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Determination of estrogens and their conjugates in water using solid-phase extraction followed by liquid chromatography–tandem mass spectrometry

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Abstract

An analytical procedure for the determination of steroid estrogens and their conjugates was developed and applied to aqueous environmental samples. The analytes of 15 compounds were solid-phase extracted and fractionated into two fractions: one containing unconjugated (free) steroids and the other containing conjugates (sulfates and glucuronides). Identification and quantification were carried out using liquid chromatography coupled with tandem mass spectrometry. The recoveries for each compound ranged from 57 to 116% and reproducibilities represented as RSD ranged from 2.9 to 17%. Some of the sulfates and free compounds were detected in environmental samples, whereas most of the conjugates were below the detection limits.

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Keywords: Water analysis; Steroids; Estrogens; Endocrine disrupters

1. Introduction

Regarding the issue of environmental endocrine disrupting chemicals, elucidation of the distribution and behaviour of endogenous and exogenous steroidal hormones in the aquatic environment is very important. In the general aquatic environment, it has been revealed that endogenous estrogens such as 17β-estradiol (β-E2) and estrone (E1) dominate estrogenic activity evaluated by screening methods, e.g. estrogen receptor binding assay or reporter gene assay [1]. For the purpose of conducting a comprehensive survey, enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) has been used for determination of endogenous estrogens in the aquatic environment, due to its relatively simple protocol and high sensitivity [2–7]. Some analytical methods for endogenous β-E2 and E1 and exogenous ethynylestradiol (EE2) using GC–EI-MS or GC–NCI-MS following derivatization have already been reported [8–13]. Although high sensitivity and selectivity were achieved by these methods, the use of GC

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limits the target compounds to unconjugated (i.e. free) estrogens. Recently, a number of studies have been reported [10,14–20] on analytical methods for steroid hormones, as well as their related compounds including conjugates, in biological and environmental samples using LC with various detectors. Although conjugates of estrogen are suspected to be transformed to unconjugated compounds in the aquatic environment, there are only a few reports on the environmental distribution and behavior of estrogen conjugates. Belfroid et al. attempted to detect glucuronides using GC–MS after enzymatic deconjugation, but almost no analyte was present at concentrations above the limits of detection which ranged from 0.1 to 2.4 ng/l [21]. In the present study, a simple and rapid analytical method for determination of 15 compounds of steroid estrogens and their sulfates and glucuronides was developed using liquid chromatography–tandem mass spectrometry (LC–MS–MS). In the course of development of the method, we optimized the analytical parameters of LC–MS–MS detection and the conditions of solid-phase extraction from water samples. The developed method was applied to environmental water samples to reveal the distribution of steroidal estrogens and their conjugates in the aquatic environment.

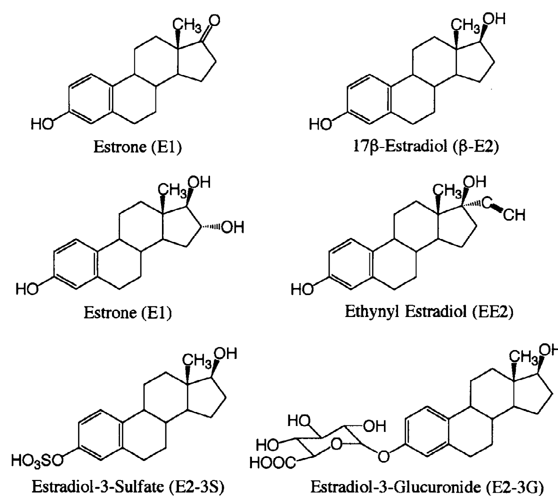

Fig. 1. Structure of steroid estrogens and conjugates.

2. Experimental

2.1. Reagents and chemicals

The chemical structures of some of the analytes appear in Fig. 1. Estrone (min. 98%), 17β-estradiol (97–103%), 17α-estradiol (min. 97%), estriol (min. 98%), and ethynylestradiol (min. 98%) were purchased from Wako Pure Chemicals (Tokyo, Japan). Estrone-β-D-glucuronide, estrone-3-sulfate, 17β-estradiol-3-(β-D-glucuronide), 17β-estradiol-17-(β-D-glucuronide), 17β-estradiol-3-sulfate (approx. 95%), estradiol-3-glucuronide-17-sulfate, estradiol-3-sulfate-17-glucuronide (min. 95%), estradiol-3,17-disulfate (approx. 95%), estriol-3-(β-D-glucuronide), and estriol-3-sulfate were purchased from Sigma–Aldrich (Tokyo, Japan). Estrone-2,4,16,16-d_4 (min. 98%), 17β-estradiol-16,16,17-d_3 (min. 98%), sodium estrone-2,4,16,16-d_4-3-sulfate (min. 99%), and sodium 17β-estradiol-2,4,16,16-d_4-3-sulfate (min. 99%) were obtained from C/D/N Isotopes (Quebec, Canada). Abbreviations of the target compounds in this study are shown in Table 1. All the solvents, methanol (MeOH), ethyl acetate, acetonitrile, dichloromethane (DCM), acetone, and *n*-hexane, used in this study were of LC grade or residual pesticide grade from Wako (Tokyo, Japan). Triethylamine (TEA) was obtained from Nacalai Tesque (Osaka, Japan). Water was purified using Milli-Q system (Millipore, Tokyo, Japan). Two different SPE (solid-phase extraction) cartridges of Autoprep EDS-1 (500 mg) and Oasis HLB (250 mg) were obtained from Showa Denko K.K. (Kawasaki, Japan) and Waters (Tokyo, Japan), respectively. A Florisil mini column, Varian Bond Elut FL (500 mg), was obtained from Varian (Tokyo, Japan).

2.2. Sample collection

Environmental water samples were collected at three locations (no. 1–3) from the middle reaches of the Tamagawa River (Tokyo, Japan) in August 2001 and at four locations (st.7, 8, 10, 13, and 14) from the shore of Lake Kasumigaura (Ibaraki, Japan) in July 2001 using a stainless steel bucket. Effluent samples from two sewage treatment plants were sampled in August (STP-1, Tokyo) and October

Table 1
MRM settings

Compound	Abbreviation	MRM ions	Cone (V)	Collision (V)	IDL (pg)
Estrone-3-sulfate	E1-3S	349>269	70	40	0.3
Estrone-3-glucuronide	E1-3G	445>269	40	40	0.3
Estradiol-3-sulfate	E2-3S	351>271	70	35	0.3
Estradiol-3-glucuronide	E2-3G	447>271	40	35	0.7
Estradiol-17-glucuronide	E2-17G	447>271	40	35	1.5
Estradiol-3-glucuronide-17-sulfate	E2-3G17S	263>253	40	30	1.9
Estradiol-3-sulfate-17-glucuronide	E2-3S17G	263>271	35	30	0.6
Estradiol-3,17-disulfate	E2-3,17DiS	215>97	35	35	0.9
Estriol-3-sulfate	E3-3S	367>287	35	30	0.1
Estriol-3-glucuronide	E3-3G	463>287	40	40	0.6
Estrone	E1	269>145	80	40	0.1
17α-Estradiol	α-E2	271>145	80	40	0.3
17β-Estradiol	β-E2	271>145	80	40	0.6
Estriol	E3	287>171	80	35	2.4
Ethynyl estradiol	EE2	295>145	80	40	0.5
Estrone-d_4-3-sulfate[a]	E1-d_4-3S	353>273	70	40	
Estradiol-d_4-3-sulfate[a]	E2-d_4-3S	355>275	70	35	
Estrone-d_4^a	E1-d_4	273>147	80	40	
Estradiol-d_3^a	E2-d_3	274>145	80	40	

[a] Internal standard.

(STP-2, Ibaraki) 2001. STP-1 discharges into the Tamagawa River and STP-2 discharges into Lake Kasumigaura. These STPs serve populations of 0.6 and 0.1 million, respectively. Mechanical settling followed by activated sludge treatment is carried out in both STPs. The water samples were stored in amber glass bottles and transported with cooling materials.

2.3. Analytical procedure

The scheme for the analytical procedure developed in this study is shown in Fig. 2. One liter of the river water, lake water and STP effluent was filtered with GF/C glass fiber filters of 1.2 μm nominal pore size (Whatman, Tokyo). The filtrate was acidified with 1 ml of acetic acid to pH 3.5–5.0. The acidified filtrate spiked with 1 ng (100 μl of 10 ng/ml) of internal standards (E1-d_4, E2-d_3, E1-d_4-3S, and E2-d_4-3S) was subjected to an Autoprep EDS-1 cartridge using Aqua Trace Automatic SPE system (GL Science, Tokyo). The EDS-1 cartridge was conditioned with 10 ml of MeOH and 10 ml of Milli-Q water prior to use. The flow-rate was set at

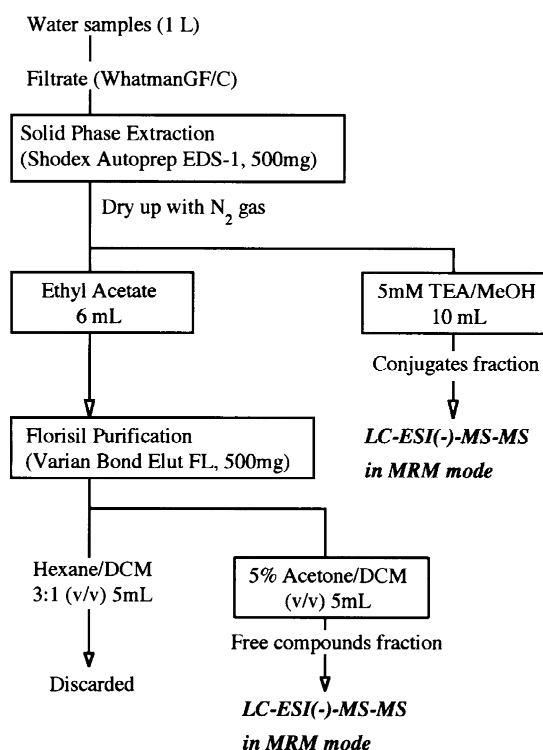

Fig. 2. The analytical procedure for estradiol and metabolites.

20 ml/min. The SPE cartridge was completely dried under vacuum for 1 h before elution to prevent incomplete fractionation. The analytes were eluted sequentially with 6 ml of ethyl acetate as free (unconjugated) compound fraction and 10 ml of 5 m*M* TEA/MeOH as conjugate fraction. The fraction of free compounds was evaporated to dryness under a gentle stream of nitrogen gas prior to purification using a Florisil column. The sample residue of free compound fraction was redissolved in 1 ml of hexane/DCM (3:1, v/v) and subjected to Florisil column conditioned with 5 ml of hexane. Five milliliters in total of hexane/DCM (3:1, v/v) was discarded, and the following 5 ml of 5% acetone/DCM (v/v) eluate was collected. Both fractions of the free compounds and conjugates were evaporated by nitrogen gas, dissolved in 100 μl–1 ml of 5% acetonitrile/H_2O (v/v) and analyzed by LC–MS–MS.

2.4. Instrumental analysis

Identification and quantification of analytes were carried out using a CapLC (Waters, USA) liquid chromatograph equipped with a Quattro Ultima (Micromass, UK) tandem mass spectrometer. The Zorbax Extend-C_{18} column (150 mm×1 mm I.D., 3.5 μm, Agilent) used for LC separation has tolerance to alkaline mobile phase. The column oven was kept at 30 °C, flow-rate was 40 μl/min, and injection volume was 10 μl. The solvents (A) acetonitrile, (B) H_2O, and (C) 100 m*M* TEA in H_2O (pH 12.2) were used as mobile phase and the gradient conditions were as follows: (A) 0%, (B) 80%, (C) 20% for initial, (A) 40%, (B) 40%, (C) 20% at 12 min, (A) 80%, (B) 0%, (C) 20% at 15 min and hold for 2 min. The mass spectrometer was operated in negative mode electrospray ionization (ESI(−)) in Multiple Reaction Monitoring (MRM) mode. The conditions for the mass spectrometer were as follows: cone gas flow: 70 l/h; desolvation gas flow: 500 l/h; ionization: negative mode electrospray; capillary voltage: 2.7 kV; multiplier voltage: 650 V. MRM monitor ions and cone and collision energies in MS–MS analysis optimized for each compound are referred to in Table 1.

3. Results and discussion

3.1. Optimizing LC–MS–MS condition

First, we examined optimal LC and MS–MS conditions for analysis of steroid estrogens and conjugates. It has been reported that sensitivities of unconjugated steroids are better when analyzed in negative mode rather than in positive mode using both atmospheric pressure chemical ionization and electrospray ionization LC–MS [22]. We found that use of the mobile phase alkalized by triethylamine (TEA) resulted in increase in the sensitivity of LC-ESI(−)-MS–MS determination of steroid estrogens and metabolites. On the other hand, the absolute abundance decreased significantly when acetate buffer, which is generally applied for a reversed-phase LC, was used as mobile phase. Four commercially available alkaline-resistant reversed-phase LC columns, TRP-100 (150 mm×1 mm I.D., 5 μm, Supelco), Asahipak ODP-40 (150 mm×1 mm I.D., 4 μm, Showa Denko K.K.), Zorbax Extend-C_{18} (150 mm×1 mm I.D., 3.5 μm, Agilent), and XTerraMS C_{18} (150 mm×1 mm I.D., 3.5 μm, Waters), were examined for response and resolution. As a result, in the case of estrogens and their conjugates analysis, Zorbax Extend-C_{18} and XTerraMS C_{18} seemed to be more appropriate for chromatographic separation of the analytes and more resistant against TEA than the other two columns. Because the retention time of α-E2 was sometimes too close to that of E1 using XTerra MS C_{18}, Zorbax Extend-C_{18} was used for sample analysis. Thus, the LC conditions, which can analyze estrogens and their conjugates, were established.

3.2. SPE extraction

Secondly, the condition of solid-phase extraction (SPE) was optimized. After examining several commercial SPE columns for trapping estrogens and conjugates from waters, we found that Autoprep EDS-1 (Showa Denko) and Oasis HLB (Waters) were the best among others such as ODS, PS2, and Polyamide to extract all of the analytes (data not shown). Both Autoprep EDS-1 and Oasis HLB are packed with polymer, which has both lipophilic and

hydrophilic functions as sorbent. To acidify the sample water, acetic acid was spiked in water samples. To improve the efficiency of elution from the cartridge, 5 m*M* of TEA was added to MeOH as an ion pair reagent. Without ion pair reagents, conjugates were not eluted effectively from the SPE cartridge by MeOH. When 1 μg of each analyte was spiked to 10 ml of purified water (100 ng/ml), subjected to the SPE cartridge, and eluted from the cartridge with 10 ml of 5 m*M* TEA/MeOH, the recoveries were higher than 80% (data not shown) for all compounds. Both of the SPE cartridges may be applicable for determination of estrogens and conjugates. Table 2 indicates the recoveries of 10 ng of standard stock solution spiked to 1 liter of the filtrate of river water (10 ng/l). The recovery tests were examined using EDS-1, and the arithmetical mean and standard deviation were calculated from the results of four replicate analysis. All the calculated recoveries were higher than 70%. When river water samples were analyzed by this method, however, the chromatographic peak, especially of β-E2, was often interfered by unknown compounds (data not shown). To solve this problem, fractionation of the analytes was examined using SPE column. EDS-1 could fractionate the extract into two fractions: free compound (unconjugated) fraction containing E1, β-E2, α-E2, and EE2 eluted with 6 ml of ethyl acetate, and conjugate fraction containing sulfates, glucuronides and E3 eluted with the following 10 ml of 5 m*M* TEA/MeOH. The recovery of the analytes, which contained 1 μg of standard in 10 ml of purified water (100 ng/ml), is shown in Table 2. The analytes were solid-phase extracted from water sample using EDS-1 and fractionated to free and conjugate fractions. The free compound fraction was subjected to a further purification step, Florisil column chromatography, using a Bond Elut FL column.

Table 2
Recovery of SPE using EDS-1 ($n=4$) and fractionation using EDS-1 ($n=1$)

	Average recovery (%) of SPE[a]	Recovery (%) of fractionation[b]	
		AcOEt fr.	MeOH fr.
E1-3S	90	1	103
E1-3G	100	2	126
E2-3S	95	1	92
E2-3G	98	0	136
E2-17G	70	0	102
E2-3G17S	115	11	98
E2-3S17G	120	0	79
E2-3,17DiS	96	1	87
E3-3S	93	1	94
E3-3G	87	3	102
E1	82	102	5
α-E2	106	98	9
β-E2	82	105	17
E3	77	35	72
EE2	94	87	5

[a] Ten nanograms of std was spiked to 1 liter of water sample taken from Lake Kasumigaura (RSD, 3–10%).
[b] One microgram of std was spiked to 10 ml of Milli-Q water.

3.3. Calibration curve, accuracy and recovery

The chromatograms of standard solutions containing 1 pg/μl of each compound using Zorbax Extend-C_{18} are shown in Fig. 3(a–b). The calibration curves for the determination of the analytes were obtained by performing a linear regression analysis on the standard solution using the ratio of standard area to internal standard area (E1-d_4 for E1, E2-d_3 for β-E2, α-E2, and EE2, E1-d_4-3S for E1-3S and E1-3G, E2-d_4-3S for E2-3S, E2-3G, E2-17G, E2-3S17G, E2-3G17S, E2-3,17DiS). The linearity of the calibration curves that were obtained from analysis of 0.5–100 pg/μl of each analyte were high and all r^2-values were higher than 0.99. The instrumental detection limits (IDL) were 0.1–2.4 pg as injected amounts, which was estimated at a signal-to-noise (S/N) ratio of 3 (Table 1). The recoveries of the analytes through the method were evaluated by spiking 10 ng of each compound to 1 liter of Milli-Q water and the filtrates of the water sample taken from Lake Kasumigaura (st. 4). The reproducibilities of this method represented as relative standard deviation (RSD) ranged from 3 to 17% when standard spiked Milli-Q water was analyzed ($n=4$). As shown in Table 3, the recoveries of four replicate analysis for each compound in Milli-Q water were higher than 75%. The recoveries of three replicate analysis for each compound spiked to the water from Lake Kasumigaura ranged from 57 to 116%. Although the recoveries of E3-3S and E2-3S17G were 57 and

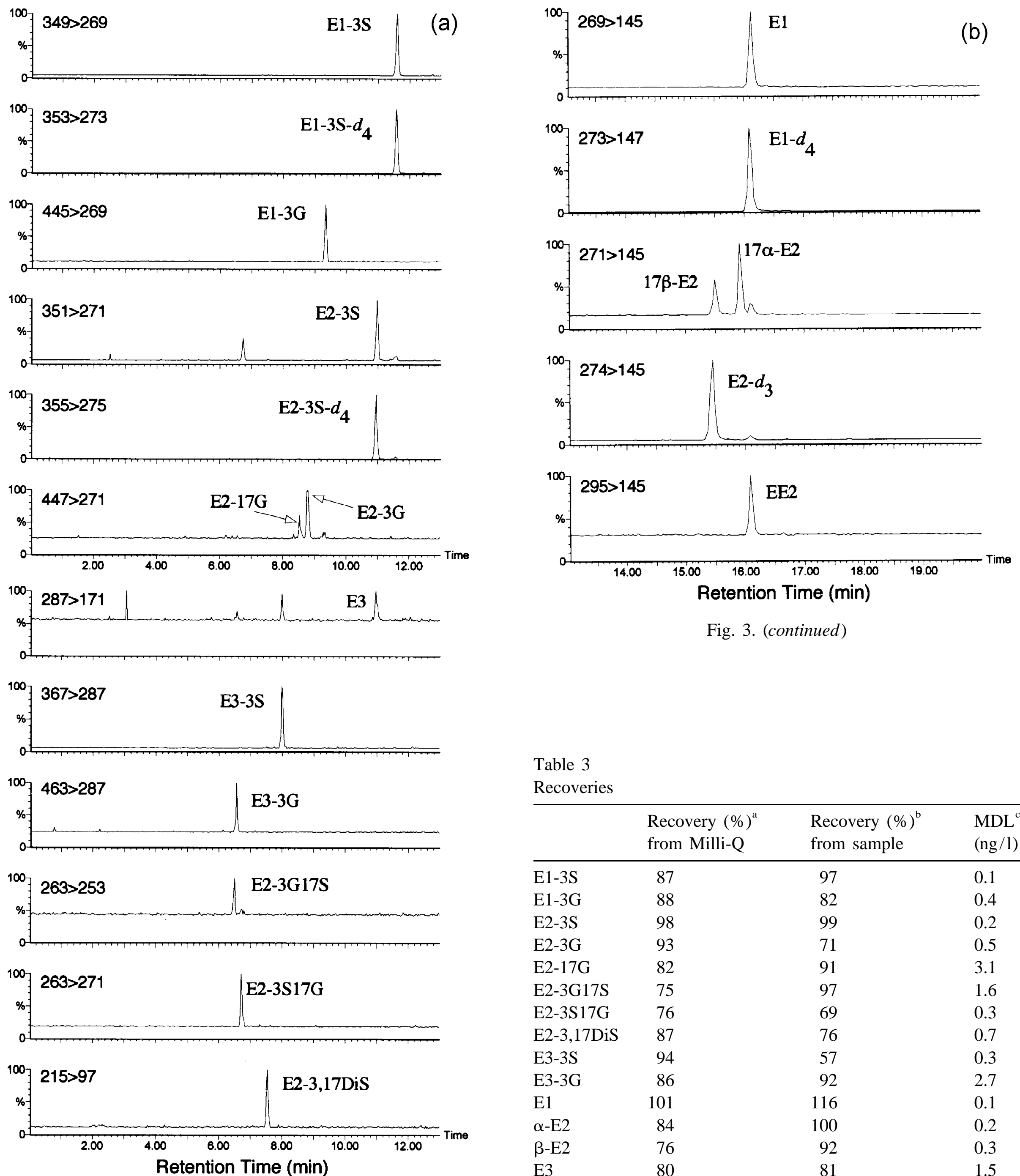

Fig. 3. (a) MRM chromatograms of E1-3S, E1-3S-d_4, E1-3G, E2-3S, E2-3S-d_4, E2-3G, E2-17G E3, E3-3S, E3-3G, E2-3G17S, E2-3S17G, and E2-3,17DiS (10-μl injection of 1 pg/μl solution using Zorbax Extend-C_{18}); (b) MRM chromatograms of E1, E1-d_4, 17a-E2, 17b-E2, and E2-d_4, EE2 (10-μl injection of 1 pg/μl solution using Zorbax Extend-C_{18}).

Fig. 3. (*continued*)

Table 3
Recoveries

	Recovery (%)[a] from Milli-Q	Recovery (%)[b] from sample	MDL[c] (ng/l)
E1-3S	87	97	0.1
E1-3G	88	82	0.4
E2-3S	98	99	0.2
E2-3G	93	71	0.5
E2-17G	82	91	3.1
E2-3G17S	75	97	1.6
E2-3S17G	76	69	0.3
E2-3,17DiS	87	76	0.7
E3-3S	94	57	0.3
E3-3G	86	92	2.7
E1	101	116	0.1
α-E2	84	100	0.2
β-E2	76	92	0.3
E3	80	81	1.5
EE2	82	90	0.2

[a] Ten nanograms of std was spiked to 1 liter of Milli-Q water ($n=4$; RSD, 3–17%).

[b] Ten nanograms of std was spiked to 1 liter of water sample taken from Lake Kasumigaura ($n=3$, RSD, 2–14%).

[c] Method detection limits.

69%, it could be assumed that reliable quantification was achieved because relatively low RSD (5 and 10%) was observed. In addition, the recoveries determined on Milli-Q water were sometimes lower than that on sample water. Although the direct cause of this phenomenon was unclear, it was partly due to the increase in background resulting from sample matrices. We defined the method detection limit (MDL) as the concentrations corresponding to those of a S/N ratio of 3 on the chromatogram of actual sample matrices. MDL, which was calculated from the above recovery experiment using lake water, is shown in Table 3. The developed method requires neither time-consuming nor complicated sample treatment steps such as liquid–liquid extraction or derivatization, and allows rapid, selective and sensitive analysis of estradiol and its metabolites in the environmental water.

3.4. Application to environmental samples

The developed analytical method was applied to some environmental waters. One nanogram of internal standards (i.e. 100 μl of 10 pg/μl) was spiked to the filtrates and solid-phase extracted as described above. The analytes were fractionated by EDS-1. The fraction of free compounds was purified with a Bond Elut FL. The concentrations of the analytes significantly detected in environmental water samples are summarized in Table 4.

In STP effluents, E1, E2, E1-3S and E2-3S were detected while all of the glucuronides were below the detection limits. Some previous reports concluded glucuronides dominated in biological samples [11,14,23]. This is consistent with the well-known pathway of the deconjugation of glucuronides during the wastewater treatment process and consequent generation of the free compounds. The trend that the glucuronides were not detectable while the sulfates and free compounds were detected was also observed in the river and lake water. The result that no detectable glucuronides were found in the environmental water is consistent with the previous result reported by Belfroid et al. [21]. E1 was the most abundant, ranging from 0.2 to 6.6 ng/l, in river and lake water. The concentrations of β-E2 in the Tamagawa River and Lake Kasumigaura, from <0.3 to 1.0 ng/l, was in the same range as that reported by Japan Ministry of the Environment or Ministry of Land, Infrastructure and Transport in other aquatic environments in Japan [24,25]. EE2, which has higher estrogenic potency, was not detected in our survey. This may be explained by the fact that the use of EE2 is not common in Japan because it had been illegal until September 1999. Almost no detectable EE2 was found in Japan [24,25], whereas it was detected in other countries [26].

Most of the targeted estrogens and conjugates in this study are probably degraded during the sewage treatment process and residual sulfates and free estrogens are discharged into the aquatic environment. Since the ranges of the sulfate concentrations were at the same level as or an order of magnitude lower than that of the free compounds, their potential contribution to estrogenic activities in the aquatic environment seems to be negligible. However, further studies on degradation rates and pathways of the conjugates are necessary because they are expected to be transformed to the free compounds. Analysis of the other conjugates that were not analyzed in this study (e.g. E3-16G) is also important.

Table 4
The concentrations of estrogens and conjugates in the environmental samples

	STP effluent (ng/l)		Tamagawa River (ng/l)			Lake Kasumigaura (ng/l)				
	STP-1	STP-2	no.1	no.2	no.3	st. 7	st. 8	st. 10	st. 13	st. 14
E1-3S	2.2	0.3	0.3	0.9	0.8	0.3	0.3	0.4	0.8	0.8
E2-3S	1.0	–	0.2	0.7	0.8	–	–	0.3	0.2	0.4
E1	34	2.5	3.4	3.8	6.6	0.7	0.2	0.6	0.8	0.8
β-E2	2.5	0.3	0.8	0.6	1.0	–	–	–	–	–

–, below the detection limit (MDLs are shown in Table 3).

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