Regular Article

ANALYTICAL AND BIOANALYTICAL CHEMISTRY RESEARCH

Published by the Iranian Chemical Society

Anal. Bioanal. Chem. Res., Vol. 6, No. 2, 353-363, December 2019.

Determination of 17-β-Estradiol in Water Samples Using Salting-out Assisted Liquid-liquid Extraction Followed by HPLC and Experimental Design for Optimization

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(Received 10 October 2018 Accepted 10 March 2019)

The aim of the present study was to extract, preconcentrate, and determine 17-β-estradiol in water samples using a simple and efficient method. To this end, salting-out-assisted liquid-liquid extraction and high-performance liquid chromatography with ultra violet detection at 210 nm were performed. Water-miscible acetonitrile, as the extractant and acetonitrile phase separation under high-salt conditions were applied to treat the samples. The extraction efficiency and method sensitivity were carefully monitored by controlling the effective factors and the optimum conditions were: sodium chloride as the salting-out agent at concentration of 1.6 g, 2.40 ml of acetonitrile as extraction solvent, 5 ml of water sample, vortexing for 2 min and centrifuging at 4000 rpm for 5 min. A central composite design was applied to optimize the hydrolysis parameters. Using optimized experimental conditions, the calibration curve was found to be linear in the range of 1-120 μg l^{-1} in water sample and the correlation coefficient (R^2), the limit of detection, and limit of quantification were >0.99, 0.25 μg ml^{-1}, and 0.83 μg l^{-1}, respectively. The enrichment factor and extraction recoveries of the selected analyte ranged from 44.96-49.57 and 89.93-99.15%, respectively. Relative standard deviations were about 5.94%. High extraction efficiency and compatibility with HPLC analysis of 17-β-estradiol in water samples are the advantages of this method.

Keyword: Salting-out-assisted, liquid-liquid extraction, 17-β-Estradiol, Central composite design

INTRODUCTION

The ability of EDCs (Endocrine-disrupting chemicals) to imitate, stop, or disturb hormones in the body has raised concerns among researchers because these chemicals lead to adverse effects on reproductive processes in animals and in humans [1]. On account of their high estrogenic activities and presence in environmental waters, estrogens have been widely explored for public and scientific purposes as a group of endocrine disrupting chemicals. Through food chain, estrogens may enter the human body and affect the normal functioning of the body's endocrine systems. They may affect the metabolism of fats, minerals, proteins, and sugars in human body. They even lead to tumors like prostate and breast cancers [2]. Research shows that significantly biological effects have been brought about by even very low doses of estrogenic compounds both in vivo and *in vitro*. Besides, these compounds are able to influence hatching times and reduce hatchability; sexual determination and expression of secondary sex characteristics in various vertebrates could be affected by these compounds as well [3].

There are two types of estrogens in nature namely natural and synthetical estrogens including estrone (E1), 17-β-estradiol (E2), estriol (E3), and 17 a-ethynylestradiol (EE2). They exist ubiquitously in the ecosystems and their endocrine disrupting effects have alarmed researchers. These estrogen hormones exist in different concentrations in

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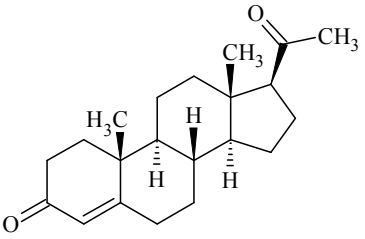


Fig. 1. Chemical structure of 17-β-estradiol.

the environment including municipal wastewater and animal manure as well as manure-applied field. Research shows that natural estrogens in the environment are mainly produced by animals and human. The most active and strong natural estrogen is E2, which is able to cause different estrogenic effects. For instance, as shown in Fig. 1, E2 casues detrimental effects on reproduction in rats at mRNA(messenger RNA) and protein level by decreasing the testosterone concentration and sperm quality. Thus, it is important to control hormone release from water samples [4].

Since it is not easy to remove hormones, it is necessary to find a way to remove these compounds before being released into surface waters to stop toxicity of the environment [5].

Conventional methods for the determination of estrogens are based on chromatography techniques, such as gas chromatography(GC) [6], high-performance liquid chromatography (HPLC) [7], and high-performance liquid chromatograph/mass spectrometry/ mass spectrometry (LC/MS/MS) [8]. HPLC-UV (high performance liquid chromatography-ultraviolet) is favored greatly because it is simple, cheap, and useful with several applications [9].

To date, many attempts have been made to separate and preconcentrate different types of drug abuse from different sample matrices using several methods including liquid-liquid extraction (LLE) [10], solid-phase extraction (SPE) [11], solid-phase microextraction (SPME) [12], liquid-phase microextraction (LPME) [13], stir-bar sorptive extraction (SBSE) [14], and dispersive liquid-liquid microextraction (DLLME) [15]. SBSE is not potentially cheap and it takes considerable time. Hence, extraction techniques; *i.e*., SPE (Solid Phase Extraction) and LLE (Liquid-Liquid Extraction)) have been gradually replaced by LPME (Liquid-Phase Microextraction) and SPME (Solid-Phase Microextraction) determination techniques. The solid-phase microextraction (SPME) is a solvent-free sample preparation technique for the simultaneous extraction of analytes from aqueous samples or the fiber-based sample headspace; however, it is pretty costly with a fragile fiber and a problematic sample carryover. As a widely-used novel sample preparation technique, the LPME uses several microliters (μl) of a water immiscible solvent as an acceptor phase for the target analytes and usually an aqueous solution as a donor phase [16].

Leong *et al.* and Rezaee et al. have explored the use of dispersive liquid-liquid microextraction (DLLME) technique to effectively determine the exposure of various human urine samples and environmental aqueous samples for different pollutants. A dispersant should be added to the extractant for the enrichment and extraction of the target analytes, leading to an increase in the interface between two immiscible solvents (liquids). The given process (*i.e.*, the addition of a dispersant), however, has two disadvantages: 1) it generally leads to a higher solvent consumption, and 2) it generally leads to a lower partitioning of especially quite polar analytes into the extractant solvent [17].

In the LLE (Liquid-Liquid Extraction) technique, organic solutions being immiscible with water are used to extract polar compounds having a rather weak function owing to their low dielectric constants. The associated compounds can be dissolved in most polar organic solvents such as acetone, acetonitrile, ethanol, and methanol; however, they cannot be applied to the traditional LLE technique because they are miscible with water [18].

Due to drawbacks of the above-mentioned procedures, salt out assisted liquid-liquid extraction (SALLE) has been introduced recently. This new extraction technique has been

used to detect various target analytes from water, food and biological matrices. Combining sample clean-up and preconcentation in a single step is one of the advantages of SALLE. Contrary to LLE, analsis in SALLE is not time-consuming. Compared to DLLME, salting-out-assisted liquid-liquid extraction (SALLE) has been employed to extract polar analytes and avoid the toxic halogenated solvents that are typically used as the extractants. Analytes are extracted from a combination of aqueous sample phase and water miscible organic solvent at high salt concentration in SALLE. Furthermore, such polar organic solvents are clearly water-miscible in all proportions. However, the addition of salts can decrease the reciprocal miscibility, and can even lead to phase separation. Thus, salt can help the polar analytes existing in the hydrous phase to be able to optionally carry into the polar organic phase. This process is named salting-out helped liquid/liquid drawing out (SALLE). This new technique is simple, sensitive, and uses less extraction solvents. The compatibility of the excrtact with the subsequent analysis by HPLC is another advantage of SALLE method. The extraction device was a glass centrifugation tube in this study [9-19].

In this paper, a simple and quick technique has been introduced to detect 17-β-estradiol in water samples. The samples are prepared directly using the SALLE technique to be analyzed by performing the high-performance liquid chromatography with UV detection (HPLC-UV) method. The liquid extraction is achieved by the salting-out effect in this technique; in addition, the extraction of molecular species to the organic phase is boosted after adding salt. Research confirms the efficiency of this mthod to determine 17-β-estradiol; 17-β-estradiol determination in water samples are perfomed fast and reliably in this technique.

EXPERIMENTAL

Instrumentation

The HPLC instrument (KNAUER, Germany) equipped with D-7000 interface, K-1000 model quarternary pump, L-2500 UV-Vis detector and a manual injector (20 µl) was used for 17-β-estradiol determination. The separation was performed on C18 column, 150 × 4.6 mm, (Foster city, USA). Ultrasonic water bath was used for degassing the mobile phase.

Reagents

HPLC-grade methanol (MeOH) (99.8%), acetonitrile (MeCN) (99.8%), and sodium hydroxide (NaOH) were purchased from Merk (Darmstadt, Germany). Sodium chloride (NaCl) was purchased from Welch Co. (Shanghai, China). Standard of 17-β-estradiol (99.6%) was purchased from Sigma-Aldrich. 100 mg l^{-1} of 17-β-Estradiol stock solution was prepared by dissolving an appropriate amount of the drug in methanol. All the stock solutions were stored in dark at -4 °C.

Chromatographic Separation of E2

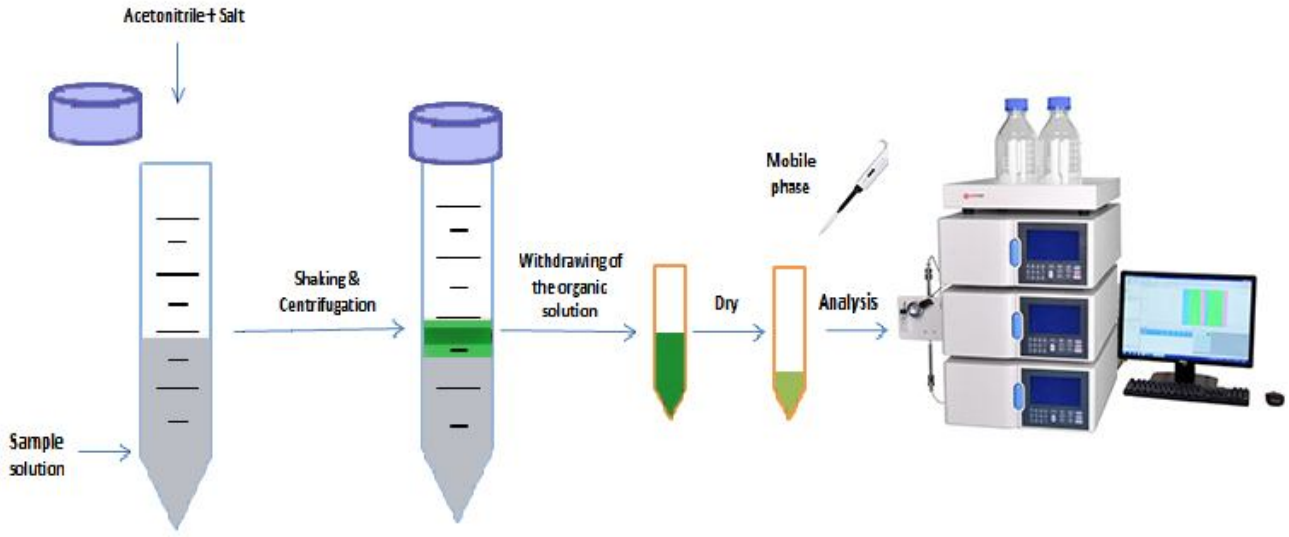
The mobile phase consisting of water and acetonitrile (30:70, v/v) at a flow rate of 1.0 ml min^{-1} was applied as a mobile phase in the isocratic elution mode. The injection volume was 20 µl for all the samples and the detection was performed at 210 nm. The prepared target analyte was separated using these chromatographic conditions.

SALLE Procedure for E2 Extraction

5 ml of the sample solution was spiked with standard solution containing the target analytes and transferred to a 15 ml screw capped test tube. The pH value of the solution was adjusted to 7.4 by adding appropriate amounts of 0.1 M NaOH, then 2.40 ml acetonitrile and 1.6 g NaCl were added. Afterwards, the solution was shaken gently for 2 min; next, it was centrifuged at 4000 rpm for 5 min leading to phase separation. Then, using 1 ml micro-syringe and quantitatively, the upper organic phase was carefully withdrawn. This volume was about 100 ± 25 µl, which poured into a vial to avoid anomalous peak in HPLC chromatogram, nitrogen stream was blown to dry it at room temperature in this stage. The final residue was reconstituted up to 100 µl through mobile phase, shaken for 2 min and filtered with a 0.2 µm nylon filter and was injected to the HPLC system. Schematic illustration of the SALLE procedure is represented in Scheme 1.

Experimental Design

In order to diminish the effects of uncontrolled factors, the optimization experiments were performed randomly. Since performing the experiments within a single day was



Scheme 1. Experiment setup employed for analysis of 17-β–Estradiol drug from water samples using salting-out-assisted liquid-liquid extraction procedure followed by HPLC technique

Table 1. The Factors Included in the Central Composite Design and their Corresponding Level

Abbreviation	Parameter	Factors' levels	
		Low	High
NaCl	Amount of salt	0.5	3
ACN	Solvent volume	1	4
pH	pH	1	10

not feasible, they were separated into two parts and each part was conducted in two sequential days to remove any variations caused by changes occurring over these intervals. According to the literature and based on the preliminary experiments, the most influential parameters affecting on the performance of SALLE process selected were amount of salt (A), extraction solvent volume (B), and pH (C). High and low set-points were chosen to obtain an orthogonal design for each variable. A CCD consisting of 20 treatments for 3 factors in 2 levels and 6 center points was utilized to achieve the best response by optimizing the values of the factors. In the CCD, random experiments were conducted to minimize the effects of uncontrolled variables and the respective design matrix is shown in Table 1.

The average extraction recovery (ER) was considered as the "experimental response" to evaluate the method performance, which was computed by Eq. (1):

$$ER = (C_{sed} \times V_{sed})/(C_0 \times V_{aq}) \times 100 \quad (1)$$

where C_{sed} is the analyte concentration in the sedimented phase, C_0 is the analyte initial concentration of the sample solution, and V_{sed} and V_{aq} are the volumes of sedimented and sample solutions, respectively.

For the assessment of the extraction efficiency, the peak area was applied as the HPLC response. To predict the dependent variable, a model of quadratic polynomials was

obtained as displayed by Eq. (2):

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n} b_{ij} x_i x_j \quad (2)$$

where Y and xi are the dependent and independent variables, b_0 represents the constant coefficient, and bi, bij, and bii indicate the coefficients of linear, interaction coefficient between independent variables and squared effects, respectively [19]. Table 1 depicts the abbreviations and levels of the factors included in design.

RESULTS AND DISCUSSION

Type of Salting-out Agent

In the salt-mediated LLE technique, salts and different salt concentrations would lead to different levels of phase separation. Addition of salt decreased the levels of aqueous-phase-based hydrophilic compounds solubility which was mediated by the salting-out effect, leading to an increase in the partitioning of analytes into the organic phase. Three different kinds of salts ($(NH_4)_2SO_4$, $Mg_3(PO_4)_2$ and NaCl, 1.6 g each) were considered as salting-out agents, whereas other experimental variables aforementioned remained unchanged (constant). The phase separation occurred for all the cases, albeit with different degrees. The results showed that the maximum peak area is achieved after adding the sodium chloride that is revealed in the HPLC-UV chromatogram as depicted in Fig. 2. Compared to other two salts, NaCl displayed sufficient phase separation between aqueous/acetonitrile (*i.e.*, ACN) and the largest efficiency of 17-β-estradiol. According to the present results, $Mg_3(PO_4)_2$ has a lower solubility compared to NaCl. Besides, $(NH_4)_2SO_4$ was not used since it made a large part of organic sedimented phase instead of a small volume of organic phase, which contributes to reduce enrichment factor. Given the higher solubility of NaCl in aqueous solutions as well as a strong salting-out capability (*i.e.*, powerful ability to salt out), it was selected for further experiments. This trend corresponds to the trend reported for salting-out Liquid-Liquid Extraction [20].

Optimization by CCD

Several parameters that may influence the SALLE performance, including extraction amount of salt, solvent volume, and sample pH should be investigated in order to obtain the maximum extraction efficiency (20 runs in total). To model the extraction efficiency of E2 obtained from the aqueous sample using salting-out-assisted liquid-liquid extraction (SALLE), a regression equation with input-coded variables was constructed and presented in Eq. (3) as follows:

$$\begin{aligned} Res = {} & -137349 + 103783[NaCl] + 91412[ACN] + \\ & 18832pH - 32131[NaCl]^2 - 18022[ACN]^2 - \\ & 1502.3[pH]^2 - 4971[NaCl]*[ACN] + \\ & 1599[NaCl]*[pH] + 340[ACN]*pH \end{aligned} \quad (3)$$

The CCD could explain the effects of the interaction with its quadratic variables, as well as the linear impacts of the factors on the response. To evaluate each interaction factor and term, analysis of variance (ANOVA) was employed in this investigation (Table 2).

All experiments were performed randomly. The percentage of NaCl in the range 0.5-3 g, the volume of ACN in the range of 1-4 ml and the sample pH in the range of 1-10 was evaluated. The lack-of-fit test showed a P-value of 0.298%, and the determination coefficient (R^2) was 99.7%, so a satisfactory fit was obtained between the experimental data and the predicted model. The surface responses of this optimization are shown in Figs. 3a, b and c. These figures showed the interaction between two interacting factors when other factors kept constant using the constructed models by Minitab Trial software Version 16 (Minitab, Inc.).

Hydration spheres are normally built up by water molecules around the salt ions in SALLE resulting in separation of organic phase which is rich in analytes. In order to separate two phases distinctly, the salt must be added adequately; however, it should be pointed out that extra salt, beyond saturation, causes analytes to be adsorbed on the solid phase.

NaCl was used as a phase separation reagent. We tested the salt concentrations of 0.5 to 3 g salt (NaCl) to obtain phase separation. Figure 3a shows that the analyte concentration increased as the salt concentration increased up to 1.6 g. Although, the solubility of the target analytes in

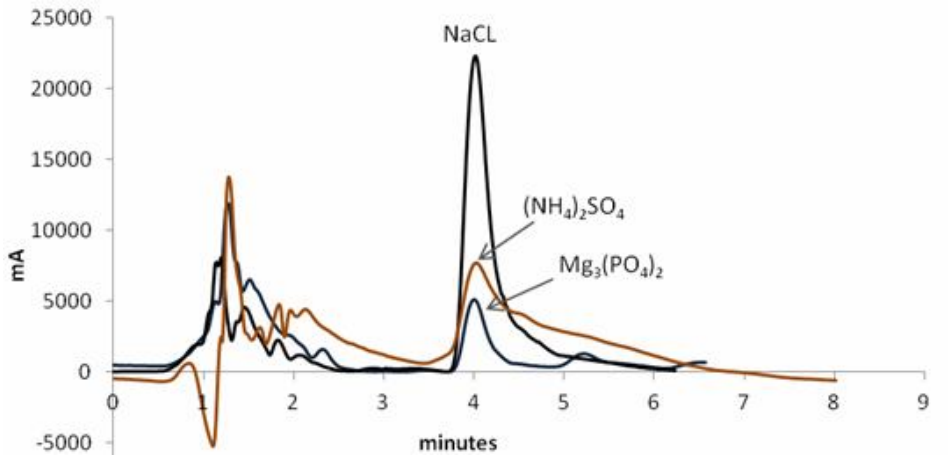


Fig. 2. Effect of salt type on the extraction of 17-β-Estradiol from aqueous sample using HPLC-UV chromatogram at λ = 210 nm; solvent: acetonitrile, salt amounts = 1.6 g.

Table 2. Analysis of Variance (ANOVA) for Response Surface Quadratic Model

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	19090958935	2121217659	1921.45	0.000
NaCl	1	124587589	124587589	112.85	0.000
ACN	1	229116980	229116980	207.54	0.000
pH	1	3473411213	3473411213	3146.30	0.000
Square	3	14980959017	4993653006	4523.37	0.000
NaCl*NaCl	1	6612180827	6612180827	5989.47	0.000
ACN*ACN	1	3062944908	3062944908	2774.49	0.000
pH*pH	1	1724053013	1724053013	1561.69	0.000
2-Way interactio	3	390150223	130050074	117.80	0.000
NaCl*ACN	1	197686728	197686728	179.07	0.000
NaCl*pH	1	184128050	184128050	166.79	0.000
ACN*pH	1	8335444	8335444	7.55	0.023
Error	9	9935706	1103967		
Lack-of-fit	4	5632336	1408084	1.64	0.298
Pure error	5	4303370	860674		
Total	18	19100894641			

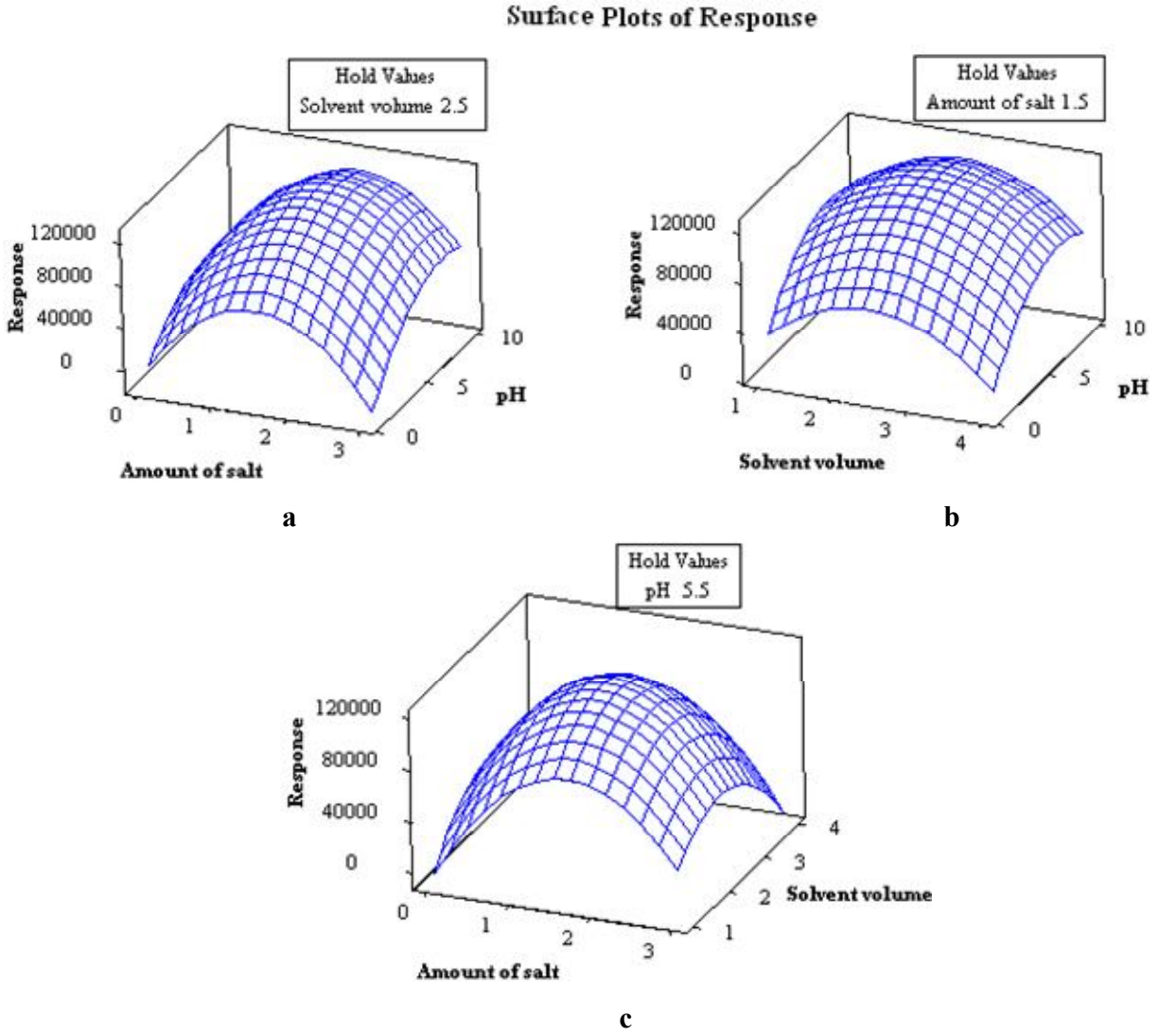


Fig. 3. Surface plots of response of predicted 17-β-Estradiol peak area as a function of (a) amount of NaCl content *versus* pH, (b) solvent volume of ACN content *versus* pH, and (c) amount of NaCl content *versus* solvent volume of ACN content, while other variables at the central point levels remain constant.

the sample solution decreased after adding salt, increasing NaCl concentration more than 1.6 g reduced the extraction efficiency to some degree. It is probably related to increase the viscosity of aqueous phase which reduces the mass transfer of the analyte from aqueous to organic phase. Thus, 1.6 g of NaCl was used in the subsequent studies.

Another important factor worth considering is the volume of extraction solvent in the SALLE technique. Therefore, some steps were taken to test various volumes of ACN ranging from 1-4 ml. As shown in Fig. 3b, changing the volume of ACN from 1-2.40 ml led to an increase of E2 recoveries. Due to indistinct interface between the acetonitrile and the aqueous phase, collecting the organic layer was not feasible at the volume lower than 2.40 ml. Moreover, with volumes above 2.40 ml, dilution of target analyte can be occurred leading to a decrease of its peak area. Based on the experimental results, 2.40 ml acetonitrile was selected as the optimum volume in all the subsequent

experiments.

Since, the performance of extraction is potentially influenced by the aqueous solution pH in SALLE, some steps were taken to test different pH of aqueous solution. From a structural standpoint, 17-β-estradiol has phenolic hydroxyl already formed in the aqueous solution may be influenced by the pH value. So, the efficacy of solution pH on extraction efficiency of 17-β-estradiol was investigated by salting-out through liquid/liquid extraction within the pH range of 1.0-10.0 and the results are displayed in Fig. 3a and 3b. It can be seen that pH had an obvious effect on the extraction of 17-β-estradiol in the range of 1.0-9.0, however, the extraction efficiency slightly decreased when pH value was above 9.0. The pKa = 10.27 value of the 17-β-estradiol would justify the aforementioned results. In the pH range of 9.0, the 17-β-estradiol was chiefly formed as ions, leading to an increase in their level of solubility in the aqueous solution and to a decrease in their extraction efficiency accordingly. Figure 3 shows the desired results. On the basis of the results, the enrichment factor of 17-β-estradiol increased with enhancing pH up to pH 7.4 and then slowly decreased up to pH of 10.0. For SALLE-mediated extraction of 17-β-estradiol, pH 7.4 was chosen for the next experiments. The same results can be derived of contour plots. Maxima are best found from the contour plots as represented in Fig. 4. The selection of optimum conditions was possible from the RMS plots which showed that the maximum recovery of 17-β-estradiol will be obtained for a 2.40 ml of acetonitrile as an extractive solvent volume, 1.6 g of NaCl and pH at about 7.4.

Analytical Performances

The established analytical method was applied to analyze the 17-β-estradiol in water samples. Figure 5 shows the full baseline separation of 17-β-estradiol in 4.1 min chromatographic running time. The dependence of the chromatographic signal on concentration of analyte was verified under the optimum conditions of SALLE and HPLC-UV chromatogram. There were no appreciable interference peaks in the HPLC chromatogram of the water sample. The results clearly suggested that HPLC technique in combination with SALLE was highly selective for the analysis of 17-β-estradiol in water samples. A linear calibration graph was obtained over the range of 1-120 μg l^{-1} of analytes by the proposed extraction method. The correlation coefficient was 0.997 for 13 concentration levels analyzed over the regression range indicating the linearity of the method. The limit of detection based on a signal-to-noise ratio (S/N) of 3 was 0.25 μg l^{-1}, also the limit of quantification, 0.83 μg l^{-1} was obtained on S/N = 10, for E2 [20,21].

Comparing SALLE with other Methods

In order to test the efficiency of the present technique for 17-β-estradiol determination, a comparison was done between the linear range, LOD, RSD, EF and extraction time obtained by SALLE with other reported methods. As shown in Table 3, the proposed method and the other methods are similar in terms of RSDs; sometimes, RSDs of the proposed method are even better. The proposed method outperforms other methods in terms of the LODs, the linear ranges and analysis time. Based on these results, it could be concluded that the proposed SALLE method is a sensitive, repeatable and simple technique. Furthermore, the selected drug in different samples can be preconcentrated and determined successfully by this technique.

Application for Analysis of Real Samples

Since water quality has been a major focus of social and environmental concerns, and manufacturers have become more aware of their product life-cycle and packaging, the environmental effects of EDCs obtained from manufactured substances will be the main subject of the future studies. Understanding how much and which biologically active compounds existing in products or bodies of water is not only important to environmentalists and scientists, but also to geneticists (a biologist studying genetics), governments, pediatricians (a medical practitioner specializing in children and their diseases), and the public at large.

To demonstrate the capabilities of the developed technique for the determination of 17-β-estradiol in real samples determination of thec target analyte in spiked water samples, prepared according to section 2.4, was carried out. Determinations of 17-β-estradiol in different water samples were assessed whose results are shown in Table 4. The accuracy of the method was evaluated by a recovery test carried out with 17-β-estradiol spiked water samples. Recovery and enrichment factor ranged from 89.93-99.15%

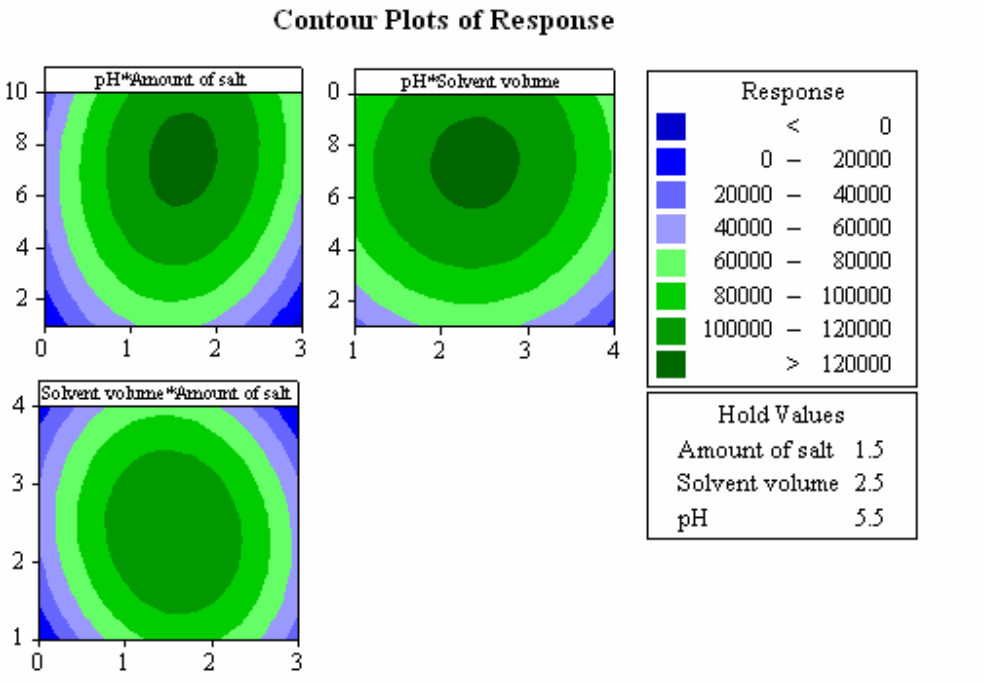


Fig. 4. Contour plots of recovery response of 17-β-Estradiol. The area of the highest acceptance is slightly located on the right upper hand of the plots.

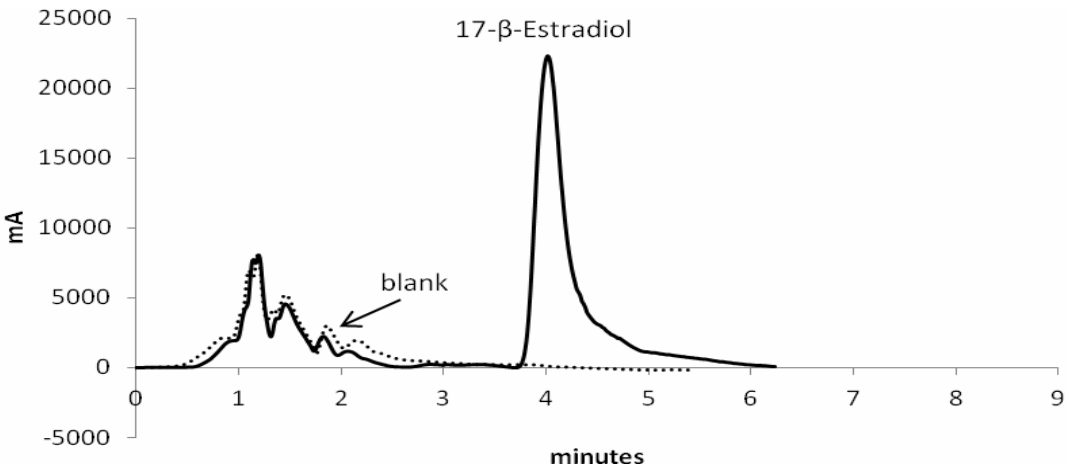


Fig. 5. HPLC chromatogram of 17-β-estradiol in spiked water sample (spiked 17-β-estradiol = 20 μg l^{-1}, NaCl = 1.6 g, pH = 7.4 and volume of acetonitrile = 2.40 ml).

and 44.96-49.57 for the 17-β-estradiol determination respectively in three water samples. Figure 5 shows the chromatograms obtained for city water and wastewater samples.

CONCLUTIONS

The efficiency of the enhanced SALLE method was

Table 3. Comparison of some Characteristics of Proposed Method with other Methods Reported for Determination of 17-β-Estradiol

Instrument	Extraction method	Linear range	LOD	RSD (%)	Determination in real samples	Recoveries (%)	Ref.
HPLC	DLLME	5-1000 ($\mu g\ l^{-1}$)	0.8 ($\mu g\ l^{-1}$)	8	Water	116	[22]
GC-MS/MS	HF-LPME	1.25-50 ($\mu g\ l^{-1}$)	0.17 ($\mu g\ l^{-1}$)	13	Milk	117	[23]
GC-MS	SPE	2.5-250 ($ug\ Kg^{-1}$)	3 ($ug\ kg^{-1}$)	22.7	Feed	76.34	[24]
HPLC	SPME	10-1000 ($\mu g\ l^{-1}$)	0.21 ($\mu g\ l^{-1}$)	7.9	Water	88.5	[25]
LC-MS-MS	LLE	5-600 ($pg\ ml^{-1}$)	-	2.5	Serum	102.8	[26]
Electrochemical	MIP	0.05-10 (μM)	0.02 (μM)	6.5	River water	97	[27]
HPLC	SBSE	1-2500 ($\mu g\ l^{-1}$)	0.28 ($\mu g\ l^{-1}$)	4.5	Water	77	[28]
HPLC	SPE	50-1000 ($ng\ l^{-1}$)	50 ($\mu g\ kg^{-1}$)	11	River water	86	[29]
Presented method	SALLE	1-120 ($\mu g\ l^{-1}$)	0.25 ($\mu g\ l^{-1}$)	5.94	Water	94.54	This work

Table 4. Recoveries and Concentrations of 17-β-Estradiol in Real Water Samples (n = 3)

Sample	Concentration ($\mu g\ l^{-1}$)	Added ($\mu g\ l^{-1}$)	Recovery (%)	Enrichment	RSD (%)
Wastewater	29.89	10	95.73	44.96	6.52
City water	4.71	10	89.93	48.91	6.11
Mineral water	Below detection limit	10	99.15	49.57	5.20

studied by selecting 17-β-estradiol. In addition to a higher enrichment factor, the technique could be used in complex matrices, such as milk, and wastewater samples, without any pretreatment or dilution. In comparison with other methods, the proposed method was simple and convenient; besides, it had a higher preconcentration factor, and its analysis took less time. The limit of detection and limit of quantification were 0.25 $\mu g\ l^{-1}$ and 0.83 $\mu g\ l^{-1}$, respectively. This study shows interesting perspectives for the application of SALLE for the monitoring of 17-β-estradiol in real samples.

ACKNOWLEDGEMENTS

Authors appreciatively acknowledge the financial support of the University of Lorestan.

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