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Determination of steroid sex hormones in water and urine matrices by stir bar sorptive extraction and liquid chromatography with diode array detection

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Abstract

In this study, stir bar sorptive extraction and liquid desorption followed by high performance liquid chromatography with diode array detection (SBSE–LD–HPLC/DAD) were combined for the simultaneous determination of nine steroid sex hormones (estrone, 17α-estradiol, 17β-estradiol, 17α-ethynylestradiol, diethylstilbestrol, mestranol, progesterone, 19-norethisterone and norgestrel) in water and urine matrices.

During the method development, it has been demonstrated that equilibrium time, ionic strength and back extraction solvents are the most important parameters to control, for determining the nine-hormones in water matrices, in which stir bars coated with 126 μl of polydimethylsiloxane were used. Assays performed on 30 ml water samples spiked at 10 μg/l levels under optimised experimental conditions, yielded recoveries ranging from 11.1 ± 4.9% (17β-estradiol) to 100.2 ± 10.4% (mestranol), showed that the methodology is well described by the octanol–water partition coefficients ($K_{PDMS/W} \approx K_{O/W}$) for the latter, while pronounced deviations to the theoretical efficiency ($K_{PDMS/W} \neq K_{O/W}$) were observed for the remaining hormones. From calibration studies, a good analytical performance for all hormones was attained, including a suitable precision (2.1–17.1%), low limits of detection (0.3–1.0 μg/l) and an excellent linear dynamic range (1.25–50.0 μg/l). Assays on environmental water and urine matrices showed recovery yields in worthy good agreement with the spiking level (10 μg/l), and suitability for profiling low μg/l levels of natural hormones in urine samples taken from pregnant women. The present methodology is easy, reliable and sensitive at the trace level, only requiring a low sample volume, showing to be a good analytical alternative to routine quality control for environmental and biomedical laboratories.

Keywords: Stir bar sorptive extraction; HPLC/DAD; Endocrine disrupters; Steroid sex hormones; Estrogens; Water analysis; Urine matrices; Trace analysis

1. Introduction

The effects of endocrine disrupter's chemicals (EDCs) are receiving a growing attention from scientific community, regulatory agencies and the public at large, as there is a continuous widespread of anthropogenic substances into the environment. EDCs have been shown to interfere with the normal functions of endocrine systems, thus affecting reproduction and development in wildlife and humans [1]. The reproductive effects of EDCs are believed to be related with mimicking endogenous hormones through the agonism and antagonism mechanisms, altering the pattern of synthesis and metabolism as well as by modifying hormone receptor levels [2].

The steroid sex hormones in particular, those regulating the differentiation and development of male and female reproductive organs, secondary sex characteristics and behaviour patterns, are important biological messengers [3]. In aquatic environments, the main sources of those estrogenic and progestational chemicals are from domestic effluents, mainly due to the widespread use of birth-control pills and other analogous drugs used for the treatment of hormonal disorders or cancers, as commonly occur during menopause. Other major sources of steroid sex hormones are the livestock wastes such as sheep, cattle, pigs, poultry and other animals, as well as growth regulators in aquaculture [4]. On the other hand, toxicological and pharmacological levels evaluated by biomedical research rely deeply on the monitoring on a restricted number of hormones in

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biological fluids, e.g. urine, and these procedures allow a more accurate medical interpretation and a rapid access to metabolic pathway information in both human and animal body. Thus, these analyses are important to monitor the action and malfunction of endocrine glands and can, therefore, be important for the diagnosis of endocrine disorders, usually associated with several diseases such as breast cancer, *anorexia nervosa*, and pseudohermaphroditism [5–7].

In the last years, several analytical strategies have been proposed to monitor estrogens or progestogens in water and biomedical matrices, based on either biological assays or chromatographic techniques [8–21]. Nevertheless, chromatographic methods have become the analytical systems of choice, since they also enable the identification of individual analytes of interest.

In addiction to gas chromatographic methodologies [19–21], high performance liquid chromatography (HPLC) is widely accepted as an advantageous technique for monitoring steroid hormones in aqueous matrices, which is more rugged and versatile without a derivatization step. The analytical procedures employed are usually based on solid phase extraction or solid phase micro-extraction (SPME) sample preparation enrichment to decrease the detection limits followed by HPLC, with UV–vis or mass spectrometry detection [8,11,12,15].

In the recent years, stir bar sorptive extraction (SBSE) has been employed as a novel sample preparation technique based on the same principles as those of SPME [22], which has been also developed for enrichment and sensitive determination of priority organic micro-pollutants in water samples [23–32], as well as in other matrices [20,33–38]. The amount of polydimethylsiloxane (PDMS) typically coating the stir bars has 24–126 μl, which is substantially higher than on a SPME fiber, usually with a maximum volume of 0.5 μl (100 μm film thickness). The lower phase ratio between the extraction medium and the sample provides an increasing capacity and much higher recoveries than those reached by SBSE. Consequently, this new sample preparation technique allows to increase the sensitivity by a factor ranging from 50 to 250 times, thus, decreasing the detection limits at the trace level.

The theory of SBSE is quite similar to that of SPME, where the partitioning efficiency of analyte into the PDMS phase of the stir bar, at equilibrium, can be reliably predicted by the octanol–water partition coefficients ($K_{O/W}$), considering the approximation to the partitioning coefficients between PDMS and water ($K_{PDMS/W} \approx K_{O/W}$), as well as by the involved phase ratio β ($=V_W/V_{SBSE}$), where V_W is the volume of the water sample and V_{SBSE} is the PDMS volume [22].

The aim of the present work is to develop a methodology combining the stir bar sorptive extraction and liquid desorption, followed by high performance liquid chromatography with diode array detection (SBSE–LD–HPLC/DAD), for the analysis of traces of steroid sex hormones and related synthetic compounds in aqueous matrices. The performance of the method was evaluated in terms of accuracy, linearity, precision and limits of detection, for which systematic assays were carried out in order to determine the most important parameters that could affect the efficiency of the SBSE–LD process. Finally, the optimised method was applied to monitor real matrices, including environmental water and urine samples.

2. Experimental

2.1. Standards and chemicals

Neat certified standard chemicals were used in this study. Diethylstilbestrol (99.5%; Lot: 0160X, No. 46207), 17α-ethynylestradiol (98.9%; Lot: 0137X, No. 46263), and 19-norethisterone (98.5%, Lot: 0264X, No. 46525) were supplied from Riedel-de-Haën (Seelze, Germany). D-(−)-Norgestrel (99.0%; Lot: 033K1165, No. 2260), progesterone (98.0%; Lot: S07092-173, No. 85,045-4), estrone (99.0%; Lot: 101K1185, No. E-9750), 17β-estradiol (Lot: 122K1269, No. E1024), 17α-estradiol (Lot: E-8750, No. 063K4048) and mestranol (Lot: 09110MI, No. 85,587-1) were supplied from Sigma–Aldrich (Steinheim, Germany). The chemical structures of the hormones are depicted in Fig. 1.

HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Riedel-de-Haën (Seelze, Germany). Sodium chloride (NaCl, 99.5%) was supplied from Merck, Darmstadt, Germany. Sodium hydroxide pellets (Analar grade, 98.0%) were obtained from BDH Chemicals, Poole, England. Hydrochloric acid (37%) was supplied from Panreac (Barcelona, Spain). Ultra-pure water was obtained from Milli-Q water purification systems.

Stock standard solutions of individual steroids (500 mg/l) were used to prepare the working and calibration standard mixtures in MeOH at the desired concentration, stored refrigerated at −20 °C and renewed monthly. Environmental water samples were obtained from a well in the metropolitan area of Lisbon (Portugal) and urine samples were collected in the morning from women and from a 37 weeks pregnant woman.

2.2. Experimental set-up

The stir bars (Twister; Gerstel, Müllheim a/d Ruhr; Germany) coated with 20 mm in length and 1.0 mm film thickness of PDMS (126 μl) were pre-conditioned before use by treating them with ACN for cleaning. In a typical assay, 30 ml of ultra-pure water spiked at the 10 μg/l concentration are introduced into a glass vial (Macherey-Nagel, Düren, Germany), a stir bar is immersed and the vial is closed with a seal using a hand crimper. For the optimization of the SBSE efficiency, assays were performed in a fifteenth agitation point plate (Variomag Multipoint) at room temperature (20 °C), and parameters such as extraction time (1–6 h), agitation speed (750, 1000 and 1300 rpm), pH (2.0, 3.2, 7.2 and 10.2), ionic strength (NaCl, 5–30%) and organic modifier (MeOH, 5–20%) were systematically studied in triplicate. To evaluate the best LD conditions, several assays using back extraction solvents such as MeOH, ACN and equimolar mixtures of both were also performed in triplicate. For liquid desorption purposes, the stir bars were removed with a clean tweezers, dried with a lint-free tissue, placed into a 2 ml glass vial filled with 1.5 ml of solvent, ensuring their total immersion, and an ultrasonic treatment

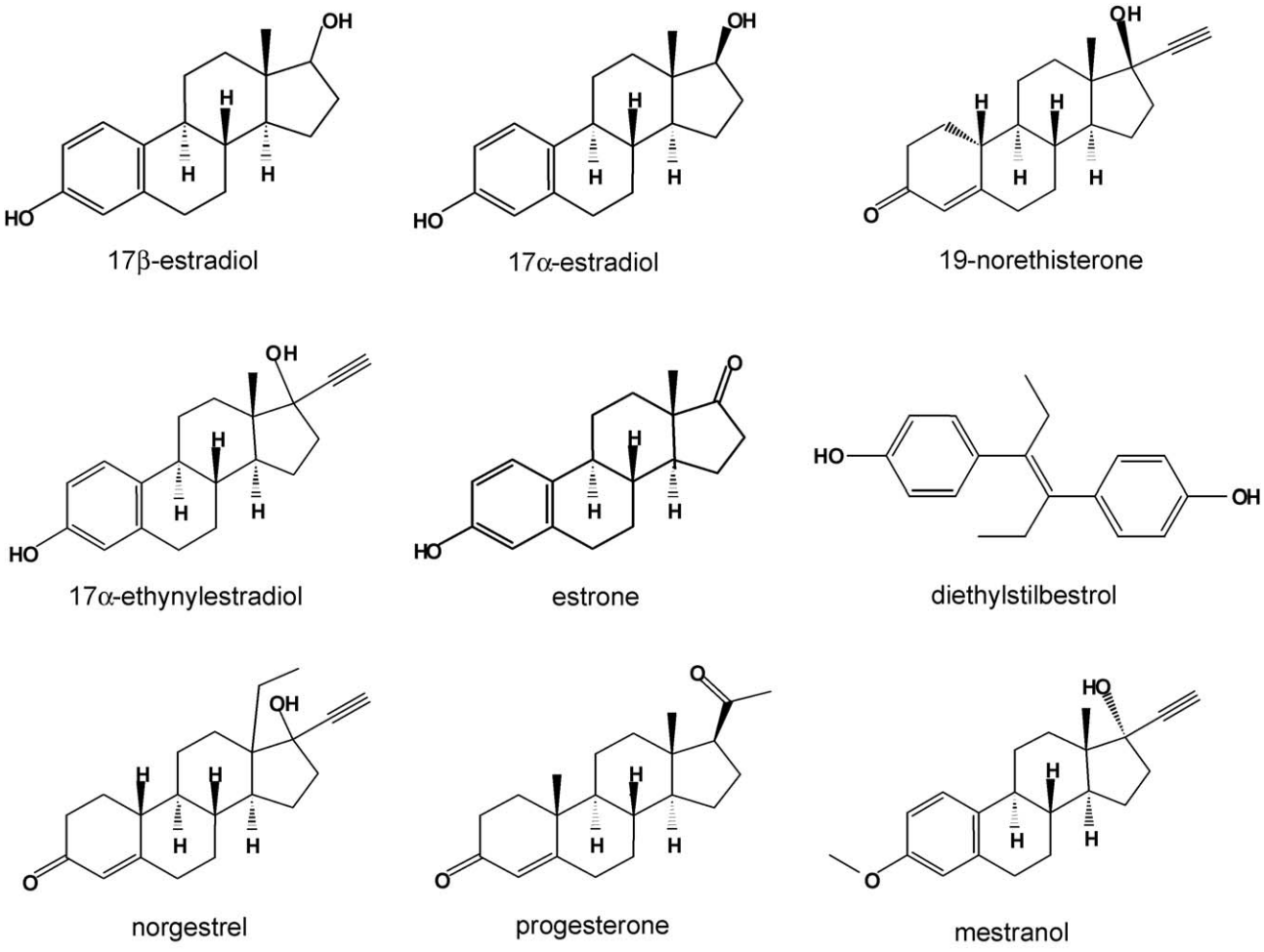

Fig. 1. Chemical structure of the nine steroid sex hormones used in the present study.

(Branson 3510) followed for 15 min at a constant temperature (25 °C).

After back extraction, the stir bars were removed by means of a magnetic rod, while the stripping solvent was evaporated to dryness under a gently stream of purified nitrogen (>99.5%). The dried residues were redissolved in 100 μl of 10% ACN in water and the vial was closed with a seal using a hand crimper and placed into the automatic liquid sampler tray for HPLC/DAD analysis. Blank assays were also performed using the same procedure as described above, employing ultra-pure water samples without spiking.

For real sample assays, 30 ml of water and 5 or 20 ml of urine diluted to 30 ml with ultra-pure water were fortified at the desired concentration or directly analysed using the same procedure as described above in triplicate.

2.3. Instrumentation

HPLC analyses were carried out on a benchtop Agilent 1100 series LC chromatographic system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser (G1322A), autosampler (G1313A), thermostated column compartment (G1316A), quaternary pump (G1311A) and a diode array detector (G1315B). Analyses were performed on a Tracer excel 120 OctaDecilSilica-A column, 150 mm × 4.0 mm, 5 μm particle size (120 ODS-A, Teknokroma).

The mobile phase consisted on a mixture of 10% (v/v) ACN aqueous solution (solvent A) and ACN (solvent B). Samples were analysed using a 60 min linear gradient, and the content of solvent B was progressively increased from 0 to 100% (1.0 ml/min). All solvents were previously filtered (150 mm diameter Whatman filters) to remove possible interference particles. The detector was set at 200 nm for 17α-estradiol, 17β-estradiol, 17α-ethynylestradiol, estrone, diethylstilbestrol and mestranol, and 240 nm for 19-noretisterone, norgestrel and progesterone. The column temperature was set at 25 °C, the injection volume was 30 μl with a draw speed of 200 μl/min.

For identification purposes, standard addition was used by spiking the samples with pure standards, as well as by comparing the relative retention time and peak purity with the UV–vis spectral reference data. For quantification purposes, calibration curves using the external standard methodology were performed. For recovery calculations, peak areas obtained from each assay were compared with the peak areas of standard controls used for spiking.

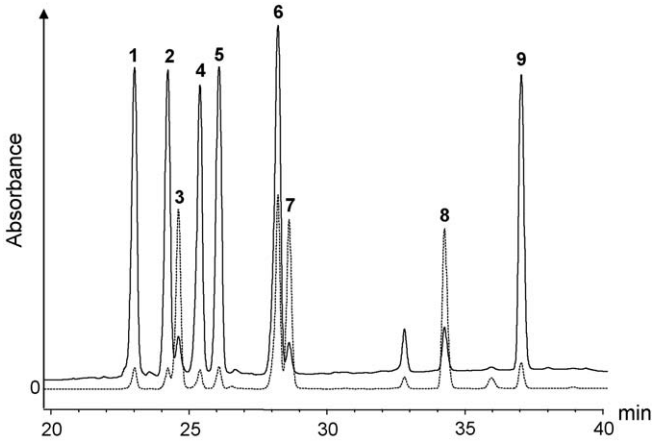

Fig. 2. Chromatogram of a standard mixture of the nine hormones (1.25 mg/l) obtained by HPLC/DAD at 200 nm (—) and 240 nm (- - -). (1) 17β-estradiol, (2) 17α-estradiol, (3) 19-norethisterone, (4) 17α-ethynylestradiol, (5) estrone, (6) diethylstilbestrol, (7) norgestrel, (8) progesterone, and (9) mestranol.

3. Results and discussion

3.1. Instrumental operating conditions

In a first approach, the HPLC/DAD conditions including the UV–vis spectral data for the detection of each hormone, as well as retention time characteristics were evaluated. It was observed that 200 nm is the best wavelength for estrogens and 240 nm for progestins absorption, in agreement with literature [12]. Fig. 2 depicts a chromatogram of a standard mixture of the nine-hormones analysed by HPLC/DAD, showing a good resolution within a suitable analytical time (<40 min), under the experimental conditions used.

Instrumental calibration was performed with standard solutions, with concentrations ranging from 0.3 to 5.0 mg/l, with the exception of 17α-ethynylestradiol, for which a range of 0.6–5.0 mg/l was used instead. From the data obtained, excellent linear responses were observed for the nine-hormones under study, with correlation coefficients higher than 0.9993. In order to evaluate the instrumental precision, repeatability injections for each calibration level were carried out, resulting relative standard deviations (R.S.D.) within 0.1 and 7.1% (estrone).

The instrumental sensitivity was also checked through the limits of detection (LODs) and quantification (LOQs), obtained by the injection of diluted calibration standards and calculated with a signal-to-noise ratio (S/N) of 3/1 and 10/1, respectively. The LODs and LOQs measured ranged from 25 to 100 μg/l and from 75 to 300 μg/l, respectively. Furthermore, no carry-over was observed by series of replicate injections (2.5 mg/l), for which the background was always below the LODs. Table 1 summarizes all instrumental data obtained for the nine-hormones under study.

3.2. Optimisation of the SBSE–LD efficiency

Since the very beginning we started to establish the experimental conditions that could enable higher recovery yields for the selected hormones by SBSE–LD. Therefore, systematic studies were carried out in water samples spiked at the 10 μg/l level, with the purpose of optimising several important parameters which could influence the SBSE–LD efficiency, particularly the extraction profile (time and agitation), the aqueous medium characteristics (pH, ionic strength and polarity) as well as the back extraction solvents. Additionally, stir bars having 126 μl PDMS were chosen, since a higher extraction capacity is attained [22].

Preliminary studies to estimate the most suitable equilibrium time were performed by making assays from 1 to 6 h for the nine-hormones, at room temperature. Fig. 3a shows the extraction profile obtained for all steroids under study, in which it can be observed that progesterone reaches a better equilibrium after 4 h. For the others hormones, the best equilibrium conditions are attained in 2 h, with mestranol reaching a maximum recovery yield. Therefore, extraction times of 4 and 2 h were selected for progesterone and for the remaining hormones, respectively. However, it is noteworthy to mention that, with the exception of mestranol and progesterone, the remaining hormones present recoveries lower than 20%, since they exhibit a smaller affinity for the PDMS phase.

According to literature [22], the agitation speed is another important parameter causing a significative effect on the SBSE efficiency. However, assays performed at 750, 1000 and 1300 rpm (20 °C) demonstrated that the differences observed

Table 1
Type, retention time, instrumental LODS and LOQs, linear dynamic range and correlation coefficients for the nine steroid hormones under study, by HPLC/DAD

Hormones	Type	RT (min)	LOD[a] (μg/l)	LOQ[b] (μg/l)	Linear range (mg/l)	R^2
17β-Estradiol	Natural estrogen	23.05	50	150	0.3–5.0	0.9995
17α-Estradiol	Natural estrogen	24.30	50	150	0.3–5.0	0.9994
19-Norethisterone	Synthetic progestin	24.70	50	150	0.3–5.0	0.9997
17α-Ethynylestradiol	Synthetic estrogen	25.50	100	300	0.6–5.0	0.9996
Estrone	Natural estrogen	26.04	50	150	0.3–5.0	0.9993
Diethylstilbestrol	Synthetic estrogen	28.40	25	75	0.3–5.0	0.9999
Norgestrel	Synthetic progestin	28.86	50	150	0.3–5.0	0.9997
Progesterone	Natural progestin	34.40	50	150	0.3–5.0	0.9995
Mestranol	Natural estrogen	37.02	50	150	0.3–5.0	0.9995

[a] S/N = 3.
[b] S/N = 10.

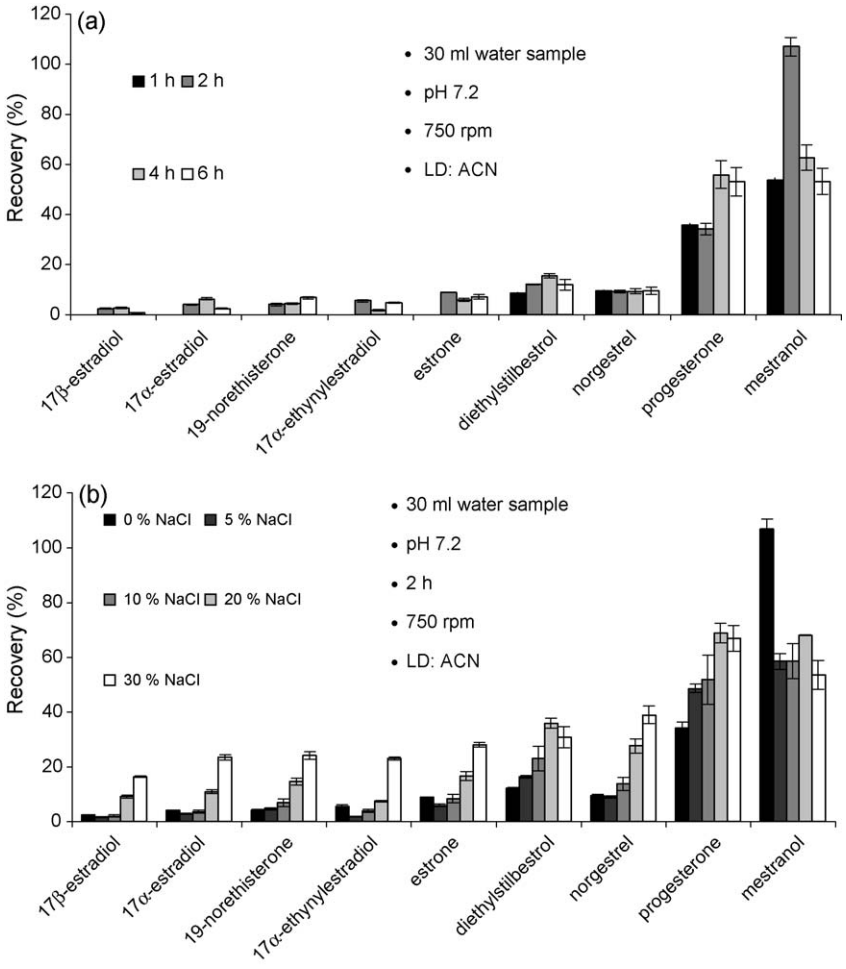

Fig. 3. Effect of the extraction time (a) and NaCl addition (b) on the recovery of the nine hormones at the 10 μg/l level by SBSE–LD–HPLC/DAD.

were negligible. Consequently, a 750 rpm agitation speed was chosen for further experiments.

During our studies, the characteristics of the aqueous medium were also investigated, particularly the pH, the ionic strength, as well as the polarity characteristics. Starting with the effect of the pH on the SBSE efficiency for the nine-hormones, four different values (2.0, 3.2, 7.2 and 10.2) were assessed at room temperature. This parameter is described to have a great relevance, because steroids are ionisable compounds, making the pH of the aqueous matrix to have a strong effect on their extraction yield [11]. While the behaviour observed shows that their recoveries at pH 7.2 are, in general, slightly higher than at other values, it can be considered that this is not a relevant parameter to be taken into account.

It is well known [22], basically for compounds with a $\log K_{O/W} < 3$ that ionic strength is a very important parameter, as it can promote a remarkable effect on the efficiency yield for this sort of analyses. Thus, an electrolyte will probably favour the migration of the organic compounds towards the PDMS phase and consequently, would increase their recovery [25,26]. Therefore, the effect of several concentrations of NaCl in the aqueous medium, ranging from 5 to 30% (w/v), was evaluated. Fig. 3b depicts that for almost all the hormones tested, the stronger the ionic strength is, the higher will be their recovery yield. Nevertheless, an exception is seen for mestranol, for which the increment of the ionic strength causes a negative effect, since its extraction efficiency decreased. This observation can probably be explained by a lesser solubility of mestranol in the new media, as according to literature [4], a smaller solubility value in relation to the other steroid hormones under study was found. At this stage, a distinct behaviour between mestranol and the other hormones was definitely observed, probably due to remarkable differences in their hydrophobic characteristics. Since NaCl enables a positive effect on the hormone recoveries, a 20% value for further assays was set, with the exception of mestranol, for which the experiments were performed in the total absence of salt. Moreover, NaCl contents above 20% were avoided, since it could damage seriously the PDMS phase, promoting its deterioration [39].

Analyte adsorption on the vial glass walls is a phenomenon that can occur. When it happed, the sorption efficiency decreases, particularly for the most hydrophobic compounds at trace levels [23]. Notwithstanding the fact that an organic modifier slightly increases the solubility of hydrophobic compounds in aqueous media, this could be an important parameter to consider, as it could help preventing undesirable adsorption on the vial glass walls, according to several authors [25,29]. Nevertheless, by increasing the amount of MeOH as organic modifier up to

Table 2
Octanol–water partitioning coefficients ($K_{O/W}$), LODs and LOQs, linear dynamic range, correlation coefficients and average recoveries achieved for the nine steroid hormones (10 μg/l) by SBSE–LD–HPLC/DAD, under optimised experimental conditions

Hormones	log $K_{O/W}$[a]	LOD[b] (μg/l)	LOQ[c] (μg/l)	Linear range (μg/l)	R^2	Recovery[d] (% ± R.S.D.; $n = 3$)
17β-Estradiol	3.94	1.0	3.0	5.0–50.0	0.9989	11.1 ± 4.9
17α-Estradiol	3.94	1.0	3.0	5.0–50.0	0.9970	16.1 ± 6.3
19-Norethisterone	2.99	0.6	1.8	2.5–50.0	0.9984	20.9 ± 5.8
17α-Ethynylestradiol	4.12	1.0	3.0	5.0–50.0	0.9974	14.8 ± 7.6
Estrone	3.43	1.0	3.0	5.0–50.0	0.9980	24.6 ± 4.4
Diethylstilbestrol	5.64	0.6	1.8	2.5–50.0	0.9925	46.5 ± 13.8
Norgestrel	3.48	0.6	1.8	2.5–50.0	0.9981	37.4 ± 6.8
Progesterone	3.67	0.3	0.9	1.25–50.0	0.9992	67.1 ± 4.0
Mestranol	4.68	0.3	0.9	1.25–10.0	0.9988	100.2 ± 10.4

[a] According to Ref. [40].
[b] S/N = 3.
[c] S/N = 10.
[d] Assays at the 10 μg/l level.

20% (v/v), a negative effect on the recovery yield of the nine-hormones was attained. In general, the addition of MeOH to the aqueous media presents disadvantages because it turns the matrix less polar, helping to solubilize the more hydrophobic ones and reducing the affinity of the target compounds for the PDMS phase of the stir bar. Consequently, further experiments were performed in the absence of MeOH in the aqueous matrices.

In SBSE–LD, the back extraction is as important as extraction set-up. Desorption solvents must have enough capacity to promote the best stripping of hormones from the stir bars and an ultrasonic treatment can further accelerate desorption [22]. In the present study, MeOH, ACN and equimolar mixtures of both solvents showed that ACN allowed a better stripping capacity, but the differences found can be considered negligible. However, an exception is progesterone, for which equimolar mixtures of MeOH/ACN allowed a more efficient stripping than with each solvent alone. Therefore, equimolar mixtures of MeOH/ACN for progesterone, and ACN for the remaining compounds, were chosen as liquid desorption solvents.

3.3. Validation of the SBSE–LD–HPLC/DAD method

After studying the most important experimental parameters that could affect the SBSE–LD efficiency, three different optimised conditions were established concerning extraction time, aqueous medium characteristics (pH and ionic strength) and back extraction solvents, respectively; 2 h (750 rpm), pH 7.2 and ACN for mestranol; 4 h (750 rpm), pH 7.2, 20% of NaCl and equimolar mixtures of MeOH/ACN for progesterone; and at least, 2 h (750 rpm), pH 7.2, 20% NaCl and ACN for the remaining steroids.

According to SBSE theory [22], the distribution coefficients of the analytes between the PDMS and the water matrix ($K_{PDMS/W}$) should be strongly correlated with the corresponding $K_{O/W}$. In the present study, the equilibrium theoretical line (% recovery versus log $K_{O/W}$) was estimated taking into consideration that 30 ml of a water sample (V_W) and a stir bar coated with 126 μl (V_{SBSE}) of PDMS were used, for which a phase ratio ($\beta = V_W/V_{SBSE}$) value of 238 was established. Thus, if a specific compound has a log $K_{O/W}$ of 2.99 (e.g. 19-norethisterone), a theoretical recovery of 80% should be expected. Additionally, the logs $K_{O/W}$ of the nine steroid hormones were determined (Table 2) according to a fragment constant estimation methodology [40].

Starting with the optimised experimental conditions, the data for the nine hormones spiked at the 10 μg/l level showed that the SBSE–LD–HPLC/DAD methodology presents a good performance, even though with recoveries within 11.1 ± 4.9 (17β-estradiol) and 100.2 ± 10.4% (mestranol), as presented in

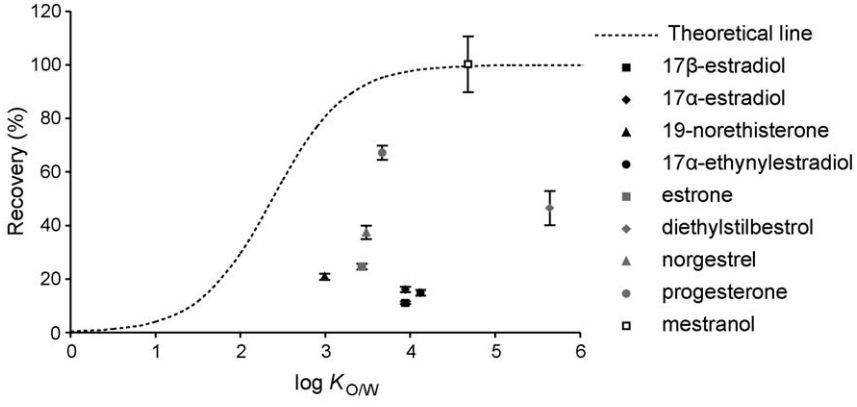

Fig. 4. Theoretical line and experimental recovery data against log $K_{O/W}$ for the nine hormones by SBSE–LD–HPLC/DAD, under optimised experimental conditions.

Table 2. Fig. 4 depicts the experimental recovery data of the nine-hormones plotted against the corresponding log $K_{O/W}$, their precision values being also denoted by means of error bars for each compound. When confronting theoretical with experimental recovery data, it can be observed that only mestranol fits the theoretical line ($K_{PDMS/W} \approx K_{O/W}$), while the remaining hormones show pronounced deviations ($K_{PDMS/W} \neq K_{O/W}$). It must be emphasized that logs $K_{O/W}$ calculated according to a fragment constant estimation methodology [40] do not account for the specific interactions between PDMS and particular analytes during the sorption equilibrium process. In case of a weak affinity, $K_{PDMS/W}$ is only a rough approximation of $K_{O/W}$. Despite this fact, the experimental data and theoretical predictable recovery profiles are similar for 19-norethisterone, estrone, norgestrel and progesterone. On the other hand, diethylstilbestrol, which has the highest hydrophobicity (log $K_{O/W} = 5.64$) and, therefore, the largest predict recovery, shows a lower yield (46.5 ± 13.8%). Nevertheless, although low recoveries are observed for the hormones under study, calibration is still possible according to literature [22,29].

The linear dynamic range of the present methodology was performed under optimised experimental conditions on 30 ml of spiked water samples having hormone concentrations between 1.25 and 50.0 μg/l. The data depicted in Table 2, shows an excellent linearity, with remarkable correlation coefficients (>0.993). Mestranol is again the exception, for which the best linearity was only achieved ranging for a 1.25–10.0 μg/l range. The precision of the present methodology was also evaluated using within- and between-day repeatability calculated as R.S.D. on six replicates, giving variations between 2.1% (progesterone) and 17.1%

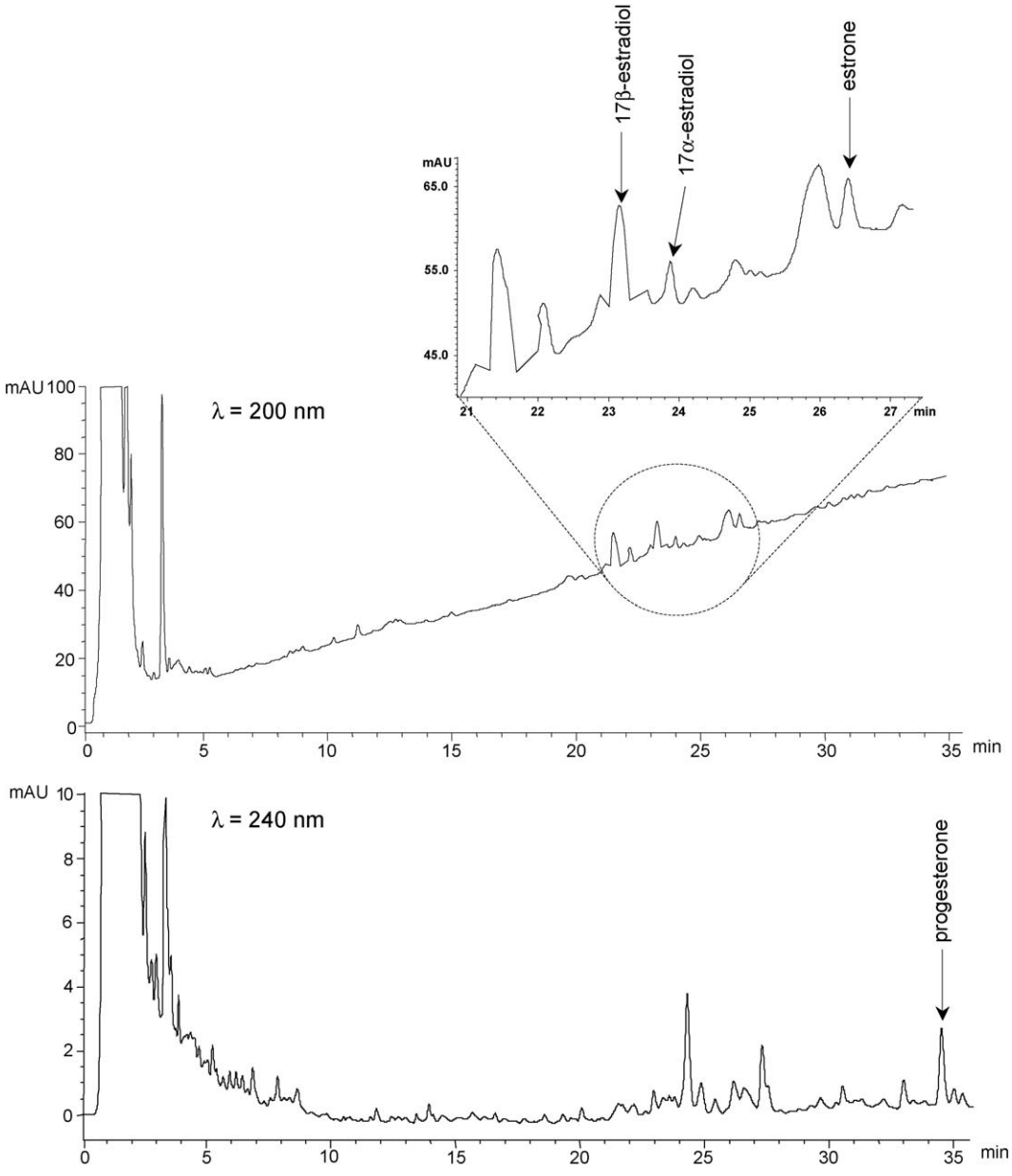

Fig. 5. Chromatogram showing the profile of a urine sample from a pregnant woman (37 weeks) obtained by SBSE–LD–HPLC/DAD, under optimised experimental conditions.

Table 3
Average concentration and standard deviation (±S.D.) for the nine hormones on environmental water and urine matrices spiked at the 10 μg/l level, measured by SBSE–LD–HPLC/DAD, under optimised experimental conditions

Hormones	Environmental water[a] (μg/l; $n=3$)	Urine[a] (μg/l; $n=3$)
17β-Estradiol	10.2 ± 0.7	10.1 ± 0.5
17α-Estradiol	10.5 ± 0.4	9.4 ± 0.4
19-Norethisterone	11.2 ± 0.4	9.7 ± 0.4
17α-Ethynylestradiol	10.1 ± 0.3	10.9 ± 1.2
Estrone	10.7 ± 0.6	10.3 ± 0.4
Diethylstilbestrol	11.0 ± 1.5	9.8 ± 0.8
Norgestrel	10.9 ± 0.4	10.3 ± 0.3
Progesterone	11.1 ± 0.3	10.9 ± 0.5
Mestranol	8.1 ± 0.9	8.6 ± 0.1

[a] Spiked at the 10 μg/l level.

(17α-ethynylestradiol). Furthermore, the sensitivity of the actual methodology was also checked through the LOD and LOQ, ranging from 0.3 to 1.0 and 0.9 to 3.0 μg/l, and measured at a signal-to-noise ratio of 3/1 and 10/1, respectively. Table 2 summarizes the log $K_{O/W}$, LODs, LOQs, linear dynamic range, correlation coefficients and average recoveries achieved for the nine-hormones at the 10 μg/l levels by SBSE–LD–HPLC/DAD, under optimised experimental conditions.

3.4. Application to real matrices

In order to demonstrate the practical ability of the present methodology, its application to real matrices, including environmental water and urine samples, was evaluated. Preliminary blank assays on two particular types of these matrices, showed hormone contents below the LODs achieved for the present methodology, under optimised experimental conditions. Subsequently, assays on both samples having the nine-hormones spiked at the 10 μg/l levels were tested. The results obtained are depicted in Table 3, showing the remarkable accuracy of the present methodology to determine steroid hormones in environmental water and urine matrices, since the experimental data obtained are quite similar to the spiking level (10 μg/l). Nevertheless, an exception is found for mestranol, for which a slightly negative deviation was observed, which can be attributed to matrix effects, as stated before. It must be emphasize that the application of the present methodology to water matrices, can play an important role to profile traces of steroid hormones in aquatic environments influenced by aquacultures, domestic effluents, livestock wastes, etc. On the other hand, once steroid sex hormone studies have been of utmost importance for biomedical interpretation of both physical and metabolic changes in human body, our goal was also to demonstrate the application of the present methodology to profile traces of such compounds in real biological matrices, which can reflect action and malfunction of endocrine glands and can, therefore, be important for the diagnosis of endocrine disorders. Owing to the fact that high releases of endogenous hormones are excreted when women are pregnant, particularly progesterone, a urine sample from a 37 weeks pregnant woman was evaluated by the present methodology. Fig. 5 depicts an overall profile of the corresponding chromatograms obtained by SBSE–LD–HPLC/DAD, under optimised experimental conditions. Although this particular urine matrix presented some complexity, low traces of natural hormones such as progesterone (3.2 ± 0.1 μg/l), 17β-estradiol (13.0 ± 1.5 μg/l), 17α-estradiol (8.4 ± 0.3 μg/l) and estrone (8.8 ± 0.2 μg/l) could be clearly detected, as well as other metabolites. In this particular sample, other steroid hormones under study are absent or present contents lower than the established LODs of this methodology. Nevertheless, it must be emphasized that the low levels detected in urine samples are referred only to the particular case of free steroid hormones, as organisms secret them in free and conjugate form by the endocrine glands [4,20].

4. Conclusions

In this work, the combination of stir bar sorptive extraction and liquid desorption followed by high performance liquid chromatography with diode array detection (SBSE–LD–HPLC/DAD) was successfully applied, for the first time, to the determination of nine steroid sex hormones in water and urine matrices, at the trace level.

Using stir bars coated with 126 μl of polydimethylsiloxane, it has been demonstrated that equilibrium time, ionic strength and back extraction solvents are the most important parameters affecting the monitorization of the tested hormones in water matrices. The experimental recovery data obtained for mestranol showed that stir bar sorptive extraction followed by liquid desorption is well described by the octanol–water partition coefficients ($K_{PDMS/W} \approx K_{O/W}$), while pronounced deviations to the theoretical efficiency ($K_{PDMS/W} \neq K_{O/W}$) were observed for the remaining hormones. A good analytical performance was attained, including a suitable precision, low detection limits and an excellent linear dynamic range. The application of this methodology to environmental water and urine matrices showed recovery yields for the tested hormones in excellent agreement with the spiking level (10 μg/l) and some performance were also achieved by profiling the occurrence of low traces of natural hormones in urine samples from pregnant women. The ability of the SBSE–LD–HPLC/DAD methodology, to accomplish the underlined objectives, should undoubtedly make it a valuable tool to monitor steroid sex hormones in real matrices, particularly in water and urine matrices. The method showed to be easy, reliable and sensitive, requiring a low sample volume.

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