

Optimization of Solid-Phase Extraction for Pretreatment of Selected Estrogens in Sewage by Response Surface Methodology

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Received: 25 November 2012

Accepted: 3 November 2013

Abstract

In order to increase the recoveries of steroid estrogens (SEs) for quantitative determination in sewage, response surface methodology (RSM) was adopted to optimize the solid-phase extraction (SPE) procedure. Quadratic models were established for predicting SE recoveries, and the effectiveness of models and the interaction of factors were analyzed. SE concentrations of typical sewage treatment plants (STPs) in Jiangsu province were detected under the optimized conditions. The optimal operating conditions of SPE were obtained with elution volume of 10.68 mL, a sample rate of 10.47 mL·min⁻¹ and elution rate of 4.07 mL·min⁻¹. The predicted recoveries were 81.63% for E1, 88.86% for E2, and 84.55% for EE2. SEs were detected in all the three STPs, and concentrations in the influents and effluents ranged from 13.4 to 78.0 ng·L⁻¹ and from 5.3 to 20.5 ng·L⁻¹, respectively.

Keywords: steroid estrogens, sewage, response surface methodology, solid-phase extraction

Introduction

Endocrine-disrupting compounds (EDCs) have aroused increasing public concern due to their adverse impacts on ecological security and human health [1, 2]. An increasing number of papers have reported the existence of EDCs in surface water [3], sea water [4], sediments of river beds [5], and even groundwater [6]. In numerous types of EDCs, steroid estrogens (SEs) are considered the most harmful ones, whose estrogenic activity is 10³-10⁶ times higher than those of the other suspected EDCs and poses a great potential threat to the reproduction of aquatic organisms and human health, even when their concentrations are as low as ng·L⁻¹ level [7]. The identified principal SEs are naturally occurring com-

pounds, such as estrone (E1) and 17 β -estradiol (E2), as well as the synthetic compound 17 α -ethynylestradiol (EE2), which is widely used for birth control and estrogen replacement therapy [8-10].

All three of these SEs are regularly excreted in human and animal urine [11] and they enter sewage systems via drainage. Presently, sewage treatment processes are not specifically designed to remove the trace level contaminants such as SEs. Therefore, the effluents of sewage treatment plants (STPs) have become an important source of SEs in aquatic environments [11-13]. Some countries, including Europe, USA, and Japan [13-15], have investigated SEs in surface waters and sewage, finding concentrations were in the range of 1.1-107.6 ng·L⁻¹ for E1, 1.4-55.12 ng·L⁻¹ for E2, and <0.2-73 ng·L⁻¹ for EE2. It can be found that SE concentrations in aquatic environments are extremely low and exceed the limitation of detection of

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Table 1. Situations of three sewage treatment plants. All the values are given as mean value.

STP	Process	HRT (h)	COD (mg·L ⁻¹)		BOD ₅ (mg·L ⁻¹)		NH ₃ -N (mg·L ⁻¹)	
			Influent	Effluent	Influent	Effluent	Influent	Effluent
a	SBR	12.8	156.6	37.8	74.8	9.6	17.4	9.5
b	A ² /O	11.8	301.7	38.9	106.7	6.2	29.4	9.0
c	OD	12	248.0	42.6	112.8	8.1	20.0	4.2

general instruments. The pretreatment of samples, such as enrichment and purification, become indispensable before quantification analysis. Solid-phase extraction (SPE) has become an effective method of extracting estrogens in the aquatic environment recently. Previous work on SPE optimization was mainly based on the classical univariate approach. However, this approach does not take into account interactive effects between factors. Response surface methodology (RSM) is a method combining mathematics and statistics to analyze the relative significance of several influence factors even in a complicated system. RSM can determine the optimal conditions and can reduce analytical time by the comprehensive analysis of mathematics and statistics [16]. Until recently, very limited information regarding the optimization of SPE for the pretreatment of SEs by RSM is available.

The objective of this study was to optimize SPE procedure for the better SE recoveries. RSM was adopted for optimization. The optimized SPE procedure was then applied in the pretreatment of samples from three typical municipal STPs, and SE concentrations were quantified by single-quad LC-MS. The experimental results would provide a strategy for SPE optimization. Furthermore, this study would supply some basic data on SE pollution levels in sewage and the effluents of STPs of Jiangsu, China.

Materials and Methods

Reagents

Chromatographic-grade estrone (E1), 17 β -estradiol (E2), and the synthetic estrogen 17 α -ethynylestradiol (EE2) were purchased from Sigma-Aldrich, USA. They were dissolved in acetonitrile (ACN) to prepare the stock solutions and further dilution was required to achieve target concentrations for the study. Chromatographic-grade methanol (MeOH) and ACN were purchased from the Chinese Pharmaceutical Group Chemical Reagent Co. Ltd.. All the reagents were analytical-grade.

SPE Extraction

Standard samples (E1, E2, EE2) were prepared in brown glass bottles and stored at 4°C. Solid-phase extraction (SPE) workstation was purchased from Caliper (USA) and extend-C18 cartridges (150 \times 4.6 mm, 5 μ m) were purchased from Agilent (USA). Aqueous samples were

extracted by C-18 SPE cartridges preconditioned with 7 mL ACN, 10 mL methanol and 10 mL water in sequence. Afterward, SPE cartridges were cleaned with 10 mL deionized water followed by 10 mL 10% methanol, and were dried with a stream of nitrogen. Then the SPE cartridges were further eluted with ACN and dried with nitrogen. Finally, samples were re-dissolved in 200 μ L ACN for concentration detection.

Water Sample Collection and Preparation

In this work, three typical STPs (a – sequencing batching reactor, SBR process, b – anaerobic-anoxic/oxic, A²/O process, and c – oxidation ditch, OD process) located in Nanjing, East China, were investigated. General treatment parameters are listed in Table 1. STP a and STP b are mainly composed of domestic sewage, while STP c is located in an industrial development zone with the influent composed of domestic sewage and industrial wastewater. From June 2010 to October 2010, the influents and effluents from the three STPs were collected monthly in sterile brown glass bottles. Collected samples were preserved by instilling 1% (v/v) formaldehyde immediately and transported to the laboratory for analysis. Water samples were adjusted to neutral pH and filtered through 0.45 μ m glass fiber filters in a glass vacuum filter. 400 mL filtrates were extracted by C-18 SPE cartridges under optimized conditions.

Estrogen Concentration Analysis

Estrogens were quantified by single-quad liquid chromatography-mass spectrometry LC-MS. LC was carried out using an Agilent LC pump Series 1100 (Agilent, CA, USA). Chromatographic separation was achieved on XTerra[®] MS C18 cartridges (3.5 μ m, 2.1 \times 100 mm, Waters, Ireland) preceded by a guard column (XTerra[®] RP 18, 3.5 μ m, Waters, Ireland). The samples were analyzed using a water:ACN (50:50, v/v) mobile phase at a flow rate of 0.2 mL·min⁻¹ for 14 min. The cartridge was then washed with water:ACN (10:90, v/v) for 2 min, followed by recondition with the initial mobile phase composition for 10 min between sample injections (10 μ L), at a flow rate of 0.2 mL·min⁻¹. Analysis of the estrogens was performed by using an electrospray interface (ESI) in negative ion mode with an Agilent Model 1100 mass spectrometer (Agilent, CA, USA). Data processing was performed with Target 4.1 software on a Windows workstation (ThermoQuest ThruPut Software, FL, USA).

Table 2. Experimental factors and levels.

Factors	Symbol	Coded Levels				
		-1.682	-1	0	1	+1.682
Elution rate (mL·min ⁻¹)	X_1	2.318	3	4	5	5.682
Elution volume (mL)	X_2	6.636	8	10	12	13.364
Sampling rate (mL·min ⁻¹)	X_3	3.272	6	10	14	16.728

Recoveries

The SEs recoveries were indicated by formula (1):

$$\eta = \frac{m_0}{m} \times 100\% \tag{1}$$

...where, m represents SE quantities before extraction and m_0 is SE quantities after extraction.

Experimental Design for SPE

Three parameters, i.e. elution rate, elution volume, and sampling rate, were considered as the most significant factors that affect SE recoveries [17]. Initially, the classical univariate method was applied to ascertain the individual effects on SPE. Afterward, optimization of the three operation parameters was conducted. RSM was used to optimize the extraction process to achieve higher SE recoveries (Y) by controlling three process variables that were indicated by X_1 , X_2 , and X_3 and encoded according to formula (2):

$$x_i = \frac{X_i - X_0}{\Delta X} \tag{2}$$

...where, x_i represents code values of independent variables, X_i represents actual values of independent variables, X_0 is independent variables at middle level, and ΔX is step length of independent variables.

The central composite design [18] was adopted to optimize the process of SPE by 20 sets of experiments. The combinations of coded variables were predetermined according to the central composite experimental design, and the specific coded values are shown in Table 2. Based on the preliminary univariate experiment, the higher SE recoveries were achieved with elution rate of 3-4 mL·min⁻¹, elution volume of 10-12 mL, and sampling rate of 10 mL·min⁻¹. Therefore, the middle level values of the independent variables in this study were fixed on elution rate of 4 mL·min⁻¹, elution volume of 10 mL and sampling rate of 10 mL·min⁻¹. SE recoveries (Y) were fitted to the empirical quadratic response surface model (3) as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \tag{3}$$

...where, Y represents response values (SEs recovery/%), b_0 is intercept, b_1 , b_2 , and b_3 are linear coefficient, b_{12} , b_{13} , and

Table 3. Experimental design and results.

Number	Code			Response values (recoveries/%)		
	x_1	x_2	x_3	E1	E2	EE2
1	1	1	-1	62.224	62.145	63.008
2	1	1	1	73.850	71.707	70.518
3	0	+1.682	0	70.309	74.880	77.589
4	0	0	0	81.279	97.764	81.369
5	-1	-1	-1	67.624	62.962	67.596
6	0	0	0	80.420	88.401	85.841
7	0	-1.682	0	60.409	61.021	65.778
8	-1	-1	1	61.024	53.469	60.500
9	-1	1	1	68.826	59.085	68.081
10	0	0	0	81.905	93.510	80.812
11	0	0	-1.682	40.658	49.873	44.784
12	+1.682	0	0	68.201	70.143	70.812
13	0	0	0	78.089	85.062	82.832
14	1	-1	-1	42.748	53.904	51.798
15	0	0	0	84.363	81.742	82.832
16	0	0	+1.682	47.213	60.183	53.341
17	-1	1	-1	63.007	67.804	53.341
18	0	0	0	77.892	83.390	90.561
19	-1.682	0	0	67.553	70.510	75.231
20	1	-1	1	54.059	59.884	67.222

b_{23} are interaction coefficient, and b_{11} , b_{22} , and b_{33} are quadratic coefficient.

Design Expert v.7.0 (Stat-Ease Inc. USA) was applied to experimental design and statistical analysis. Data were analyzed by the analysis of variance (ANOVA), and the mean values were considered the significant difference when $p < 0.05$. The optimum operation parameters were estimated by the three-dimensional response surface analysis of the independent variables and the dependent variable.

Table 4. ANOVA for the response surface quadratic model for EE2 recovery.

Source	Sum of squares	d.f. ^a	Mean square	F value	p- value prob>F
Model	2815.17	9	312.80	14.59	0.0001
Residual	214.38	10	21.44		
Lack of fit	148.14	5	29.63	2.24	0.1988
Pure error	66.24	5	13.25		
Cor total	3029.55	19			
	R=0.964		R ² =0.929		

^aDegree of freedom

Table 5. Coefficient of regression model and their significance for EE2 recovery.

	Coefficient estimate	d.f. ^a	Standard error	F value	p-value prob>F
Intercept	84.06	1	1.89	-	-
x_1	-0.32	1	1.25	0.066	0.8021
x_2	2.03	1	1.25	2.62	0.1366
x_3	3.29	1	1.25	6.91	0.0252
x_1x_2	2.65	1	1.64	2.62	0.1369
x_1x_3	1.91	1	1.64	1.36	0.2701
x_2x_3	1.74	1	1.64	1.13	0.3127
x_1x_1	-4.03	1	1.22	10.94	0.0079
x_2x_2	-4.51	1	1.22	13.65	0.0041
x_3x_3	-12.50	1	1.22	105.11	< 0.0001

^aDegree of freedom

Results and Discussion

Model Fitting and Significance Test

In order to optimize the SPE process parameters, 20 sets of experiments were conducted for the response surface modeling, as shown in Table 3, and the order of experiments was arranged randomly. This approach was used to give broader insight to the SPE procedure. E1 recoveries ranged from 40.658% to 84.363%, E2 recoveries ranged from 49.873% to 97.764%, and EE2 recoveries ranged from 44.784% to 90.561% with respect to variations in extraction conditions.

EE2 recoveries were taken as an example to demonstrate the effectiveness of the models. The following quadratic polynomial equation (4) was fitted by multiple regressions of data, which was written in terms of actual factors:

$$Y = -94.55679 + 13.92898X_1 + 16.07706X_2 + 12.36699X_3 - 4.03340X_1^2 - 1.12668X_2^2 - 0.78152X_3^2 + 1.32378X_1X_2 + 0.47780X_1X_3 + 0.21754X_2X_3 \quad (4)$$

Table 4 presents the analysis of variance (ANOVA) for the quadratic model (4). From Table 4, the regression analy-

sis showed a significant probability of F value ($F = 14.59$ and $p = 0.0001$) in estimating Y values, which means that the three independent variables had significant effects on Y . A lack-of-fit value of 2.24 implies difference was not significant, indicating that the residual error was caused by random when p value is 0.1988 (> 0.05), and this also confirms the fitness of the model. Coefficient of determination (R^2) is defined as the ratio of the explained variable to the total variation and a measure of the degree of fit. When R^2 is close to 1, the model fits the actual experimental data better. The proposed model had a sufficiently high R-square value ($R^2=0.929$) to indicate that the Y data were adequately explained. Therefore, the model can be used as an estimate of tendency.

Table 5 shows the regression test for coefficient significance. X_3 (sampling rate, $p=0.0252 < 0.05$) significantly affected experimental results, while the influence of X_1 (elution rate, $p=0.8021$) and X_2 (elution volume, $p=0.1366$) was not significant. The influence of quadratic term X_1X_1 ($p=0.0079$), X_2X_2 ($p=0.0041$), and X_3X_3 ($p<0.0001$) significantly affected experimental results. However, the influence of interaction terms, X_1X_2 ($p=0.1369$), X_1X_3 ($p=0.2701$), and X_2X_3 ($p=0.3127$) could be negligible. The results showed that sampling rate was the main factor that

affected EE2 recoveries. The order of priority among the main affecting factors could be obtained based on coefficient estimated values $x_1 = -0.32$, $x_2 = 2.03$, $x_3 = 3.29$, that is sampling rate > elution volume > elution rate.

The models of E1 and E2 recoveries were gained in the same way as EE2 recoveries. The following quadratic polynomial equations (5) and (6) were fitted for E1 and E2 recoveries, respectively, by multiple regressions of data.

$$Y = -49.22 - 2.37633X_1 + 14.05893X_2 + 10.84708X_3 - 3.69093X_1^2 - 1.14524X_2^2 - 0.75971X_3^2 + 2.25505X_1X_2 + 0.74122X_1X_3 + 0.19896X_2X_3 \quad (5)$$

$$Y = -233.47718 + 37.31809X_1 + 36.58838X_2 + 10.50474X_3 - 6.69980X_1^2 - 1.88497X_2^2 - 0.75680X_3^2 + 0.60035X_1X_2 + 1.05481X_1X_3 + 0.068048X_2X_3 \quad (6)$$

Models of E1 and E2 recoveries also can be estimated through analysis of variance. For E1, $F=20.60$, $p<0.0001$ and $R^2=0.903$, while for E2, $F=15.68$, $p<0.0001$, and $R^2=0.934$. The results indicated that the two models can be used as an estimate of tendency. From the regression test it could be concluded that elution volume was the key factor affecting E1 and E2 recoveries, different from EE2, and the order of factors for E1 and EE2 are all elution volume > sampling rate > elution rate.

Verification of the Models

Based on the fit models, elution volume was the most important factor influencing E1 and E2 recoveries, while sampling rate was the most important one for EE2. The maximum of predicted SE recoveries were 81.63% for E1, 88.86% for E2, and 84.55% for EE2 based on the function of Design Expert v.7.0 when extracted simultaneously. The SPE parameters were set as follows: elution rate of 4.07 mL·min⁻¹, elution volume of 10.68 mL, and sampling rate of 10.47 mL·min⁻¹, respectively. Three sets of experiments were conducted to identify the effectiveness of models. The results showed that the relative errors between predicted values and actual values of SE recoveries were all below 5%, suggesting that it was feasible to optimize SPE conditions with RSM.

Response Surface Analysis

Three-dimensional surfaces and contour plots are graphical representations of regression equations for the optimization of SPE conditions and are the most useful approach for revealing the conditions of the extraction system. E1 recoveries were taken as an example to analyze the interaction of the model. The three-dimensional surfaces and contour plots that account for the influence of elution rate, elution volume, and sampling rate on E1 recoveries (Y) were produced by the above regression equation and are presented in Fig. 1. The plots in Fig. 1 were produced for each pair of factors, whereas the third factor was taken as a constant at its middle level.

Fig. 1 (a and b) represents the interactive effects of sampling rate and elution volume on E1 recovery. The maximum E1 recovery was obtained with sampling rate between 10.0 and 11.0 mL·min⁻¹ and elution volume between 10.5 and 11.5 mL. When beyond the range, increasing sampling rate or elution volume could not improve E1 recovery. Fig. 1 (c and d) illustrates the effect of elution volume and elution rate on E1 recovery. The maximum E1 recovery was obtained with elution volume between 10.5 and 11.0 mL and elution rate between 4.0 and 4.2 mL·min⁻¹. When elution rate exceeded 4.2 mL·min⁻¹, E1 recovery displayed an inverse relationship with the elution rate. This might be attributed to the inadequate elution, because the contact time between eluent and extraction column was not enough when elution volume was small and elution rate was high. When elution volume was more than 10.5 mL and elution rate was less than 4.0 mL·min⁻¹, E1 recovery increased with the increase of elution rate. It was because the elution force was enhanced with the increase of elution rate when elution volume was enough. The effects of elution rate and sampling rate on E1 recovery are shown in Fig. 1 (e and f). In a specific range, increasing either sampling rate or elution rate could enhance E1 recoveries. The maximum E1 recovery could be obtained with sampling rate around 10.3 mL·min⁻¹ and elution rate between 3.5 and 4.0 mL·min⁻¹.

Analysis of Real Samples

The developed SPE procedure: elution rate of 4.07 mL·min⁻¹, elution volume of 10.68 mL, and sampling rate of 10.47 mL·min⁻¹, was applied to the pretreatment of samples from three typical STPs in Jiangsu province, China (seen in Table 1) from June 2010 to October 2010. SE recoveries and detected concentrations are shown in Figs. 2 and 3, respectively.

From Fig. 2 the recoveries ranged from 60.8% to 83.4% for E1, from 67.2% to 83.4% for E2, and from 64.3% to 83.0% for EE2, respectively. SE recoveries of wastewater samples were slightly lower than that in pure water, which might be attributed to the impurities in sewage. Pedrouzo et al. [19] reported recoveries in 500 mL river water spiked with 100 ng·L⁻¹ estrogens were 46-87%, slightly lower than the results in our case. Xiao et al. [20] analyzed 21 estrogens spiked into clean groundwater using SPE, the recoveries of which were 84-116% at the 10 ng·L⁻¹ level, and Wang et al. [21] also obtained comparable recoveries of estrogens in real water (ranging from 85 to 112%).

Fig. 3 presents SE concentrations in the influents and effluents of the investigated STPs. The concentrations of E1, E2, and EE2 in the influents were found to be in the range of 36.8-89.0, 34.9-78.0 and 18.9-45.0 ng·L⁻¹ in SBR, 27.8-58.1, 21.6-67.4 and 13.4-23.9 ng·L⁻¹ in A²/O, and 24.1-28.4, 20.4-27.4, 14.4-19.9 ng·L⁻¹ in OD, respectively. SE concentrations in the OD process were significantly lower than the other two STPs. This might be ascribed to the higher proportion of industrial wastewater in the influents of the plant (nearly 50%). Belfroid et al. [22] also suggested that estrogen concentrations were higher in domestic STPs than

those in industrial STPs. From Fig. 3 one can see (that although all three STPs had the capacity for SE removal) SEs were still detected in the effluents, and most of the selected SEs exceeded 10 ng·L⁻¹. Compared with SE concentrations in STP effluents in Germany [3] and Paris [14], experimental results in this work were remarkably higher. This may be caused by climate, population density, different

processes, operating conditions, and other factors. SE concentrations in the effluents of all three STPs exceeded predicted no effect concentration (PNEC, 1 ng·L⁻¹) [23], which may pose a crucial threat to ecological security and human health. Therefore, further studies on the occurrence and fate of SEs in STPs are indispensable for enhancing the removal effects.

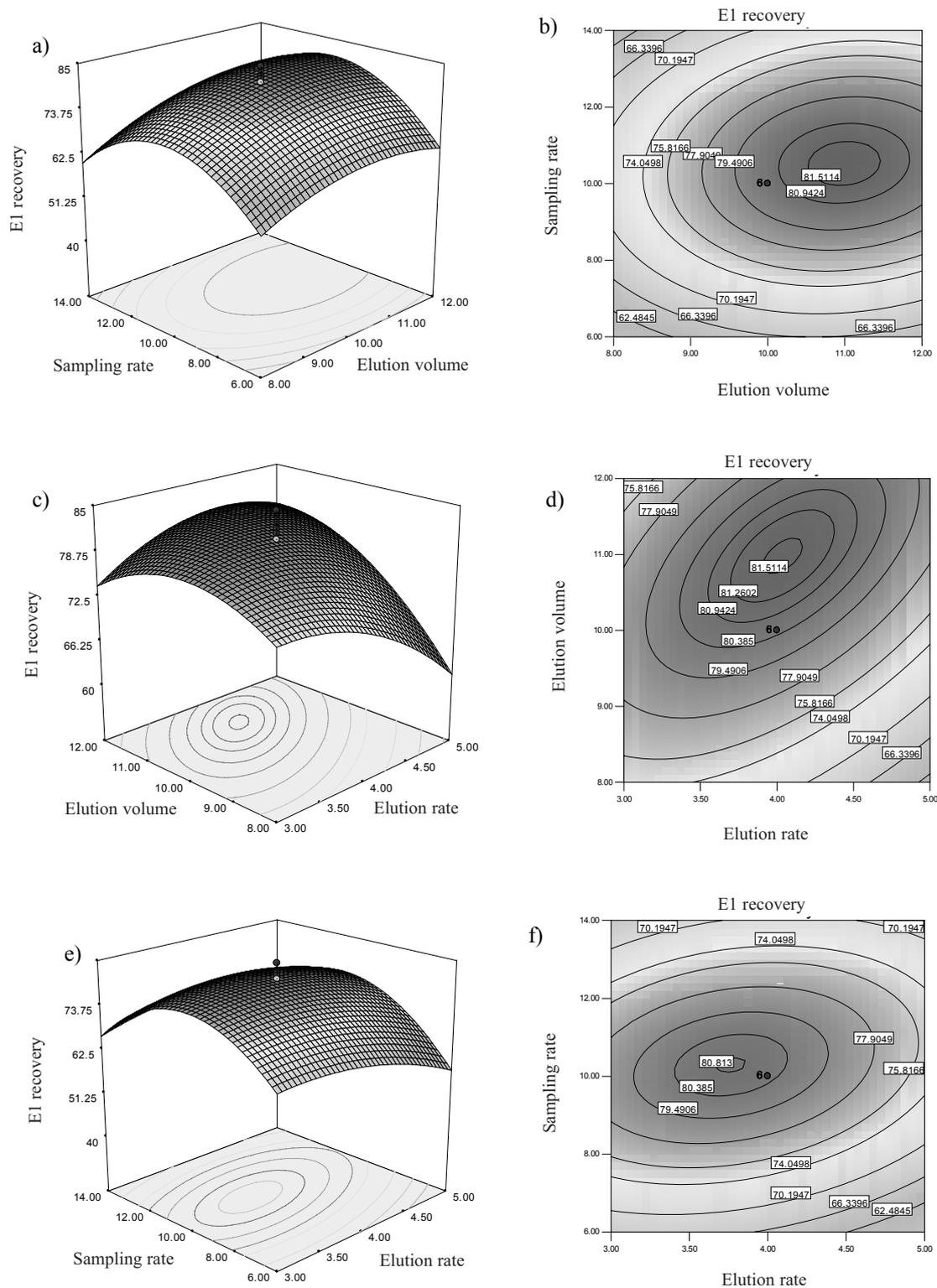


Fig. 1. Response surface and contour plots for the effects of variables on E1 recovery (Y) in water: (a and b) elution volume and sampling rate; (c and d) elution rate and elution volume; (e and f) elution rate and sampling rate.

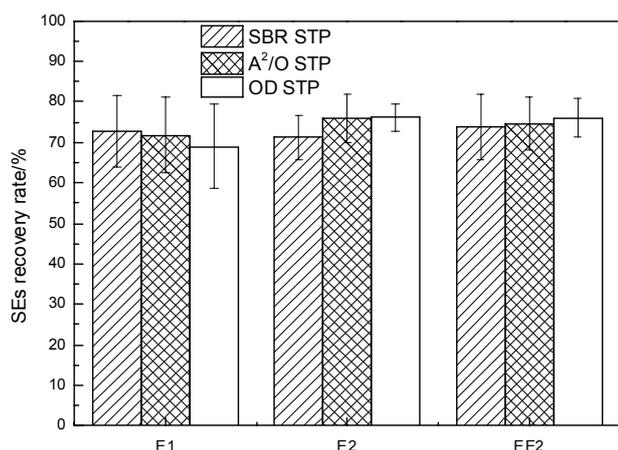


Fig. 2. SE recoveries of samples from real STPs.

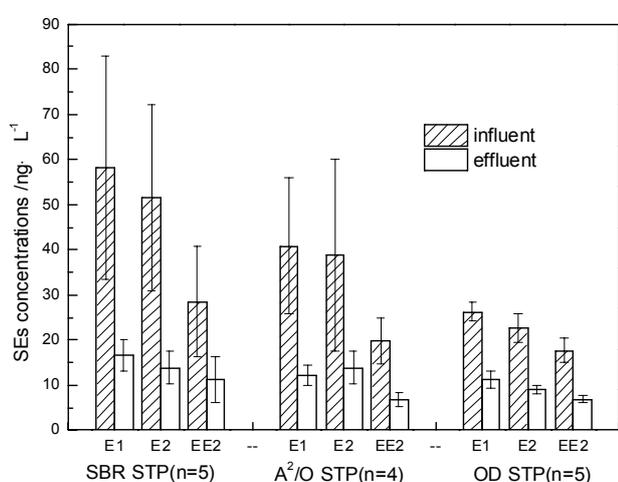


Fig. 3. SE concentrations in the influents and effluents of different STPs.

Conclusions

Solid-phase extraction operating conditions for SEs in sewage were optimized by response surface methodology and quadratic polynomial models of E1, E2, and EE2 recoveries were established. The optimal conditions for extracting E1, E2, and EE2 simultaneously were determined with elution rate of 4.07 mL·min⁻¹, elution volume of 10.68 mL, and sampling rate of 10.47 mL·min⁻¹. Determinations of SEs in all three STPs, with the process of SBR, A²/O and OD, respectively, all achieved good recoveries, that is, in the range from 60.8% to 83.4%. SEs were detected in all three STPs, and the concentrations in influents were 24.1–89.0 ng·L⁻¹ for E1, 20.4–78.0 ng·L⁻¹ for E2, and 13.4–45.0 ng·L⁻¹ for EE2. SE concentrations in the effluents still reached 5.3–20.5 ng·L⁻¹, which is several times higher than the predicted no-effect concentration.

Acknowledgements

The authors would like to acknowledge financial support from the National Natural Science Foundation of

China (No. 51008064 and No. 51109038), the Priority Academic Program Development of Jiangsu Higher Education Institutions (Grant No. 1105007001), and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry.

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