Scientific paper

Determination of Estrogens in Water Samples Using Dispersive Liquid Liquid Microextraction and High Performance Liquid Chromatography

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Abstract

A new method for the analysis of estrogens including estrone (E1), 17β- estradiol (E2) and diethylstilbestrol (DES) in aqueous samples was performed using dispersive liquid liquid microextraction (DLLME) and high- performance liquid chromatography (HPLC). In order to optimize DLLME some important parameters such as type and volume of extraction and disperser solvent, extraction time, ionic strength and pH of sample were studied and optimum condition was obtained. Under optimum condition (extraction solvent: 80 μL CCl_4; dispersive solvent: 1.25 mL acetone; NaCl: 12% (w/w) and pH of sample = 10.0), the enrichment factors and extraction recoveries were 71.0–78.5 and 85.2–94.2 respectively. Linearity was observed in the range of 0.02–500.0 μg L^{-1} for DES and 0.03–500.0 μg L^{-1} for E1 and E2. Limits of detection were 0.008 μg L^{-1} for DES and 0.010 μg L^{-1} for E1 and E2. The relative standard deviations (RSDs) for determination of estrogens in water were in the range of 2.4–3.2% (n = 5).

Keywords: Dispersive liquid liquid microextraction, Estrogens, Water samples, HPLC.

1. Introduction

The endocrine- disrupting phenomenon became a relatively new area of concern. Endocrine- disrupting compounds (EDC_s) are environmental contaminants that disturb normal endocrine function.[1,2] Among the wide range of substances with endocrine- disrupting properties, natural and synthetic estrogens are of particular interest due to their high estrogenic potency.[3,4] Estrogens mimic such as alkylphenols, nonylphenols, bisphenol A and alkylphenol polyethoxylates.[5–9] The major estrogenic components that have been identified as the natural estrogens, include estrone (E1) and 17β-estradiol (E2) which are either produced endogenously by animals or used as pharmaceutical products in both human and veterinary medicine.[10,11] One of the synthetic forms of the estrogen hormone, diethylstilbestrol (DES) is used as a growth promoter in domestic animal.[12] More than 30 years of research have confirmed that DES is a teratogen, an agent that can cause malformations of an embryo. It is reported that exposure to synthetic estrogen during critical stages of child development in the uterns increases the risk of abnormalities which can result in structural, functional or long term pathological changes including cancer. E1, E2 and DES have shown estrogenic effects in fish at very low concentration.[13,14] Thus the very low environmental concentrations expected for these estrogens require a sensitive, selective and simple method to monitor them in water.[15] Before determination of these materials in water samples they require a pretreatment technique. Many different pretreatment techniques, such as liquid liquid extraction (LLE),[16–18] solid phase extraction (SPE),[8,19,20] solid phase microextraction (SPME),[21–23] stir bar sorptive extraction (SBSE),[24] cloud point extraction (CPE)[25] and liquid phase microextraction techniques[26–30] were used for the extraction of estrogens. Unfortunately, the traditional methods such as LLE and SPE require a large consumption of organic solvents, sample volume and are time consuming. Although SPME and SBSE are both solvent-free techniques, but the fibers of SPME are fragile, expensive and have limited life time and sample carry over is the other problem of this technique. For SBSE an additional desorption step is required when it couples with HPLC. CPE uses surfactants for extraction thus the choices of the surfactants often bring the nuisance to the analysis of analytes using GC and

HPLC.[31–35] Dispersive liquid liquid microextraction (DLLME) is a novel microextraction method which was developed by Assadi and co-workers.[36] It is based on a ternary component solvent system and uses microliter volumes of extraction solvent. In this method, the appropriate mixture of extraction solvent and disperser solvent is injected into aqueous sample by syringe, rapidly. Thereby, cloudy solution is formed. It has been widely used for trace analysis in different matrices (water, serum and urine etc).[37–39] DLLME is a rapid, simple and low cost method with high recovery and enrichment factor.[40–43] The aim of this work was to use DLLME-HPLC-UV for simultaneous determination of three estrogens in water samples. The effects of various experimental parameters on the extraction of estrogens from water samples were studied and optimized. The optimized method was applied to determine estrogens in river tap and well water.

2. Experimental

2. 1. Materials and Solutions

Estrone (99.0%), 17β-estradiol (98.0%) and diethylstilbestrol (99.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloroform (analytical grade), carbon tetrachloride (spectrophotometric grade) and acetonitrile (HPLC-grade) were purchased from Fluka (Buchs, Switzerland). Dichloromethane (analytical grade), acetone and methanol (HPLC-grade), sodium hydroxide, hydrochloric acid, sodium chloride and tetrahydrofuran (THF) were obtained from Merck (Darmstadt, Germany). Water used was double distilled deionized. Stock solutions of estrogens (500.0 mg L^{-1}) were prepared in methanol and stored in the dark at 4 °C. The working solutions were prepared daily by an appropriate dilution of the stock solution in water. All solutions were filtered through 0.45 μm membrane filters (Millipore, Bedford, MA) prior to use.

2. 2. Instrumentation

Chromatographic measurements were carried out using a HPLC system equipped with a series 10 LC pump, UV detector model LC-95 set at 245 nm and model 7125i manual injector with a 20 μL sample loop (Perkin-Elmer, Norwalk, CT, USA). Adjustment of pH of solutions was done by a 3030 Jenway pH meter (Leeds, UK). Column used was C_{18} (250 × 4mm, 5μm particle size) from waters (Milford, MA, USA). Mobile phase used was a mixture of acetonitrile / water /THF (50:48:2 v/v) at a flow rate of 1.0 mL min^{-1} and room temperature.

2. 3. Extraction Procedure

For the DLLME, 5.0 mL of aqueous sample was placed in a 10 mL screw cap glass test tube with conical bottom and spiked at level of 10.0 μg L^{-1} of target analytes. 1.25 mL of acetone (as disperser solvent) containing 80 μL CCl_4 (as extraction solvent) were rapidly injected into sample solution and the mixture was gently shaken. In this step, a cloudy solution was formed and the analytes in the water sample were extracted into fine droplets. The mixture was centrifuged for 10 min at 4000 rpm and CCl_4 was sedimented in the bottom of the conical test tube (about 60 μL). The sedimented phase was removed by microsyringe, placed into a vial and evaporated by slight heating to dryness. The residue was reconstituted by 50μL methanol and 20 μL of this solution was injected into HPLC.

2. 4. Calculation of Enrichment Factor and Extraction Recovery

Enrichment factor (EF) was defined as the ratio of the analyte concentration in the sedimented phase (C_{sed}) to the initial concentration of analyte (C_0) within the sample:

$$EF = \frac{C_{sed}}{C_0} \quad (1)$$

C_{sed} for each estrogen compound was obtained from calibration curves of standard solutions. The extraction recovery (ER) and it's relationship with EF are as the following.

$$ER = \frac{C_{sed} V_{sed}}{C_0 V_0} \times 100 \quad (2)$$

$$ER = EF \times \frac{V_{sed}}{V_0} \times 100 \quad (3)$$

Where V_{sed} and V_0 are the volumes of sedimented phase and aqueous phase, respectively.

3. Results and Discussion

There are various parameters affecting the DLLME performance, including type and volume of extraction and disperser solvent, ionic strength, extraction time and pH of sample. These parameters were investigated and the optimal condition was selected.

3. 1. Optimization of DLLME

3. 1. 1. Selection of Extraction and Dispersive Solvents

The selection of an appropriate extraction and dispersive solvents are very important for the DLLME process. Some of significant parameters in selection of ex-

traction solvent are (a) higher density than water (b) good chromatographic behavior (c) extraction capability of interested compounds and (d) low solubility in water. In addition, dispersive solvent should be miscible with both water and the extraction solvent. In this study, all combinations of extraction solvents (dichloromethane: CH_2Cl_2, chloroform: $CHCl_3$ and carbon tetrachloride: CCl_4) and disperser solvents (methanol, acetonitrile and acetone,) were tested. The experimental procedure was done by injecting each combinations of 1.0 mL of dispersive solvent containing 50 μL of extraction solvent into 5.0 mL water sample. In the case of CH_2Cl_2, as extraction solvent, a two-phase system was not observed with any studied dispersive solvents. In the case of $CHCl_3$ just acetone and acetonitrile were formed two-phase system but were not stable. With CCl_4 as extraction solvent, a stable two-phase system was formed with all three dispersive solvents. These cloudy solutions centrifuged and sedimented phases were injected to HPLC. Among these dispersive solvents, acetone gave the highest recovery with CCl_4. Thereby, CCl_4 and acetone were selected as the best extraction and dispersive solvent, respectively. Results are shown in Fig.1.

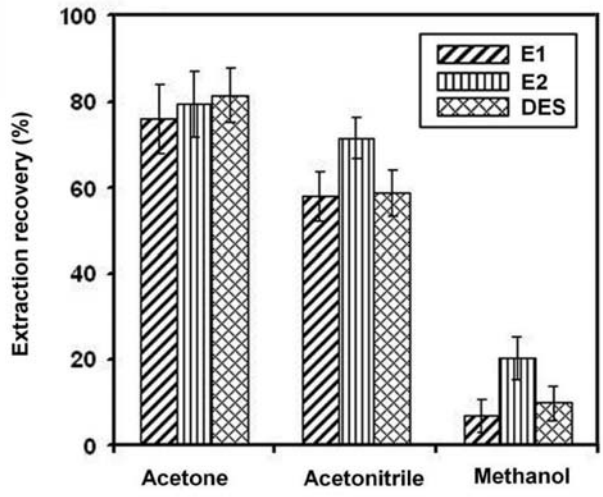

Figure 1. Effect of different dispersive solvent on the extraction recovery of estrogens (n = 3). Extraction condition: sample volume, 5.0 mL; dispersive solvent volume (acetone, acetonitrile and methanol), 1.0 mL; extraction solvent, 50 μL CCl_4.

3. 1. 2. Effect of Extraction Solvent Volume

In order to study the effect of extraction solvent volume, a series of experiment were performed using 1.0 mL acetone containing different volumes of CCl_4 (20–120 μL). Fig. 2 shows the ER versus volume of extraction solvent. It is obvious that ER gradually increases by increasing the volume of extraction solvent up to 80 μL of CCl_4 because the capability of sample extraction into organic phase is increased. After this volume the ER was almost constant and 80 μL of CCl_4 was chosen as the optimum of extraction volume.

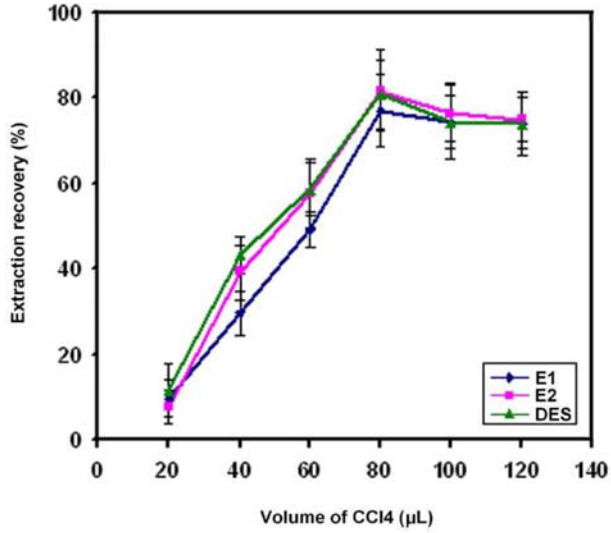

Figure 2. Effect of the volume of extraction solvent (CCl_4) on the extraction recovery of estrogens (n = 3). Dispersive solvent: 1.0 mL of acetone. Other conditions as Fig. 1.

3. 1. 3. Effect of Dispersive Solvent Volume

Volume of the dispersive solvent is one of the important factors which must be considered in DLLME. At low volume, acetone can not disperse extraction solvent properly, and cloudy solution does not form completely, and the extraction recoveries are low. On the contrary, the solubility of analytes in water sample increases at high volu-

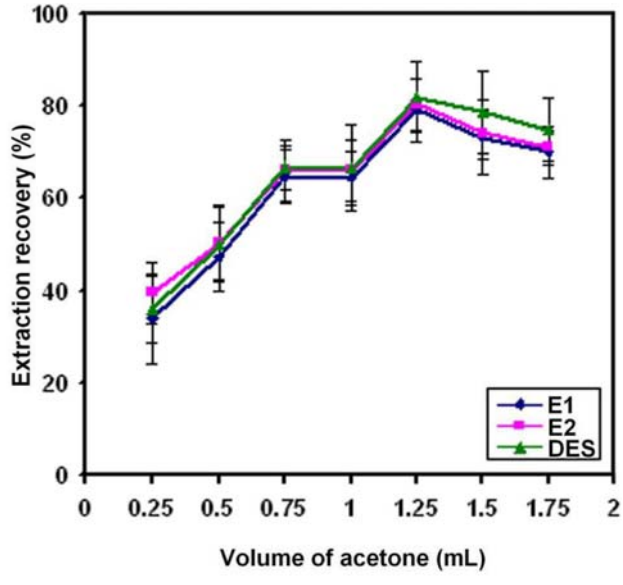

Figure 3. Effect of the volume of dispersive solvent (acetone) on the extraction recovery of estrogens (n = 3). Extraction solvent (CCl_4) volume: 80 μL. Other conditions as Fig. 2.

me of acetone, therefore, extraction recovery decreases too. In this work various volumes of acetone were tested (0.25–1.75 mL) with optimum amount of CCl_4 (80 μL) and results are shown in Fig. 3. It was observed that the extraction recovery was increased by increasing the volumes of acetone up to 1.25 mL and then decreased. Thus, 1.25 mL of acetone was chosen as the optimum volume.

3. 1. 4. Effect of Ionic Strength

Generally, increasing the ionic strength of sample solution decreases the solubility of the analyte and enhances extraction recovery. To investigate the influence of ionic strength on the extraction recovery, various experiments were performed by adding different amounts of NaCl 0.0–18.0% (w/w). Fig. 4 shows the effect of the ionic strength on the extraction recovery of estrogens. It was found that addition of NaCl up to 12.0% (w/w) causes to increase extraction recovery due to salting out effect and moving of analytes into the organic droplets. At higher concentration of NaCl (higher than 12.0%) interaction between salt ions and analytes reduce the ability of analyte to move into organic droplets and decrease the extraction recovery. Therefore 12.0% (w/w) of NaCl was selected as the optimum of ionic strength.

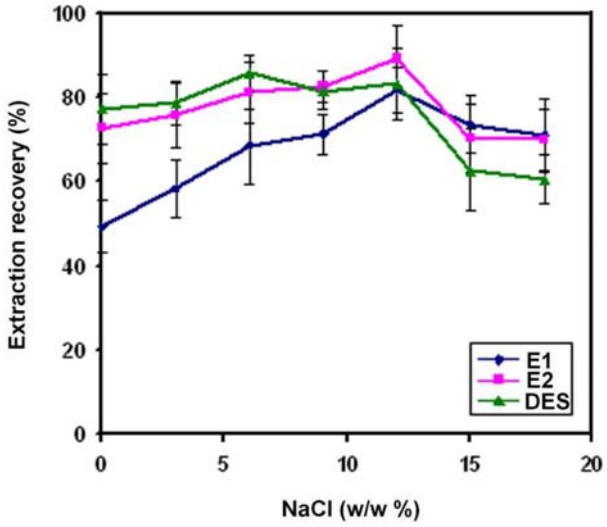

Figure 4. Effect of the ionic strength (addition of NaCl to sample solution) on the extraction recovery (n = 3). Extraction conditions: sample volume, 5.0 mL; extraction solvent (CCl_4): 80 μL; dispersive solvent (acetone): 1.25 mL.

3. 1. 5. Effect of Extraction Time

In DLLME, extraction time is defined as interval time between injecting the mixture of disperser solvent (acetone) containing of extraction solvent (CCl_4) and before starting to centrifuge. The effect of extraction time was examined in the range of 0–60 min. Results showed that extraction time has little influence on the extraction recovery. It is revealed that after formation of the cloudy solution, the surface area between extraction solvent and water sample is infinitely large and shows that the transition of analytes from water sample to extraction solvent is fast and equilibrium state is achieved quickly. Thus, centrifugation procedure could be done immediately after forming cloudy solution. Preliminary consideration of centrifugation time in the range 2 to 15 minutes showed that 10 minutes centrifugation leads to better aggregation of sedimented phase at bottom of the conical test tube and provided the highest recovery values for estrogens. So, in this method the most time-consuming step is the centrifuging of sample solution in the extraction procedure, which is 10 min.

3. 1. 6. Effect of Sample pH

To examine the effect of sample pH on extraction recovery, hydrochloric acid and sodium hydroxide were used to adjust acidity (pH = 5.0–7.0) and basicity (pH = 8.0–12.0), respectively. Fig. 6 shows the curve of recoveries of estrogens versus sample pH (5.0–12.0). Results show that extraction recoveries increased with increasing pH value in the range of 5.0 to 10.0, and then decreased at higher pH values (because ionized forms of these compounds at pH higher than 10). Thus pH = 10.0 was selected as the optimum of sample pH.

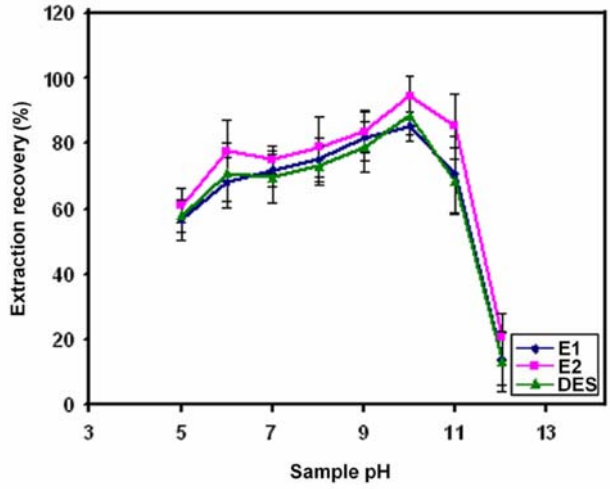

Figure 5. Effect of pH on the extraction recovery of estrogens (n = 3). NaCl: 12% (w/w).Other conditions as Fig. 4.

3. 2. Optimization of Chromatographic Conditions for Separation of Estrogens

In order to select a suitable mobile phase composition for separation of estrogens, several preliminary experiments were carried out. Methanol and acetonitrile (ACN) were examined as organic modifiers where ACN provide better resolution and peak shapes. It is observed

that variation of mobile phase pH had no significant effect on resolution also addition of a small amount of THF to the mobile phase reduced the tailing of estrogens peak without loss of resolution between them. Investigation of different volume percentage of acetonitrile (45–60% v/v), THF (1.0–3.0% v/v) on resolution between analytes peaks and matrix peaks showed that the appropriate mobile phase composition for the separation of estrogens was a mixture of acetonitrile / water /THF (50:48:2 v/v). At this condition estrogen peaks in the chromatograms had no interferences with other compounds extracted from water sample. Chromatogram of spiked water samples with standard of estrogens were shown in Fig. 6 and 7.

3. 3. Figures of Merit

Under the optimum condition, limits of detection (LOD_s), limits of quantification (LOQ_s), linear range (LR), correlation coefficient (r), intra-day precision (repeatability) and inter-day precision (reproducibility), extraction recovery and enrichment factor of the DLLME method for the estrogens were obtained and shown in Table 1. LOD and LOQ was determined based on $3S_b/m$ and $10S_b/m$, respectively, where S_b is the standard deviation of blank and is equal to Peak-Peak noise when only mobile phase was passing through the column for 45 min and »m« is the slope of calibration curve. Limits of detection were 0.008 μg L^{-1} for DES and 0.010 μg L^{-1} for E1 and E2. Linear range was in the range of 0.020–500.0 μg L^{-1} for DES and 0.030–500.0 μg L^{-1} for E1 and E2. Correlation coefficient (r) ranged from 0.9997 to 0.9998. Relative standard deviations (RSD) were used to determine the intra-day precision and inter-day precision of the method. In this way, consecutive extraction of five aqueous samples spiked at 20 μg L^{-1} (working standard solution) was performed in a day and five continual days to evaluate the intra-day and inter-day precision of the estrogens recovery. Results are shown in Table 1.

Table 1. Quantitative results of estrogens determined by DLLME-HPLC-UV from water samples [a].

Parameters	Compounds		
	E1	E2	DES
Limit of detection (LOD, μg L^{-1})	0.010	0.010	0.008
Limit of quantification (LOQ, μg L^{-1})	0.030	0.030	0.020
Linear range (LR, μg L^{-1})	0.030–500.0	0.030–500.0	0.020–500.0
Correlation coefficient (r)	0.9997	0.9997	0.9998
Intra-day precision (RSD %, n = 5)	2.4	3.2	2.7
Inter-day precision (RSD %, n = 5)	4.9	3.5	4.1
Extraction recovery (ER %)	85.2	94.5	88.4
Enrichment factor (EF)	71.0	78.5	73.5

[a] Extraction conditions: water sample volume, 5.0 mL 20 μg L^{-1} of each estrogen; extraction solvent (CCl_4) volume, 80 μL; disperser solvent (acetone) volume, 1.25 mL; NaCl: 12% (w/w); pH = 10.0. HPLC conditions: Mobile phase: water/acetonitrile/THF (50:48:2 v/v); flow rate: 1.0 mL min^{-1}; column: C_{18} (250 × 4 mm, 5μm); room temperature; λ = 245 nm.

3. 4. Real Water Analysis

River and well water from (Babolsar and Babol city, Iran) and tap water from our laboratory were collected and extracted using the optimized DLLME method. The extracts were analyzed by HPLC-UV. The results for well and tap water showed that any of the estrogens were detected above the detection limits of the method. In Babolsar and Babol river water samples E1, E2 and DES were detected and they were confirmed by spiking with standards of estrogens. Concentration of analytes in both river waters was determined using standard addition method. Recoveries and concentration of estrogens in Babolsar and Babol river, well and tap water are shown in Table 2. Figs. 6 and 7 show the chromatograms obtained from Babol and Babolsar river, respectively.

Table 2. Determination of estrogens and their recoveries in water samples [a].

Compounds	Sample	Concentration (μg L^{-1}) Mean ± SD n = 5 [b]	Recovery (%) [c] Mean ± SD n = 5 [b]
E1	Babolsar river	7.3 ± 0.4	84.7 ± 6.4
	Bobol river	2.7 ± 0.2	83.6 ± 5.4
	Well water	ND[d]	86.9 ± 3.9
	Tap water	ND	90.5 ± 6.8
E2	Babolsar river	9.7 ± 0.3	93.4 ± 3.1
	Bobol river	2.2 ± 0.2	92.5 ± 5.8
	Well water	ND	91.8 ± 4.6
	Tap water	ND	89.9 ± 6.1
DES	Babolsar river	6.1 ± 0.2	89.6 ± 5.1
	Bobol river	4.3 ± 0.1	88.3 ± 5.4
	Well water	ND	88.2 ± 2.5
	Tap water	ND	95.8 ± 7.3

[a] Extraction and HPLC conditions: as Table 1.
[b] n: Number of replication
[c] Spiked at 10 μg L^{-1}
[d] Non detected

4. Conclusions

Determination of estrogens in tap, river and well water samples were performed using DLLME-HPLC-UV. DLLME provide a simple, inexpensive, repeatable and easy to use method for extraction and preconcentration of

Table 3. Comparison of DLLME-LC-UV with other methods for the determination of estrogens.

Method	Compound	Recovery (%)	LOD (μg L^{-1})	LR (μg L^{-1})	Extraction Time (min,)	References
SPE-LC-UV	E1	90–102	0.0162	0.1–400	NA [a]	[19]
	E2	85–105	0.0781	0.1–300	NA	
	DES	86–112	0.00098	0.01–300	NA	
SBSE-LC-UV	E1	24.6	1.0	0.005–0.050	120	[24]
	E2	11.1	1.0	0.005–0.050	120	
CPE-LC-UV	E1	98	0.25	1–192	60	[25]
	E2	81.2–92	0.32	1–90	60	
HF-LPME-LC-UV [b]	DES	87.1–92.6	0.5	1–100	40	[26]
DLLME-SFO-LC-UV [c]	E1	89–100	1.0–1.6	5–1000	0	[29]
	E2	102–116	0.8–1.4	5–1000	0	
DLLME-LC-UV	E1	84.7–90.5	0.01	0.03–500.0	0 [d]	Present study
	E2	89.9–94.5	0.01	0.03–500.0	0	
	DES	88.2–95.8	0.008	0.02–500.0	0	

[a] Non available [b] Hollow fiber-mediated liquid phase microextraction [c] Dispersive liquid–liquid microextraction with solidification of a floating organic drop [d] See text for extraction time definition

estrogens in environmental water samples. In comparison with other method for extraction and determination of estrogens the presented method has high recovery, low limit of detection and good linearity with a short extraction time as shown in Table 3.

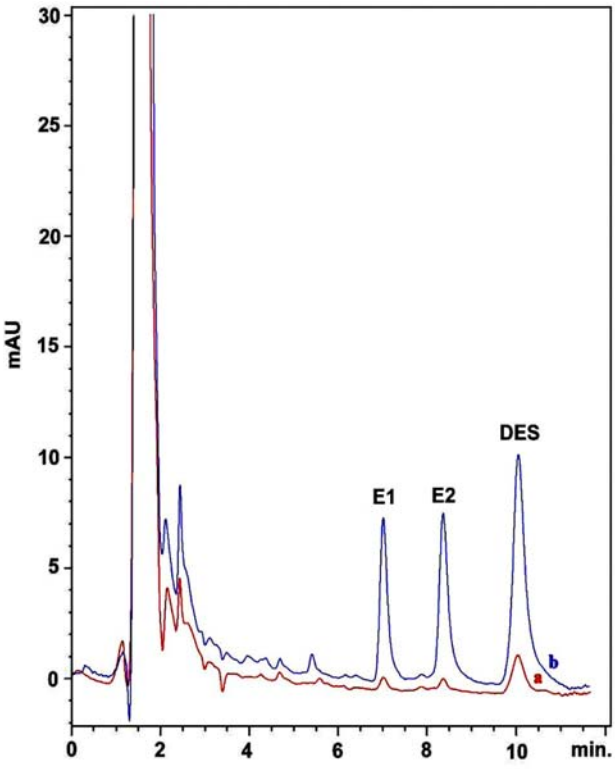

Figure 6. Chromatograms of estrogens at optimum extraction condition a) Babol river water b) spiked Babol river water (10 μg L^{-1}). Mobile phase: acetonitrile /water /THF (50:48:2 v/v); flow rate: 1.0 mL min^{-1}; column: C_{18} (250 × 4 mm, 5μm); room temperature; λ = 245 nm.

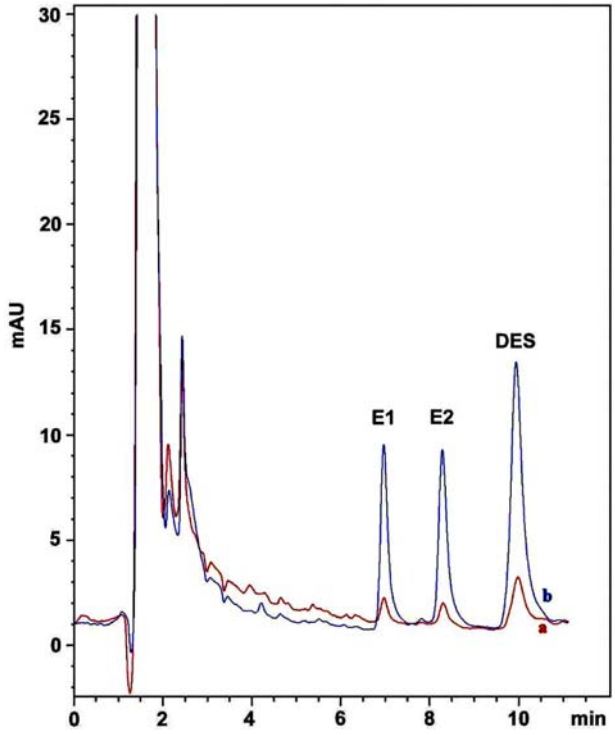

Figure 7. Chromatograms of estrogens at optimum extraction condition a) Babolsar river water b) spiked Babolsar river water (10 μg L^{-1}). Other Condition as Fig. 6.

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Povzetek

Izdelana je bila nova metoda za določevanje estrogenov (estrona- E1, 17 β – estradiola- E2 in dietilstilbestrola –DES) v vodnih vzorcih, ki temelji na disperzijski mikroekstrakciji tekoče-tekoče (DLLME) in visokoločljivostni tekočinski kromatografiji (HPLC). Pogoji ekstrakcije so bili določeni na osnovi izbire topil, njihovega volumna, ekstrakcijskega časa, ionske moči in pH vrednosti. Pri optimalnih pogojih (ekstrakcijsko topilo 80 µL CCl_4, disperzijsko topilo 1,25 mL acetona, 12 % raztopina NaCl in pH 10) so bili doseženi koncentracijski faktorji od 71do 78,5 in izkoristki ekstrakcije med 85,2 in 94,2 %. Linearnost metode je bila v območju od 0,02 do 500 µg L^{-1} za DES in od 0.03 do 500.0 µg L^{-1} za E1 in E2. Dosežene meje zaznave so bile 0.008 µg L^{-1} za DES in 0.010 µg L^{-1} zaE1 in E2. Relativni standardni odmik pri določevanju estrogenov v vodnih vzorcih je bil od 2.4 do 3.2 % (n = 5).