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Stir bar sorptive extraction with in situ derivatization and thermal desorption–gas chromatography–mass spectrometry in the multi-shot mode for determination of estrogens in river water samples

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Abstract

A novel method for the trace analysis of natural and synthetics estrogens, such as estrone (E1), 17β-estradiol (E2) and 17α-ethynylestradiol (EE), in river water sample was developed, which involved stir bar sorptive extraction (SBSE) with in situ derivatization followed by thermal desorption (TD)–gas chromatography–mass spectrometry (GC–MS). The derivatization conditions with acetic acid anhydride and the SBSE conditions such as sample volume and extraction time were investigated. In addition, the single and multi-shot modes in TD were investigated. The detection limits of E1, E2 and EE in river water sample were 0.2, 0.5 and 1 pg ml^{-1} (ppt), respectively, in the multi-shot mode using five stir bars. The calibration curves for E1, E2 and EE were linear and had correlation coefficients >0.99. The average recoveries of E1, E2 and EE from all sample volumes were higher than 90% (R.S.D. < 10%) with correction using an added surrogate standard such as estrone-$^{13}C_4$, 17β-estradiol-$^{13}C_4$ or 17α-ethynylestradiol-$^{13}C_4$. This simple, accurate, sensitive and selective analytical method may be applicable to the determination of trace amounts of estrogens in water samples.

Keywords: Estrogens; Stir bar sorptive extraction; Derivatization, GC; Water analysis; Thermal desorption

1. Introduction

The detection of endocrine-disrupting chemicals (EDCs) in river water has led to much concern worldwide and increased awareness that animal, and perhaps human, health and function in the entire ecosystems may be adversely affected by the continued release of EDCs into the environment. Some of the most potent EDCs include both natural and synthetic estrogens, which are either produced endogenously by animals or used as pharmaceutical products in both human and veterinary medicine [1]. Compounds of concern include the natural estrogen, 17β-estradiol (E2), its oxidation product, estrone (E1), and the synthetic contraceptive additive, 17α-ethynylestradiol (EE). Although these compounds are degraded biologically, they have been detected in river waters at ppt levels [2–5]. Recent work has shown that although normally only female fish produce vitellogenin, an increase in plasma vitellogenin levels was detected in wild male fish thriving in rivers polluted by EDCs [6–8]. Accordingly, it is highly possible that these compounds may leach into the environment.

Highly reliable methods are required for the detection of trace compounds with estrogenic activity. Enzyme-linked immunosorbent assay (ELISA) has been recently reported to be a sufficiently sensitive technique for the determination of estrogens [9–12]. However, ELISA may give erroneous results due to non-specific binding to the antibody, as evi-

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denced by the overestimation of trace amounts of estrogens [11,12]. Many analytical methods for the determination of estrogens in water samples have been reported, including liquid chromatography (LC) with ultraviolet detection (UV) [13,14], electrochemical detection (ED) [14], fluorescence detection (FD) [15], mass spectrometry (MS) [16,17] and tandem mass spectrometry [18–21]. However, the LC method has low resolution and the sample matrix is frequently affected. On the other hand, gas chromatography–mass spectrometry (GC–MS) was initially used for the determination of estrogens even though derivatization was required [22–26]. The derivatization led to sharper peaks and hence to better separation of and higher sensitivity for the phenols; however, it required much time and effort. In order to overcome this problem, we choose in situ derivatization, which has been developed by various groups and involves the simple addition of a reagent to a liquid sample.

Such analytical procedures as liquid–liquid extraction (LLE) [27] and solid-phase extraction (SPE) [13,15–27] have been developed for the determination of estrogens. However, LLE requires large volumes of organic solvents and additional clean-up steps, and although SPE requires small volumes of organic solvents, the manual version necessary for concentrating large sample volumes still takes 8–10 h. Recently, a new sorptive extraction technique that uses a stir bar coated with 50–300 μl of polydimethylsiloxane (PDMS) was developed [28] and is known as stir bar sorptive extraction (SBSE). The analytical method for the determination of EDCs, such as aldrin, dieldrin, 4,4′-DDE and 4,4′-DDT, has been reported [29]. On the other hand, SBSE with in situ derivatization has been successfully used for the determination of phenolic compounds in various samples [30–34]. We have determined phenolic xenoestrogens in water samples by means of SBSE with in situ derivatization [35]. Moreover, the determination of steroids including estrone in human urine samples by SBSE with in situ derivatization has been reported [36]. However, to our knowledge, the simultaneous analysis of estrogens in an environmental sample by SBSE with in situ derivatization has not been reported so far.

In general, after pretreatment by SBSE, one PDMS-coated stir bar is thermally desorbed in the thermal desorption (TD) system, and this is followed by GC–MS. On the other hand, the simultaneous TD of five stir bars at a maximum can be carried out in the "multi-shot" mode. Therefore, by carrying out simultaneous TD of two or more stir bars, high-sensitivity analysis can be achieved.

The aim of this study was to determine trace amounts of estrogens in river water samples by SBSE with in situ derivatization, followed by TD–GC–MS in the multi-shot mode. The developed method was applied to river water samples.

2. Experimental

2.1. Materials and reagents

Estrone (E1), 17β-estradiol (E2) and 17α-ethynylestradiol (EE) of biological grade were purchased from Wako Pure Chemical Inc. (Osaka, Japan). Estrone-$^{13}C_4$ (E1-$^{13}C_4$), 17β-estradiol-$^{13}C_4$ (E2-$^{13}C_4$) and 17α-ethynylestradiol-$^{13}C_4$ (EE-$^{13}C_4$) surrogate standards were purchased from Hayashi Pure Chemical Inc. (Osaka, Japan). The chemical structures are shown in Fig. 1. Acetic acid anhydride for trace analysis was purchased from Kanto Chemical Inc. (Tokyo, Japan). Other reagents and solvents were of pesticide or analytical grade and purchased from Wako Pure Chemical Inc. (Osaka, Japan). The water purification system used was Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA,

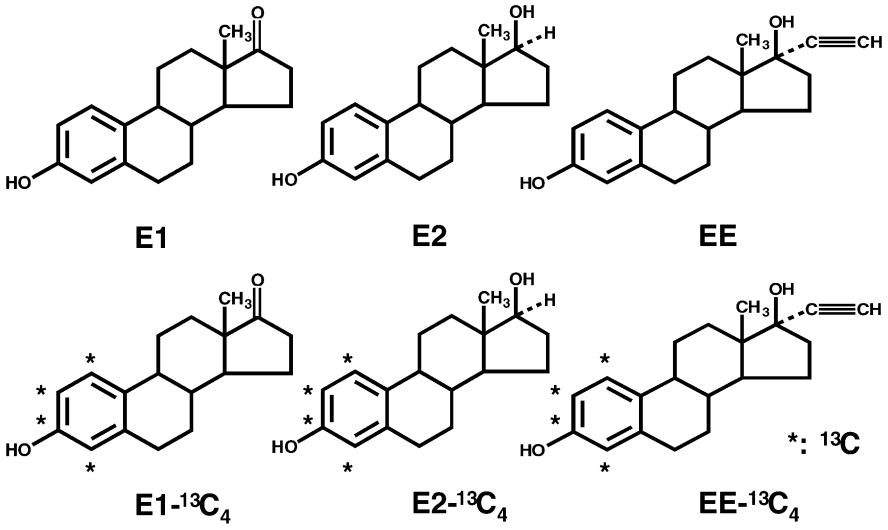

Fig. 1. Chemical structures of E1, E2, EE, E1-$^{13}C_4$, E2-$^{13}C_4$ and EE-$^{13}C_4$.

USA). The EDS polisher was a new filter purchased from Millipore, Japan.

2.2. Standard solutions

Standard solutions (1.0 mg ml^{-1}) of E1, E2 and EE were prepared as required by the addition of purified water. Calibration was performed daily for all samples with a surrogate standard.

2.3. Water samples

River water was sampled from three sites (points A, B and C) at Tama River, Tokyo, Japan. All samples were stored at 4 °C prior to use.

2.4. Instrumentation

Stir bars coated with 500-μm thick (24 μl) PDMS were obtained from Gerstel (Mullheim an der Ruhr, Germany). Prior to use, the stir bars were conditioned for 4 h at 300 °C in a flow of helium. The stir bars could be used more than 100 times with appropriate re-conditioning. For the extraction, 20 and 100 ml headspace vials from Agilent Technologies (Palo Alto, CA, USA) and GL Science (Tokyo, Japan), respectively, were used. TD–GC–MS analysis was performed using a Gerstel TDS 2 thermodesorption system equipped with a Gerstel TDS-A autosampler and a Gerstel CIS 4 programmable temperature vaporization (PTV) inlet (Gerstel), and an Agilent 6890 gas chromatograph with a 5973 mass-selective detector (Agilent Technologies). The TD system can carry out simultaneous TD of five stir bars at a maximum.

2.5. TD–GC–MS conditions

The temperature of TDS 2 was programmed to increase from 20 °C (held for 1 min) to 280 °C (held for 5 min) at a rate of 60 °C min^{-1}. The desorbed compounds were cryofocused in CIS 4 at −150 °C. After desorption, the temperature of CIS 4 was programmed to increase from −150 to 300 °C (held for 10 min) at a rate of 12 °C s^{-1} to facilitate injection of the trapped compounds into the analytical column. Although a blank run of the stir bar was always performed after an analysis, memory effects were never detected. Injection was performed in the splitless mode. Separation was accomplished on a DB-5MS fused silica column (30 m × 0.25 mm i.d., 0.5 μm film thickness, Agilent Technologies). The oven temperature was programmed to increase from 60 to 300 °C (held for 4 min) at a rate of 15 °C min^{-1}. Helium was used as the carrier gas at a flow rate of 1.2 ml min^{-1}. The mass spectrometer was operated in the selected ion-monitoring (SIM) mode with electron impact ionization (ionization voltage: 70 eV). Nine ions were monitored in the SIM mode (*m/z* 270, 271 for E1; *m/z* 272, 213 for E2; *m/z* 296, 338 for EE; *m/z* 274 for E1-$^{13}C_4$; *m/z* 276 for E2-$^{13}C_4$; *m/z* 300 for EE-$^{13}C_4$. The underlined number is the *m/z* of the ion used for quantitation).

In the quantitative procedure, standard solutions of the compounds were prepared by dissolving the compounds in purified water to cover the calibration range. Quantitative analysis was performed in the SIM mode in order to maximize sensitivity. The concentrations were calculated relative to the surrogate standards added to the sample prior to analysis.

2.6. SBSE with in situ derivatization of estrogens from river water samples

River water sample (10 or 50 ml) was placed in a headspace vial with a surrogate standard. To the 10 or 50 ml sample were added sodium carbonate (106 or 530 mg) as the pH adjustment agent (pH 11.5), and acetic acid anhydride (100 or 500 μl) as the derivatization reagent. One stir bar was added to each vial and the vial was crimped with a Teflon-coated silicone septum. SBSE was performed at room temperature for 0 to 5 h while stirring at 500 rpm. After the extraction, the stir bar was easily removed with forceps (due to magnetic attraction), rinsed with purified water and dried with lint-free issue. In the single-shot mode, one stir bar was placed in a glass TD tube. On the other hand, two or more stir bars were placed in a glass TD tube in the multi-shot mode. Then, the TD tube was placed in the TD unit. The stir bar was thermally desorbed in the TD system, and this was followed by GC–MS.

3. Results and discussion

3.1. Derivatization of estrogens

In the mass analysis of standard solutions using electron impact ionization (EI)–MS, *m/z* 270, 272 and 213 were observed as the main peaks of E1, E2 and EE, respectively. For the surrogate standards, E1-$^{13}C_4$, E2-$^{13}C_4$ and EE-$^{13}C_4$; their main peaks were detected at *m/z* 274, 276 and 213, respectively. The main peaks of the standard compound and the surrogate standard were overlapped in the case of EE. Therefore, the monitoring ions of EE and EE-$^{13}C_4$ were set at *m/z* 296 and 300, respectively (Fig. 2). The mass spectrometer was operated in the SIM mode. Nine ions were monitored (*m/z* 270, 271 for E1; *m/z* 272, 213 for E2; *m/z* 296, 338 for EE; *m/z* 274 for E1-$^{13}C_4$; *m/z* 276 for E2-$^{13}C_4$; *m/z* 300 for EE-$^{13}C_4$. The underlined number is the *m/z* of the ion used for quantitation).

3.2. Theoretical recovery of SBSE

Table 1 shows log $K_{o/w}$ and the theoretical recoveries of the compounds investigated in this work. The $K_{o/w}$ values were calculated with the log *P* predictor, which is available from Interactive Analysis Inc. (Bedford, MA, USA). Theoretical recoveries were calculated with the following

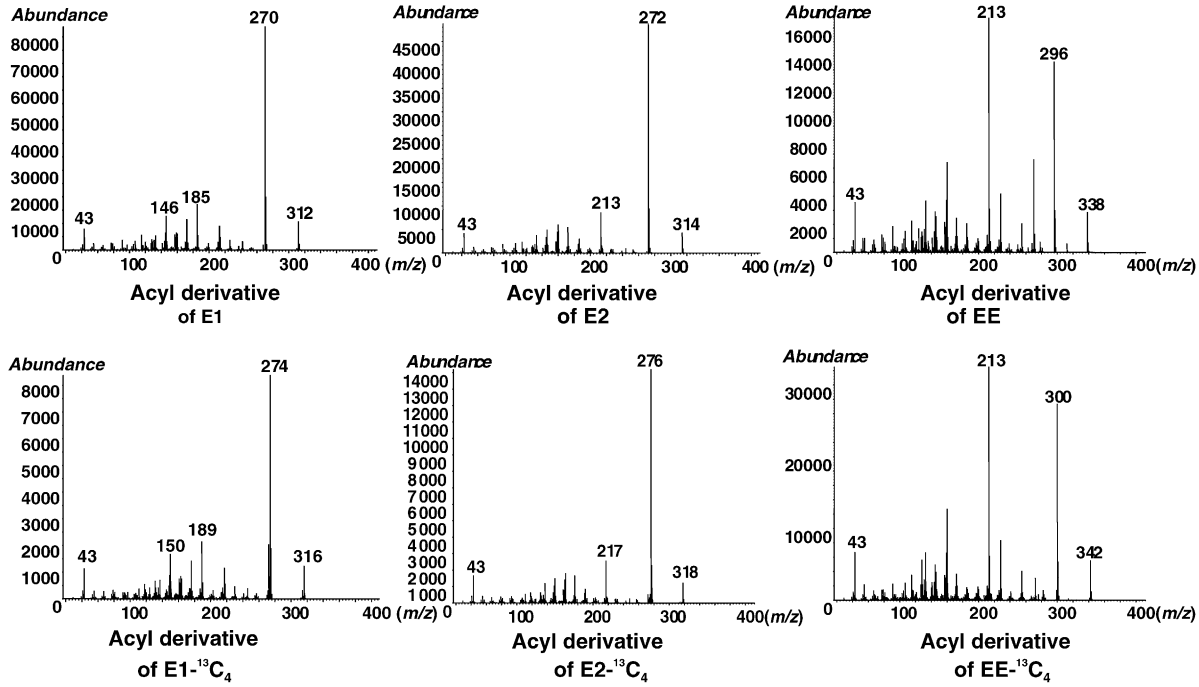

Fig. 2. Mass spectra of acyl derivatives of E1, E2, EE, E1-$^{13}C_4$, E2-$^{13}C_4$ and EE-$^{13}C_4$.

equations:

$$\text{Theoretical recovery} = \frac{K_{o/w}/\beta}{1 + K_{o/w}/\beta} = \frac{1}{\beta/K_{o/w} + 1}$$

where $\beta = V_w/V_{PDMS}$, V_{PDMS} is the volume of PDMS and V_w, the volume of water. The theoretical recoveries of SBSE were calculated based on 10 and 50 ml sample volumes and a stir bar with a 500-um thick PDMS coat (24 μl of PDMS). When the sample volume was increased, the recovery of the estrogens was decreased. However, the recovery of the acyl derivatives of the estrogens did not change markedly even when the sample volume was increased. Moreover, as a result of calculating theoretical recovery from the formula when same sample volume, it turns out that the recoveries of acyl derivative of estrogens were superior to that of estrogens. Therefore, the results showed that the theoretical recoveries of the estrogens were increased by the derivtization.

3.3. Optimum time for SBSE with in situ derivatization

One important parameter affecting SBSE was the extraction time. Moreover, it has been reported that the impact of water/PDMS phase ratio, volume of PDMS, and sampling time on recovery were important [37]. To optimize the extraction time, a 10 ng ml^{-1} standard solution of E1, E2 or EE was used. The extraction time profiles (equilibration curves) of the acyl derivatives of the estrogens in 10 and 50 ml standard solutions using SBSE with in situ derivatization and the estrogens in 10 ml standard solution using SBSE without derivatization were determined by TD–GC–MS, and are shown in Fig. 3. The acyl derivatives of the estrogens in 10 and 50 ml standard solutions using SBSE with in situ derivatization reached equilibrium after approximately 2 and 4 h, respectively. This was proof that the extraction time was increased when sample volume was increased. These condi-

Table 1
log $K_{o/w}$ and theoretical recoveries of estrogens and their acyl derivatives by SBSE

Compound	CAS number	log $K_{o/w}$[a]	Theoretical recovery (%)	
			Sample volume (ml)	
			10	50
Estrone (E1)	[53–16–7]	3.42	86.3	55.8
Acyl derivative of estrone	[901–93–9]	3.94	95.4	80.7
17β-Estradiol (E2)	[50–28–2]	3.50	88.4	60.3
Acyl derivative of 17β-estradiol	[4245–41–4]	4.37	98.3	91.8
17α-Ethynylestradiol (EE)	[57–63–6]	3.31	83.1	49.5
Acyl derivative of 17α-ethynylestradiol	[5779–47–5]	4.38	98.3	92.0

[a] log $K_{o/w}$ values for all compounds as predicted from "the log P predictor" as well as the calculated recoveries.

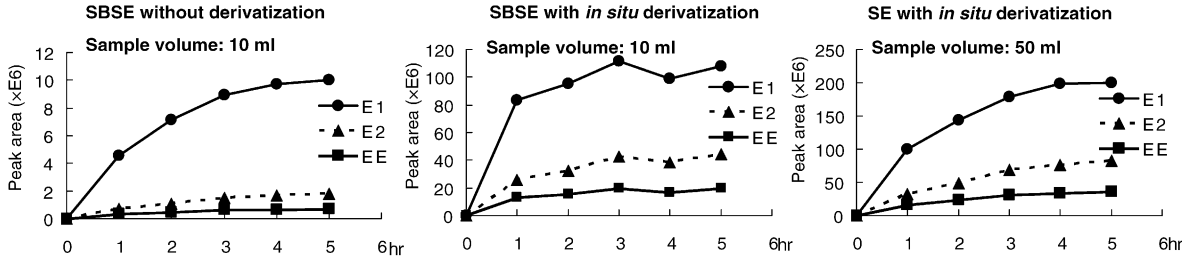

Fig. 3. Extraction time profiles of estrogens in water samples using stir bar. A stir bar coated with polydimethylsiloxane (PDMS) and derivatization reagents were added to 10 ng ml^{-1} standard solution and stirring was commenced for 0–5 h at room temperature (25 °C) in a glass vial. The extract was then analyzed by TD–GC–MS.

tions were therefore used for the determination of estrogens in liquid samples. On the other hand, the estrogens reached equilibrium after approximately 4 h for the case without derivatization.

3.4. Figure of merit of SBSE with in situ derivatization and TD–GC–MS in single and multi-shot modes

When sample volume was 10 ml, the calculated detection limits (LODs) of E1, E2 and EE in river water sample with in situ derivatization were 1, 2 and 5 pg ml^{-1}, respectively, by SBSE–TD–GC–MS when the signal-to-noise ratio (S/N) was 3. On the other hand, the calculated LODs of E1, E2 and EE without the derivatization were 20, 100 and 200 pg ml^{-1}, respectively. In addition, the calculated limits of quantification (LOQs) of E1, E2 and EE when S/N > 10 were 5, 10 and 20 pg ml^{-1} with in situ derivatization, respectively, and 100, 500 and 1000 without the derivatization, respectively. The in situ derivatization method exhibited approximately 20- to 50-fold higher sensitivity than the method without derivatization in all the analytes. The chromatogram of estrogen standard solution (10 ng ml^{-1}) subjected to SBSE with in situ derivatization was compared with that subjected to SBSE without derivatization, and an increase in sensitivity was observed in the former (Fig. 4). Moreover, when sample volume was increased to 50 ml, the LODs of the acyl derivatives of E1, E2 and EE were 0.5, 1 and 2 pg ml^{-1}, respectively, for samples with in situ derivatization. On the other hand, SBSE with in situ derivatization was performed after adding stir bars to each of the approximately 10 ml water samples (10 ng ml^{-1}). The simultaneous TD of the five stir bars was performed in the multi-shot mode. When the number of stir bars was increased, higher peak responses were obtained (Fig. 5). The LODs of E1, E2 and EE were 0.2, 0.5 and 1, respectively, when TD was performed in the multi-shot mode using five stir bars. Therefore, the determination of estrogens in five 10 ml water samples in the multi-shot mode showed higher sensitivity than the determination of estrogens in 50 ml water sample in the single-shot mode. This can be explained in terms of the difference in theoretical recovery. The peak area ratios with respect to each surrogate standard were plotted and the response was found to be linear over the calibration range with correlation coefficients (r^2) higher than 0.99. The figure of merit is summarized in Table 2.

The recovery and precision of the method were assessed by replicate analysis (n = 6) of river water samples spiked with surrogate standards at 0.1 and 1.0 ng ml^{-1}. The non-spiked and spiked samples were analyzed by SBSE with in situ derivatization and TD–GC–MS. The recoveries were calculated by subtracting the results for the non-spiked samples from those for the spiked samples. The results were obtained

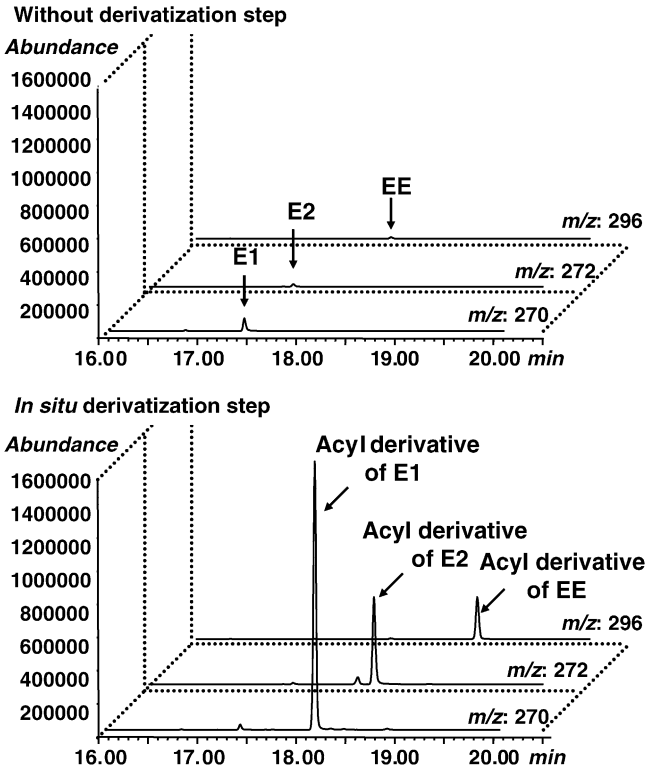

Fig. 4. Comparison of chromatograms of estrogens subjected to SBSE with in situ derivatization with those of estrogens subjected to SBSE without derivatization. For SBSE with in situ derivatization: A PDMS-coated stir bar and derivatization reagents were added to 10 ml of estrogen standard solution (10 ng ml^{-1}) and stirring was commenced for 2 h at room temperature (25 °C) in a glass vial. The extract was then analyzed by TD–GC–MS. For SBSE without derivatization: The same procedure was performed except that no derivatization reagents were added.

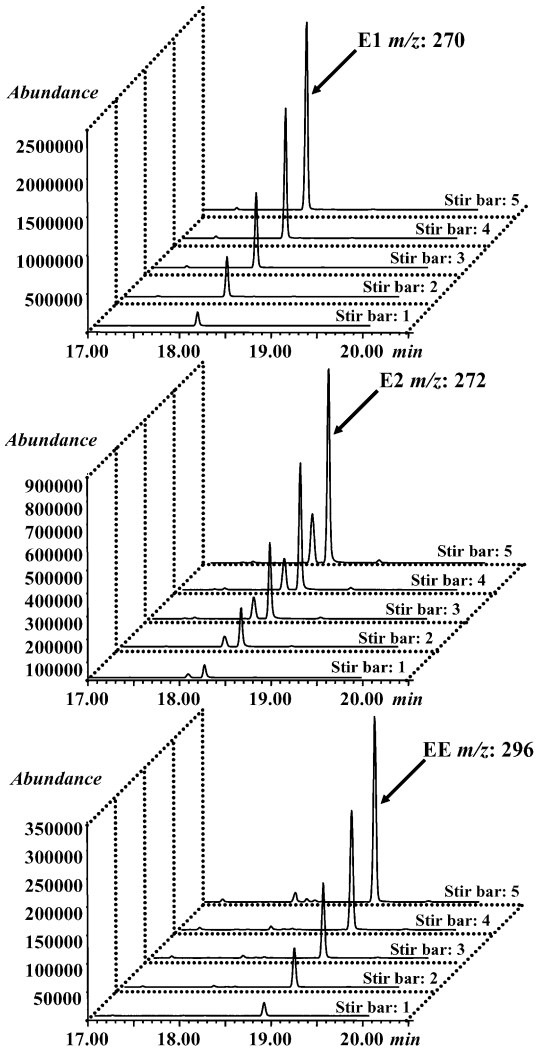

Fig. 5. Chromatograms of acyl derivatives of estrogens (10 ng ml^{-1}) subjected to SBSE with in situ derivatization and TD–GC–MS in the single or multi-shot mode.

Table 3
Recoveries of estrogens in spiked river water samples

Compound	Sample volume (ml)	Amount spiked			
		0.1 ng ml^{-1}		1.0 ng ml^{-1}	
		Recovery (%)	R.S.D. (%)[a]	Recovery (%)	R.S.D. (%)[a]
E1	10	101.5	3.5	98.5	3.6
	50	99.4	2.6	97.3	3.6
E2	10	90.3	2.8	95.7	3.9
	50	103.3	2.9	101.7	4.5
EE	10	105.7	1.3	97.2	2.9
	50	101.9	3.4	105.1	9.3

[a] The recoveries and precision were also examined by replicate analysis (n = 6) of river water samples.

Table 4
Concentrations of estrogens in river water samples

Compound	Tama river (pg ml^{-1})		
	A	B	C
E1	8.7	13.6	19.7
E2	5.3	4.8	4.2
EE	N.D.[a]	N.D.	N.D.

[a] N.D. indicates E1, E2 and EE concentrations lower than 1, 2 and 5 pg ml^{-1}, respectively.

by using calibration curves of the standard solutions with surrogate standards. The recoveries corrected by surrogate standards were equal to or higher than 90% (R.S.D. < 10%) for all river water samples (Table 3). Therefore, the method is applicable to the precise determination of trace amounts of estrogens in river water samples.

3.5. Application of the analytical method

We measured the concentrations of estrogens in three water samples collected from Tama River in the multi-shot mode using five stir bars, and the results are shown in Table 4. Typical chromatograms of the river water samples (point C) are

Table 2
Figure of merit of SBSE and TD–GC–MS

Compound	SBSE method	Mode	Stir bar	Sample volume (ml)	LOD[a] (pg ml^{-1})	LOQ[b] (pg ml^{-1})	Correlation coefficient (r^2)
E1	Without derivatization	Single-shot	1	10	20	100	0.999 (100–10000)[c]
	In situ derivatization	Single-shot	1	10	1	5	0.999 (5–10000)
	In situ derivatization	Single-shot	1	50	0.5	2	0.999 (2–10000)
	In situ derivatization	Multi-shot	5	10 × 5	0.2	1	0.999 (1–10000)
E2	Without derivatization	Single-shot	1	10	100	500	0.999 (500–10000)
	In situ derivatization	Single-shot	1	10	2	10	0.999 (10–10000)
	In situ derivatization	Single-shot	1	50	1	5	0.999 (5-10000)
	In situ derivatization	Multi-shot	5	10 × 5	0.5	2	0.999 (2–10000)
EE	Without derivatization	Single-shot	1	10	200	1000	0.999 (1000–10000)
	In situ derivatization	Single-shot	1	10	5	20	0.999 (20–10000)
	In situ derivatization	Single-shot	1	50	2	10	0.999 (10–10000)
	In situ derivatization	Multi-shot	5	10 × 5	1	5	0.999 (5–10000)

[a] LOD: limit of detection (S/N = 3).
[b] LOQ: limit of quantification (S/N > 10).
[c] Values in parentheses are the linear ranges of the calibration curves (pg ml^{-1}).

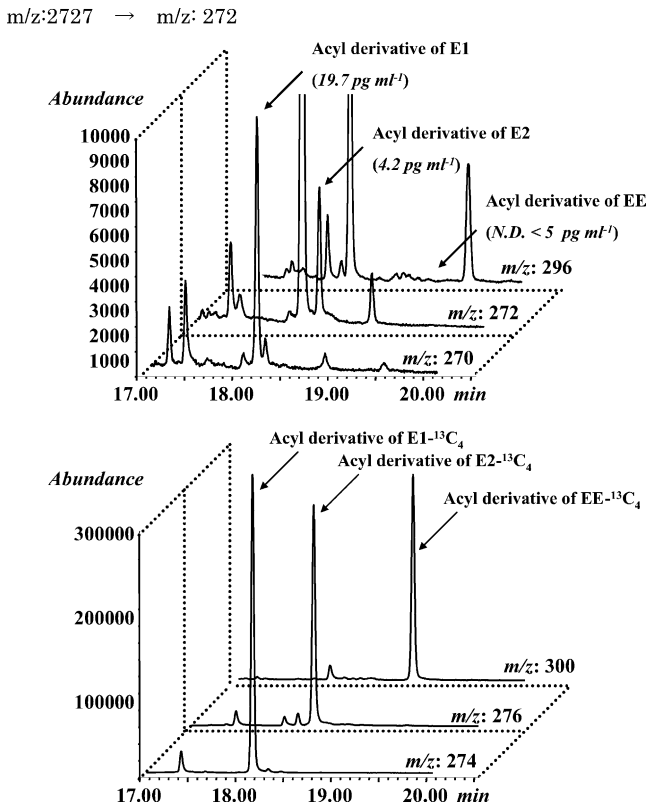

Fig. 6. Chromatograms of acyl derivatives of estrogens in river water samples (point C). Five PDMS-coated stir bars, surrogate standards and derivatization reagents were added to each of the five 10 ml river water samples and stirring was performed for 2 h at room temperature (25 °C) in a glass vial. Then, the five stir bars were simultaneously analyzed by TD–GC–MS.

shown in Fig. 6. E1 and E2 in the river water samples were detected. In addition, the concentration of E1 was found to be higher than that of E2.

One group has reported morphological abnormalities in milt in approximately 30% of the male carp population downstream of sewage treatment plants in Tama River, Japan, during the period from 1997 to 1998 [38]. Subsequently, elevated levels of vitellogenin were observed in the male carp [39]. Therefore, this river is a good study site for assessing estrogenic activity in the aquatic environment in Japan. In addition, the determination of estrogens in Tama River water samples by LC–MS with off-line SPE was conducted [40]. According to previous reports, the concentrations were N.D. (<0.2 pg ml^{-1}) to 27 pg ml^{-1} for E1 and N.D. (<10 pg ml^{-1}) for E2. However, the SPE method required a large sample volume (4–20 l) for realizing high sensitivity. In the present study, the combined use of SBSE with in situ derivatization and TD–GC–MS in the multi-shot mode enabled the successful determination of trace amounts of estrogens in a small volume of water sample (50 ml).

4. Conclusions

The determination of trace amounts of estrogens in water samples using SBSE with in situ derivatization followed by TD–GC–MS in the single or multi-shot mode was investigated. The proposed method has many practical advantages such as a small sample volume (10 or 50 ml) and simplicity of extraction. It is also solvent-free and has high sensitivity. The LODs of E1, E2 and EE were 0.2, 0.5 and 1 pg ml^{-1}, respectively, in the multi-shot mode using five PDMS-coated stir bars. In addition, the LOQs were 1, 2 and 5 pg ml^{-1}, respectively. The average recoveries were between 90.3 and 105.7% with acceptable precision (R.S.D. 1.3–9.3%) for river water samples spiked with these compounds at concentrations of 0.1 and 1.0 ng ml^{-1} and corrected by isotopically labeled surrogate standards. This simple, accurate and highly sensitive method is expected to have potential applications in various water samples.

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