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Solvent Bar Microextraction Method Based on a Natural Deep Eutectic Solvent and Multivariate Optimization for Determination of Steroid Hormones in Urine and Water

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Abstract: Steroid hormones may pose potential risks to both human health and wildlife, primarily through the consumption of medication or polluted food and water; efforts are being made to monitor their levels in the human body and regulate and minimize their releases to the environment. In this study, a simple and environmentally friendly sample preparation method was developed to simultaneously determine three steroid hormones in urine and water samples. A monoterpene (menthol) and a fatty acid (lauric acid) were combined in various ratios to form a hydrophobic deep eutectic (HDE) solvent as an extraction solvent in solvent bar microextraction (SBME). Using a univariate strategy, a menthol-to-lauric acid HDE ratio of 4:1 and a pH 7 of the sample solution resulted in the highest extraction efficiency (EE%) of the selected steroids. The computational methods have been employed to predict a 4:1 HDE interaction with chosen steroids. Additionally, chemometric approaches suggested that the optimal extraction conditions involved HDEs as extract solvent confined within three SBME devices directly immersed into a 20 mL sample solution with a 30 min extraction time, followed by ultrasonication within 200 μ L of elution solvent for a 5 min elution time. Under optimized conditions, the method calibration graph for the spiked selected steroids in the water and urine samples showed good linearity with $R^2 \geq 0.994$ with limits of detection/quantification lower than 0.40/1.35 μ g L⁻¹ and repeatability/reproducibility (RSD%, n = 5) lower than 5.09/7.11. The developed method allows a safe, rapid, and reliable analysis of three steroid hormones in human urine and water samples without using toxic volatile organic solvents.

Keywords: hydrophobic deep eutectic solvent; solvent bar microextraction; steroid hormones; multivariate analysis; HPLC-DAD; urine and water



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1. Introduction

The steroid hormones of β -estradiol (BES), testosterone (TES), and progesterone (PRO) shown in Figure 1 play crucial roles in the development and functioning of the reproductive system, as well as in other physiological processes [1]. The presence of selected steroids at appropriate levels is necessary for normal physiological functioning [2]. Nevertheless, excessive levels, whether due to medical conditions, hormone imbalances, or external factors like steroid abuse, can have detrimental effects on health and potentially increase the risk of toxic effects [3]. Furthermore, steroid hormones such as BES, TES, and PRO can act as endocrine-disrupting chemicals and interfere with the normal functioning of the endocrine system, which is responsible for regulating various physiological processes in organisms, including reproduction, development, metabolism, and homeostasis [4]. It is important to note that the presence of steroid hormones chemicals in the environment is a

complex issue with multiple sources, such as industrial pollutants, agricultural runoff, and the improper disposal of pharmaceuticals [5]. Efforts are being made to monitor steroids in biological fluids and to regulate and minimize the release of these compounds into the environment to mitigate their impact on wildlife and human health.

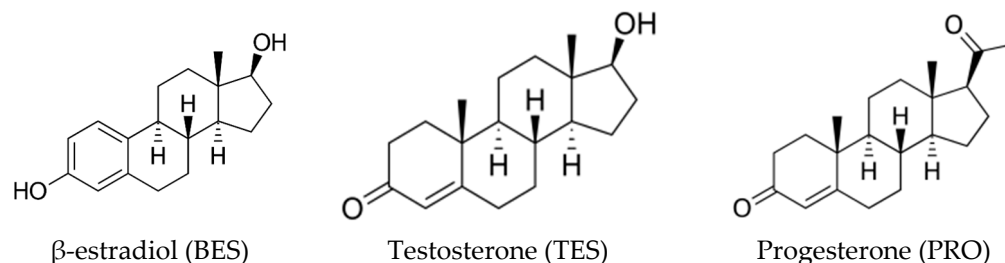


Figure 1. The structure of selected steroid hormones under study.

The analytical methods used to assess natural steroid hormones in life sciences (bioanalytics, ecology, medicine, and pharmacology) have to overcome challenges such as the low concentration of these compounds and the presence of interfering substances in biological matrices. Hyphenated techniques such as high-performance liquid chromatography (HPLC) or gas chromatography (GC) coupled with mass spectrometry (MS) [6–11] are commonly used to achieve high selectivity and sensitivity. However, sample preparation steps, including solid-phase extraction (SPE) [12,13], liquid–liquid extraction (LLE) [14], or derivatization [15], are required to enhance analyte stability or detectability. While LLE and SPE techniques have proven to be effective methods, they encountered certain drawbacks related to their time-consuming, complex, high organic solvent consumption, and loss of trace-level analytes during extraction [16]. The demand to overcome these limitations has promoted significant endeavors to modify extant sample preparation techniques and devise novel approaches.

Solvent bar microextraction (SBME) is a technique that offers an alternative to the hollow fiber–liquid phase microextraction (HF-LPME) procedure [17]. This method uses a hydrophobic porous HF microtube as a carrier for the extraction solvent. The extraction phase relies on an organic solvent, typically immiscible with water. Compared to other extraction techniques, SBME offers advantages in the setting process as it requires only a small volume of organic solvent in the microliters range, along with a few milliliters of the sample within a shorter time to being analyzed [18]. However, it is important to note that traditional organic solvents used in SBME often have high volatility and exhibit instability, which can affect the accuracy and reliability of the extraction process. Additionally, some organic solvents are known to be toxic, posing risks to human health and the environment.

Hydrophobic deep eutectic solvents (HDEs) are a relatively new class of solvents that have garnered significant attention as alternative media for liquid–liquid equilibria with water in living organisms [19]. The unique properties and advantages of HDEs, such as stability, relatively high viscosity, low toxicity, high biodegradability, and good extraction efficiency, qualify them as promising replacements for conventional extraction solvents and make them more suitable for sustainable and environmentally friendly standpoints [20]. The preparation of deep eutectic solvents results in a high yield in production with simple synthesis steps without generating significant waste. Usually, it depends on the establishment of hydrogen bonds between a hydrogen bond donor (HBD) and the other as a hydrogen bond acceptor (HBA) [21]. Concerning the hydrophobicity of the DES, the present study has selected menthol and lauric acid as the hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD), respectively, in varying ratios [22]. These hybrid HDEs exhibit advantageous solvation characteristics for both polar and nonpolar substances and demonstrate enhanced stability at elevated temperatures, rendering them suitable for extracting diverse organic compounds [22–25].

Therefore, based on those mentioned above, this study aims to develop a simple, cheap, and green sample preparation procedure based on SBME using synthesized HNDEs (containing various ratios of menthol and lauric acid) for the simultaneous extraction and preconcentration of steroid hormones BES, TES, and PRO at trace levels from human urine and real waters and subsequent measurement by HPLC-DAD. The parameters that impact the EE% were carefully optimized by utilizing response surface methodology (RSM) founded on central composite design (CCD) multivariate analysis.

2. Materials and Methods

2.1. Reagent and Materials

The compounds of β -estradiol ($\geq 98\%$), testosterone ($\geq 99\%$), progesterone ($\geq 99\%$), menthol (99.7%), lauric acid (98.7%), hydrochloric acid (37%), and sodium hydroxide were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol and water HPLC-grade quality were purchased from Carlo Erba Reagents (Milan, Italy). The polypropylene hollow fiber microtube (Q3/2, ACCUREL, 600 m i.d., 200 m wall thickness, and 0.2 m pore size) was supplied from Membrana (Wuppertal, Germany).

2.2. Synthesis of HDEs

The previously described technique was used to synthesize the HDEs [26]. Menthol and lauric acid were combined in molar ratios of 2:1, 3:1, 4:1, and 5:1 and then heated at 70 °C for 30 min at air pressure with stirring at 500 rpm until liquids formed. The produced liquids were then cooled to room temperature, homogenized by sonication for three minutes, and kept in a desiccator until used.

2.3. Preparation of Standard Solutions

The BES, PRO, and TES stock solutions were prepared with a concentration of 1 mg L⁻¹ and kept in the dark at 4 °C. The working solutions were diluted with 20 mL of water and then immediately exposed to the HDE-SBME without further processing. All calibration solutions were freshly prepared by diluting the appropriate volume of the stock solution with 10 mL of absolute methanol.

Steroid-free urine samples were prepared using the previously reported method [27] with slight modifications. Following the written agreement, 100 mL of urine samples were collected from volunteers and centrifuged at 3000 rpm for 3 min at 4 °C. The centrifuged urine samples were then percolated through Bond elut C18 cartridges to retain urinary steroids and other potential interfering compounds. The unretained fraction was collected to be used as the matrix for steroid spikes in method validation processes.

2.4. Urine and Water Samples

Morning urine samples were collected from two healthy volunteers, a young boy and a pregnant woman aged 14 and 29, respectively. The samples were stored at 4 °C until further analysis. Two samples of urine, each with a volume of 20 mL, were used for the analysis using the HDE-SBME method without any pretreatment.

Water samples, groundwater, and tap water were collected in 1 L amber glass bottles and stored in the dark at 4 °C until used. HDE-SBME and analysis procedures were then carried out the same way as for urine samples.

2.5. HDE-SBME Procedure

The HF microtube made of polypropylene was manually and meticulously cut into 2.5 cm lengths, submerged in HDEs for one-minute while being sonicated. The lumen of the HF microtube was cleaned with tissue paper, then filled with HDEs and heat-sealed on both ends to seal the contents. The prepared HDE-SBME device was added to 20 mL of a sample solution containing the chosen analytes with agitation using a magnetic stirrer bar. The HDE-SBME device was moved to a micro-vial containing methanol once the extraction process was complete in order to desorb the analyte using ultrasonication before being

injected into the HPLC-DAD for analysis. Each experiment employed a freshly constructed HDE-SBME device to rule out the potential of a memory effect.

2.6. Instrumentation

The specific analytes were examined using a UHPLC (Ultimate 3000/Dionex, Germering, Germany) system equipped with a binary pump and a UV/DAD detector. The separation was performed on an Inertsil ODS-3, C18 (150 mm × 4.6 mm × 5 µm) column (Merck Germany, Darmstadt, Germany). The isocratic elution program was based on 80% methanol as the mobile phase at a flow rate of 1 mL min^{−1}, with a running time of 6 min. The detection wavelength was set at 268 nm, and the column temperature was maintained at 32 °C with an injection volume of 20 µL. Under the above chromatogram conditions, the retention times of BES, TES, and PRO were approximately 4.08 min, 4.49 min, and 5.42 min, respectively. The solution's pH was determined using a Metrohm pH-meter model 691 (Herisau, Switzerland). Infrared spectra were captured using a V70 FT-IR spectrophotometer (Bruker Optic GmbH, Ettlingen, Germany) utilizing a single reflection ATR cell (DuraDisk, fitted with a diamond crystal). The spectral data were obtained at an ambient temperature in the 400 to 4000 cm^{−1} range. Each sample underwent 290 scans at a resolution of 4 cm^{−1}, and five duplicate spectra were obtained for reproducibility assessment. The ¹H NMR experiments were conducted using an NMR spectrometer model Ascend 400, 400 MHz (Bruker Biospin, Rheinstetten, Germany). The HDE mixture samples were created on 5 mm NMR tubes based on weight, using around 30 mg of eutectic mixture. Subsequently, 0.5 mL of deuterated chloroform (CDCl₃) was added. The sample was thoroughly mixed using vortex mixing to ensure uniformity.

2.7. Method Validation

The consideration of matrix effects is crucial during the development of a method, as they can have an impact on both reproducibility and accuracy. To assess matrix effects, the results obtained from using the developed HND-SBME-HPLC-DAD method for extracting selected steroids spiked at 1, 100, and 10⁴ µg L^{−1} from ultrapure water were compared to those obtained from groundwater and tap water samples spiked at the exact quantities using the same method. The absolute matrix effect was determined by calculating the following [28]:

$$\text{Matrix Effect (\%)} = C_{\text{matrix}} / C_{\text{ultrapure water}} \times 100 \quad (1)$$

where C_{matrix} is the measured concentration of each steroid in the groundwater and tap water sample, and $C_{\text{ultrapure water}}$ is the measured concentration of each steroid in ultrapure water. In particular, when the matrix effect value is closer to 100%, the interference caused by the matrix is minimal.

The proposed method was validated for accuracy, precision, and robustness. This included testing its linear dynamic range, correlation coefficient, limit of detection (LOD), quantification (LOQ), reproducibility, and recovery for three analytes in ultrapure water and steroids-free urine samples. The method calibration curves were evaluated for linearity in the 0.5–10⁴ µg L^{−1} range for each analyte. The limits of detection (LOD) and quantification (LOQ) were determined based on three times and ten times the standard deviation of the blank divided by the slope, respectively [29]. Inter- and intra-precision were estimated by measuring the recoveries of selected steroids spiked at three concentrations (1, 100, and 1000 µg L^{−1}) in water and steroid-free urine over one and three days, respectively. The method's robustness was evaluated in ultrapure water and steroid-free urine samples (spiked at 100 µg L^{−1} for each analyte) by varying four parameters of the HDE-SBME method: pH, extraction time, elution volume, and ultrasonication time. The ranges for these parameters were 6.8–7.2 for pH, 28–32 min for extraction time, 190–210 µL for elution volume, and 4–6 min for ultrasonication time. Each experiment, including these variation parameters, was triplicated to ensure consistency in the results.

2.8. Computational Methods

Computations were completed with Hyperchem (release 6.03 professional, Hypercube Inc., Waterloo, ON, Canada). The initial molecular geometries of the studied molecules were obtained using their X-ray diffraction data [30–34]. Several initial geometries of HDEs, containing a molar menthol-to-lauric-acid ratio of 4:1, were built by randomly placing four menthol molecules (mixtures of D-and L-isomers) around lauric acid. Each initial structure was energy minimized with molecular mechanics, employing the AMBER force field and the conjugate gradient algorithm (0.01 kcal mol^{−1} Å^{−1} gradient), followed by further minimization using the semiempirical PM3 (Parametric Method 3). The most stable HDE structure from above was used to build several starting geometries of HDE–hormone complexes by placing the hormone molecule at different positions around HDEs; those structures were minimized with the above protocol.

2.9. Experimental Design

The SBME of BES, TES, and PRO is subject to numerous influencing factors, necessitating an optimized experimental design to determine the optimal combination of factors with minimal experimental effort. For this purpose, chemometric approaches using CCD-based RSM using Chemoface[®] v1.66 [35] were applied at three levels for four test factors listed in the Supplementary Table for Materials (Table S1). Midpoints were usually replicated to estimate experimental errors. Experiments were then run on these design points, and the EE% response variable was used to measure each combination of factor levels listed in Table 1. Then, the obtained experimental data listed in Table 1 were used to fit a mathematical (quadratic or polynomial) model equation describing the relationship between factors and responses [35]. The essential factors and their optimal values can be estimated by analyzing the model.

Table 1. The CCD experimental setup at three levels for steroid hormones optimization.

Test	Influence Factors				Analyte Response (EE%)		
	A: Number of HDE-SBME Devices	B: Extraction Time (min)	C: Volume of Elution (μL)	D: Elution Time (min)	BES	TES	PRO
1	5	10	300	2	27.33	32.50	32.51
2	1	10	300	2	17.11	19.18	22.35
3	3	30	200	8	70.21	82.92	81.36
4	3	50	200	5	70.63	86.65	83.21
5	1	10	100	8	11.28	13.41	14.23
6	3	30	200	5	73.26	90.39	87.92
7	5	50	100	8	61.89	75.45	71.70
8	3	30	200	5	73.26	90.39	87.92
9	3	30	200	5	73.26	90.39	87.92
10	5	10	300	8	32.43	39.44	40.01
11	1	50	300	8	13.42	16.02	17.01
12	5	50	300	2	59.42	67.45	65.67
13	5	50	300	8	67.11	82.63	81.47
14	3	30	200	5	73.26	90.39	87.92
15	1	50	100	8	32.43	39.21	40.23
16	1	10	300	8	9.67	11.44	12.83
17	3	30	200	2	68.38	73.41	70.73
18	1	50	300	2	8.77	10.52	11.74
19	3	30	300	5	70.26	88.45	83.25
20	1	50	100	2	6.11	8.47	9.35
21	3	10	200	5	37.74	42.59	43.38
22	5	30	200	5	72.26	88.63	85.65
23	1	10	100	2	3.26	5.74	6.623
24	5	10	100	8	35.66	40.59	40.03

Table 1. Cont.

Test	Influence Factors				Analyte Response (EE%)		
	A: Number of HDE-SBME Devices	B: Extraction Time (min)	C: Volume of Elution (μ L)	D: Elution Time (min)	BES	TES	PRO
25	5	10	100	2	30.14	36.51	35.16
26	3	30	200	5	73.26	90.39	87.92
27	5	50	100	2	29.33	34.76	34.61
28	3	30	100	5	61.89	75.45	71.70
29	1	30	200	5	27.23	29.18	34.84

3. Results and Discussion

To obtain the best extraction performance of the HDE-SBME method, two approaches were used to optimize the influence of different experimental parameters. A univariate approach based on chromatographic peak area was used to evaluate the effect of using various types of synthesized HDEs and the effect of sample solution pHs, while for reduced labor and experimental effort, experimental parameters, including the number of SBME devices, extraction time, and elution conditions, were optimized using the multivariate methodology. These experiments used spiked ultrapure water (20 mL) with various BES, PRO, and TES concentrations. All experiments were run in triplicate, and the average of the results was used for optimization.

3.1. Selection of HDEs

At the core of the SBME technique is achieving equilibrium in target analyte diffusion and migration between the sample and extraction phases. Therefore, choosing an extraction solvent with a high affinity for the analyte is essential in the mass transfer and separation process. For this mission, HDEs were utilized as both the extraction phase filled in the lumen and impregnated in the wall pores of the HF microtube. Synthesized HDEs using various ratios of menthol–lauric acid (2:1, 3:1, 4:1, and 5:1) were tested. The results indicated that the HDEs with a ratio of 4:1 (menthol to lauric acid) showed the highest peak area for the three target compounds, especially for PRO and TES, as listed in Table 2. As the quantity of menthol increases, the viscosity of HDEs gradually decreases [26], thereby diminishing the mass transfer hindrance of the desired analytes into the extraction phase. Meanwhile, by increasing the molar ratio of menthol above 4:1, the peak area of PRO and TES started to decrease slowly. As a result, menthol–lauric acid (4:1 ratio) HDE was most appropriate for extracting the target hormones and was used for further optimization experiments.

Table 2. The effect of using HDEs with various ratios of menthol and lauric acid in the SBME * technique.

HNDE Ratio of Menthol–Lauric Acid	Peak Area		
	BES	TES	PRO
2:1	1.081	5.309	3.864
3:1	1.757	4.467	5.236
4:1	1.760	4.608	5.523
5:1	1.741	4.184	5.308

* SBME conditions were set as follows: 10.0 μ g L^{−1} of selected steroids, 20.0 mL of water as a sample solution using one SBME device stirred at 250 rpm for 30 min extraction time, and 150 μ L of methanol as elution solvent sonicated for 5.0 min, n = 3.

3.1.1. HDE Characterization

The HBD and HDA interaction results in the well-known mixture HDE. In order to determine if there is a hydrogen bond interaction between the oxygen atom of the carboxylic group located on lauric acid as HBA and the hydroxyl group of menthol as HBD, the FT-IR spectra and ¹H NMR spectra of the HDE (menthol–lauric acid with a molar ratio of 4:1)

were recorded. As shown in Supplementary Figure (Figure S1), the FT-IR characteristic absorption peaks of lauric acid remained unchanged after the addition of menthol, while the stretching vibration of the C=O peak in lauric acid shifted from 1692 cm^{-1} to 1739 cm^{-1} in the HDE. The peak at 3241 cm^{-1} , corresponding to menthol's O–H stretching vibration, shifted to 3384 cm^{-1} .

The menthol and lauric acid chemical signals show significant differences with 1:4 HDE in ^1H NMR spectra. The menthol spectrum (Figure 2A) shows a doublet at $\delta = 3.1\text{ ppm}$, while the HDE spectrum (Figure 2C) shows a singlet without significant chemical displacement. As expected, the proton bonded to the same carbon as the hydroxyl group in menthol is also a hydrogen bonding signal that appears as a multiplet with a resonance of -H-at a chemical shift of $2.0\text{--}2.2\text{ ppm}$. However, in the HDE ^1H NMR spectrum, no detectable change is observed, and the signals are no longer well-defined multiplets, suggesting that hydrogen bond interactions between the parent molecules influence the hydrogen of menthol. The absence of the hydroxyl group of lauric acid (appears at $\delta = 11.7\text{--}11.9\text{ ppm}$, as shown in Figure 2B) in the HDE ^1H NMR spectrum supports hydrogen bonding. The physical state of the compounds further confirms this proof, as a transparent liquid is obtained after the formation of HDE solvent from initially solid compounds. Overall, the data strongly indicate the formation of hydrogen bonds between the hydroxyl groups of menthol (hydrogen bond donor) and the carboxyl group of lauric acid (hydrogen bond acceptor).

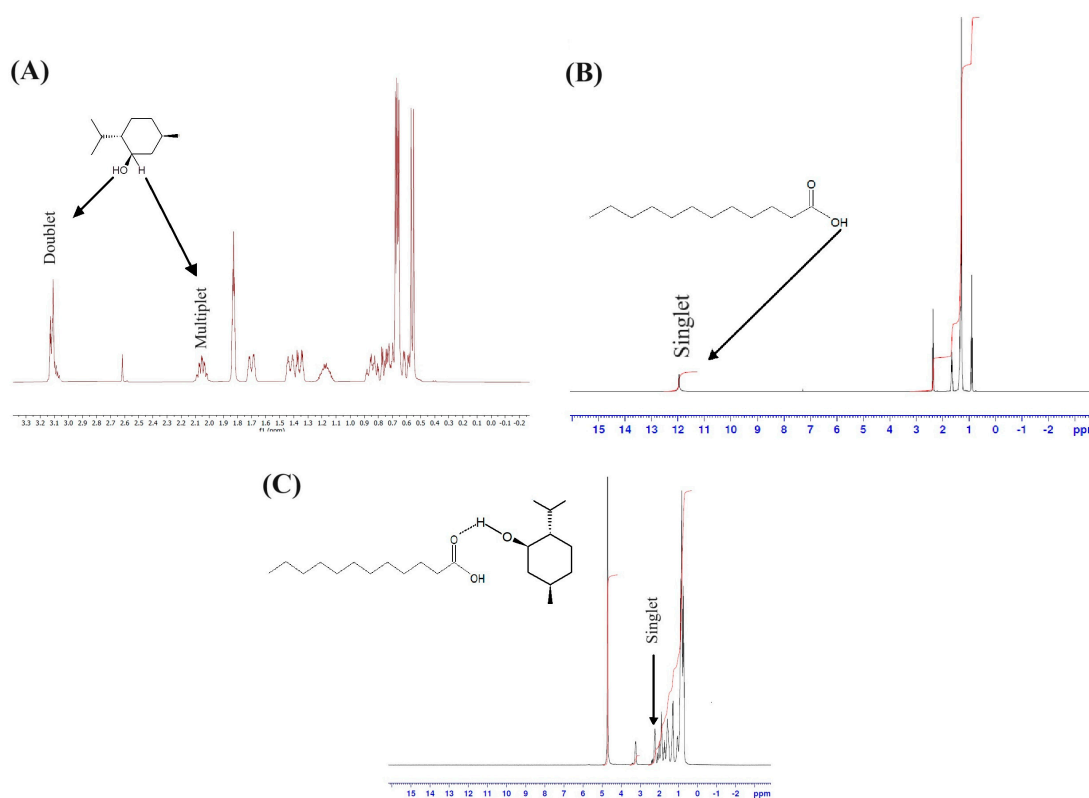


Figure 2. The spectra ^1H NMR for (A) menthol, (B) lauric acid, and (C) for synthesized HDE.

3.1.2. Interaction of the 4:1 HDEs and Selected Steroids Based on Computational Methods

The most stable structure of the 4:1 HDEs (Figure 3) shows that two menthol molecules form hydrogen bonds with the carboxylic group of lauric acid, while the alkyl chain of lauric acid interacts via van der Waals interactions with the hydrophobic moieties of the surrounding menthols.

Among the many starting geometries of the molecular complexes of each steroid hormone with 4:1 HDEs that were used as input for the computational work, two significant modes of binding of the hormone with HDEs (Figure 4) are obtained. The optimized

geometry of binding mode 1 of each complex reveals the hydroxyl/carbonyl group of the hormone situated within the polar pocket of HDEs and forming a hydrogen bond with one of the menthol molecules, while the hydrophobic moieties are in close contact with the hydrophobic region of HDEs. Furthermore, for binding mode 2, the hormone molecules are located in the hydrophobic pocket composed of the alkyl chain of lauric acid and the hydrophobic moieties of menthol molecules, while the polar groups are in close proximity to the hydroxyl groups of the menthol molecules. PM3 estimates of the interaction energies are listed in Table 3 and show that both TES and PRO interact more strongly with HDEs than BES in both binding modes. The lower interaction energy in the case of the BES complex might be a direct result of the presence of an aromatic ring in BES, which reduces its ability to adapt to a more favorable conformation. The interaction strength of BES with HDEs is nearly the same for both binding modes, whereas TES and PRO binding mode 2 is more favorable than binding mode 1 by 1.9 and 2.5 kcal·mol^{−1}, respectively.

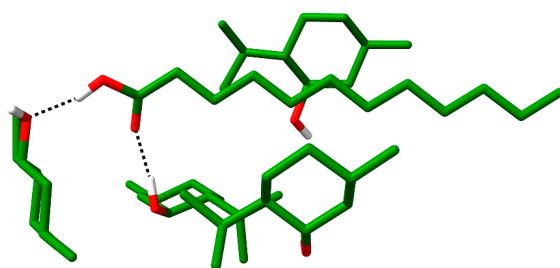


Figure 3. PM3-optimized structure of 4:1 HDEs.

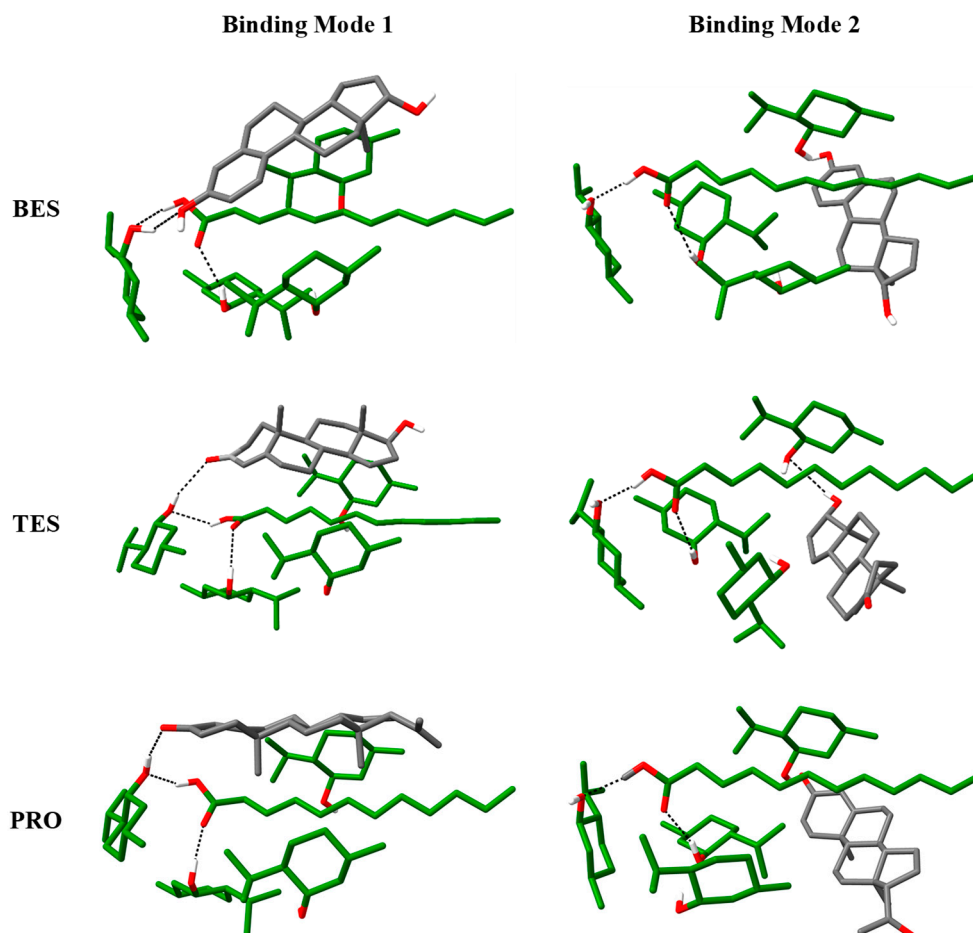


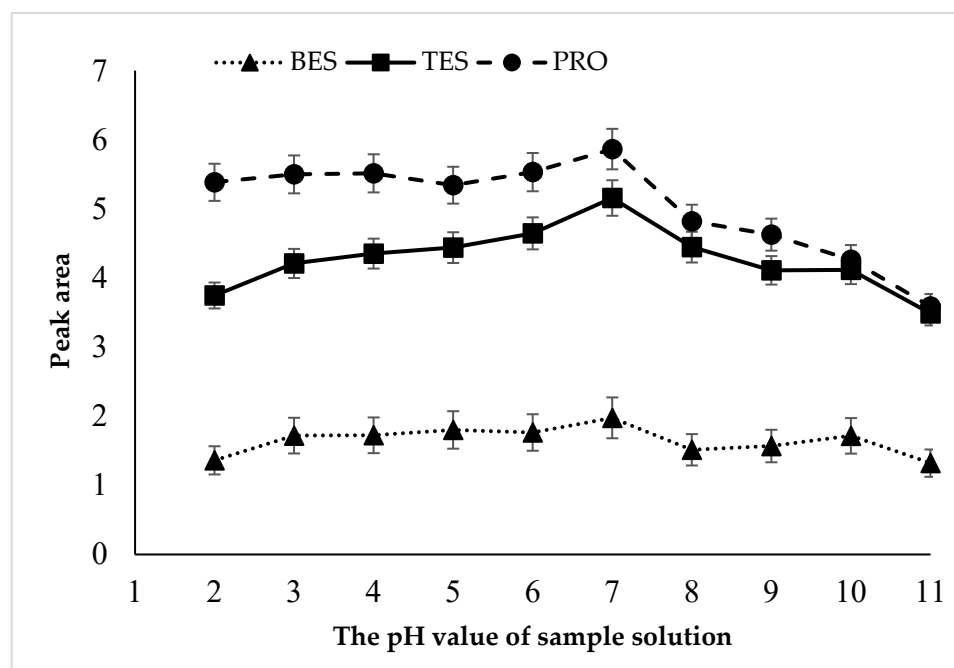
Figure 4. PM3-optimized structures of the studied steroid hormones (in grey) with HDEs (in green).

Table 3. PM3 estimates the interaction energies (in kcal.mol^{−1}) of the studied steroid hormones with 4:1 HDEs.

	BES	TES	PRO
Binding Mode 1	−11.5	−13.9	−13.3
Binding Mode 2	−11.4	−15.8	−15.8

3.2. Optimize the Sample pH

The pH of aqueous sample solutions often alters the efficiency of the SBME method by changing the structure of analytes. The selected BES, TES, and PRO analytes are hormones that can be hydrolyzed under certain conditions, such as acidic or alkaline pH levels [36]. However, the extent and rate of hydrolysis depend on the specific hormone and pH conditions, so the pH of the sample solution should be optimized. For this purpose, the pH was tested over the range of 2–11 and adjusted with 0.1 M HCl or NaOH solutions. As demonstrated in Figure 5, the efficiency of the SBME method was dependent on pH, and the optimum was reached at 7. However, a decrease in analytical signal was observed above and below pH 7 due to the hydrolysis of selected hormones [36,37]. Thus, pH 7 was selected as the best sample solution pH for further optimization steps.

**Figure 5.** The influence of pH sample solution on the peak area of the extracted analytes using HDE-SBME. Other extraction conditions as listed in Table 1. Error bars correspond to standard deviation.

3.3. Optimize Parameters Using a Chemometric Approaches

The results from the CCD were fitted to a quadratic polynomial using several regressions to obtain the following quadratic equation from the encoded data.

$$\begin{aligned} \text{EE\% BES} = & 71.9718 + 15.9039A + 8.0273B + 1.8629C + 4.6801D + 4.5483AB + \\ & 2.0862AC + 1.2078AD + 0.79794BC + 3.7513BD - 3.9001CD - 21.147A^2 - \\ & 16.705B^2 - 4.8185C^2 - 1.5980D^2 \quad (R^2 = 0.9239) \end{aligned} \quad (2)$$

$$\begin{aligned} \text{EE\% TES} = & 87.151 + 19.155A + 9.9871B + 2.1136C + 6.2539D + 5.4244AB + \\ & 2.7738AC + 1.9213AD + 0.77775BC + 5.0723BD - 3.9554CD - 25.545A^2 - \\ & 19.826B^2 - 2.4962C^2 - 6.2847D^2 \quad (R^2 = 0.9108) \end{aligned} \quad (3)$$

$$\begin{aligned} \text{EE\% PRO} = & 84.79295 + 17.64394A + 9.32583B + 2.40044C + 6.11878D + \\ & 5.21419AB + 2.79081AC + 1.93831AD + 0.52206BC + 4.91131BD - 3.83681CD \quad (4) \\ & - 21.93324 A^2 - 18.87824B^2 - 4.70274C^2 - 6.13174D^2 \quad (R^2 = 0.9177) \end{aligned}$$

where EE% is the extraction efficiency for selected steroid hormones, A is the number of HDE-SBME, B is the extraction time, C is the volume of the elution solvent, and D is the elution time.

In Equation (2), the observed positive value, 71.9718, is the intercept or baseline value of the BES EE% response when all factors are at their low levels. The coefficients for factors A, B, C, and D are positive, indicating that increasing their level will positively affect the EE% response. The quadratic terms for A^2 , B^2 , C^2 , and D^2 factors are negative, indicating that their effect on the response becomes weaker as their level increases beyond a certain point. Furthermore, it is noteworthy that the coefficients pertaining to the interaction terms AB, AC, AD, BC, and BD exhibit a positive value, signifying that the cumulative impact of these factors surpasses the summation of their individual effects. Conversely, the combination of CD yields a negative effect, implying that the collective influence of these factors is inferior to the summation of their individual effects. Equation (3) with a baseline value of and Equation (4) with a baseline value of 84.79295 have the same behavior between all tested factors, quadratic terms, and their interaction terms (A, B, C, D, A^2 , B^2 , C^2 , D^2 , AB, AC, AD, BC, and BD) with the EE% response for TES and PRO, respectively.

The analysis variances (ANOVA) for the selected steroids EE% of the HDE-SBME method are shown in Tables S2–S4. Regarding the estimated p -value derived from the ANOVA outcomes, it can be inferred that the impact of extraction conditions on the response of all chosen steroids was deemed significant ($p < 0.0001$). The regression equations obtained showed that the coefficients of determination (R^2) were 0.9239, 0.9108, and 0.9177 for BES, TES, and PRO, respectively. These estimate the fraction of overall variation in the data accounted for by the steroid models. Thus, all models can explain 92.39%, 91.08%, and 91.77% of the variation in response for BES, TES, and PRO, respectively. Also, the ‘Model F-value’ of BES = 25.27, TES = 21.34, and PRO = 23.30 implied that the models were significant. As shown in Tables S2–S4, the independent variables, A, B, and D; interaction terms, AB and BD; and quadratic of A^2 and B^2 had a significant linear effect on the EE% response ($p < 0.05$) for the selected steroids. However, the effects of independent variable C, interaction terms AC, AD, BC, and CD, and quadratic of C and D were not significant ($p > 0.05$).

Based on the coefficients of the terms in the model (refer to Equations (2)–(4)), it can be observed that the interaction between the number of HDE-SBME devices and extraction time (AB) holds the most significant main effect in the model. To elaborate, elevating the values of these two variables from lower to higher values increases the EE% of the chosen steroids.

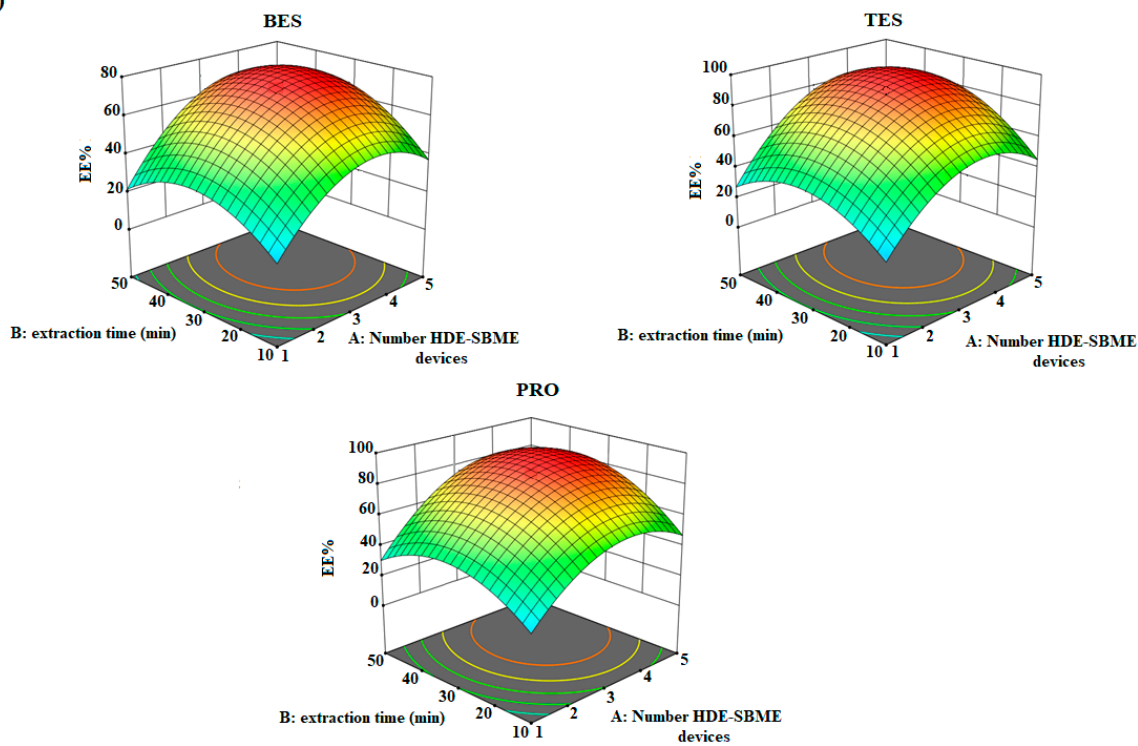
The three-dimensional graph of RSM for the positive significant interaction effect of the extraction time and number of HDE-SBME devices on the EE% response is shown in Figure 6A. It depicts that increasing the extraction time and number of HDE-SBME devices enhances the performance of the microextraction process. The maximum response was obtained in 30 min using three HDE-SBME devices. Meanwhile, by increasing the extraction time to more than 30 min, all selected analytes start to deplete from the HDE-SBME devices [38].

Figure 6B shows the positive effect of decreasing the number of HDE-SBME devices and volume of methanol as an elution solvent on the efficiency of the developed method. The EE% of the process increased with an increase in the volume of methanol used as the elution solvent from 100 to 200 μL ; however, by increasing the volume to more than 200 μL , the response state decreased, which may be attributed to the dilution effect of the analytes.

The interaction effect of the number of HDE-SBME devices and elution time positively affects the EE% of the developed method. As shown in Figure 6C, by increasing the ultrasonic time from 2 to 5 min, the EE% of selected steroids were increased. Then, the EE% started to decrease with an increase from 5 to 8 min, and this could be attributed to the

chemical reaction of ultrasound on methanol during prolonged times resulting in produced free radicals, which may lead to the oxidation of selected steroids and the reduction in their EE% [39].

(A)



(B)

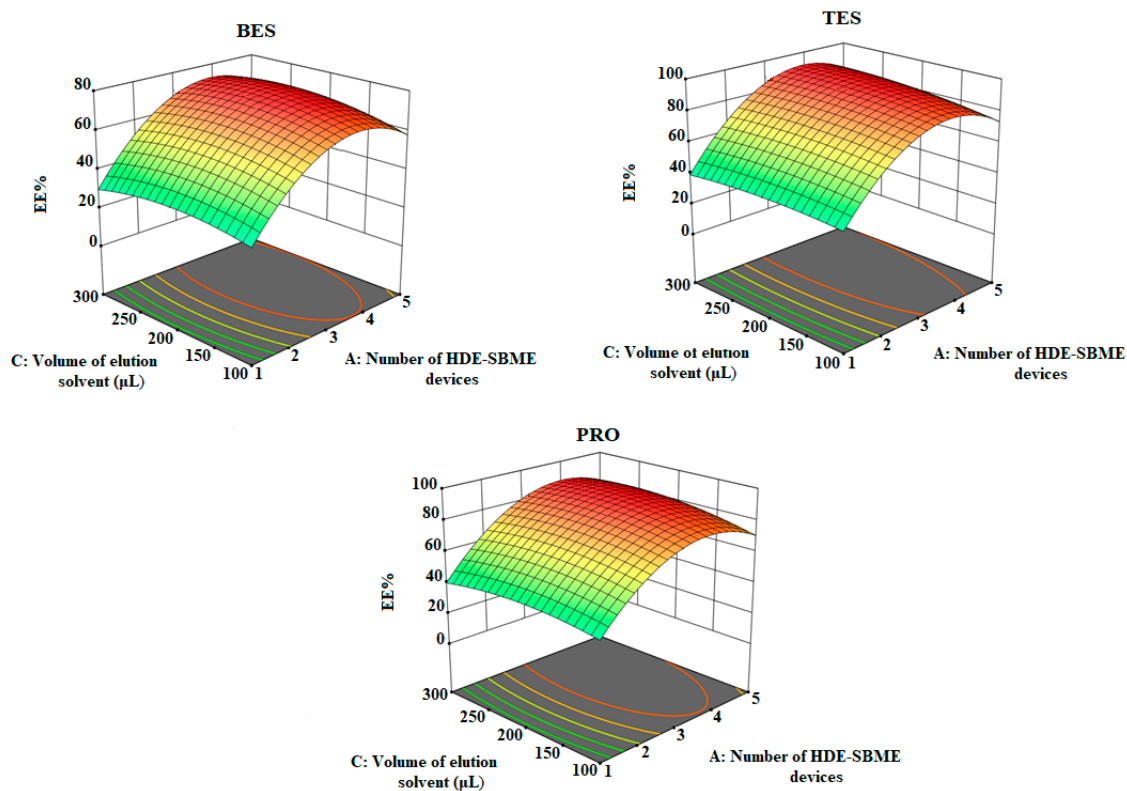


Figure 6. Cont.

(C)

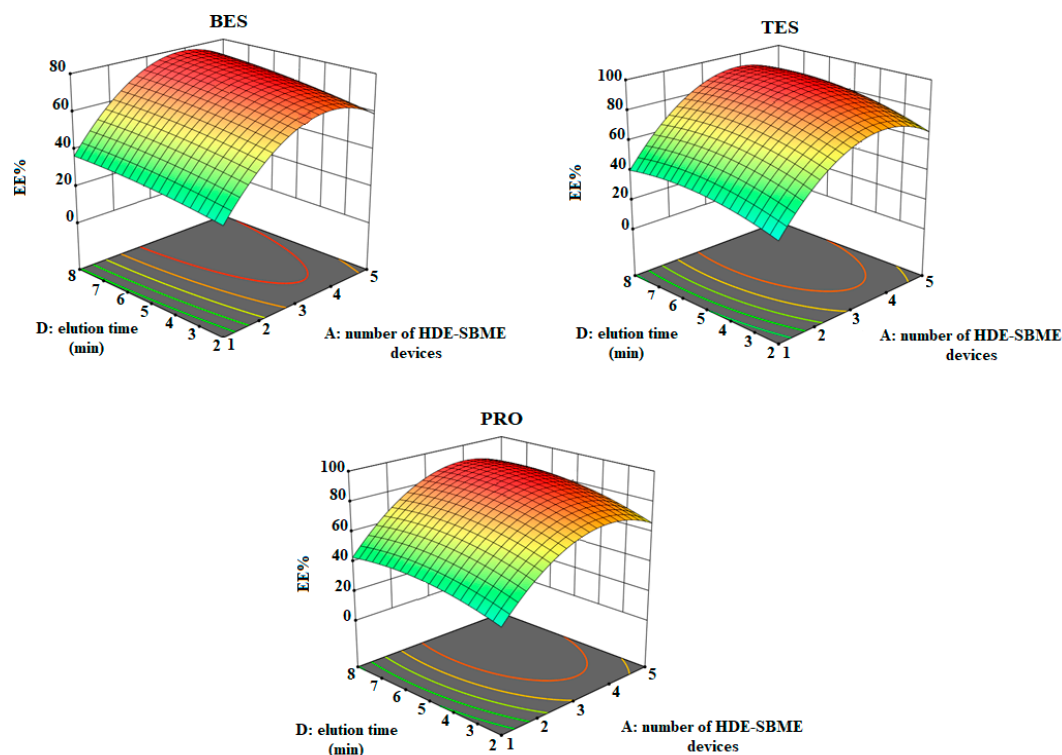


Figure 6. RSM plots showing the effect of selected steroid hormones EE% in process variables on (A) the number of HDE-SBME devices and time of extraction, (B) the number of HDE-SBME devices and volume of elution, and (C) the number of HDE-SBME devices and elution time.

As a result, the RSM based on the CCD optimization model recommended the following experimental conditions, which were adopted. Three HDE-SBME devices, 30 min extraction time, 200 μL methanol as elution solvent, and sonication for 5 min as elution time.

3.4. Method Performance and Application

In order to assess the analytical efficacy of the proposed methodology, several significant parameters were tested, namely the linear dynamic range, correlation coefficient, limit of detection (LOD), limit of quantification (LOQ), reproducibility, recovery, and robustness. The insignificance of the matrix effects of groundwater and tap water is evident from the findings presented in Table S5. Consequently, the examination of selected steroids spiked in ultrapure water and steroids-free urine samples was carried out to generate calibration curves and analyze the validation parameters of the developed method. As summarized in Table 4, the linear dynamic ranges of BES, TES, and PRO in water/steroid-free urine are $0.899\text{--}10^4/1.357\text{--}10^4$, $1.124\text{--}10^4/1.337\text{--}10^4$, and $0.573\text{--}10^4/0.929\text{--}10^4$ $\mu\text{g L}^{-1}$, respectively. The correlation coefficients of determination (R^2) exceeded 0.994 for all steroids in both tested matrixes, indicating a robust linear relationship between concentration and response. The LOD/LOQ values were calculated to be $0.269/0.899$ $\mu\text{g L}^{-1}$ for BES, $0.337/1.124$ $\mu\text{g L}^{-1}$ for TES, and $0.171/0.573$ $\mu\text{g L}^{-1}$ for PRO in spiked water samples. In spiked steroid-free urine samples, the LOD/LOQ were $0.407/1.357$ $\mu\text{g L}^{-1}$, $0.401/1.337$ $\mu\text{g L}^{-1}$, and $0.278/0.929$ $\mu\text{g L}^{-1}$ for BES, TES, and PRO, respectively. Table 4 shows good repeatability and reproducibility with RSDs ranging from 1.43% to 5.09% and 1.98% to 7.11%, respectively.

The robustness results presented in Table 5 revealed slight discrepancies in the collected data. They confirmed that the test conditions did not significantly affect the results of the HDE-SBME-HPLC-DAD analysis. The recovery yields of the selected steroids exceeded 91.7% in water and steroid-free urine matrixes.

Table 4. Validation parameters * of HDE-SBME-HPLC-DAD method for selected steroids spiked in water and steroid-free urine samples.

Parameters	Water			Urine		
	BES	TES	PRO	BES	TES	PRO
Calibration curve	PA = 0.5917x – 0.1226	PA = 1.2458x + 0.0538	PA = 1.7164x – 0.3684	PA = 0.5779x – 0.1481	PA = 1.2149x + 0.0359	PA = 1.6629x – 0.3086
Correlation coefficient (R ²)	0.999	0.997	0.998	0.995	0.996	0.994
Standard deviation, n = 3	1.07–3.44	2.13–3.42	1.54–4.08	1.52–3.81	2.76–3.90	1.82–5.13
Inter precision (RSD%, n = 5)	2.71–3.04	1.90–3.67	1.43–4.21	3.51–4.32	2.22–4.46	2.13–5.09
Intra precision (RSD%, n = 5)	2.43–5.32	2.71–5.83	1.98–5.11	2.71–7.11	3.06–6.21	2.78–6.53
LOD (µg L ^{−1})	0.269	0.337	0.171	0.407	0.401	0.278
LOQ (µg L ^{−1})	0.899	1.124	0.573	1.357	1.337	0.929
Linear range (µg L ^{−1})	0.899–10 ⁴	1.124–10 ⁴	0.573–10 ⁴	1.357–10 ⁴	1.337–10 ⁴	0.929–10 ⁴

* A 5-point calibration line was estimated by regressing the peak area (PA) against drug concentration for each solute.

Table 5. Robustness of the HDE-SBME-HPLC-DAD method in spiked water and steroids-free urine matrixes with three operation levels.

Matrix	Level	Spiked Concentration (µg L ^{−1} , n = 3)	Steroids	Mean Extracted Concentration (µg L ^{−1} , n = 3)	Accuracy RE %	Recovery %
Water	1	10	BES	698.2	−4.68	95.32
			TES	880.3	−2.58	97.41
			PRO	852.9	−2.98	97.01
	2	10	BES	704.3	−3.84	96.16
			TES	890.9	−1.43	98.58
			PRO	862.2	−1.93	98.06
	3	10	BES	687.8	−6.09	93.91
			TES	876.6	−2.99	97.00
			PRO	839.0	−4.57	95.43
Steroids-free urine	1	10	BES	675.6	−7.75	92.24
			TES	852.3	−5.68	94.31
			PRO	814.2	−7.38	92.61
	2	10	BES	688.8	−5.95	94.04
			TES	862.4	−4.57	95.42
			PRO	828.7	−5.73	94.26
	3	10	BES	673.1	−8.09	91.90
			TES	845.1	−6.48	93.51
			PRO	806.2	−8.29	91.70

1: The pH = 7.20, extraction time 32 min, volume of methanol for elution 210 µL and ultrasonicated for 6 min.
 2: The pH = 7.00, extraction time 30 min, volume of methanol for elution 200 µL and ultrasonicated for 5 min.
 3: The pH = 6.80, extraction time 28 min, volume of methanol for elution 190 µL and ultrasonicated for 4 min. RE: Relative error.

After the optimization and validation conditions were established, tests were performed on actual samples to demonstrate the practical application of the current methodology. For this purpose, the developed HDE-SBME-HPLC-DAD method was applied to measure the selected steroids in urine samples and real water samples such as ground-water and tap water. The lack of significant interference in the retention times of selected steroids in both the real water and urine matrices, as shown in Figure 7, indicates that the developed method does not interfere with other pre-existing components and can be effectively identified and measured from the selected analytes independently. The presence of steroid hormones was not discerned in any water samples. Furthermore, the method's efficacy was assessed on biological samples, specifically urine samples. Two urine samples were procured in the morning from a healthy young boy and a woman in the fifth month

of gestation. The BES and PRO were detected in the pregnant woman's urine sample at $11.2 \mu\text{g L}^{-1}$ (RSD 4.31%) and $5.31 \mu\text{g L}^{-1}$ (RSD 5.22%), respectively. Only TES $1.79 \mu\text{g L}^{-1}$ (RSD 3.81%) was detected in a urine sample from a young healthy man.

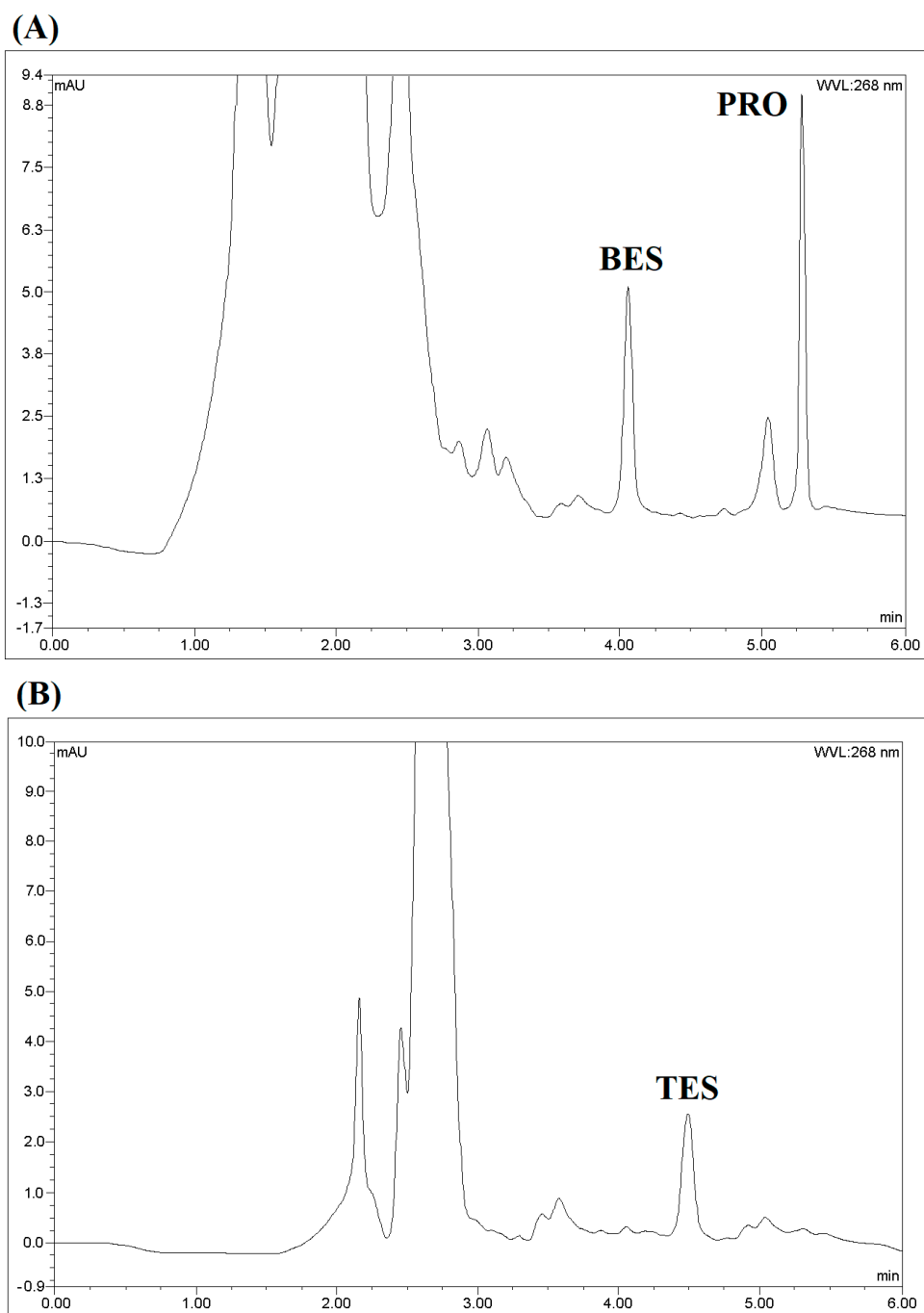


Figure 7. Cont.

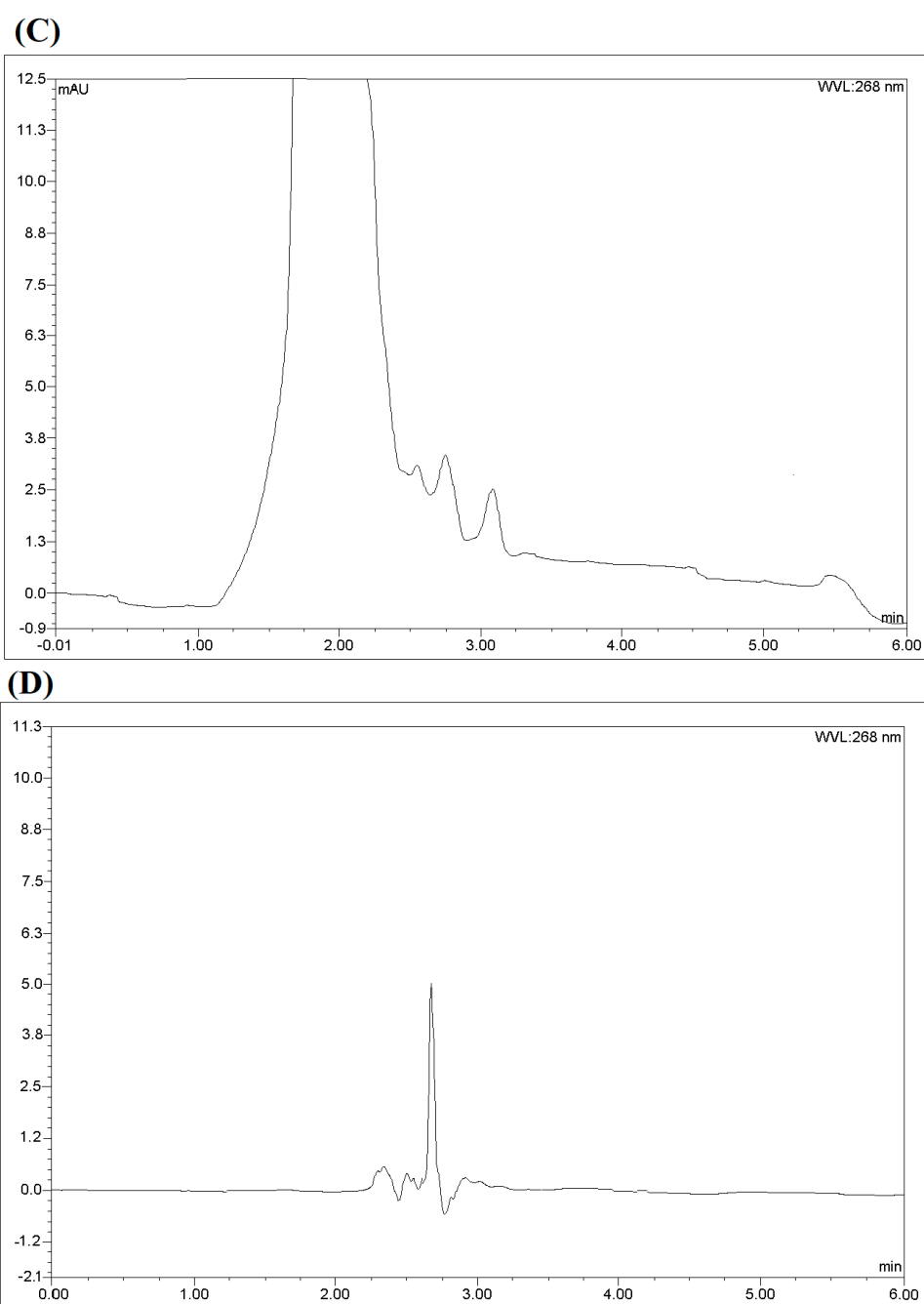


Figure 7. Cont.

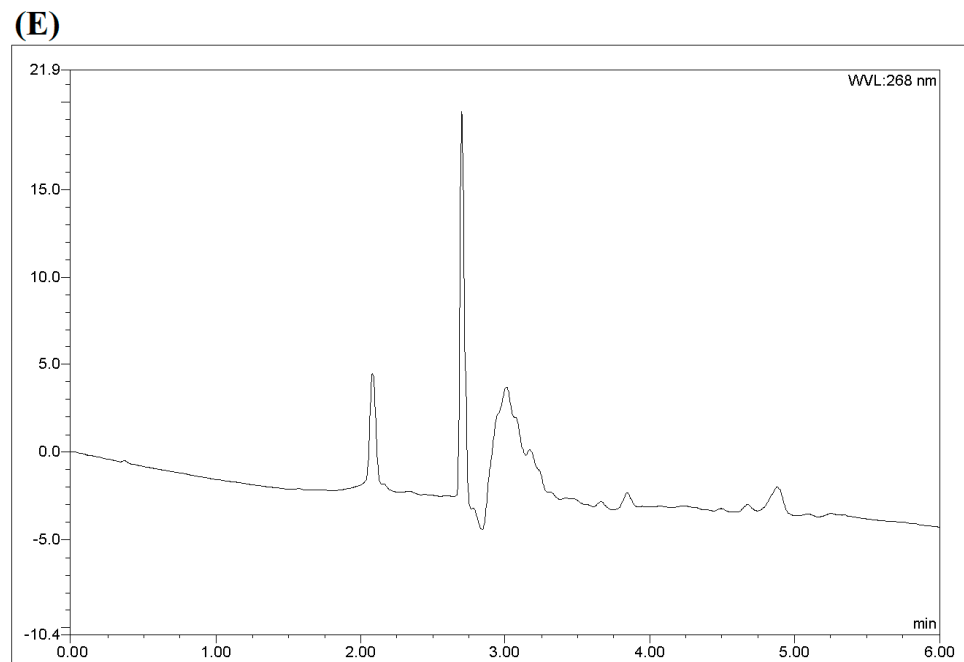


Figure 7. The chromatograms of the HDE-SBME method under optimized conditions for (A) pregnant women’s urine, (B) boy’s urine, (C) steroid-free urine, (D) groundwater samples, and (E) tap water samples.

3.5. Method Comparison

Ultimately, a comparative analysis was conducted between the efficacy of the established method and several prior techniques for measuring the selected steroids in both the water and urine samples, and the results of this evaluation are presented in Table 6. It is worth noting that the proposed method involved the direct immersion of HDE-SBME devices into urine and water samples without any pre-treatment. On the other hand, most of the other methods listed in the literature necessitate multiple procedures before the extraction process. The proposed method offers a broader linear range (0.57 to $10^4 \mu\text{g L}^{-1}$), satisfactory LOD ($\leq 0.407 \mu\text{g L}^{-1}$) and LOQ ($\leq 1.357 \mu\text{g L}^{-1}$) values, and comparable extraction recovery ($\geq 91.70\%$) for all chosen analytes, except for those obtained from the expensive LC-MS/MS methods [9,14,40], when compared to the referenced techniques. In general, most of the methods in the literature use volatile toxic organic solvents for extracting and eluting selected steroids from the urine and water samples. The developed method, however, uses HDE in the extraction process and limits the use of organic solvents during elution, making it more straightforward, cost-effective, and environmentally friendly. Furthermore, only 29 extraction tests were necessary to determine the optimal extraction conditions for the optimized HDE-SBME-HPLC-DAD method using CCD. Moreover, due to the similarity of most steroid hormones in their physical properties and the utilization of highly flexible HPLC-DAD for analysis, the proposed method can efficiently extract a range of steroid hormones, including the targeted ones.

Table 6. A Comparison with published extraction methods for simultaneous determination of the selected steroids in urine and water samples.

Method	Matrix	Steroid	Linear Range ($\mu\text{g L}^{-1}$)	LOD ($\mu\text{g L}^{-1}$)	Recovery (%)	Refs.
HDE-SBME-HPLC-DAD	Water	BES	0.89–10 ⁴	0.269	93.9–96.1	This work
		TES	1.12–10 ⁴	0.337	97.0–98.5	
		PRO	0.57–10 ⁴	0.171	95.4–98.0	
	Urine	BES	1.357–10 ⁴	0.407	91.9–94	
		TES	1.337–10 ⁴	0.401	93.5–95.4	
		PRO	0.929–10 ⁴	0.278	91.7–94.2	
^a PVOHD-GC-MS/MS	Water	BES	0.5	0.5–100	-	[40]
		BES	1	1–100	-	
		PRO	1	1–100	-	
^b AMED-MMF-SPME-HPLC-DAD	Water and urine	BES	0.10–200	0.027	78.1–115	[41]
		TES	0.50–200	0.085	77.3–113	
		PRO	0.50–200	0.12	77.0–116	
^c MISPE-HPLC-DAD	Water	BES	5 × 10 ³ –10 ⁵	39.6–76.1	>90	[42]
		TES	5 × 10 ³ –10 ⁵	24.0–89.8	>90	
		PRO	5 × 10 ³ –10 ⁵	59.4–77.7	>90	
^d MISPE-LC-DAD	Urine	BES	0.05–0.5	0.003	97–107	[43]
		TES	0.005–0.5	0.002	93–107	
		PRO	0.025–0.75	0.008	87–107	
^e UHPLC-DAD	Urine	BES	0.116–10.447	0.034	-	[44]
		TES	0.089–4.456	0.026	-	
		PRO	0.102–9.029	0.030	-	
^f LLE-UHPLC-MS/MS	Urine	BES	0.2–200	0.40	103	[9]
		TES	0.2–200	0.20	109	
		PRO	0.2–200	0.33	94	
^g LLE-LC-MS/MS	Serum and urine	BES	0.04	0.14–200	93.3–120	[14]
		TES	0.14	0.46–200	113–119	
		PRO	0.15	0.5–200	117–141	

^a Polyvinyl alcohol hydrogels disk-gas chromatography–tandem mass spectrometry. ^b Multiple monolithic fiber solid-phase microextraction high-performance liquid chromatography–diode array detection. ^c Molecularly imprinted solid-phase extraction–high performance liquid chromatography–diode array detection. ^d Molecularly imprinted solid-phase extraction–liquid chromatography–diode array detection. ^e Ultra high-performance liquid chromatography–diode array detection. ^f Liquid–liquid-extraction–ultra-high performance liquid chromatography/triple-quadrupole mass spectrometry. ^g Liquid–liquid extraction liquid chromatography–electrospray ionization–tandem mass spectrometry.

4. Conclusions

A list of HDEs that can be readily used as extraction solvents for SBME for the simultaneous determination of three selected steroid hormones in urine and aqueous environments was investigated. Among them, HDEs consisting of menthol and lauric acid at a ratio of 4:1 was selected and characterized as effective for pre-concentrating the target steroids based on the univariate method. PM3 estimates of the interaction energies show that both TES and PRO interact more strongly with 4:1 HDEs than BES in both binding modes. The effects of the pH solution were also examined using the univariate method, and pH 7 was found to be the optimum. Furthermore, a multivariate approach RSM based on CCD was employed to optimize the affected variables. By conducting only 29 extraction tests using CCD, the optimal extraction conditions for the chosen steroids were determined. The final optimized method, which involved using HND-SBME followed by HPLC-DAD, exhibited a linear response over a wide range and was found to be precise and accurate based on method validation. Furthermore, the method was successfully applied to both real water and urine samples. Overall, the results demonstrate that the proposed method

is simple, easy to use, reliable, and does not require toxic volatile organic solvents for the sample preparation procedure.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app14114438/s1>, Figure S1: The spectra (a) FT-IR and (b) ¹H NMR for HDE and its individual components, respectively; Table S1: Levels of factors used for the CCD experimental design; Table S2: The ANOVA data of the model obtained from CCD for EE% of BES; Table S3: The ANOVA data of the model obtained from CCD for EE% of TES; Table S4: The ANOVA data of the model obtained from CCD for EE% of PRO; Table S5: The effect of groundwater and tap water matrices on the analysis of selected steroids using HDE-SBME.

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