An alternative method for the determination of estrogens in surface water and wastewater treatment plant effluent using pre-column trimethylsilyl derivatization and gas chromatography/mass spectrometry

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Abstract A procedure using pre-column trimethylsilyl derivatization and gas chromatography/mass spectrometry (GC/MS) was developed and applied in determining trace estrogens in complex matrix. Main conditions were optimized, including pH value, salinity of water sample, elution reagents, clean procedure, derivative solvent and temperature. The optimized method was used to determine steroid estrogens in surface water and effluents of wastewater treatment plant (WWTP). Low detection limits of 0.01, 0.03, 0.03, 0.07, 0.09 and 0.13 ng/l for DES, E1, E2, EE2, E3 and E_V, respectively were obtained under optimum condition. No apparent interferences appeared in chromatography in comparison with ultrapure water blank. Mean recovery ranged from 72.6% to 111.0% with relative standard deviation of

1.1–4.6% for spiked surface water, and from 66.6% to 121.1% with relative standard deviation of 1.5–4.7% for spiked effluent of WWTP. The results suggested that the optimized method provides a robust solution for the determination of trace steroid estrogens in complex matrix.

Keywords Determination · Estrogen · Derivatization · Gas chromatography mass spectrometry · Water

Introduction

It has been long concerned that the environmental impacts of the discharge of sewage effluents to water body. Attention in recent years has been focused on substances in sewage, which contain endocrine disrupting properties to wildlife and human (Feigelson and Henderson 1996; Graham et al. 2000; Xiao et al. 2001).

Steroid estrogens of natural and anthropogenic origin have been identified as the majority contributors to endocrine-disrupting activity in both sewage effluent and surface water (Desbrow et al. 1998; Lai et al. 2000; Solé et al. 2001). Estrogens are extremely potent compounds and estrogenic effects have been observed in laboratory studies down to 1 ng/l (Desbrow et al. 1998; Zha et al. 2008). So it is very important to determine the concentration of estrogens in environmental

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water. Considering low volatility of steroid estrogens, LC/MS (Ferguson et al. 2001; López and Barceló 2001; Hu et al. 2005; Cui et al. 2006) and LC/MS/MS (Zhang and Henion 1999; Baronti et al. 2000; Johnson et al. 2000; Xu et al. 2004, 2005, 2006; Mauricio et al. 2006; Matejcek et al. 2007) methods have recently been developed for the determination of these estrogenic compounds. However, at present, the application of LC/MS and LC/MS/MS in environmental analyses of estrogenic steroids appears to be limited for their complex operation and capital costs. Therefore, as a sensitive analysis technique for determining trace estrogens in water, GC/MS is used widely in environmental analyses (Desbrow et al. 1998; Larsson et al. 1999; Rodgers et al. 2000; Zhou et al. 2007).

It is necessary for estrogens to be derivative before analyzed by GC/MS. A range of derivatives used for the determination of the steroid estrogens has been included in a review by López and Barceló (2001). Silylanized derivatives are predominantly used to facilitate determination by GC (Alda and Barceló 2001). Since GC/MS has been an available tool, *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) are widely used in the derivatization of estrogenic steroids by reason of its facile and low cost, which leads to the formation of trimethylsilyl derivative (Xiao et al. 2001; Jeannot et al. 2002; Zuo and Zhang 2005; Zhou et al. 2007). However, a great deal of research has recently reported that formation of multiple trimethylsilyl derivatives in the derivatization of 17 α -ethinylestradiol with BSTFA or MSTFA followed by gas chromatography-mass spectrometry determination (Shareef et al. 2004; Zuo and Zhang 2005; Zhou et al. 2007). Fortunately, it has been found that it is an effective solution by adding trimethylsilylimidaz (TMSI) in BSTFA or MSTFA reagent (Zhou et al. 2007) or using pyridine as derivatization solvent (Zuo and Zhang 2005). Even so, the determination of trace estrogens in complex matrix such as effluents of WWTP is still a challenge for environmental monitoring.

A pre-column trimethylsilyl derivatization and gas chromatography/mass spectrometry method for the determination of estrogens had been built

in our previous research (Zhou et al. 2007). In order to realize much lower detection limit and higher recovery for environmental water sample, in this study, the pretreatment conditions were optimized to assure better recovery and less matrix disruptor, and the conditions of derivatization were modified to enhance sensitivity. Based on the development of the previously proposed method, a more sensitive and precise method was built.

Materials and methods

Chemicals and materials

All solvents were of HPLC grade, purchased from J.T. Baker (Philipsburg, NJ, USA). Derivatization reagents, BSTFA+1%TMCS and TMSI, were both purchased from Aldrich (Milwaukee, WI, USA). Estrogen standards, including diethylstilbestrol (DES), estrone (E1), 17- β -estradiol (E2), ethinylestradiol (EE2), estriol (E3) and estradiol-17-valerate (E_V), were all obtained from Aldrich (Milwaukee, WI, USA), whose structures and physicochemical properties were shown in Table 1. Surrogate standard 17- β -estradiol-16,16,17-*d*₃ (E2-*d*₃) was produced by C/D/N Isotopes (Montreal, Canada). Pyrene-*d*₁₀ obtained from Supelco Inc. (Bellefonte, PA, USA) was used as the internal standard (IS). Anhydrous sodium sulfate was purchased from Beijing chemical plant (Beijing, China) and roasted at 450°C for 12 h before used. Hydrochloric acid and sodium hydroxide were purchased from Beijing chemical plant (Beijing, China). Stock standard solutions were prepared by dissolving solid standard in methanol. Calibration standards were evaporated to dryness under a gentle flow of nitrogen. Then 80 μ l derivative reagent BSTFA + 1%TMCS + 0.5%TMSI was added and diluted to 200 μ l using hexane, and reacted for 30 min at air bath of 40°C.

Silica gel (60/200 mesh, ultra pure) and neutral aluminum oxide (50/200 mesh, ultra pure), which were obtained from Acros Organics (New Jersey, USA), heated at 180°C and 250°C for 12 hr, respectively, and then both cooled in a desiccator and de-activated with 3% of deionized (DI) water. The Oasis HLB cartridge (6 cc, 500 mg) was purchased from Waters Corp.

Table 1 Structure and physico-chemical properties of analytes

Compound	Structure	Abbreviation	CAS number	Molecular weight ^a	Water solubility ^a (mg/l)	LogK _{ow} ^a	Vapor pressure ^a (mm Hg)	Henry's law constant ^a (atm·m ³ /mole)	pK _a ^b
Diethylstilbestrol		DES	000056-53-1	268.36	12	5.07	1.41E-008	5.8E-012	na
Estrone		E ₁	000053-16-7	270.37	30	3.13	1.42E-007	3.8E-010	10.4
17β-estradiol		E ₂	000050-28-2	272.39	3.6 (27 °C)	4.01	1.26E-008	3.64E-011	10.23
Ethinyl estradiol		EE ₂	000057-63-6	296.41	11.3 (27 °C)	3.67	1.41E-008	7.94E-012	10.21
Estriol		E ₃	000050-27-1	288.39	441	2.45	1.97E-010	1.33E-012	na
17β-estradiol acetate		Ev	na ^d	365.50	na	6.41 ^c	na	na	na

^aRefer to Kuster et al. (2004, 2005)^bRefer to Yu et al. (2004)^cRefer to Lai et al. (2000)^dNot available

(Milford, MASS, USA). Visi-prepTM-DLSPE Vacuum Manifold with disposable flow control valve liners and a solid-phase extraction (SPE) system were purchased from Supelco Inc. (Bellefonte, PA, USA).

Extraction and clarifying

Water samples were pre-filtered using 0.8–2.0 μm APFF fiberglass filters (Millipore, Bedford, MA, USA) to eliminate particulate matter, and then they were spiked surrogate standard (E2-*d*₃). Adjust pH value using 6 mol/l hydrochloric acid or 6 mol/l sodium hydroxide before extraction. The Oasis HLB cartridge was conditioned with 5.0 mL *tert*-butyl methyl ether, 5.0 ml methanol and 5.0 ml DI water respectively. Water sample (4.0 l) was passed through cartridge at a flow rate of 10.0 ml/min. Then cartridge was dried under a gentle stream of nitrogen. The cartridge was then washed by 5.0 ml of methanol/water (25/75, v/v), 5 ml DI water, 5 ml methanol/ammonia/DI water (10/2/88, v/v), then dried under a gentle stream

of nitrogen. Subsequently, 10.0 ml of organic solvent was used to elute SPE cartridge. Elution was gathered by KD concentrator and dehydrated by anhydrous sodium sulfate, then reduced to 0.5 ml under a gentle stream of nitrogen with 40°C water bath.

The concentrated solution was cleaned up using column chromatography. At first, concentrated solution was subjected to a glass column (10 mm i.d.) containing 10 g of 1:1 alumina/silica gel, and 2.0 ml of methanol/acetone (50/50) was used to wash KD concentrator. Transfer the solution to the glass column. Then 10.0 ml organic solvent was used to elute the column. The elution was evaporated to dryness under a gentle stream of nitrogen.

Derivatization and analysis

The dry residues extracted from sample were re-dissolved in 100 μl organic solvent, then 80 μl derivative reagent BSTFA+1%TMCS+0.5%TMSI was added and diluted to 200 μl. This mixture

solution reacted in air bath at 40°C for 30 min. Derivatized samples were analyzed using an Agilent 6890 gas chromatograph and 5973 quadrupole mass spectrometer equipped with a non-polar HP-5 MS 30 m × 0.25 mm capillary column with 0.25 µm film (Agilent, USA). The injector was set at 300°C, and the oven temperature was programmed at 80°C for 1 min, ramped at 20°C/min to 200°C, then ramped at 10°C/min to 300°C and maintained the temperature for 7 min. The carrier gas was helium with a constant flow rate at 1 ml/min. The mass spectrometer was operated in the electron impact ionization mode at 70 eV with an interface temperature of 280°C and a source temperature of 230°C. Positive fragment ions were analyzed over 50–550 *m/z* mass range in SCAN mode for qualitative analysis. All control of GC and MS parameters and analysis of data were performed by MSD Productivity Chemstation Software Rev. D.00.00.

Qualification and quantitation

Qualitative analysis of target compounds was carried out at the basis of retention time and mass spectrum together in a full SCAN mode.

Quantitative analysis was performed in a selected ion monitoring (SIM) mode using internal standard calibration. Retention time, characteristic ions and quantitative ion of DES, E1, E2, EE2, E3, E_v were shown in Table 2.

Initially, a series of injections of target compounds in the concentration range from 20 ng/ml to 10 µg/ml with constant internal standard 100 ng/ml were performed to determine the lin-

ear concentration range. Calibration curves were performed by linear regression analysis.

Method validation

The recoveries and overall method reproducibility were determined from triplicate analyses (N = 3) of spiked samples. Surface water and effluent of a wastewater treatment plant (WWTP) were spiked with 10 ng/l of the composite standard solution of six kinds of estrogen 24 h before analysis (to prevent microorganism growth, 0.5% volume methanol was added). Then the samples were analyzed by the method described above. Surface water was taken from a fish pool about 12,000 m², which lies in Haidian district in Beijing and effluent (TOC 6.46 mg/l, TSS 5.40 mg/l, pH 7.28) was collected from Lugouqiao WWTP donating about 219700 m² and designed to process 0.2 millions m³ wastewater per day, which lies in Fengtai district in Beijing.

The limit of detection (LOD) and limit of quantification (LOQ) for this method were achieved by concentrating 4 L water, and they were calculated as the minimum amount of a compound present in the sample that produced a signal to noise ratio of three to nine, based on an injection of a 1 µl aliquot of the final 0.2 ml extract solution.

Results and discussion

Optimization of the pre-concentration procedure

The sorbent of SPE cartridge and elution reagent were very important factors for SPE method ef-

Table 2 Parameters of quantification used for selected estrogens

Compounds	Abbreviation	Retention time (min)	Characteristic ions (<i>m/z</i>)	Quantification ion (<i>m/z</i>)
Internal standard	IS	12.04	106, 212	212
<i>cis</i> -diethylstilbestrol	<i>cis</i> -DES	12.65	412, 397, 383	412
<i>trans</i> -diethylstilbestrol	<i>trans</i> -DES	13.47	412, 397, 383	412
Estrone	E1	15.57	342, 218, 257	342
17-β-estradiol	E2	15.99	285, 416, 129	416
Surrogate Standard	SS	15.99	132, 419	419
17-α-ethynylestradiol	EE2	16.81	285, 425, 440	425
Estriol	E3	17.42	129, 345, 504	504
Estradiol-17-valerate	E _v	18.97	428, 231, 244	428

IS Internal standard (pyrene-*d*₁₀ being as internal standard), SS surrogate standard (17-β-estradiol-16,16,17-*d*₃ being as surrogate standard)

iciency. In previous studies, C_{18} SPE cartridge was applied (Ternes et al. 1999; Rodgers et al. 2000; López and Barceló 2001) due to its small interference of complex matrix sample. However, during the concentration procedure, the cartridge might be dry, which would affect the recovery efficiency. As a result, it is difficult to obtain good reproducibility using C_{18} cartridge. In comparison, Oasis HLB cartridge was a very good choice because of its hydrophobic and hydrophilic groups, which were more beneficial for enrichment of steroid estrogen (Benijts et al. 2004; Hernando et al. 2004; Beck et al. 2005; Peck et al. 2007).

Acidity of water sample would change the distribution of target substances between water and cartridge. It was necessary to optimize pH condition of the sample to achieve higher recovery of target compounds. The effect of pH was examined in the range of 1.3–13.4, and 50 ng/l target compounds were fortified according to estrogen composite standards. It was shown that the recovery of DES increased from pH 1.3 to 5.0 and decreased after pH 9 (Fig. 1a). Increasing pH value led to an increase recovery of E2. Lower recovery of E_v was observed for pH 5–8. However, the recoveries of E1, EE2 and E3 were pH-independent evidently.

HLB cartridge contains hydrophobic and hydrophilic function groups, which interacted with steroid estrogen, including absorption and partition. The adjustment of water pH value would change the speciation of estrogens and surface

property of sorbent, which would affect absorption or partition of analytes on cartridge. Table 1 suggested that the pK_a of steroid estrogens was about 10.0. When pH value varied between 3 and 4, steroid estrogens might present at neutral form in water. These forms were beneficial for hydrophobic partitioning on cartridge. Meanwhile, when water pH was higher than 4 or lower than 3, it would lead to analyte and cartridge carrying negative or positive charges respectively, which would affect the absorption of analytes on sorbent. Therefore, the pH condition was optimized from pH 3 to pH 4. Previous studies also showed that better retention could be acquired by acidified water sample (Ternes et al. 1999; Isobe et al. 2003; Quintana et al. 2004; Bila et al. 2007).

Salinity may also affect the extraction efficiency of organic pollutant by either liquid–liquid extraction (LLE) or solid phase extraction. Effect of salinity was evaluated by adding sodium chloride in this study. Sodium chloride was added 100 and 200 g/l respectively to evaluate their effects with control. Figure 1b showed that the recoveries of estrogens did not change with the concentration of sodium chloride except for E3. It was found that residual sodium chloride induced the dehydration of cartridge more difficult, though the increase concentration of sodium chloride enhanced the recovery of E3. Incomplete removal of water led to the fail of derivative reaction between steroid estrogen compounds and BSTFA. In order to re-

Fig. 1 Effect of **a** pH and **b** salinity on the recovery of analytes (methanol was used as elution)

Table 3 Comparison of recovery by using different elution for silicon gel filling column ($n = 2$)

Solvent	Recovery (%)					
	DES (mean $\pm$ SD)	E1 (mean $\pm$ SD)	E2 (mean $\pm$ SD)	EE2 (mean $\pm$ SD)	E3 (mean $\pm$ SD)	EV (mean $\pm$ SD)
Ethyl acetate	132 $\pm$ 5	143 $\pm$ 3.8	101 $\pm$ 4.4	104 $\pm$ 3.2	0	106 $\pm$ 4.9
Acetone/hexane (65/35, v/v)	103 $\pm$ 2.4	122 $\pm$ 3.2	86.1 $\pm$ 2.1	85.7 $\pm$ 2.5	38.2 $\pm$ 6.1	83.9 $\pm$ 2.2
Dichloromethane	0	0	0	0	0	0

duce operation process and guarantee the success of derivative reaction, no variation of the salinity was performed.

HLB cartridge would capture a lot of compounds during the concentration procedure due to coextraction. The eluting reagent was optimized to ensure a better recovery of target compound

and less matrix disrupting. Quintana et al. (2004) reported 70–107% of recovery could be obtained using ethyl acetate as elution, and 80–109% of recovery was realized by mixture elution of *tert*-butyl methyl ether and methanol (1/9, v/v) (Jeannot et al. 2002). Therefore, comparing the two-elution strategies, mixture solvent of *tert*-

Fig. 2 GC-MS chromatograms of **a** crude extract, **b** clarified with silica gel, **c** clarified with silica gel and neutral aluminum oxide, **d** clarified with neutral aluminum oxide in SCAN Mode

Table 4 Comparison of recovery using different elution for neutral aluminium oxide filling column ($n = 2$)

Solvent (acetone/methanol)	Recovery (%)					
	DES (mean $\pm$ SD)	E1 (mean $\pm$ SD)	E2 (mean $\pm$ SD)	EE2 (mean $\pm$ SD)	E3 (mean $\pm$ SD)	EV (mean $\pm$ SD)
3/1	27.7 $\pm$ 3.4	89.2 $\pm$ 5.7	95.0 $\pm$ 7.3	79.2 $\pm$ 4.6	0	89.4 $\pm$ 5.3
1/1	76.5 $\pm$ 2.2	118 $\pm$ 3.5	122 $\pm$ 4.6	83.2 $\pm$ 3.1	52.6 $\pm$ 1.2	93.5 $\pm$ 4.1

butyl methyl ether/ methanol (1/9, v/v) was selected as elution reagent in this study. The coelution was tested with two different solvents above and the results suggested that there was less coelution in *tert*-butyl methyl ether/ methanol (1/9, v/v).

Optimization of the clarification procedure

The effect of sorbent (10 g) of cleaning column and elution reagents on recovery and clarifying efficiency were investigated in this study. And different elution solvents were selected to test silicon gel and neutral aluminum oxide. Ethyl acetate, acetone/hexane (65/35, v/v) and dichloromethane (10 ml solvent) were tested for their recoveries in the silicon gel test. Table 3 showed that in the experiment of recovery efficiency that used ethyl acetate as elution, higher recoveries of $132 \pm 5.0\%$, $143 \pm 3.8\%$, $101 \pm 4.4\%$, $104 \pm 3.2\%$, $106 \pm 4.9\%$ for DES, E1, E2, EE2, E_v respectively were obtained except for E3. Acetone/hexane (65/35, v/v) could enhance the recovery of E3 to $38.2 \pm$

6.1, but decreased the recoveries of E2, EE2, E_v to $86.1 \pm 2.1\%$, $85.7 \pm 2.5\%$, $83.9 \pm 2.2\%$. Dichloromethane could not provide good recoveries for these estrogens at all. However, as shown in Fig. 2b, the silicon gel could not remove matrix disrupting effectively.

When neutral aluminum oxide was used as sorbent, the acetone/methanol ratios of 3/1 (v/v) and 1/1 (v/v) were tested respectively. Table 4 suggested that acetone/methanol of 3/1 could not elute E3 at all and made a lower recovery for DES (27.7 ± 3.4). When 1/1 acetone/methanol was used as elution, the recovery of E3 could attain $52.6 \pm 1.2\%$ and the recovery of DES, E1, E2, EE2 and E_v were 76.5 ± 2.2 , 118 ± 3.5 , 122 ± 4 , 83.2 ± 3.1 and 93.5 ± 4.1 respectively. As shown in Fig. 2d, neutral aluminum oxide could eliminate the matrix disrupting completely. The lower recoveries might be due to the strong absorption of polar estrogens on aluminum oxide.

Fig. 3 Effect of the reaction temperature on peak area of analytes (hexane used as solvent and reaction half an hour) **Fig. 4** Effect of reaction solvent on peak area of analytes (reaction half an hour at 40°C air bath)

Fig. 5 **a** GC-MS chromatograms of TMS derivatives of analytes in SCAN Mode (injection 1 μ l 1000 ng/ml mixture standard) **b** GC-MS chromatograms of trimethylsilyl (TMS) derivatives of analytes, surrogate and internal standard in SIM Mode (injection 1 μ l 50 ng/ml mixture standard)

Combining the characteristics of silicon gel and neutral aluminum oxide, a cartridge with silicon gel and neutral aluminum oxide 1:1 as sorbent were designed. Effluent of WWTP was used to check the recovery and clarification efficiency. Higher recovery and better matrix elimination were obtained simultaneously when acetone/methanol (1:1) was used to elute cartridge. Less disrupting peaks were found in Fig. 2c comparing with Fig. 2b, which was clarified only with silica

gel. The recovery of DES, E1, E2, EE2, E3 and E_v are 97.5 ± 4.2 , 108 ± 3.6 , 98.2 ± 6.5 , 94.2 ± 2.0 , 88.1 ± 1.6 and 92.6 ± 2.8 .

Optimization of the derivative condition

It was a novel solution by adding trimethylsilylimidaz (TMSI) in BSTFA or MSTFA reagent to deal with the formation of multiple trimethylsilyl derivatives in the derivatization of 17 α -ethinylestradiol

Fig. 5 (continued)

with BSTFA followed by gas chromatography-mass spectrometry determination (Zhou et al. 2007). To obtain higher sensitivity and broader linearity, the effects of temperature and solvent on derivative reaction were examined. In the investigation of the effects of temperature on chromatographic peak area, hexane was chosen as reaction solvent. Figure 3 showed that the peak area of analytes decreased when derivative reaction temperature increased in the range of 40°C to 80°C except for E₂, which was temperature-independent. In the experiments of effects of temperature, the greatest variation of peak area was found for E₁. The ratios of variation of peak area were about 1.36, 2.70, 1.17, 1.55, 1.31, 2.63 for DES, E₁, E₂, EE₂, E₃ and E_v respectively. The peak area of analytes did not change with time after half an hour reaction at 40°C. The reaction

temperature was set to 40°C and the sensitivity was enhanced about two times. The optimal temperature in this study was obviously lower than those that used in previous work with different derivative reagent from 60°C to 100°C (Jeannot et al. 2002; Shareef et al. 2004). Further lower temperature was not suitable for the reaction. A standard with 1,000 ng/ml EE₂ was injected to test whether undesirable TMS derivative would be produced, and it was suggested that no undesirable by-products of EE₂ were traced in SCAN Mode.

At 40°C, different solvent, including hexane, ethyl acetate, dichloromethane, acetone and acetone/hexane (1:1, v/v) was tested on media effects. As shown in Fig. 4, the largest peak area of analyte was achieved when hexane was used as solvent. Ethyl acetate led to the smallest peak area for DES, EE₂ and E₃. Undesirable TMS derivative

Fig. 6 Mass spectra of TMS derivative of **a** di-trimethylsilyl (TMS) diethylstilbestrol (DES), **b** mono-TMS estrone (E1), **c** di-TMS 17 β -estradiol (E2), **d** di-TMS 17 α -ethinylestradiol (EE2), **e** tri-TMS estriol (E3), **f** mono-TMS 17 β -estradiol acetate (E_v)

Fig. 6 (continued)

Table 5 Average recoveries and relative standard (RSD) for 6 estrogens spiked 10 ng/l in surface water and effluent of WWTP ($n = 3$)

Estrogens	Surface water		Effluent of WWTP	
	Mean recoveries (%)	RSD (%)	Mean recoveries (%)	RSD (%)
DES	80.7	4.5	66.6	3.3
E1	111.0	2.4	90.4	4.7
E2	81.3	4.4	85.3	1.5
EE2	107.5	3.7	121.1	2.6
E3	72.6	1.1	87.5	4.3
E _v	90.9	4.6	74.8	2.1

of EE2 was not present in above reactions. Therefore, hexane was selected as reaction solvent.

Method performance

According to the optimized condition, water samples were pre-filtered using 0.8–2.0 μm APFF fiberglass filters to remove particulate matter, and then were spiked surrogate standard (E2- d_3). The pH value was adjusted to 3–4 using 6 mol/l hydrochloric acid or 6 mol/l sodium hydroxide before extraction. The Oasis HLB cartridge was conditioned with 5.0 mL *tert*-butyl methyl ether, 5.0 ml methanol and 5.0 ml DI water respectively. Water sample (4.0 l) was passed through cartridge at a flow rate of 10.0 ml/min. Then cartridge was dried under a gentle stream of nitrogen. The cartridge was then washed by 5.0 ml of methanol/water (25/75, v/v), 5 ml DI water, 5 ml methanol/ammonia/DI water (10/2/88, v/v), then dried under a gentle stream of nitrogen. Subsequently, 10.0 ml of methanol/ *tert*-butyl methyl ether (1/9, v/v) was used to elute SPE cartridge. Elution was gathered by KD concentrator and dehydrated by anhydrous sodium sulfate, then reduced to 0.5 ml under a gentle stream of nitrogen

with 40°C water bath. The concentrated solution was cleaned up using column chromatography. At first, concentrated solution was subjected to a glass column (10 mm i.d.) containing 10 g of 1:1 alumina/silica gel, and 2.0 ml of methanol/acetone (50/50) was used to wash KD concentrator. Transfer the solution to the glass column. Then 10.0 ml methanol/ acetone (1/1, v/v) was used to elute the column. The elution was evaporated to dryness under a gentle stream of nitrogen. The dry residues were redissolved in 100 μl hexane, then 80 μl derivative reagent BSTFA + 1%TMCS + 0.5%TMSI was added and diluted to 200 μl . This mixture solution was reacted in air bath at 40°C for 30 min and then analyzed by GC/MS.

Figure 5 showed the chromatograms of analytes in SCAN and SIM mode. Figure 6 explained the corresponding mass spectra on detail. Good chromatography separation was realized and characteristic ion was obtained. The trimethylsilyl (TMS) derivatives of DES, E1, E2, EE2, E3 and E_v were (a) di-TMS DES, (b) mono-TMS E1, (c) di-TMS E2, (d) di-TMS EE2, (e) tri-TMS E3, (f) mono-TMS E_v. No undesirable TMS derivative of EE2 was found. Instead, two isomers of DES (*cis*-diethylstilbestrol and *trans*-diethylstilbestrol)

Table 6 Calibration curve, limit of detection (LOD) and limit of quantification (LOQ) for six estrogens fortified at 20, 40, 60, 80, 100, 500, 1,000 ng/ml

Compound	Calibration curve	R^2	LOD ^a (ng/l)	LOQ ^b (ng/l)
DES	$y = 4173.5x$	0.9979	0.01	0.03
E1	$y = 1521.6x$	0.9987	0.03	0.09
E2	$y = 1454.0x$	0.9951	0.03	0.09
EE2	$y = 948.5x$	0.9968	0.07	0.21
E3	$y = 558.3x$	0.9981	0.09	0.27
E _v	$y = 343.4x$	0.9981	0.13	0.39

^aValues estimated on a minimal S/N of 3

^bValues estimated on a minimal S/N of 9

Table 7 Range, mean and standard deviation (SD) in surface water and effluent of WWTP (ng/l)

Estrogens	Surface water			Effluent of WWTP		
	Range	Mean	SD	Range	Mean	SD
DES	nd	–	–	nd–0.30	0.15	0.21
E1	1.60–2.00	1.80	0.30	1.90–3.00	2.45	0.78
E2	0.30–0.50	0.40	0.10	nd–2.00	1.00	1.41
EE2	nd–1.60	0.80	1.10	1.90–2.50	2.20	0.42
E3	nd	–	–	0.60–1.90	1.25	0.92
E _v	nd	–	–	nd	–	–

nd No detection

were presented in Fig. 5b, while only the peak of *trans*-DES appeared in Fig. 5a due to very small ratio of *cis*-DES in total DES. Calculating its concentration on the basis of the correlation of total peak area and total concentration, good linearity was observed with correlation coefficients above 0.99 for concentrations from 0 to 1000 ng/ml and a better linearity and instrument detection limit (IDL) were obtained.

The procedure was applied in spiked surface water and effluents of WWTP. Mean recovery ranged from 72.6 to 111.0% with relative standard deviation 1.1–4.6% for spiked surface water, and 66.6% to 121.1% with relative standard deviation 1.5–4.7% for spiked effluent of WWTP. Detailed results were shown in Table 5. The limit of detection (LOD) was similar with the value calculated from instrument detection limit (IDL). LOD of analytes could even attain 0.01 ng/l (Table 6).

The method was applied to determine estrogens concentrations in surface water and wastewater samples. The mean concentrations of E1, E2, and EE2 in surface water ranged from 1.6 to 2.0, 0.3 to 0.5, no detection (nd) to 1.6 ng/l, respectively. No DES, E3 and E_v were detected in surface water. The mean concentrations of DES, E1, E2, EE2 and E3 in effluents of WWTP ranged from nd to 3.0, 1.9 to 3.0, nd to 2.0, 1.9 to 2.5 and 0.6 to 1.9 ng/l respectively. Detail referred to Table 7. Previous study widely reported the presence of steroid estrogens in surface water and effluent of WWTPs. In river water sample, concentration of E1, E2 and EE2 were changed from nd to 74 ng/l in United Kingdom (Fawell et al. 2001), Italy (Baronti et al. 2000) and China (Chen et al. 2007). In effluent of WWTPs, common detected value were nd to 82, nd to 64, nd to 42 and 0.4 to 39.1 ng/l for E1, E2, EE2 and E3 in Netherlands (Vethaak et al. 2005), Italy (Baronti

et al. 2000), U.K. (Desbrow et al. 1998; Johnson and Sumpter 2001), Germany and Canada (Ternes et al. 1999), Sweden (Larsson et al. 1999), Japan (Nakada et al. 2007) and China (Wang et al. 2005; Chen et al. 2007; Sun et al. 2008). Our results in this study were within the range of previous report.

Conclusions

An optimized condition for determining trace estrogen in surface water and wastewater treatment plant effluent had been obtained in the current work. The pH value of water sample was adjusted to 3–4, and salinity was not altered. HLB cartridge was conditioned with 10 mL methanol/ *tert*-butyl methyl ether (1/9, v/v). Extract was clarified using a glass column (10 mm i.d.) containing 10 g of 1:1 alumina/silica gel with 10 mL acetone/methanol elution, then dried under gentle nitrogen. The dried residuals were derivatized by TMSI and BSTFA in hexane solvent at 40°C in air bath, then analyzed by GC/MS.

Correlation coefficient of 0.9951 to 0.9987 was determined. Detection limits were 0.01, 0.03, 0.03, 0.07, 0.09, 0.13 ng L⁻¹ for DES, E1, E2, EE2, E3 and E_v, based on a 4.0-l samples. Mean recovery ranged from 72.6 to 111.0% with relative standard deviation of 1.1–4.6% for surface water spiked, and 66.6% to 121.1% with relative standard deviation of 1.5–4.7% for effluent of WWTP spiked. Therefore, this method provided a robust solution for the determination of trace steroid estrogen in surface water and effluent of WWTP.

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