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Short communication

Application of dispersive liquid–liquid microextraction for estrogens' quantification by enzyme-linked immunosorbent assay

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ABSTRACT

Estrogens, such as 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), are the major responsible for endocrine-disrupting effects observed in aquatic environments due to their high estrogenic potency, even at concentrations ranging from pg L^{-1} to ng L^{-1}. Thus, it is essential to develop analytical methodologies suitable for monitoring their presence in water samples. Dispersive liquid–liquid microextraction (DLLME) was used as a pre-concentration step prior to the quantification of E2 and EE2 by enzyme-linked immunosorbent assay (ELISA). First, an evaluation of the effect of DDLME on the E2 and EE2 ELISA calibration curves was performed. Since the extraction procedure itself had an influence on the ELISA optical density (OD), it became necessary to subject, not only the samples, but also all the standards to the DLLME process. Working ranges were determined, being between 1.2 and 8000 ng L^{-1}, for E2, and between 0.22 and 1500 ng L^{-1}, for EE2. The influence of organic matter, both in the extraction and quantification, was evaluated and it was observed that its presence in the solution did not affect considerably the calibration curve. Recovery rates were also determined, ranging from 77% to 106% for ultrapure water and from 104% to 115% for waste water samples, the most complex ones in what concerns matrix effects. Results obtained when applying the proposed method to real water samples can be considered quite satisfying. Moreover, the obtained working ranges encompass values generally reported in literature, confirming the practical use of the method for environmental samples.

1. Introduction

General concern about endocrine disrupting chemicals (EDCs) has been increasing in the last decades due to their toxicity and effects on aquatic life. EDCs comprise a wide range of substances, such as estrogens, progestogens, phytoestrogens and a variety of other organic pollutants. Estrogens, like the endogen 17β-estradiol (E2), and the synthetic 17α-ethinylestradiol (EE2), have often been identified as the main responsible for endocrine-disrupting effects observed in aquatic environments due to their high estrogenic potency, even at concentrations ranging from pg L^{-1} to ng L^{-1} [1–4]. It has been proven that E2 concentrations as low as 1–5 ng L^{-1} have the ability to induce the production of a female-specific egg-yolk protein precursor in male fish [2,5]. These effects, even at such low concentrations, led the European Union to establish Environmental Quality Standards (EQS), with the annual average AA-EQS for E2 being 0.4 ng L^{-1} and for EE2 0.035 ng L^{-1} [6], which makes imperative the use of an extremely sensitive method. Generally, gas chromatography coupled with mass spectrometry (GC–MS) [7] and GC–MS/MS [8], as well as liquid chromatography coupled with mass spectrometry (LC–MS) [9] and LC–MS/MS [10] are the most sensitive techniques and thus have experienced impressive progress in the last decades and been indicated as techniques of choice for environmental analysis of steroid hormones [11]. Even though these methods are extremely sensitive, they require a pre-concentration step, commonly solid-phase extraction (SPE). The expensive instrumentation, high maintenance costs and the requirement of specifically trained analysts make these methods unattractive due to financial restrictions. On the other hand, immunoassays, such as enzyme-linked immunosorbent assay (ELISA),

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based on the selectivity and affinity of an antibody (Ab) for its antigen, provide a valid alternative approach for the estrogen quantification in water samples, as presented by previous works [3,12,13]. These methods present several advantages such as high specificity, reduced time of analysis, high throughput of samples, low detection limits and cost effective detection [14]. However, pre-concentration methodologies are generally required in order to achieve environmentally relevant limits of detection. Huang and Sedlak [8] developed an SPE procedure followed by ELISA for the determination of E2 and EE2 in secondary wastewater effluent and surface water samples where concentrations ranged from 0.2 to 4.1 ng L^{-1}. A similar approach, with slightly lower sensitivity, was described by Shishida et al. [15] for the determination of E2 in wastewater from a pilot-scale plant; after SPE, the method detection limit was 10 ng L^{-1}. Dorabawila and Gupta [5] analyzed the presence of E2 in water samples from ponds, rivers, sewage treatment plants (STPs) and coastal bays. Samples were filtered and E2 extracted by C18 cartridges and analyzed by ELISA. Concentrations varied between 1.9 and 6.0 ng L^{-1} and detection limit was 0.5 ng L^{-1}. Several other authors refer to SPE as the pre-concentration step of choice prior to ELISA [16–18]. However, SPE is time-consuming, implies not only a high consumption of organic solvents, but also expensive cartridges, and requires high sample volume. As an interesting and valid alternative, dispersive liquid–liquid microextraction (DLLME) is a simple and fast microextraction technique based on a ternary component solvent system. In a previous work [19], DLLME was successfully used to pre-concentrate E2 and EE2 present in water samples prior to quantification by high performance liquid chromatography with fluorescence detection (HPLC-FLD). This extraction method presents several advantages over SPE, such as simplicity, low quantity of organic solvents, low cost, high recovery and enrichment factors, and also the possibility of a large number of samples to be extracted in parallel [20,21].

The main purpose of this work was to combine the key advantages of the DLLME procedure already developed [19] with the advantages provided by ELISA. Interference in ELISA due to the extraction had to be evaluated and overcome in order to apply the method to E2 and EE2 quantification in diverse water samples.

2. Material and methods

2.1. Reagents and standards

Polyclonal Ab sera and enzyme conjugates (tracers, T) were provided by BAM Federal Institute for Materials Research and Testing, Berlin, Germany. The production of the antisera and the enzyme conjugate preparation were described elsewhere [3,12,22].

Steroid hormones E2 (≥ 97%) and EE2 (≥ 98%), chlorobenzene (99.9%), tetramethylbenzidine (TMB, puriss.), tetrabutylammonium borohydride (TBABH, > 97%), dimethylacetamide (DMA), tris(hydroxymethyl) aminomethane (TRIS, p.a.), bovine serum albumin (BSA, for electrophoresis, 98%) and commercial humic acids (HA) (technical) were all supplied by Sigma. Acetone (for HPLC, 99.9%) was from Carlo Erba. Sodium phosphate dibasic dihydrate (> 99%), sodium phosphate monobasic dihydrate (> 99%), potassium sorbate (> 99%), potassium dihydrogen citrate (> 99%), hydrogen peroxide (30%), Tween™ 20 and sulfuric acid (95–97%) were from Fluka. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, p.a.) and sodium chloride (99.5%) were from Panreac. Sodium azide was from Riedel-de Haën.

For the immunoassay the following buffers were used: washing buffer concentrate (60 ×) (43 mmol L^{-1} KH_2PO_4, 375 mmol L^{-1} K_2HPO_4, 1.33 mmol L^{-1} sorbic acid potassium salt and 3% Tween™ 20, pH 7.6), phosphate buffer solution (PBS) (10 mmol L^{-1} $NaH_2PO_4 \cdot 2H_2O$, 70 mmol L^{-1} $Na_2HPO_4 \cdot 2H_2O$, 145 mmol L^{-1} NaCl, pH 7.6), coating buffer (15 mmol L^{-1} Na_2CO_3, 35 mmol L^{-1} $NaHCO_3$, 3 mmol L^{-1} NaN_3, pH 9.6) and citrate buffer (220 mmol L^{-1} $C_6H_7KO_7$, 0.5 mmol L^{-1} $C_6H_7KO_2$, pH 4.0). Final substrate solution was freshly prepared for each run and consisted in 540 μL stabilized TMB solution (prepared according to Frey et al. [23], using 41 mmol $L^{-1}C_{16}H_{20}N_2$ (TMB), 8 mmol $L^{-1}C_{16}H_{40}BN$ (TBABH), in 10 mL DMA), 22 mL citrate buffer and 8.1 μL H_2O_2. Sample buffer consisted in 1 mol L^{-1} $C_4H_{11}NO_3$, 1.5 mol L^{-1} NaCl, 107 mmol L^{-1} $Na_2EDTA \cdot 2H_2O$, 1% (w/v) BSA, pH 6.4.

Transparent 96 flat-bottom well microtiter plates with high binding capacity (MaxiSorp™) were purchased from Nunc (Thermo Scientific). Washing steps were carried out using an automatic 8-channel plate washer (Atlantis, Asys Hitech). Plates were shaken using a plate shaker (Titramax 100, Heidolph). Absorbance was measured at 450 nm and referenced to 620 nm with a plate reader (UVM 340, Asys Hitech).

Individual standard stock solutions of E2 and EE2 were prepared in methanol at a concentration of 1000 mg L^{-1}. Each solution was further diluted to the appropriate concentration using ultrapure water, obtained from a Milli-Q water purification system from Millipore Corp. A stock HA solution of 1000 mg L^{-1} at pH 9 (in 1 mol L^{-1} ammonium hydroxide) was also prepared.

2.2. DLLME procedure

The optimization of the applied DLLME procedure is described elsewhere [19]. Briefly, 8 mL aliquots of E2 and EE2 standards or samples 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 added to each tube and immediately shaken using a vortex during 30 s. After the formation of the cloudy solution, as a result of the dispersion of fine droplets of chlorobenzene in aqueous sample, the tubes were centrifuged at 4000 rpm for 5 min. Chlorobenzene organic phase, which was sedimented at the bottom of the conical centrifuge tube, was transferred to a 2 mL vial, dried under a nitrogen stream and redissolved using 160 μL of ultrapure water. The redissolved fraction was then analyzed by ELISA.

2.3. ELISA procedure

The E2 and EE2 ELISA procedures applied in this study were previously developed by Schneider et al. [3,12]. Detailed information about further assay optimization has been described in Silva et al. [13]. Concisely, microtiter plates were coated with polyclonal Ab serum diluted 1:10,000 for E2 and 1:50,000 for EE2 in coating buffer (200 μL per well). After overnight incubation, plates were washed two times with washing buffer concentrate, diluted 1:60. Sample buffer was added (25 μL per well), followed by standards/samples (100 μL per well) and incubated for 30 min. This was followed by the addition of T (100 μL per well), diluted 1:50,000 for E2 and 1:100,000 for EE2 in PBS and incubated 10 and 15 min for E2 and EE2, respectively. After a second two-cycle washing step, the final substrate solution was added (200 μL per well) and incubated for 30 min. The enzyme reaction was stopped by the addition of 1 mol $L^{-1}H_2SO_4$ (100 μL per well). The SoftMax Pro Software (Version 5.3, Molecular Devices) was used for data analysis.

ELISA calibration curves were fitted to a four-parametric logistic function (4PL):

$$y = \left[\frac{A-D}{[1+(x/C)^B]}\right] + D$$

where y is the optical density (OD); x, the analyte concentration; A, the OD for an infinitely small analyte concentration (“blank”); B, the slope at the inflection point; C, the concentration value at

the inflection point; and *D*, the OD for an infinite analyte concentration ("excess" standard).

2.4. Evaluation of the DLLME on ELISA

In order to evaluate the influence of the extraction procedure on the ELISA performance, several standards and one blank sample (ultrapure water) were subjected to the DLLME procedure and quantified by the previously developed ELISA procedure [13].

Also, to evaluate the extraction procedure, two ELISA calibration curves were obtained, with and without the DLLME procedure. Standards between 0.1 and 1×10^6 ng L^{-1} were analyzed directly by ELISA. Standards between 2×10^{-3} and 2×10^4 ng L^{-1} were subjected to DLLME, resulting in concentrations 50 times higher after the extraction (between 0.1 and 1×10^6 ng L^{-1}). Theoretically, similar curves should be obtained.

2.5. Analytical performance

To determine the quantification range (defined as the highest and the lowest concentration that can be determined with a given degree of precision), 16 standards were used ($n=6$). The relative error of the E2 and EE2 concentrations was calculated in order to obtain the precision profile as described by Ekins [24]. A relative error of 30% was established as the maximum allowable error for the quantification of both estrogens, as explained in Silva et al. [13].

2.6. Matrix effects

The application of the developed method for E2 and EE2 quantification in real water samples could be problematic due to matrix effects that may affect both the extraction and also the ELISA. As reported previously [13], 1% BSA buffer can be used to overcome matrix effects observed in ELISA, due to organic matter. It was considered relevant to also evaluate the organic matter influence in the extraction procedure itself. Therefore, standards prepared in ultrapure water and in 30 mg L^{-1} HA were subjected to DLLME and analyzed by ELISA. Also, to evaluate the effect of organic matter present in real water samples, ultrapure, surface and waste waters were spiked with 25 and 50 ng L^{-1} E2 or EE2, subjected to DLLME procedure and analyzed by ELISA.

2.7. Application to environmental water samples

In order to evaluate the applicability of the proposed method, several samples (250 mL) from public fountains providing potable water (samples 1–7) and surface water samples (samples 8–18) were collected (April 2013), in the north and center of Portugal using dark glass containers (previously washed 3 times with the sample to be collected). Also, wastewater samples from two STPs (samples 19 and 20) were collected. 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. Evaluation of the DLLME on ELISA

Extremely high recovery results obtained for the standards subjected to DLLME and quantified by ELISA, demonstrated an overestimation of the E2 and EE2 concentrations. Moreover, the high E2 and EE2 concentrations obtained for the blank (ultrapure water) corroborated the recovery results.

Two ELISA calibration curves, with and without the DLLME procedure, were obtained and are presented in Fig. 1. A decrease in the signal (associated to an increase of concentration) is observed for both compounds, which can explain the overestimation observed previously.

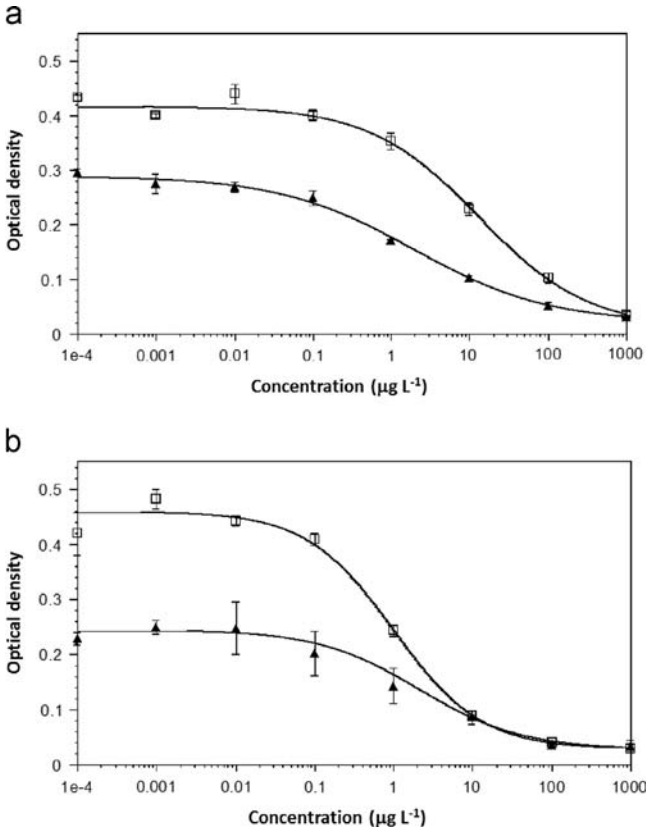

Fig. 1. Evaluation of DLLME effect on the E2 (a) and EE2 (b) ELISA calibration curve. Standards not subjected to DLLME (open squares); standards subjected to DLLME (solid triangles); E2 ELISA conditions: Ab 1:10,000; T 1:50,000 incubated 10 min; EE2 ELISA conditions: Ab 1:50,000; T 1:100,000 incubated 15 min.

Table 1
Effect of water sample matrix on the extraction recovery of E2 and EE2 ($n=3$).

Water samples	Recovery (%) Spiking level = 25 ng L^{-1}		Recovery (%) Spiking level = 50 ng L^{-1}	
	E2	EE2	E2	EE2
Ultrapure water	88 ± 5	79 ± 18	77 ± 17	106 ± 29
Surface water	86 ± 2	120 ± 7	78 ± 17	112 ± 45
Waste water	104 ± 22	107 ± 19	115 ± 17	111 ± 12

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

If the extraction efficiency was not satisfactory, a calibration curve shifted to higher OD values would be obtained. However, exactly the opposite was observed. This can be explained by the use of an organic solvent in the DLLME procedure that, although evaporated, can still influence the ELISA (known to be influenced by organic solvents). Even though, the DLLME can be used as a preconcentration step prior to ELISA quantification if both samples and standards are subjected to the same procedure (proved by good recovery results obtained for standards in ultrapure water, presented in Table 1).

3.2. Analytical performance

In order to determine the quantification range for both estrogens, the precision profiles were obtained using standards with

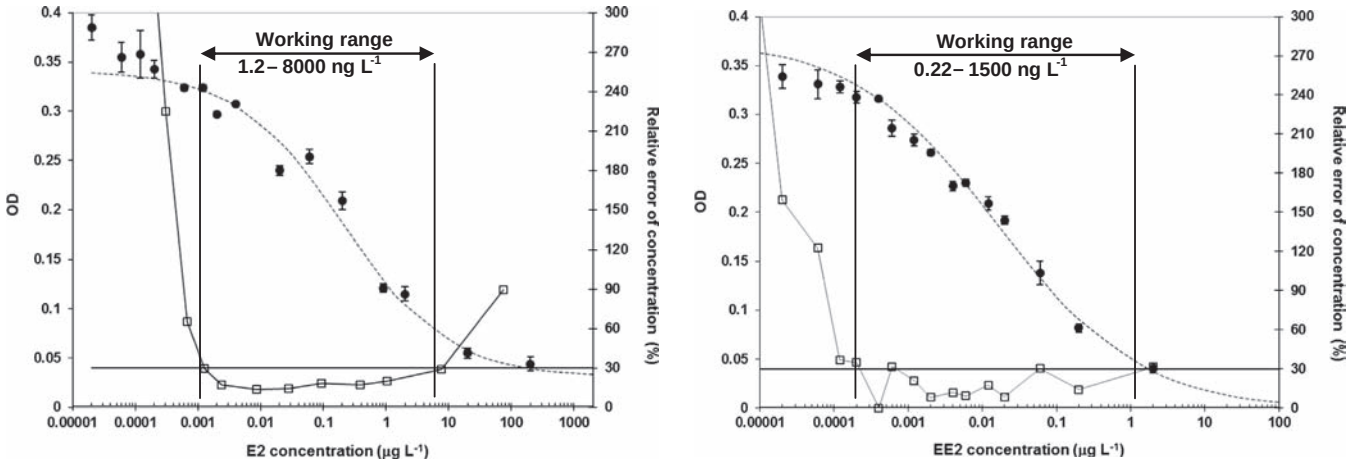

Fig. 2. Calibration curve (black marks) of E2 (A=0.342; B=0.512; C=0.203; D=0.0306; r^2=0.981) and EE2 ELISA (A=0.377; B=0.446; C=0.0161; D=0; r^2=0.992) and precision profile (gray marks), in presence of BSA buffer at pH 6.4. E2 ELISA conditions: Ab 1:10,000; T 1:50,000 incubated 10 min; EE2 ELISA conditions: Ab 1:50,000; T 1:100,000 incubated 15 min. The precision profile and determination of the relative error of concentration were calculated in accordance with Ekins [24].

concentrations between 2×10^{-2} and 2×10^{5} ng L^{-1} for E2 and between 2×10^{-3} and 2×10^{4} ng L^{-1} for EE2 (Fig. 2).

Considering the maximum relative standard deviation allowed of 30%, a 1.2–8000 ng L^{-1} working range for E2 and a 0.22–1500 ng L^{-1} working range for EE2 were obtained. Comparing the working ranges obtained with DLLME-ELISA with the ones obtained for ELISA without the prior extraction (30–2×10^{5} ng L^{-1} for E2 and 20–1×10^{4} ng L^{-1} for EE2) [13], it is possible to conclude that this simple extraction procedure developed decreases the lower limit of both working ranges, approximately 30 times for E2 and 100 times for EE2. The working ranges obtained, in the present conditions, easily allow the quantification of these estrogenic disruptors in surface waters, where the expected concentrations are extremely low.

3.3. Matrix effects

Calibration curves for both estrogens in the presence and absence of HA are presented in Fig. 3.

As it can be seen, the presence of HA does not affect the extraction considerably and consequently, the calibration curve. It is important to highlight that this behavior is only possible if the organic-aqueous interphase, that contains a small amount of analyte, is extracted together with the organic phase. However, to confirm that organic matter present in the water samples does not affect the quantification, ultrapure, surface and waste waters were spiked with different concentrations of E2 or EE2, subjected to DLLME procedure and analyzed by ELISA. Recovery results are presented in Table 1.

For ultrapure water, recovery rates ranged from 77% to 106%, while for waste water samples (the most complex samples in what concerns matrix effects) recoveries ranged from 104% to 115%. Recovery results can be considered acceptable and the developed method suitable for application in real water samples.

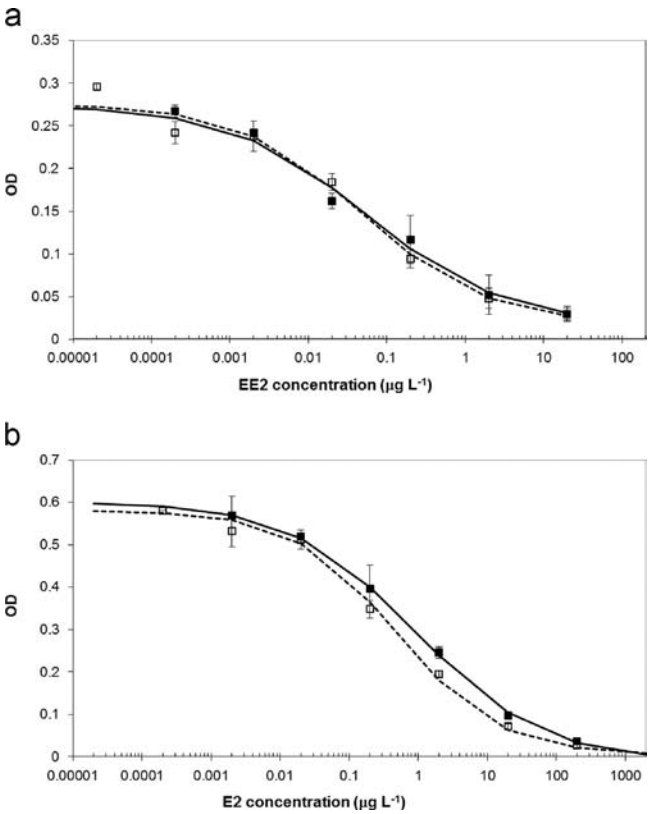

Fig. 3. Evaluation of the organic matter effect on the DLLME and ELISA calibration curve of E2 (a) and EE2 (b). Standards prepared in ultrapure water – 0 mg L^{-1} HA (—) and in 30 mg L^{-1} HA (···). E2 ELISA conditions: Ab 1:10,000; T 1:50,000 incubated 10 min; EE2 ELISA conditions: Ab 1:50,000; T 1:100,000 incubated 15 min.

3.4. Application to environmental water samples

Several samples were collected in public fountains providing potable water (samples 1–7). Only one sample (sample 4) contained E2 in a quantifiable amount (Table 2); however, EE2 was quantified in three (samples 4–6) of the seven samples tested. Results obtained for surface water samples (8–18) collected in several rivers, small streams and ponds were slightly different. In this case, E2 concentrations were in general higher than those of EE2, just as expected. Concentrations ranged from 4 to 34 ng L^{-1} for E2 and from 0.3 to 24 ng L^{-1} for EE2. In what concerns waste water samples, results demonstrated that in both samples analyzed E2 concentration is higher than EE2 concentration.

Also, it is important to note that the quantified concentrations are in accordance with values generally obtained and reported by other authors [25–27], confirming the applicability of the developed method.

Table 2
Determination of E2 and EE2 in water from public fountains and in surface and waste water samples, subjected to DLLME and analyzed by ELISA ($n=3$).

Samples[a]	Concentration (ng L^{-1})	
	E2	EE2
1	< lod	< lod
2	< lod	< lod
3	< lod	< lod
4	2.0 ± 0.1	1.32 ± 0.8
5	< lod	0.37 ± 0.05
6	< lod	0.5 ± 0.4
7	< lod	< lod
8	< lod	0.4 ± 0.2
9	4 ± 1	< lod
10	< lod	< lod
11	34 ± 11	2.4 ± 1.1
12	< lod	< lod
13	17.8 ± 0.9	< lod
14	8 ± 2	24 ± 6
15	< lod	5 ± 2
16	30 ± 1	16 ± 3
17	< lod	0.8 ± 0.2
18	33.1 ± 0.1	0.3 ± 0.1
19	77 ± 33	6 ± 1
20	21 ± 19	8.5 ± 0.6

[a] Samples 1–7 – from public fountains with potable water; Samples 8–18 – surface water samples; Samples 19 and 20 – waste water samples.

Table 3
Comparison of DLLME-ELISA with chromatographic methods used for the quantification of E2 and EE2 in water samples after DLLME.

Method	Compounds	Recovery (%)	LOD (ng L^{-1})	Reference
DLLME-HPLC-UV	E2	89.9–94.5	10	[28]
DLLME-HPLC-FLD	E2, EE2	86–120	2–6.5	[19]
DLLME-ELISA	E2, EE2	77–115	0.22–1.2	This study

3.5. Comparison of DLLME-ELISA with DLLME-chromatographic analysis

In order to compare the method developed with DLLME-chromatographic analysis already reported in literature, Table 3 is presented. When compared with HPLC, ELISA provides several advantages, such as sensitivity, specificity, simplicity and high throughput of samples. In what concerns limit of detection, generally ELISA presents lower detection limits, when compared to chromatographic techniques. As it can be seen in Table 3 the limit of detection for E2 using DLLME-HPLC-UV is 10 ng L^{-1}, while using DLLME-HPLC-FLD is 2 ng L^{-1}. However, if DLLME-ELISA is used the reliable working range extends to 1.2 ng L^{-1}. The improvement observed for EE2 is even higher, from 6.5 ng L^{-1} with DLLME-HPLC-FLD to a lower working range limit of 0.22 ng L^{-1} using DLLME-ELISA. Thus, besides the advantages enumerated previously, ELISA quantification after DLLME allows the quantification of E2, and especially of EE2 in water samples at lower concentrations.

4. Conclusions

The main objective of this work was to combine DLLME and ELISA procedures in order to quantify E2 and EE2 in concentrations as low as few ng L^{-1}. However, some problems due to the extraction procedure interference in ELISA had to be solved, since the extraction procedure itself yielded an influence on the ELISA OD, leading to an overestimation of the concentration. As a solution, the standards for ELISA calibration curves were subjected to DLLME prior to ELISA in order to eliminate ELISA signal differences due to extraction. No influence of organic matter was observed in the extraction and quantification; recovery rates obtained were in the ranges 77–106% using ultrapure water and 104–115% using waste water samples. The simple extraction procedure adopted decreased the working range approximately 30 times for E2 and 100 times for EE2, comparing with the working ranges obtained without DLLME. Lower working range limits were 1.2 ng L^{-1} for E2 and 0.22 ng L^{-1} for EE2. Results for E2 and EE2 determination in water samples collected were between 2 and 77 ng L^{-1} for E2 and between 0.3 and 24 ng L^{-1} for EE2.

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