Determination of Three Estrogens in Environmental Water Samples Using Dispersive Liquid-Liquid Microextraction by High-Performance Liquid Chromatography and Fluorescence Detector

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Abstract In this work the dispersive liquid-liquid microextraction technique (DLLME) is presented as an important alternative to the classical extraction methods and was used to extract and concentrate estrogens’ before its quantification by HPLC in environmental water samples. For the evaluation of the analytical methodology, the following conditions were used: sample volume 8 mL, extraction solvent 200 μL of chlorobenzene, and dispersive solvent 2000 μL of acetone. The enrichment factor (EF) was 140 for estrone, 202 for 17β-estradiol (E2), and 199 for 17α-ethinylestradiol (EE2). Limit of detection was 20 ng L⁻¹ for E1, 3.1 ng L⁻¹ for E2, and 2.7 ng L⁻¹ for EE2. Repeatability and intermediate reproducibility presented values of relative standard deviation lower than 10%. Finally, recovery tests were performed to evaluate the water matrices’ effects on the extraction performance, resulting in recoveries between 76 and 110% in surface water and between 84 and 109% in wastewater.

Keywords Pharmaceuticals · Estrogens · High-performance liquid chromatography · Dispersive liquid-liquid microextraction · Water

1 Introduction

In recent decades, there has been an expressive increase in pharmaceuticals and personal care product production and consumption, and although it has improved the population life quality, it has also brought environmental consequences (de Jesus Gaffney et al. 2015). Since then, there has been a growing concern about the presence of these compounds due to their possible effects on ecosystems and public health.

Endocrine disruptors (EDs) are substances capable of interfering or modifying the endocrine system of humans and animals, causing adverse health effects (Dodgen et al. 2017). Estrogens represent an important group of steroid EDs and are classified as natural (17β-estradiol (E2) and estrone (E1)) and synthetic (17α-ethinylestradiol (EE2)) compounds (Becker et al. 2017; Rodenas et al. 2017). The presence of steroid hormones in water may be indicative of anthropogenic contamination due to discharges of contaminated...
According to studies by Berenger et al. (2017), many of these substances are difficult to remove at wastewater treatment plants (WWTPs), and thus persisting in the aquatic environment. Depending on the concentration and time of exposure, it is possible that these substances may be related to diseases such as breast, testicular, and prostate cancer; polycystic ovaries; and reduced female fertility (Di Donato et al. 2017; Sifakis et al. 2017). The presence in the environment has been related to inhibition of egg production in fish, proving its potential risk to the environment (Thrupp et al. 2018). E1, E2, and EE2 were detected and determined in domestic sewage and in WWTPs by several investigators (Becker et al. 2017; do Nascimento et al. 2018), showing that anthropogenic activities are an important contribution for aquatic environment contamination. These hormones are generally present in the aquatic environment at very low concentrations (ng L$^{-1}$), demanding the development of sensitive techniques for their detection and quantification.

Due to the presence in the environment at very low concentrations, the determination of E1, E2, and EE2 in water samples requires sensitive instruments such as gas chromatography coupled to mass spectrometry (GC-MS) (Česen and Heath 2017) and liquid chromatography coupled to mass spectrometry (LC-MS) (Kleywegt et al. 2011). These techniques have been used in the last decades for hormone analysis in environmental samples. However, its use has several requirements, such as the need of specifically trained analysts, expensive instrumentation, and high-cost of maintenance. Due to financial constraints, high costs may be impracticable for the use of these analytical techniques in various laboratories around the world. On the other hand, high-performance liquid chromatography (HPLC) is a widely available, low-cost, easy to use, faster, and simple technique. It can be coupled to diode array (DAD), fluorescence (FLD), and/or UV-visible detectors. Due to their low sensitivity, preconcentration methodologies, previously to the analysis, are required. Among preconcentration methodologies, solid-phase extraction (SPE) (Razmkhah and Sereshki 2018), liquid-liquid extraction (LLE) (Fredj et al. 2015), solid-phase microextraction (SPME) (Liao et al. 2016), and liquid-phase microextraction (LPME) may be applied. LLE and SPE consume high volumes of organic solvents, being SPME an alternative to SPE. However, SPME is expensive, has the problem of sample carryover, and has limited application. LPME, on the other hand, overcomes many disadvantages caused by LLE, SPE, and SPME, since it focuses on simplicity, fast application, low cost, and low solvent volume required for the extraction (Lima et al. 2013).

Among LPME techniques, dispersive liquid-liquid microextraction (DLLME) has emerged as an alternative to meet these objectives. It was introduced in 2006 by Rezaee et al. and consists of the preconcentration of analytes in aqueous matrices. Since the emergence of DLLME, numerous innovations have improved its performance, such as automation (Alves et al. 2016), the use of less toxic and safer extractive solvents (e.g., ionic liquids and surfactants (Tuzen et al. 2017)), the use of solvents with lower density than water (Seebunnueg et al. 2014), and the absence of a dispersive solvent (Galuszka et al. 2013; Spietelun et al. 2014), being recently the focus of analytical chemistry.

DLLME is a preconcentration method where some microliters of a suitable organic extraction solvent together with a dispersive solvent are injected into the sample, causing the formation of small droplets which are dispersed throughout the water sample. After the formation of a cloudy solution, the equilibrium state is readily reached. The mixture is then centrifuged, and the sedimented/ floating phase containing the analyte of interest is collected (Lima et al. 2013). It is a simple and fast technique, with many advantages over conventional extraction methods, among them are the low volume of solvents (μL) used, high surface area between the extraction solvent, and the aqueous sample and the high enrichment factor (Kocúrová et al. 2012). Also, advantages, such as simplicity of operation, speed, environmentally friendly, and easy connection to the various methods of analysis (Rezaee et al. 2006), are referred.

Therefore, the objective of this work was to develop and validate an analytical method, based on a microextraction technique, to quantify E1, E2, and EE2 estrogens in different environmental water samples by HPLC with fluorescence detection.

2 Materials and Methods

2.1 Reagents and Solutions

Estrone (99.9%), 17β-estradiol (≥ 97%), 17α-ethinylestradiol (≥ 98%), and chlorobenzene ((C₆H₅Cl) 99.9%) were provided by Sigma Aldrich. Acetonitrile
and acetone (99.9% HPLC grade) were supplied by HiperSolv Chromanorm and Carlo Erba, respectively. Ultrapure water was obtained through Millipore’s Milli-Q water system.

The individual stock solutions of E1, E2, and EE2 were prepared in acetonitrile at a concentration of 100 mg L\(^{-1}\). The working solutions were obtained by dilution of the stock solution with ultrapure water until the desired concentrations.

2.2 Instrumentation

The chromatographic system used for the analysis of E1, E2, and EE2 was a Shimadzu® LC 20AT Prominance (model DGU-20A5) with a high-pressure pump coupled to a fluorescence detector (model RF-20 A XS) also from Shimadzu®. The detection was performed using an excitation wavelength of 280 nm and an emission of 310 nm. A Shimadzu LCsolution® software was used to control the equipment and to obtain the data. The column used for the separation was an ACE C18-PFP (5 \(\mu\)m, 150 mm × 4.6 mm) connected to an ACE 5 C18 4.6-mm i.d. guard column. Both the column and cell were maintained at 25 °C temperature. The mobile phase consisted of a water:acetonitrile (50:50, v/v), at a flow rate of 0.8 mL min\(^{-1}\) with an injection volume of 20 \(\mu\)L. The water and acetonitrile were pretreated by filtration with Whatman 0.2-\(\mu\)m polyamide membrane filters.

A Lab Dancer Mini Vortex from VWR International was used to perform the agitation during the DLLME extraction procedure.

2.3 DLLME Procedure

The extraction conditions used were based on the work developed by Lima et al. (2013) for application in E2 and EE2. These conditions were tested in this work for the quantification of E1 together with E2 and EE2 in environmental samples.

The extraction procedure consisted on 8 mL of ultrapure water spiked with E1, E2, or EE2 added to 12 mL conical bottom tubes. Then, a mixture containing 2000 \(\mu\)L of acetone and 200 \(\mu\)L of chlorobenzene was added rapidly to each tube and vortexed for 30 s. After the formation of the cloudy solution, the tubes were centrifuged at 4000 rpm for 5 min. After centrifugation, the organic drop was formed and the organic phase sedimented at the bottom of the tube was collected with micropipette and transferred to a 2 mL vial, then dried under a stream of nitrogen and reconstituted using 40 \(\mu\)L acetonitrile. The reconstituted aliquot was analyzed using the HPLC conditions described previously.

2.4 Sampling and Sample Preparation

An estuarine water sample was collected in the Ria de Aveiro (40° 38′ 18″ N 8° 38′ 43″ W), Aveiro, Portugal, in the city center. This sample is known to have high levels of salinity and high content of organic matter (da Silva et al. 2017). Another sample was collected at the WWTP in the center region of Portugal. The collections were carried out in June 2018, and the predominant season was summer. In Brazil, tests were performed on samples from of the Bacanga River (2° 33′ 02″ S 44° 16′ 58″ W) located in São Luís, Maranhão. The samples were collected in August 2018, and the predominant season was summer.

For the collection, the sampling rules were considered, deepness, flow, and cleaning of the materials used. The container was sanitized, and the samples were collected and transferred to an amber flask with 1 L volume, then stored and refrigerated at approximately 4 °C. Samples were filtered using a Millipore® assembly, with 0.45 \(\mu\)m Chrom Tech® filters.

2.5 Method Validation

After the optimization of the conditions, linear range, determination coefficient (\(r^2\)), linearity, limit of detection (LOD), extraction recovery (ER), and extraction efficiency (EF) were calculated. EF is defined as the ratio of the concentration of analyte determined by HPLC to the initial concentration of analyte in the sample. The ER is given by

\[
ER(\%) = EF \times \frac{V_{ACN}}{V_{sample}} \times 100\% 
\]

where \(V_{ACN}\) is the volume of acetonitrile used to redissolve the dry sediment phase and \(V_{sample}\) is the volume of sample used in the extraction (Lima et al. 2013, 2018; Rezaee et al. 2006). The performance of the DLLME-HPLC-FLD method was calculated based on the values of \(r^2\), LOD, and linearity (Lin (%) = 100-RSD\(b\), where RSD\(b\) is the relative standard deviation of slope of the curve) (Peters et al. 2007). The 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) (Lima et al. 2013). The program used was GraphPad Prism 5.
2.6 Evaluation of Matrix Effects

The DLLME method was applied directly to the environmental samples. The evaluation of the water matrix influence on the extraction was performed by spiking known amounts of E1, E2, and EE2 on water samples analyzed previously. Concentration levels used were adjusted to the calibration curve obtained for each hormone and to the expected environmental levels. Thus, for E2 and EE2, the concentration spiking was performed in order to obtain a final concentration of 0.05 and 0.3 μg L⁻¹, while for E1, the final concentration was 2 and 20 μg L⁻¹. At least five analyzes were performed for each of the two levels of fortification studied. The recovery percentage was calculated by the ratio between the experimentally determined average concentration and the corresponding expected concentration.

3 Results and Discussion

3.1 Analytical Performance of the HPLC-FLD Method

External standard calibration curves using HPLC-FLD without DLLME were constructed. For this, six levels of concentration 1 to 200 μg L⁻¹ for E2 and EE2 and 20 to 160 μg L⁻¹ for E1 were prepared in triplicate for the hormones E1, E2, and EE2. Values of r² higher than 0.99 for the three curves were obtained and considered satisfactory. The instrumental detection limits were 19 μg L⁻¹, 12 μg L⁻¹, and 9 μg L⁻¹ for E1, E2, and EE2, respectively.

3.2 Analytical Performance DLLME-HPLC-FLD

Six concentration levels ranging from 10 to 500 ng L⁻¹ for E2 and EE2 and 40 to 40,000 ng L⁻¹ for E1 were prepared and subjected to the DLLME procedure described above (five replicates each concentration). The DLLME-HPLC-FLD analytical curve was obtained, and LOD, r², linearity, EF, and ER were calculated (Table 1).

The DLLME-HPLC-FLD resulted in optimum linearity in the range of 10 to 500 ng L⁻¹ for E2 and EE2 and 40 to 4000 ng L⁻¹ for E1. The results of r² were above 0.99, showing a very strong correlation. The low detection limits, between 2.7 and 20 ng L⁻¹, confirm the possible applicability of the method to detect these hormones in aquatic environmental samples. Also, the method allows to obtain an enrichment factor between 140 and 202. The LOD was in the range of ng L⁻¹, demonstrating, therefore, satisfactory parameters for the detection and quantification of these analytes at low concentrations and in complex matrices.

3.3 Application to Environmental Samples

The optimized method was applied to different environmental samples: Ria Aveiro (water with high salinity), WWTP (high concentration of organic matter), and Bacanga River (highly polluted river), and results are presented in Table 2.

The Bacanga River is located in an area subjected to siltation, mangrove urbanization, garbage, littering the banks, deforestation, burning, water contamination, and eutrophication. This may explain the high concentrations obtained for all the hormones analyzed in the water samples from that river. In Portugal, the results obtained were lower than LOD values, suggesting a better water treatment than in Brazil, where the sewage treatment is scarce. In fact, estrones are often detected in Brazilian waters. For example, Sodré et al. (2010b) quantified hormones E1, E2, and EE2 in surface water in the order of 39 ng L⁻¹,

### Table 1

| Analyte | Linear range (ng L⁻¹) | Determination coefficient (r²) | Linearity (%) | Detection limit (ng L⁻¹) | Extraction recoverya (%) | Enrichment factora |
|---------|----------------------|-------------------------------|---------------|--------------------------|--------------------------|-------------------|
| E1      | 40–4000              | 0.998                         | 99.89         | 20                       | 72 ± 2                  | 140 ± 2           |
| E2      | 10–500               | 0.997                         | 99.76         | 2.7                      | 101 ± 7                 | 202 ± 3           |
| EE2     | 10–500               | 0.999                         | 99.97         | 3.1                      | 99 ± 2                  | 199 ± 5           |

*a Mean value ± standard deviation (n = 5) obtained for a concentration of 100 ng L⁻¹ for E2 and EE2 and 400 ng L⁻¹ for E1. Extraction conditions: 8 mL of standard E1, E2, or EE2; extraction solvent 200-μL chlorobenzene; 2000 μL of acetone as dispersive solvent; extraction time 30 s
7.3 ng L$^{-1}$, and 25 ng L$^{-1}$, respectively. In environmental waters from Rio de Janeiro, hormone concentrations in the 1.97 to 3.50 ng L$^{-1}$ range were determined (Kuster et al. 2009). E1 and E2 were also determined in drinking water in Campinas (Brazil) at concentrations of 70 ng L$^{-1}$ and 100 ng L$^{-1}$ (Sodré et al. 2010a). Torres et al. (2015) determined E1, E2, and EE2 in drinking water in São Paulo (Brazil) at concentrations of 28 ng L$^{-1}$, 137 ng L$^{-1}$, and 194 ng L$^{-1}$, respectively. Pessoa et al. (2009) detected the occurrence of estrogens hormones in Brazilian WWTP; the effluent concentrations were 242 ng L$^{-1}$, 48 ng L$^{-1}$, and 124 ng L$^{-1}$ for E1, E2, and EE2.

In Portugal, for water samples from Ria Formosa (Rocha et al. 2013a) and Sado River Estuary (Rocha et al. 2013b), estrogens concentrations of 1.4 ng L$^{-1}$ and 4.2 ng L$^{-1}$ for E1, 5.9 ng L$^{-1}$ and 7.4 ng L$^{-1}$ for E2 and 17.3 ng L$^{-1}$ and 1.6 ng L$^{-1}$ EE2 were determined, respectively.

### Table 2 Hormonal concentrations found

| Concentration found (ng L$^{-1}$) | E1 | E2 | EE2 |
|-----------------------------------|----|----|-----|
| Portugal                          |    |    |     |
| Ria Aveiro                        | n.d| 120| n.d |
| Bacanga River                     | 590| 640| 820 |
| Brazil                            |    |    |     |
| n.d, not detected                 |    |    |     |

3.4 Evaluation of Matrix Effects

Spikes of 0.05 and 0.3 μg L$^{-1}$ of E2 and EE2 and 2 and 20 μg L$^{-1}$ of E1 were performed into two different types of samples: surface water with saline characteristics and wastewater (Brazil and Portugal) which has a high concentration of organic matter. All samples were submitted to the optimized DLLME method and analyzed by HPLC-FLD. Results obtained are presented on Table 3.

The recovery results ranged from 76 to 114% in surface water and from 84 to 109% for wastewater. The selectivity of the method was verified by the analysis of possible experimental interferents at the same retention time of estrogens under study. Recovery results demonstrated that even for complex water matrices as wastewater samples, the DLLME procedure can be applied.

Selectivity was evaluated comparing the chromatograms obtained for the matrix with and without fortification. Chromatograms of a surface water sample, before and after fortification, and subjected to the DLLME procedure, are presented in Fig. 1. It is possible to see the increase of the peaks attributed to E2, EE2, and E1 in the fortified sample that were absent in the surface water sample analyzed, except for E2 showing concentration of 120 ng L$^{-1}$ (Table 2).

The results were considered satisfactory since no interfering compounds were observed at the same retention time of the analyzed analytes. This confirms that the sample preparation method was effective in eliminating interferents that could interfere with the quantification of

### Table 3 Effect of water matrices on DLLME recovery

| Recoveries (%)$^a$ | E2 | EE2 | E1 |
|--------------------|----|-----|----|
|                    | 50 ng L$^{-1}$ | 300 ng L$^{-1}$ | 50 ng L$^{-1}$ | 300 ng L$^{-1}$ | 200 ng L$^{-1}$ | 2000 ng L$^{-1}$ |
| Portugal           |    |     |    |    |    |    |
| Surface water      | 102 ± 7 | 102 ± 1 | 104 ± 10 | 76 ± 6 | 110 ± 3 | 99 ± 7 |
| Wastewater         | n.d | n.d | 109 ± 12 | 84 ± 5 | 85 ± 2 | 96 ± 5 |
| Brazil             |    |     |    |    |    |    |
| Surface water      | 100 ± 8 | 92 ± 6 | 77 ± 9 | 101 ± 3 | 114 ± 5 | 86 ± 1 |

$^a$ Mean value ± standard deviation ($n = 5$). Extraction conditions: 8 mL of fortified water sample; extraction solvent 200-μL chlorobenzene; 2000 μL of acetone as dispersant solvent; extraction time 30 s

n.d, not determined
the analytes in study or even damage the chromatographic system.

3.5 Comparison with Other Methods

Table 4 allows the comparison of the DLLME-HPLC-FLD method here developed with other methods presented in literature used for quantification of hormones in water samples. The parameters used for comparison were LOD (ng L$^{-1}$), enrichment factor, time of extraction and sample volume.

Comparing with SPE (Melo and Brito 2014; Wang et al. 2008), DLLME has many advantages: easier to implement, low extraction time, and low sample volume. Comparing with fabric-phase sorptive extraction (FPSE) (Kumar et al. 2014) and SPME (Peñalver et al. 2002), the sample volume is similar; however, the extraction time is significantly reduced and a better enrichment factor is achieved. Wu et al. (2012), Hadjmohammadi and Ghoreishi (2011) and Lima et al. (2013) used the DLLME-HPLC and concluded that the method is advantageous in relation to the others due to the low detection limits, lower extraction volumes, and less use of organic solvents. In our work, the extraction time was reduced when comparing with the work developed by Hadjmohammadi and Ghoreishi (2011) and the

Table 4 Comparison of DLLME-HPLC-FLD with other methods used for estrogens’ quantification in environmental samples

| Methodology            | Hormones      | LOD (ng L$^{-1}$) | Enrichment factor | Extraction time | Sample volume (mL) | Reference                   |
|------------------------|---------------|------------------|-------------------|-----------------|-------------------|-----------------------------|
| FPSE-HPLC-FLD          | E2, EE2       | 20, 36           | 14.4, 14.7        | 10 min          | 10                | Kumar et al. (2014)         |
| SPE-HPLC-UV            | E1, E2        | 12.78            | 248, 269          | N.A             | 50                | Wang et al. (2008)          |
| DLLME-HPLC-DAD-FLD     | E1, E2, EE2   | 50, 10, 8        | 162, 276, 308     | 20 min          | 5                 | Wu et al. (2012)            |
| DLLME-HPLC-UV          | E1, E2        | 10, 10           | 71, 78.5          | 10 min          | 5                 | Hadjmohammadi and Ghoreishi (2011) |
| CPE-HPLC-UV            | E1, E2        | 250, 320         | 152, 73           | 5 min           | 10                | Wang et al. (2006)          |
| SPME-HPLC-UV-ED        | E1, E2, EE2   | 700, 700, 700    | N.A               | 35 min          | 3.5               | Peñalver et al. (2002)      |
| SPE-HPLC-FLD           | E2            | 650              | N.A               | N.A             | 100               | Melo and Brito (2014)       |
| DLLME-HPLC-FLD         | E2, EE2       | 2, 6.5           | 72, 89            | 30s             | 8                 | Lima et al. (2013)          |
| SPME-GC-MS             | E1, E2, EE2   | 25, 20, 40       | N.A               | N.A             | 500               | Dévier et al. (2013)        |
| DLLME-GC-MS            | E2, EE2       | 3140, 4830       | N.A               | 15 s            | 7.9               | Koçoğlu et al. (2019)       |
| SPME-LC-MS             | E1, E2, EE2   | 18, 12, 5        | N.A               | N.A             | 250               | Pessoa et al. (2009)        |
| DLLME-HPLC-FLD         | E1, E2, EE2   | 20, 3.1, 2.7     | 140, 202, 199     | 30s             | 8                 | This study                  |

FPSE, fabric-phase sorptive extraction; SPE, solid-phase extraction; FLD Fluorescence detector; DAD diode array; CPE cloud point extraction; SPME solid-phase microextraction; N.A, not applied
enrichment factors were higher. Comparing with CPE-HPLC-UV (Wang et al. 2006), our work reached lower limits of detection, shorter extraction time, smaller sample volume, and the analysis of three hormones using only one detector. The DLLME technique resulted in detection limits similar to those obtained using SPME (Dévier et al. 2013; Pessoa et al. 2009) and has several advantages: low use of solvents, simple, and low-cost technique. When compared with high maintenance cost of analytical equipment, such as GC-MS and or LC-MS, the hereby optimized method proved to be superior, reaching lower detection limits (Koçoğlu et al. 2019).

Overall, the proposed method has several advantages over other extraction techniques, showing that it is a suitable pre-treatment method for the determination of hormones in water samples.

4 Conclusions

The DLLME method has many advantages over the traditional methods of sample preparation for hormone determination in water samples, among them we can mention good recovery percentages and high enrichment factors. This work presents the development of a robust and selective analytical method for the determination of E1, E2, and EE2 in aqueous samples. The DLLME-based methodology, coupled with HPLC-FLD, provided high extraction recovery rates (76 to 114% in surface water and 84 to 109% in wastewater samples). The high HPLC separation capacity along with the high sensitivity of the fluorescence detector, coupled with the fast and efficient sample preparation method, offers several advantages in the determination of estrogens and can be used as a routine method in several laboratories. The DLLME-HPLC-FLD strategy is a fast and economical methodology to determine and quantify E1, E2, and EE2 in different aquatic effluents.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval Not applicable.

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