Iranian Journal of Analytical Chemistry
Volume 11, Issue 2, September 2024 (173-181) Original Research Article
مجله ایرانی شیمی تجزیه
دوره ۱۱، شماره ۲، مهرماه (۱۸۱-۱۷۳)

Extraction and Quantification of 17-β-Estradiol in Wastewater Samples via Coacervative Phase Formation with β-Cyclodextrin and High-Performance Liquid Chromatography

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Received: 18 August 2024 **Accepted:** 19 September 2024
DOI: 10.30473/IJAC.2025.74335.1318

Abstract
A simple, rapid, and cost-effective method for the determination of 17-β-estradiol in water samples was developed. The method is based on the extraction of the 17-β-estradiol–β-cyclodextrin complex using a coacervate phase composed of reverse micelles of decanoic acid, followed by high-performance liquid chromatography with ultraviolet detection for quantification. The effects of key parameters, including decanoic acid concentration (50–200 mg in a 30 mL total volume), tetrahydrofuran concentration (1–15% v/v), β-cyclodextrin to 17-β-estradiol molar ratio (1:1–1:4), ionic strength (0–1 M) NaCl, pH (1–4), and extraction time (0–30 min), on recoveries and enrichment factors were studied and optimized. The optimal extraction conditions involved stirring a 20 mL water sample containing 50 mg of decanoic acid with 3 mL of THF, using a 1:1 molar ratio of 17-β-estradiol to β-cyclodextrin complex, for 10 minutes, followed by centrifugation, 10 min at 4000 rpm. Recoveries and enrichment factors of 17-β-estradiol was primarily influenced by the decanoic acid and THF concentrations that form the coacervate phase but remained independent of the ionic strength of the sample solution. The recovery rate, enrichment factor, limit of detection, and relative standard deviation for 17-β-estradiol were 95%, 284, 0.19 µg/L, and 4.34%, respectively. This method was applied to analyze 17-β-estradiol in city water, mineral water, and pastewater samples. No 17-β-estradiol was detected in mineral water, while its concentration in city water and pastewater was found to be 1.9 µg/L and 33.67 µg/L, respectively.

Keywords
17-β-Estradiol; β-Cyclodextrin; Micellar Phase; Coacervative Extraction; Enrichment Factor.

1. INTRODUCTION

Recently, endocrine disrupting chemicals (EDCs) are considered as one of the major classes of environmental contaminants which may interfere with the function of the endocrine system. Indeed, EDCs have gained more attention by the researchers in the field of environmental sciences because of their release in the environment without treatment [1]. These estrogen hormones have been widely detected at varying concentrations from various sites in the environment, i.e., municipal wastewater and animal manure as well as manure-applied field. It is believed the natural estrogens produced and excreted by animals and human constitute the primary source of estrogens present in the environment [2].

Among EDCs, 17-β-estradiol (Fig. 1) is a naturally occurring steroidal estrogen [3]. It can cause many effects in the endocrine system of humans and wild animals by interfering with the normal physiological processes and may create many deleterious effects [4]. Due to the high activity of 17-β-estradiol, even at low concentration (<ng/L) it can be toxic, carcinogenic and can disturb the function of male reproductive system also induce an abnormality of growth [5]. The main routes of excretion are via urine and feces, which are the main sources of these compounds in wastewaters [6].

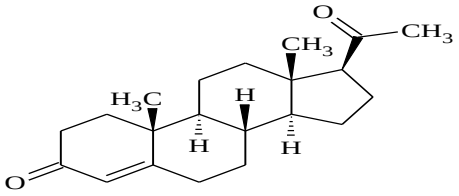

Fig. 1. Chemical Structure of 17-β-Estradiol.

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Animal manures generally contain fiber, nitrogen, phosphorus, potassium, protein, amino acid, and fat, and are thus often land applied as fertilizers. This increases the possibility of estrogen entrance into the terrestrial and aquatic environments. It is found that E2, at concentration as low as 0.1 ng/L, can be highly bioactive, whereas the typical concentrations detected in the environment are at ng/L or ng/g levels [7-8]. Freshwater ecosystems, particularly those exposed to EDCs, may experience sexual alterations in fish (e.g., feminization), reproductive abnormalities, and developmental toxicity. These effects are especially pronounced in ecosystems receiving high levels of untreated wastewater treatment plant (WWTP) effluents. Given that estrogen contamination cannot be fully eliminated, various treatment processes in sewage treatment plants (STPs) have been optimized and studied to determine the fate and distribution of steroid hormones and their conjugates in the environment [9-10]. Therefore, the development of a sensitive method for the analysis and detection of this compound in environmental samples is of paramount importance. Various methods for the quantification of 17-β-estradiol at low concentration levels were reported in literature such as high performance liquidchromatography (HPLC) [11-12], Gas Chromatography combined with Mass Spectrometry (GC/MS) [13], LC-MS/MS [14], Capillary Electrophoresis [15], spectrophotometry-UV [16] and electrochemical [17], molecularly imprinted polymer [18]. But these methods are time consuming because of multistage operation, harmful for human and environment due to consumption of large amounts of toxic organic solvents, low preconcentration factors, expensive and require a complicated instrument. One approach to improve the solubility of a poorly soluble compound can be by the use of a solubility enhancer such as cyclodextrins (CD) (Fig. 2). CDs are oligosaccharides with a hydrophobic central cavity and a hydrophilic external surface. If a molecule's dipole moment and size correspond to the central cavity of the CD, an inclusion complex can be formed which increases the total solubility of the binding molecule [19].

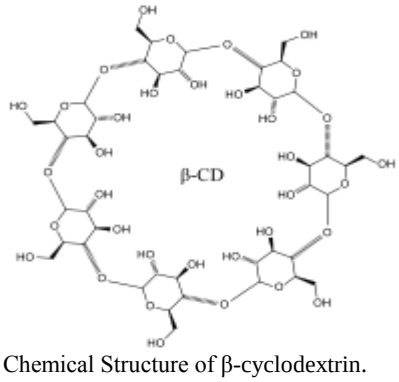

Fig. 2. Chemical Structure of β-cyclodextrin.

The use of CDs-guest supramolecular complexes will add new dimensions to the analysis to provide greater selectivity. The purpose of the present study is to combine these advantages and to develop a simple, economical, selective, and sensitive method of determining small quantities of 17-β-estradiol. So, we have embarked on the investigations of the supramolecular interaction between a new β-cyclodextrin derivatives—βCD and 17-β-estradiol [20].

Coacervative extraction (CAE) was introduced by Giokas et.al in 2005 is a fast, cheap and safe sample preparation method based on phase separation occure between an aqeous phase and supramolecullar assemblies called coacervates [21]. It has also been used for wastewater treatment and protein purification. Their application to analytical extraction processes has invariably involved the use of coacervates made up of supramolecular assemblies [22-23]. Coacervates have intrinsically useful properties which have attracted enormous attention from academic and industrial researchers alike. They have excellent solvation properties for a lot of organic and inorganic substrates [24]. This paper describes, the coacervation processes produced in ternary systems made up of alkyl carboxylic acids (chain length C8-C18), tetrahydrofuran, and water and explores their capability for the extraction of organic compounds prior to liquid chromatography.

Application of the coacervation phenomenon to analytical extraction processes has invariably involved the use of dehydrating agent-induced coacervates made up of aqueous micelles. Nonionic surfactant micelle-based coacervates, induced by temperature changes, have been by far the most used ones. This approach, initially reported by Watanabe et al [25]. Moreover, lipid-core micelles have a relatively low critical micelle concentration (CMC) and are more stable than conventional low- molecular- weight surfactants [26]. However, this technique is based on the coacervate extraction have gained more interest recently as tools for 17-β-estradiol determination due to the various advantages that they offer such as low cost, fast response, simple and short analysis time. Having in mind the risks that steroid estrogens pose to humans and wildlife, the monitorization of these chemicals in the environment is of crucial importance and claim for development of valid and robust analytical methods. Therefore, the main purpose of the present paper is to provide a simple and universal analytical method for determination 17-β-estradiol in types of real samples. We discuss chromatographic conditions, detection and sample preparation and their influence on separation efficiency, selectivity, and method sensitivity. Our

DOI: 10.30473/IJAC.2025.74335.1318

aim was to find a method with the best characteristics (i.e. fast, selective and with the high sensitivity).

2. EXPERIMENTAL

2.1. Instrumentation and materials

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 Xterra ODSH-Optimal, 150×4.6 mm, 5 µm (Waters, Ireland). Ultrasonic water bath was for degassing of the mobile phase.

Reagents and reference samples

HPLC grade tetrahydrofuran (THF), methanol (MeOH), acetonitrile (ACN), ethanol (EtOH), were purchased from Merk. Capric (decanoic) acid ≥ 99 % from Alfa Aesar. Standard of 17-β-estradiol was purchased from Sigma–Aldrich. The 15 mg Lit^{-1} stock solution of 17-β-estradiol was prepared by dissolving appropriate amount of the drug in methanol.

2.2. Chromatography conditions

The chromatographic measurements were carried out with HPLC system equipped D-7000 interface, K-1000 model quarternary pump, L-2500 UV–Vis detector and a manual injector (20 µl) set at 220 nm, Separation was done by an isocratic elution on a Shim-Pack CLC-Optimal analytical column (150 • 4.6 mm, 5 lm) from Shimadzu (Kyoto, Japan). Mobile phase was a mixture of acetonitrile, water, ethanol and β-cyclodextrin (65:35, v/v) with flow rate of 1.0 ml min^{-1}. A Hettich Rotanta centrifuge (Tuttlingen, Germany) was used for sample preparation. Adjustment of pH was made by model 3030 Jenway pH meter (Leeds, UK). Handmade centrifuge tubes which were specially designed for easing of withdrawing coacervate phase after measuring its volume, were used for extraction.

2.3. Extraction procedure

For the coacervate extraction (CAE), aliquots of 20.0 mL of the sample or standard solution containing the17-β-estradiol (15 µg L^{-1}) were placed into the glass centrifuge tube. The pH of the solution was adjusted to pH 2.0 via addition of hydrochloric acid (HCL), 5 mL of 725 µg L^{-1} β-cyclodextrin solution and 1mL of 1mol L^{-1} sodium chloride (NaCl) were added into the sample solution. Further, a binary solution containing 3 mL of tetrahydrofuran and 50mg of decanoic acid was injected into the sample solution using a syringe. Then, the mixture was gently shaken. After shaking, the solution became turbid and water immiscible coacervate made up of decanoic acid reverse micelles was formed. The extraction was accomplished in 10 min under shaking. Next, the mixture was centrifuged at 4000 rpm for 10 min to accelerate the separation of the coacervate from the bulk solution. The aqueous phase was then separated completely by a syringe. Later, the coacervate phase was dried under nitrogen flow and then added 70µl mobile phase. Eventually, aliquots of the coacervate phase were withdrawn using a microsyringe and directly injected into the HPLC-UV system for analysis.

3. RESULTS AND DISCUSSION

3.1. Optimization of the coacervate extraction

Effects of experimental parameters including concentration of THF (1-15% v/v) and capric acid (50-200 mg in 30 ml total volume), molar ratios of 17-β-estradiol /βCD (1:0.5- 1:4), pH (1-4), ionic strength (0-1 M NaCl), and extraction time (0-30 min) on recoveries (Rs) and enrichment factors (EFs) were evaluated. All the extractions were carried out according to extraction procedure section using aqueous standard solution containing 15 µgL^{-1} of 17-β-estradiol. The percent recovery can be expressed by the following equation;

$$R(\%) = \frac{D\times 100}{D+Vaq/Vc} \quad (1)$$

where D is distribution coefficient and Vaq and Vc are the volumes of aqueous solution and coacervate phase obtained after the extraction step, respectively. Enrichment factor (EF), defined as the ratio of analyte concentration in coacervate to original sample was used as a criterion for the selection of the experimental conditions as follows:

$$EF = \frac{R(\%)Vo}{100Vc} \quad (2)$$

where V_o is the volume of original aqueous solution prior to the extraction step.

3.2. Effect of THF and decanoic acid concentration

Concentration of both decanoic acid and THF as the main components making up the coacervate was found to be a highly influential parameter on recoveries and enrichment factors. For all the 17-β-estradiol good recovery were obtained at capric acid amount 50 mg in 30 ml of the total volume (Fig. 3). Since the volume of coacervate increased with capric acid concentration, a 50 mg of capric acid was selected as the optimal condition for further experiments.

The influence of THF concentration (1-15% v/v) on recovery of 17-β-estradiol shows that the maximum recovery was obtained at 10% of THF for 17-β-estradiol and was selected as the optimal condition for the extraction of the compounds from water samples. Dissolution of a portion of coacervate phase in the THF/water bulk solution occurs at high THF percentages. So the lower recovery at percentages higher than 10% THF are probably due to a change in the composition of coacervate.

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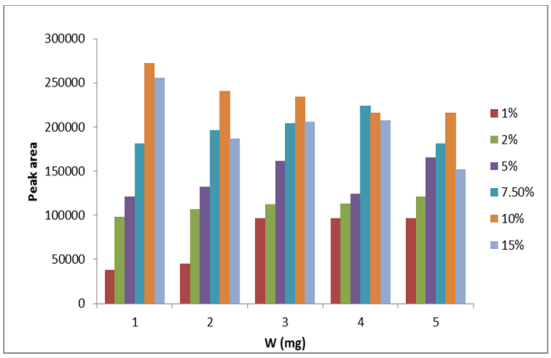

Fig. 3. Effect of capric acid and THF concentration on recovery of 17-β-estradiol, THF = 10%; pH = 2; extraction time: 30 min

3.3. Effect of β-CD concentration

The effect of different molar ratios of 17-β-estradiol /βCD (1:0.5, 1:1, 1:2, 1:3 and 1:4) were investigated through adjusting the amount of 17-β-estradiol by fixing 7.3 × 10-10 molar of 17-β-estradiol. The results indicated that the maximum extraction could reach at molar ratio of 1:3 (17-β-estradiol /βCD) (Fig 4). Thus, further optimization was carried out with molar ratio 1:3 17-β-estradiol /βCD complex.

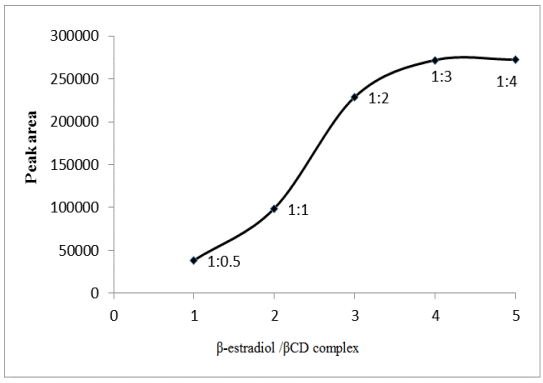

Fig. 4. Effect of βCD concentration on the 17-β-estradiol extraction, Conditions: 50 mg capric acid, THF = 10%; pH = 2; extraction time: 30 min, (the points in this figure have the ratios of 17-β-estradiol /βCD).

3.4. Effect of pH

Coacervation process occurs only in solutions containing protonated capric acid molecules (pKa = 4.8 ± 0.2). Thus the effect of pH was examined by varying pH between 1 and 4. Recovery of 17-β-estradiol obtained were affected at pH range of 1-4 (Fig 5). When the pH value is adjusted in lower ranges, extraction of 17-β-estradiol compound increases. This is due to the formation of 17-β-estradiol /βCD-complex and the extraction of these compounds into the micellar phase. Also17-β-estradiol /βCD complex are easily dissolved and extracted in aqueous micellar solution. So pH = 2 was used for the extraction of 17-β-estradiol from water sample solutions.

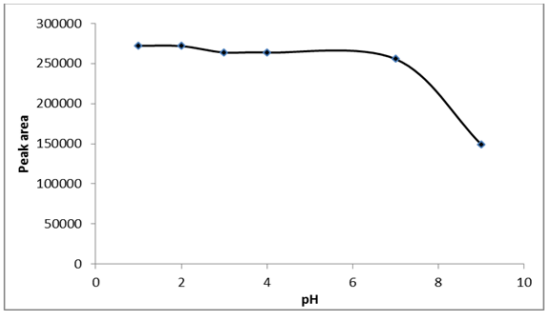

Fig. 5. Effect of pH on the 17-β-estradiol extraction, Conditions: 50 mg capric acid, THF = 10%, molar ratio of 1:4 (17-β-estradiol /βCD), extraction time: 30 min.

3.5. Effect of ionic strength

For investigating the influence of ionic strength on the performance of CAE, various experiments were performed by adding different amounts of NaCl (0.0-1.0 mol L^{-1}). Other experimental conditions were kept constant. The results (Fig 6) showed that within experimental error limits and low salt concentration, ionic strength had no appreciable effect upon extraction efficiency.

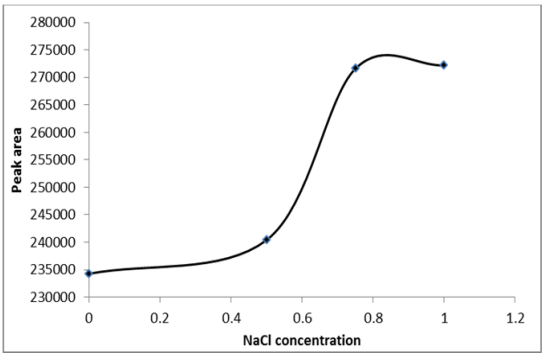

Fig. 6. Effect of ionic strength on the 17-β-estradiol extraction, Conditions: 50 mg capric acid, THF = 10%, molar ratio of 1:4 (17-β-estradiol /βCD), pH=2, extraction time: 30 min.

3.6. Effect of extraction time

The efficiency of micelle-mediated extractions based on non-ionic surfactants has been reported to depend on the time that analytes interact with micelles and get into their core. In order to determine the effect of this parameter on recovery and enrichment factors of 17-β-estradiol the following experiment was carried out. The mixture

DOI: 10.30473/IJAC.2025.74335.1318

containing the bulk sample solution and the coacervate phase were mixed by Ultrasonic water before centrifugation (4000 rpm, 10 min). (Fig 7) Changing the extraction time between 10 and 30 min had no significant effect on recoveries and enrichment factors for 17-β-estradiol.

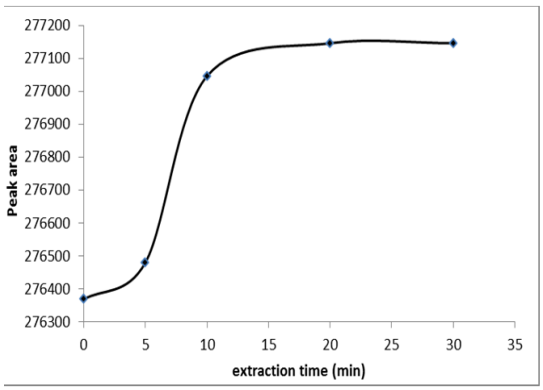

Fig. 7. Effect of extraction time on the 17-β-estradiol extraction, Conditions: 50 mg capric acid, THF = 10%, molar ratio of 1:4 (17-β-estradiol /βCD), pH=2.

3.7. Comparing CAE with other methods

A comparison of the presented method with other reported preconcentration methods for 17-β-estradiol determination in water samples is given in Table 1. The presented method has low LOD, good enrichment factor and RSD and these characteristics are comparable or even better than most of the other methods in Table 1. All these results indicate that CAE is a reproducible, simple, and low cost technique that can be used for the preconcentration of estrogen hormones from various samples. A comparison of the proposed method with other reported preconcentration techniques for 17-β-estradiol determination in water samples is presented in Table 1. The proposed method demonstrates low limit of detection (LOD), high enrichment factor, and reliable relative standard deviation (RSD), with performance that is comparable to or superior to most existing methods listed in Table 1. These findings confirm that coacervate-assisted extraction (CAE) is a reproducible, simple, and cost-effective technique suitable for preconcentration of estrogen hormones from different sample matrices.

3.8. Method validation

Under the optimized conditions, characteristics of the calibration curves including linear dynamic range, limit of detection, reproducibility, squared correlation coefficient and enrichment factor were investigated. The calibration graph was linear in the range of 1–140 µg $L^{-}1$ of 17-β-estradiol. The limit of detection (defined as a signal to noise ratio of 3) was 0.19 µg L^{-1}. Percentage relative standard deviation (RSD) was 4.34 %. The values of the correlation coefficient were good ($R^2 > 0.997$) and excellent linearity was obtained in the range studied. The Recovery, enrichment factors of 17-β-estradiol were 95% and 284, respectively.

3.9. Application of the method

Determinations of 17-β-estradiol in different water samples were assessed whose results are shown in Table 2. The accuracy of the method was evaluated by a recovery test carried out with 17-β-estradiol - spiked water samples. Recovery, enrichment factors were between 82.88-95.5% and 248-286 for the 17-β-estradiol determination in three water samples. Figures 8 & 9 shows some of the chromatograms obtained for standard solution, city water, mineral water and waste water samples.

Table 1. Comparison of some characteristics of proposed method with other methods reported for determination of 17-β-estradiol.

Method	Linear range	LOD	RSD (%)	Determination in real samples	extraction method	Recoveries %)	Reference
HPLC	5-1000 (μgL^{-1})	0.8 (μgL^{-1})	8	water	DLLME	116	[27]
GC-MS/MS	1.25-50 (μgL^{-1})	0.17 (μgL^{-1})	13	milk	HF-LPME	117	[28]
GC-ms	2.5-250 ($ugKg^{-1}$)	3 ($ugKg^{-1}$)	22.7	feed	SPE	76.34	[13]
HPLC	10-1000 (μgL^{-1})	0.21 (μgL^{-1})	7.9	water	SPME	88.5	[29]
LC-ms-ms	5-600 (pg mL^{-1})	-	2.5	serum	LLE	102.8	[30]
Electrochemical	0.05-10 (µM)	0.02 (µM)	6.5	river water	MIP	97	[18]
HPLC	1-2500 (μgL^{-1})	0.28 (μgL^{-1})	4.5	water	SBSE	77	[2]
HPLC	50-1000 (ngL^{-1})	50 ($\mu g\ kg^{-1}$)	11	river water	SPE	86	[10]
Presented method	1-140 (μgL^{-1})	0.19 (μgL^{-1})	4.34	water	CE	95	This work

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DOI: 10.30473/IJAC.2025.74335.1318

Table 2. Recoveries and concentrations of 17-β-estradiol in real water samples (n = 3).

Sample	Concentration (µg/L)	added (µg/L)	Recovery (%)	Enrichment	RSD (%)
Wastewater	34.84	20	82.88	248	13.32
City water	1.9	20	87.45	262	8.21
Mineral water	Below detection limit	-	-	-	-
Mineral water	"	50	97.23	298	5.68

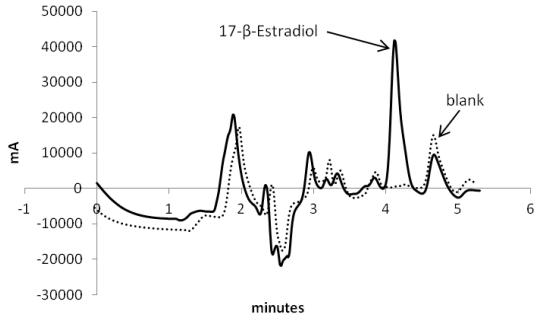

Fig. 8. HPLC chromatogram, a) blank, b) standard solution with 25 µg/L of 17-β-estradiol.

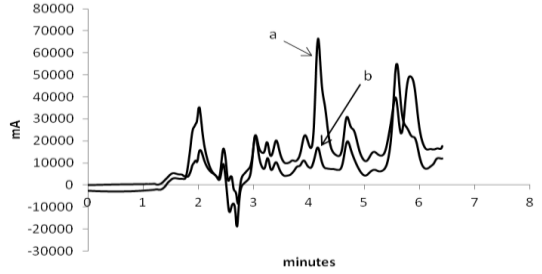

Fig. 9. HPLC chromatogram in, a) city water, b) wastewater for 17-β-estradiol

4. CONCLUSIONS

For the above analysis, we conclude that there are several possibilities of extending the range of applications for coacervate-based extraction techniques. Modification of the extraction medium is one possibility. The effects of various extraction parameters, including tetrahydrofuran (THF) and decanoic acid concentration, pH, ionic strength, and extraction time, on the efficiency of 17-β-estradiol extraction were examined. Obtained results shows that water solutions had the highest extraction efficiency with the following optimum conditions: 50 mg of capric acid, 10% THF, pH =2, 0.2 mol L^{-1} NaCl salt and 30 min extraction time, using an ultrasound bath assisted extraction procedure. The proposed method results in an increase in extraction efficiency. Results obtained from the present work provide essential background knowledge to introduce the application 17-β-estradiol determination based on coacervate extraction in food, pharmaceutical, and cosmetic products. Coacervate-based extraction techniques offer a viable alternative to conventional preconcentration methods, which typically require large amounts of chemical reagents. High recoveries and low LODs provide excellent opportunities applying coacervate-based extraction techniques to preconcentrate organic compounds, metal ions and NPs. By achieving such results, coacervates can play an important role in chemical analysis and environmental monitoring.

ACKNOWLEDGMENT

This research is part of a Ph.D. thesis by Mohammad Hasannejad and is financially supported by Lorestan University and the Faculty of Pharmacy at Tabriz University of Medical Sciences.

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DOI: 10.30473/IJAC.2025.74335.1318

استخراج و تعیین کمیت ۱۷-β-استرادیول در نمونه های فاضلاب از طریق تشکیل فاز کواسرویتیو با β-سیکلودکسترین و کروماتوگرافی مایع با کارایی بالا

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تاریخ دریافت: ۲۸ مرداد ۱۴۰۳ **تاریخ پذیرش:** ۲۹ شهریور ۱۴۰۳

چکیده

یک روش ساده، سریع و مقرون به صرفه برای تعیین ۱۷-β-استرادیول در نمونه های آب توسعه داده شد. این روش مبتنی بر استخراج کمپلکس ۱۷-β-استرادیول - β-سیکلودکسترین با استفاده از یک فاز کواسروای متشکل از میسل های معکوس اسید دکانوئیک و به دنبال آن کروماتوگرافی مایع با کارایی بالا با تشخیص اشعه ماوراء بنفش برای تعیین مقدار است. اثرات پارامترهای کلیدی، از جمله غلظت اسید دکانوئیک (۵۰ تا ۲۰۰ میلی‌گرم در حجم کل ۳۰ میلی‌لیتر)، غلظت تتراهیدروفوران (۱-۱۵ درصد حجمی)، نسبت مولی بتا سیکلودکسترین به ۱۷-β-استرادیول (۱:۱-۱:۴)، قدرت یونی ۰-۱ مولارNaCl، pH ۱-۴ و زمان استخراج (۰-۳۰دقیقه) روی شرایط استخراج بهینه و فاکتور تغلیظ مطالعه شد. شرایط استخراج بهینه نمونه آب ۲۰ میلی لیتری حاوی ۵۰ میلی گرم اسید دکانوئیک با ۳ میلی لیترTHF، با استفاده از نسبت مولی ۱۷-β-استرادیول به ۱۷-β-استرادیول (۱:۳) برای مدت ۱۰ دقیقه و به دنبال آن سانتریفوژ ۴۰۰۰ دور در دقیقه بود ۱۷-β-استرادیول در درجه اول تحت تأثیر غلظت اسید دکانوئیک و THF بود که فاز کواسروای را تشکیل می دهند، اما مستقل از قدرت یونی محلول نمونه باقی ماند. نرخ بازیابی، فاکتور غنی‌سازی، حد تشخیص و انحراف استاندارد نسبی برای ۱۷-β-استرادیول به ترتیب ۹۵ درصد ، ۲۸۴، µg/L ۰,۱۹ و ۴,۳۴ درصد بود. این روش برای تجزیه و تحلیل ۱۷-β-استرادیول در نمونه های آب شهری، آب معدنی و آب شیرین استفاده شد. هیچ ۱۷-β-استرادیول در آب معدنی شناسایی نشد، در حالی که غلظت آن در آب شهری و آب شیرین به ترتیب ۱,۹ µg/L و ۳۳,۶۷ µg/L بود.

کلید واژه ها

۱۷β-استرادیول؛ β -سیکلودکسترین؛ فاز میسلی؛ استخراج کواسروای؛ فاکتور غنی‌سازی.

DOI: 10.30473/IJAC.2025.74335.1318