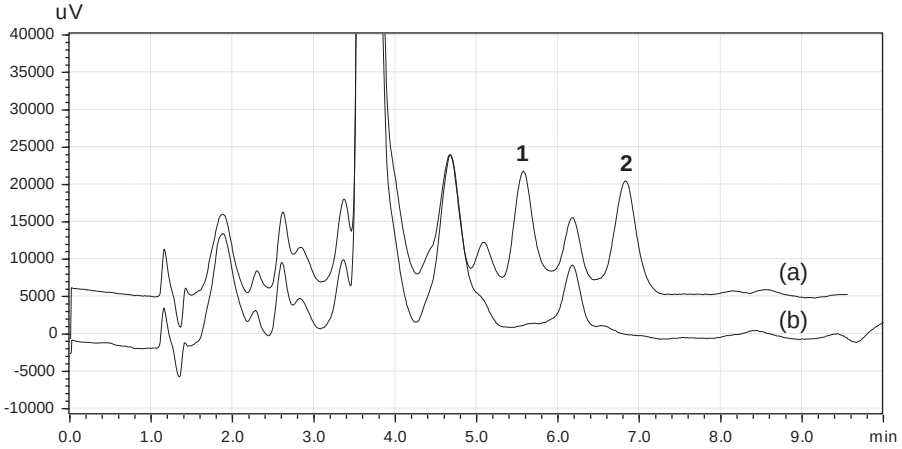

Fig. 6. Chromatogram of a surface water sample (a) with and (b) without a 0.1 μg L^{-1} spike of E2 and EE2 subjected to DLLME–HPLC–FLD. peaks: 1—E2; and 2—EE2. Extraction conditions: 8 mL of sample; extracting solvent: 200 μL of chlorobenzene; 2000 μL of acetone as dispersive solvent; extraction time: 30 s.

Table 4
Comparison of DLLME–HPLC–FLD with other methods used for the quantification of E2 and EE2 in water samples.

Method	Compounds	Recovery (%)	LOD (ng L^{-1})	Extraction time	Sample volume (mL)	Reference
SPE–HPLC–UV	E2	85–105	78.1	N.A.	50	[34]
SPE–HPLC–FLD	E2, EE2	72–100	3–15	N.A.	500–1000	[35]
SPME–HPLC–UV	E2, EE2	N.A.	300–400	45 min	3.5	[36]
SPME–HPLC–FLD	EE2	80.9–81.6	5	N.A.	1	[37]
DLLME–HPLC–UV	E2	89.9–94.5	10	In seconds	5	[30]
DLLME–HPLC–FLD	E2, EE2	86–120	2–6.5	In seconds	8	This study

N.A.—Non-available.

sample was obtained near to a STP discharge point, which can explain the 12.05 ± 0.08 ng L^{-1} concentration obtained for E2 and 11 ± 2 ng L^{-1} concentration obtained for EE2. Considering the three waste water samples analyzed, both E2 and EE2 were only detected after primary decantation with concentrations of 32 ± 3 ng L^{-1} for E2 and 18 ± 3 ng L^{-1} for EE2. Concentrations obtained are within the values generally obtained by other authors [31–33], confirming the applicability of the developed method.

3.5. Comparison with other methods

In order to compare this work with other methods reported in literature, Table 4 is presented. Comparing with SPE, DLLME presents the following advantages: easier to implement, less time consuming and lower sample volume needed. When comparing with SPME, similar sample volumes are used, but extraction time is significantly reduced. Overall, the DLLME proposed presents several advantages over other extraction techniques, showing that it is a suitable pre-treatment method for determination of E2 and EE2 in water samples. Hadjmohammadi and Ghoreishi [30] used DLLME–HPLC–UV for the determination of E2, estrone and diethylstilbestrol and also concluded that the extraction procedure was the most advantageous. Our work (DLLME–HPLC–FLD) is an improvement, since a lower limit of detection (from 10 to 2 ng L^{-1}) was achieved for E2, using a fluorescence detector; it must also be highlighted that this work presents results for EE2 DLLME, which was not developed in the referred work [30].

4. Conclusions

A methodology based on dispersive liquid–liquid microextraction followed by high-performance liquid chromatography with fluorescence detection (DLLME–HPLC–FLD) was developed and optimized for the analysis of estrogens in water samples. The optimized DLLME–HPLC–FLD methodology provides low detection limits (2.0 ng L^{-1} for E2 and 6.5 ng L^{-1} for EE2), high enrichment factors (145 ± 8 for E2 and 178 ± 7 for EE2) and also high extraction recoveries (72 ± 4 for E2 and 89 ± 3 for EE2) for the detection and quantification of E2 and EE2 in tap, surface and waste water samples. Also, recovery tests proved that the water samples matrix does not interfere in the extraction efficiency.

DLLME is fast, inexpensive and easy-to-use, allowing the extraction and pre-concentration of a large number of environmental samples in parallel. Besides, it is an environmentally friendly technique, due to the low volume of toxic organic solvents used.

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