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Fully automated analysis of estrogens in environmental waters by in-tube solid-phase microextraction coupled with liquid chromatography–tandem mass spectrometry

K. Mitani, M. Fujioka, H. Kataoka *

School of pharmacy, Shujitsu University, Nishigawara, Okayama 703-8516, Japan

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Abstract

A simple, rapid and sensitive method for the determination of five estrogens, estrone, 17β-estradiol, estriol, ethynyl estradiol, and diethylstilbestrol, was developed using a fully automated method consisting of in-tube solid-phase microextraction (SPME) coupled with liquid chromatography–tandem mass spectrometry (LC/MS/MS). These estrogens were separated within 8 min by HPLC using an XDB-C8 column and 0.01% ammonia/acetonitrile (60/40, v/v) at a flow rate of 0.2 mL/min. Electrospray ionization conditions in the negative ion mode were optimized for MS/MS detection of the estrogens. The optimum in-tube SPME conditions were 20 draw/eject cycles of 40 μL of sample using a Supel-Q PLOT capillary column as an extraction device. The extracted compounds were easily desorbed from the capillary by passage of the mobile phase, and no carryover was observed. Using the in-tube SPME LC/MS/MS method, good linearity of the calibration curve ($r \geq 0.9996$) was obtained in the concentration range from 10 to 200 pg/mL for all compounds examined. The limits of detection ($S/N = 3$) of the five estrogens examined ranged from 2.7 to 11.7 pg/mL. The in-tube SPME method showed 34–90-fold higher sensitivity than the direct injection method (5 μL injection). This method was applied successfully to the analysis of environmental water samples without any other pretreatment and interference peaks. Several surface water and wastewater samples were collected from the area around Asahi River, and estriol was detected at 35.7 pg/mL in the effluent of a sewage treatment plant. The recoveries of estrogens spiked into river waters were above 86%, except for estriol, and the relative standard deviations were below 0.9–8.8%.

Keywords: In-tube solid-phase microextraction; Automated sample preparation; Liquid chromatography–tandem mass spectrometry; Estrogens; Environmental water samples

1. Introduction

Endocrine-disrupting chemicals (EDCs) are known as industrial and environmental contaminants, and have found in the waters of many rivers, lakes, seas, etc. EDCs interfere with the function of the endocrine systems of both wildlife and humans [1]. It was reported that these compounds affect ecosystems, e.g., feminization of wild fishes living downstream from wastewater effluent [2,3]. EDCs detected in environmental waters are derived from factory effluent, wastewater from treatment plants, and residential wastewater. Some environmental estrogens have a natural origin. 17 β-Estradiol (E2) and its main metabolites, estriol (E3) and estrone (E1), along with their conjugates (mainly sulfates and glucuronides) are naturally present at higher levels in females than in males. Synthetic estrogens, such as the potent estrogen ethynyl estradiol (EE2), are used extensively for contraceptive and therapeutic purposes (management of menopausal syndrome and in a wide range of cancers, mainly prostate and breast cancer) [4,5]. Diethylstilbestrol (DES) has also been used extensively in estrogenic hormone therapy in the prevention of miscarriage in humans and as a growth promoter in livestock [6]. However, its use for promoting growth is controversial and it has been banned in some countries. These natural and synthetic estrogens can-

* Corresponding author. Tel.: +81 862517943; fax: +81 862942974.
E-mail address: hkataoka@shujitsu.ac.jp (H. Kataoka).

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not be removed completely in wastewater treatment plants, and are discharged into environmental waters where they can reach concentrations at the nanogram per liter level [7–10]. It is not yet known whether estrogens in water may have adverse biological effects in wildlife or humans at this level because of the presence of many other pollutants with estrogen activity in the aquatic medium. A sensitive, selective, and simple method to monitor these estrogens in water is therefore required.

Analysis of estrogens has been carried out mainly by enzyme-linked immunosorbent assay (ELISA) [11] and gas chromatography/mass spectrometry (GC/MS) or gas chromatography/tandem mass spectrometry (GC/MS/MS) [11–19]. ELISA is simple and sensitive, but the results using ELISA kits tend to overestimate the concentrations, because of the influence of coexisting materials. GC/MS and GC/MS/MS are also highly sensitive methods, but time-consuming sample pretreatment and derivatization steps are required prior to the analysis. In most cases, extraction and pre-concentration steps are required for analysis of environmental samples, because the analysis of these compounds is influenced by the complexity of the environmental matrix. Solid-phase extraction (SPE) [12–17], solid-phase microextraction (SPME) [18], and stir bar sorptive extraction (SBSE) [19] are used as sample preparation techniques for GC/MS or GC/MS/MS analysis. These extraction techniques are also described in detail in some reviews [20,21].

Liquid chromatography/mass spectrometry (LC/MS) [22–24] and liquid chromatography–tandem mass spectrometry (LC/MS/MS) [25,26] are now widely used as sensitive methods instead of GC/MS. LC/MS and LC/MS/MS have some benefits over GC/MS for analysis of estrogens in environmental water. LC/MS and LC/MS/MS can be coupled with on-line devices for sample preparation and preconcentration techniques, such as SPE, and estrogens can be analyzed without derivatization. LC/MS and LC/MS/MS analyses of estrogens are usually carried out with an electrospray ionization (ESI) interface operated in the negative ion mode for detection and quantification, because the sensitivity is considerably better than that in the ESI positive ion mode. Recently, Barcelo et al. [27] reported a novel, fully automated LC/MS/MS method based on on-line SPE, with which the analysis of estrogens in water samples could be completed within 60 min per sample.

Off-line and on-line SPE techniques have been used for extraction and concentration of estrogens in environmental water samples. However, most of these techniques require large sample volumes. The in-tube SPME technique [28], using an open tubular fused-silica capillary with an inner surface coating as the SPME device, is simple and can be easily coupled on-line with HPLC and LC/MS. In-tube SPME allows convenient automation of the extraction process, which not only reduces the analysis time, but also provides better accuracy, precision, and sensitivity than off-line manual techniques. We have developed the in-tube SPME method for determination of various compounds, such as drugs and EDCs, by coupling with HPLC [29–32] and LC/MS [33–36]. The details of the in-tube SPME technique and its applications have also been summarized in a number of reviews [37–42]. In the present study, we developed a fully automated on-line in-tube SPME LC/MS/MS method for the determination of estrogens in environmental water to achieve high throughput analysis. Target estrogens included the natural estrogens, E1, E2, and E3, as well as the synthetic estrogens, EE2 and DES. Although these estrogens are excreted primarily in conjugated forms, the free forms were analyzed in the present study, because conjugated estrogens are expected to be relatively short-lived in the environment [9,43]. Our method was also applied to the determination of estrogens in several environmental water samples.

2. Experimental

2.1. Materials

Fig. 1 shows the structures of the five estrogens examined in the present study. Estrone (E1), 17 β-estradiol (E2), estriol (E3), ethynyl estradiol (EE2), and diethylstilbestrol (DES) were purchased from Sigma-Aldrich (Saint Quentin Fallaviers, France). Each compound was dissolved in methanol to make a stock solution at a concentration of 1 mg/mL. The solutions were stored at 4 °C and diluted to the required concentrations with pure water prior to use. Acetonitrile and water used as mobile phases were of HPLC grade, and were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade.

2.2. Sample collection and preparation

Surface water samples were collected in amber glass bottles, and filtered through 0.2-μm nylon syringe filters 13 mm in diameter (Tosoh, Tokyo, Japan), if necessary. Sampling was performed at six sites at Asahi River and Sasagase River in Okayama City. The samples were stored in the dark at 4 °C and then analyzed within 48 h. One-millilitre aliquots of each sample were transferred into 2-mL screw-cap autosampler vials equipped with silicon/PTFE septa, and the vials were set onto the sample tray in the autosampler.

2.3. Instrument and analytical conditions

The HPLC system was a Model 1100 series (Agilent Technologies, Boeblingen, Germany), which consisted of a binary pump, an on-line-degasser, an autosampler, a column compartment, a diode array detector, and an HP ChemStation. An XDB-C_8 column (50 mm × 2.1 mm, particle size of 5 μm; Agilent Technologies) was used for HPLC separation. Chromatography was performed by isocratic reverse phase separation with 0.01% ammonia/acetonitrile (60/40, v/v) at a flow-rate of 0.2 mL/min.

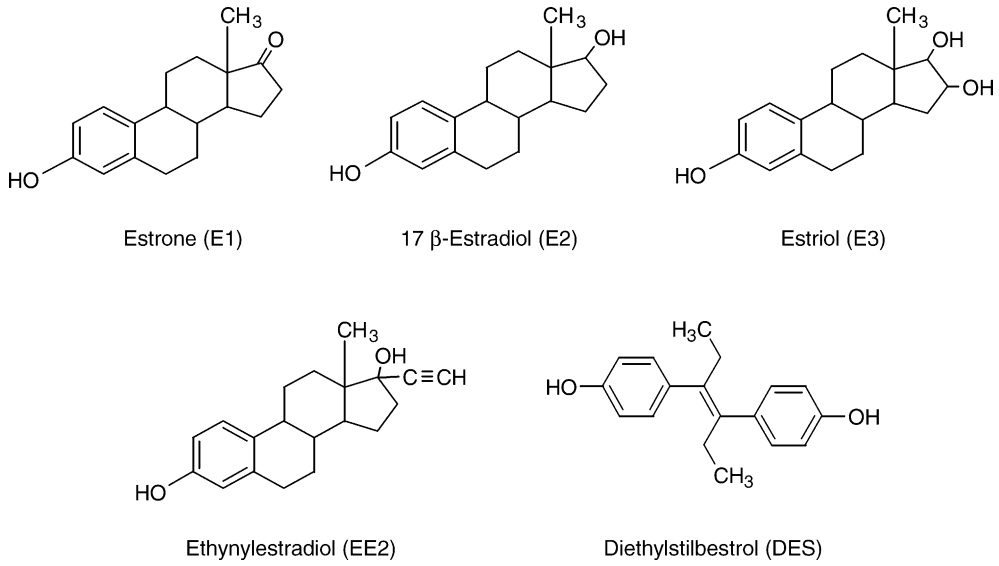

Fig. 1. Chemical structures of natural and synthetic estrogens used in this study.

The MS/MS system was an API 4000 triple quadruple mass spectrometer (Applied Biosystems, Foster City, CA, USA), equipped with a turbo ion spray interface. The turbo ion spray interface was operated in the negative ion mode at 5500 V and 600 °C. Nitrogen as the nebulizing and drying gas was generated from compressed air using a Kaken N_2 generator (System Instruments Co., Ltd., Tokyo, Japan). The ion sources gas 1 (GS1) and 2 (GS2) were set at 50 and 80 L/min, respectively. The curtain gas (CUR) flow was set at 40 L/min and the collision gas (CAD) at 4.0 L/min. Quantification was performed by multiple reaction monitoring (MRM) of the deprotonated precursor molecular ions $[M-H]^-$ and the related product ion for estrogens. Quadrupoles Q1 and Q3 were set on unit resolution. MRM in the negative ion mode was performed using a dwell time of 200 ms per transition to detect ion pairs. Table 1 shows the optimized MS/MS conditions for each compound. LC/MS/MS data were processed by Analyst Software 1.3.1 (Applied Biosystems, Foster City, CA, USA).

2.4. In-tube solid-phase microextraction

As shown in Fig. 2, a Supel-Q porous layer open tubular (PLOT) capillary column (60 cm × 0.32 mm i.d., film thickness of 12 μm; Supelco, Bellefonte, PA, USA) was used as the in-tube SPME device. The column was placed between the injection loop and injection needle of the autosampler. The injection loop was retained in the system to avoid fouling of the metering pump. Capillary connections were facilitated by the use of a 2.5-cm sleeve of 1/16-in. polyetheretherketone (PEEK) tubing at each end of the capillary (1 in. = 2.54 cm). PEEK tubing with an internal diameter of 330 μm was suitable to accommodate the capillary used. Normal 1/16-in. stainless steel nuts, ferrules, and connectors were used to complete the connections. The autosampler software was programmed to control the in-tube SPME extraction, desorption, and injection. Vials (2 mL) were filled with 1 mL of sample for extraction, and set into the autosampler programmed to control SPME extraction and desorption. In addition, 2-mL autosampler vials with a septum, one containing 1.5 mL of methanol and another containing 1.5 mL of water were set into the autosampler. The capillary column was washed and conditioned by two repeated draw/eject cycles (40 μL each) of these solvents, and then a 50-μL air plug was drawn prior to the extraction step. The extraction of five estrogens onto the capillary coating was performed by 20 repeated draw/eject cycles of 40 μL of sample at a flow rate of 100 μL/min with the six-port valve in the LOAD position (Fig. 2A). After

Table 1
Optimum conditions for MS/MS (ESI negative ion mode) analysis of estrogens

	MW	*m/z*		DP (V)	EP (V)	CE (V)	CXP (V)
		Q1 mass	Q3 mass				
E3	288	287.156	171.050	105	10	52	13
E2	272	270.995	145.000	140	10	54	9
EE2	296	295.056	145.050	140	10	58	13
E1	270	269.004	145.150	115	10	52	11
DES	268	267.009	237.050	105	10	40	19

DP: declustering potential, EP: entrance potential, CE: collision energy, CXP: collision cell exit potential.

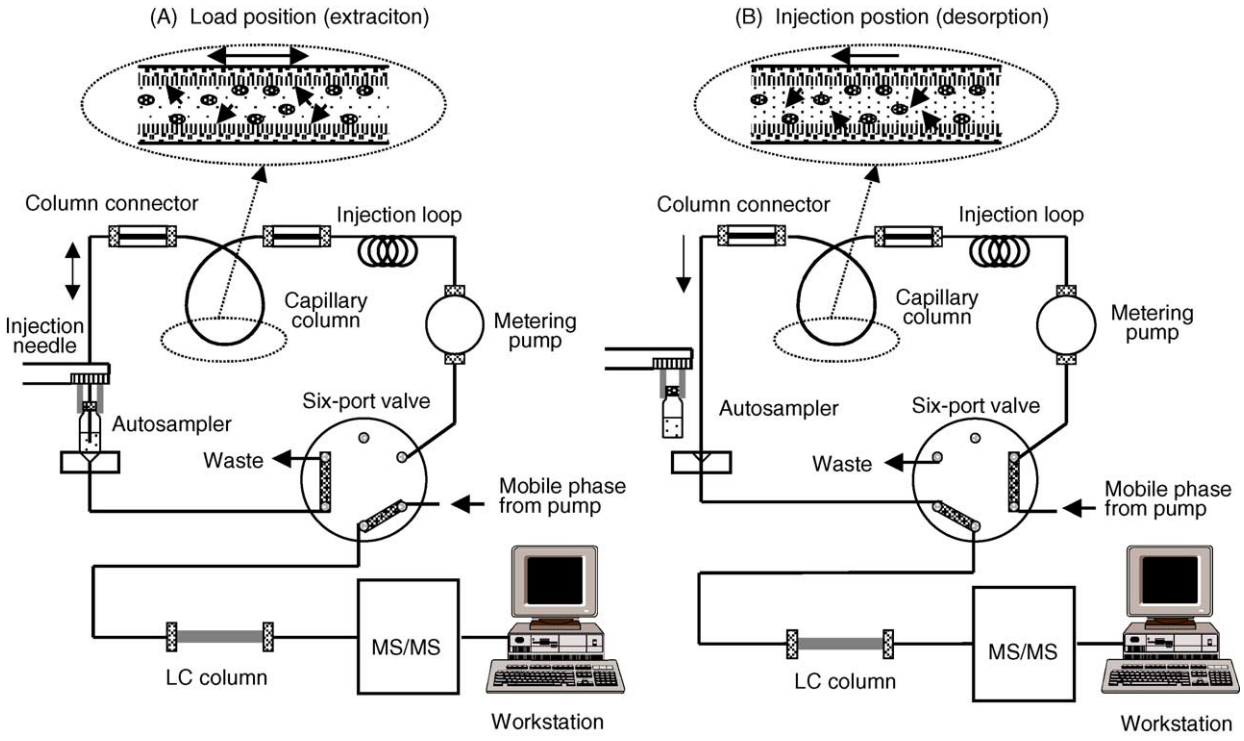

Fig. 2. Schematic diagram of the on-line in-tube SPME/LC/MS/MS system.

washing the tip of the injection needle by one draw/eject cycle of 2 μL of methanol, the extracted compounds were desorbed from the capillary coating and transported to the LC column by switching the six-port valve to the INJECT position (Fig. 2B), and detected by the MS/MS system. During the analysis, the Supel-Q capillary was washed and conditioned with mobile phase for the next extraction.

3. Results and discussion

3.1. LC/MS/MS analysis of estrogens

For the MS/MS operation, APCI and ESI were evaluated for determination of estrogens in both positive and negative ion modes. The ESI negative ion mode was most effective for the ionization of these estrogens. A solution of 100 ng/mL in 50% methanol containing 1% acetic acid infused at a flow rate of 5 μL/min produced a signal of appropriate size in the ESI negative ion mode for the deprotonated precursor ion $[M-H]^-$ using turbo ion spray. Parameters, including nebulizer gas stream, curtain gas, and ion spray voltage, were optimized by flow injection analysis (FIA) with a mobile phase flow of 0.2 mL/min. Tuning was processed using the automatic tuning tool of the Analyst software to determine the declustering and focusing potentials, fragmentation pattern, collision energy, and collision cell exit potential. Table 1 shows the optimum MS/MS conditions of estrogens. The most abundant fragments were separated sufficiently by Q3 in unit resolution for quantification. To determine the optimal composition, different mobile phases consisting of acetonitrile–ammonia were tested. The best signals were achieved using 0.01% ammonia/acetonitrile (60/40, v/v). LC separation of the five estrogens examined here was performed using an XDB-C_8 column. The development of the chromatographic system focused on short retention times and co-elution of estrogens, paying attention to matrix effects as well as good peak shapes. A high proportion of organic solvent was used to co-elute each substance. Thus, an increased flow rate of 0.2 mL/min produced a good peak shape and made a runtime of 8 min possible (Fig. 3).

3.2. Optimization of in-tube solid-phase microextraction and desorption

To optimize the extraction of estrogens by in-tube SPME, several parameters such as the stationary phase of the in-tube SPME capillary column and number and volume of draw/eject cycles were investigated. In the present study, in-tube SPME conditions were optimized with standard solution (100 ng/mL of each) using a UV detector (200 nm). Four different capillary columns (Omegawax 250, DB-17, DB-1, and Supel-Q PLOT) were evaluated for extraction efficiency. As shown in Fig. 4, the extraction efficiency of the porous polymer-type capillary column (Supel-Q PLOT) was better than that of the liquid-phase type capillary columns (Omegawax 250, DB-17, and DB-1). As the Supel-Q PLOT column has a large adsorption surface area, the extracted

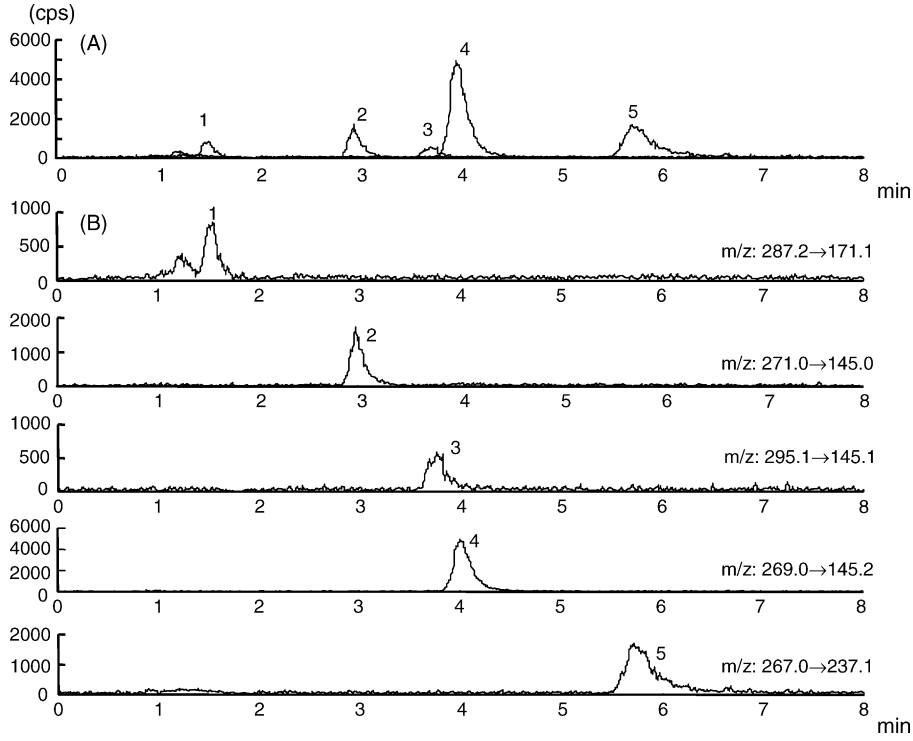

Fig. 3. Chromatograms obtained from 50 pg/mL standard estrogens by in-tube SPME LC/MS/MS in negative ion mode. (A) Total ion chromatogram. (B) MRM chromatograms. Peaks: (1) = E3, (2) = E2, (3) = EE2, (4) = E1, (5) = DES. LC/MS/MS conditions: see Section 2.

amount was greater than that with liquid-phase type columns. The sample load and the amounts of compounds extracted increased with increasing internal diameter and film thickness of the column. However, the area of contact of the liquid phase with the sample under constant draw/eject conditions was found to have a greater effect on extraction efficiency than the film thickness of the column.

In in-tube SPME, the extraction time, flow-rate, and sample pH are related to the amounts of extracted compounds. To monitor the extraction time profiles of estrogens by in-tube SPME, the number of draw/eject cycles was varied from 0 to 20 using a Supel-Q PLOT capillary column. As shown in Fig. 5, extraction equilibrium of these compounds was not obtained with 20 cycles. However, analysis could be performed reproducibly because the extraction conditions were fixed using an autosampler. A draw/eject rate of 100 μL/min was optimal for extraction. Below this level, extraction required an inconveniently long time, while above this level, bubbles forming inside the capillary reduced the extraction efficiency. The sample matrix pH was shown to have no effect on the extraction of estrogens by in-tube SPME. The absolute amounts of five estrogens extracted by the SPME capillary column were calculated by comparing peak area counts with the corresponding direct injection of the sample solution onto the LC column. At sample concentrations of 10 ng/mL, 3.5 ng (34.5%) of E1, 3.7 ng (36.8%) of E2, 1.3 ng (12.6%) of E3, 3.9 ng (38.6%) of EE2, and 4.7 ng (46.7%) of DES were extracted onto the Supel-Q PLOT column by in-tube SPME. Although the extraction yields of these compounds were low, their reproducibility was good (RSD < 10%) due to the use of an autosampler.

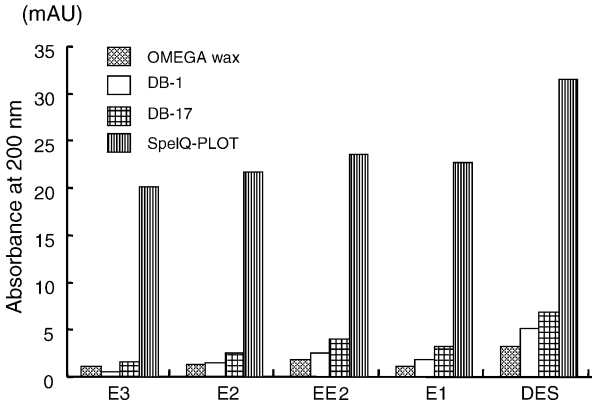

Fig. 4. Effects of capillary coatings on the in-tube SPME of estrogens. Each compound was extracted by 20 draw/eject cycles of 40 μL of standard solution (50 ng/mL of each) at a flow rate of 100 μL/min.

The mobile phase was found to be suitable for desorption of estrogens extracted into the stationary phase of a capillary column. Dynamic desorption of these compounds from the capillary was readily achieved by switching the six-port valve (Fig. 2B). The desorbed estrogens were transported to the LC column by mobile phase flow. No carryover was observed because the capillary column was washed and conditioned by draw/eject cycles of methanol and mobile-phase prior

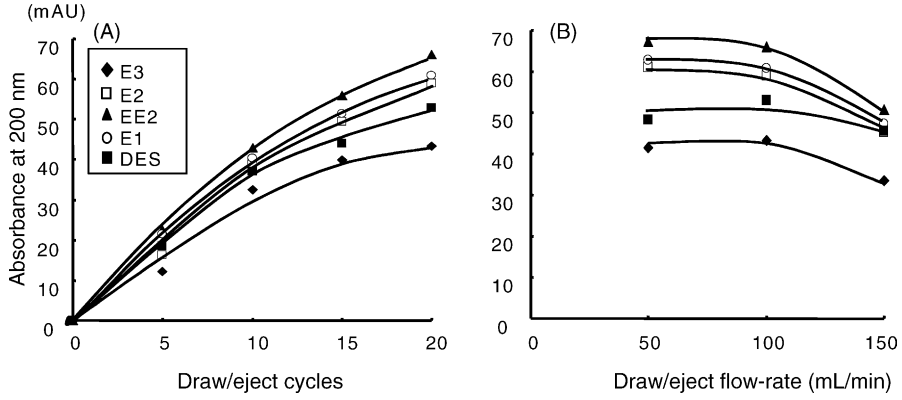

Fig. 5. Effects of (A) draw/eject cycle and (B) flow rate on the in-tube SPME of estrogens. Each compound was extracted by draw/eject of 40 μL of standard solution (100 ng/mL of each) using a Supel-Q PLOT capillary.

Table 2
Linear regression data and detection limits of estrogens by in-tube SPME/LC/MS/MS

Compound	Range (pg/mL)	Regression line		Correlation coefficient (r)[a]	Detection limits (pg/mL)	
		Slope	Intercept		Direct injection (5 μL)	In-tube SPME
E3	10–200	15.3	−20.8	0.9996	395	11.7
E2	10–200	30.5	−80.4	0.9997	394	7.4
EE2	10–200	11.9	−40.4	0.9996	941	10.5
E1	10–200	115.3	−329.6	0.9997	203	2.7
DES	10–200	42.2	−53.5	0.9999	370	6.9

[a] $n = 15$.

to extraction. The extraction and desorption of estrogens by the in-tube SPME method were accomplished automatically within 30 min, and automated analysis of about 48 samples per day was possible by overnight operation.

3.3. Limits of detection and calibration curves

The limits of detection of the five estrogens examined ranged from 2.7 to 11.7 pg/mL (Table 2), with signal-to-noise ratios of 3:1 under our LC/MS/MS conditions. The in-tube SPME method showed 34–90-fold higher sensitivity than the direct injection method (5 μL injection), because these compounds in the sample solution were concentrated in the capillary column during draw/eject cycles. To test the linearity of the calibration curve, various concentrations of the five estrogens ranging from 10 to 200 pg/mL were analyzed. Calibration curves were constructed from the peak area counts. As shown in Table 2, a linear relationship was obtained for each compound in this range (five-point calibration). The correlation coefficients ranged from 0.9996 to 0.9999, and relative standard deviations were 0.7–8.8% ($n = 3$).

3.4. Application to the analysis of environmental waters

Several environmental water samples were analyzed. Estrogens were detected in only one sample, which was collected downstream from wastewater effluents. In this sample, E3 was detected at 35.7 pg/mL (Fig. 6). To confirm the validity of this method, known amounts of five estrogens were spiked into river water, and their recoveries were calculated. As shown in Table 3, the overall recoveries of these compounds were above 86%, and the relative standard deviations were below 0.9–8.8%.

Table 3
Recoveries of estrogens spiked to environmental water

Compound	Spiked (pg/mL)	Recovery (%) Mean ± SD[a]	Spiked (pg/mL)	Recovery (%) Mean ± SD[a]
E3	10	86.1 ± 1.8	50	87.1 ± 4.9
E2	10	100.1 ± 8.8	50	94.8 ± 2.2
EE2	10	86.6 ± 0.8	50	100.8 ± 2.8
E1	10	106.8 ± 5.1	50	106.8 ± 4.1
DES	10	87.1 ± 3.4	50	96.0 ± 4.8

[a] $n = 3$.

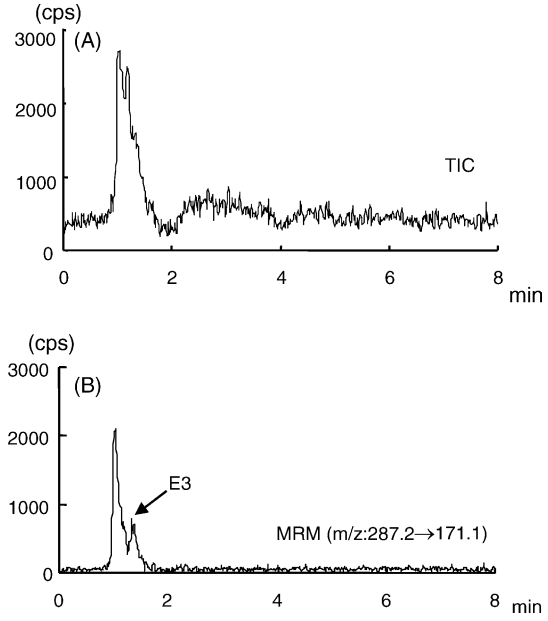

Fig. 6. Total ion and MRM chromatograms obtained from river water by in-tube SPME LC/MS/MS. (A) Total ion chromatogram. (B) MRM chromatograms.

4. Conclusions

The on-line in-tube SPME/LC/MS/MS method developed in the present study can continuously perform extraction of five estrogens from aqueous samples without any other pretreatment, which can then be analyzed by LC/MS/MS. This method is fully automated, simple, rapid, selective, and sensitive, and can be applied easily to the analysis of environmental waters. We believe that this method is a very useful tool for the monitoring and determination of estrogens in environmental water.

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