Analysis and occurrence of estrogen in wastewater in Japan

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Abstract This paper describes an analytical procedure for free estrogens and their conjugates in domestic wastewater. The procedure demonstrated in this study is innovative in terms of levels of detection and quantification of the following substances: estrone (E1); 17β-estradiol (E2); 17α-ethynylestradiol (EE2); estriol (E3); estrone-3-sulfate (E1-S); β-estradiol 3-sulfate (E2-S); estriol 3-sulfate (E3-S); estrone β-D-glucuronide (E1-G); β-estradiol 17-(β-D)-glucuronide (E2-G); estriol 3-(β-D)-glucuronide (E3-G); β-estradiol 3-sulfate 17-glucuronide (E2-S&G); and estradiol 3,17-disulfate (E2-diS). The detection limits of this method ranged from 0.1 to 1.4 ng/l. The recovery efficiencies of the estrogens in the analysis from influent and effluent of the secondary settling tank in a wastewater treatment plant (WWTP) were higher than 94% for the free estrogens, but were less than 50% for the conjugated estrogens. The field study using this method was conducted at twenty WWTPs in Japan. The median concentrations of the estrogens ranged from ND to as high as >100 ng/l. In the influent and secondary effluent samples, the concentrations of E1, E2 and E3 were the same levels as those previously reported. We found that the conjugated estrogens exist at higher concentrations in the influent and the secondary effluent than in the other studies, and that the concentrations of the conjugated estrogens were higher than those of the free estrogens.
Keywords Endocrine disrupters; 17β-estradiol; estrogen; LC/MS/MS; wastewater; water analysis

Introduction

In recent years a new problem has emerged in our water environment, namely, endocrine disruptors (EDs) that may adversely affect the reproductive functions of human beings and wildlife. In Japan the EDs issue has arisen since the book *Our Stolen Future* (Colborn *et al*., 1996) was introduced in 1997. Contamination of water with EDs poses new and potential environmental (and social) problems.

The Japan Environmental Agency (JEA) published strategic programs on environmental endocrine disruptors (SPEED'98), in which basic policies and specific approaches to the problem are documented (JEA, 1998). In this document, the JEA listed more than 70 chemicals that are suspected of causing abnormalities in animals at extremely low levels. The Ministry of Land, Infrastructure and Transport (MLIT) of Japan has decided to establish ED conditions in the water environment, conducting extensive studies of major rivers and WWTPs (MLIT, 2001a). Among over 70 suspected substances, the MLIT selected 27 compounds for the river studies and 25 substances for the WWTPs studies, based on the annual production of the chemicals and the levels detected in the environment. The MLIT was particularly concerned about female hormones originating from humans and animals. The study by the MLIT, thus far, has found that estrogen represented by 17β-estradiol (E2) exists in river water and wastewater (including treated wastewater) at significant levels (MLIT, 2001a; Tanaka *et al*., 2001b, 2003).

Analytical methods currently available for EDs are limited in their applications to certain chemicals. The method for the analysis of E2 in the early stage of the MLIT survey

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had been based on enzyme-linked immunosorbent assay (ELISA), which can detect E2 as low as 0.2 ng/l. However, due to the potential "cross-reaction" problem, ELISA is limited in its applications to certain conditions when it is applied to domestic wastewater. Recently, estrone (E1) has emerged as a serious ED in the water environment (MLIT, 2001b; Goda *et al*., 2001), and many other estrogen-like chemicals appear to have estrogenic effects on fish. Furthermore, naturally occurring estrogens (e.g., E1 and E2) tend to have higher estrogenic potentials than do synthetic, industrial chemicals (Yakou *et al*., 1999; Tanaka *et al*., 2001b). Although E2 and 17α-ethynylestradiol (EE2) can be analyzed simultaneously using the GC/MS method (Huang *et al*., 2001), this method is rather cumbersome in requiring a derivatization process. In this study, we refined the analytical method developed by Komori *et al*. (2001) for the analysis of specific estrogens (i.e., E2, E1, and EE2) present in wastewater. This method uses LC/MS/MS, but the derivatization process is not required.

Estrogens are excreted by male as well as female animals. Prior to excretion, most estrogens are hydroxylated and conjugated to glucuronides, sulfates, and acetates. Because very few analytical methods (Ternes *et al*., 1999a; Belfroid *et al*., 1999) are capable of analyzing estrogenic compounds, relatively little work has been directed toward investigating the effects and occurrence of estrogens in the water environment. The objectives of this study are two-fold: 1) based on the method by Komori *et al*. (2002), refining an analytical procedure that allows routine analysis of estrogens and their conjugates (i.e., glucuronides and sulfates conjugates) in wastewater; and 2) determining the occurrence of estrogens and their conjugates in wastewater by applying the developed method to the WWTP performance evaluation.

Materials and methods

LC/MS/MS method for estrogens in wastewater

In this study, the analytical method by Komori *et al*. (2002) was refined for the analysis of estrogens and their conjugates in wastewater. Sample preparation for this method consists of solid-phase extraction with an Oasis HLB cartridge (for the filtrate), supersonic liquid extraction by methanol (for suspended matter), and cleaning with Sep-Pak Plus Florisil and Sep-Pak Plus NH_2. The pretreated (cleaned-up) sample was analyzed using LC/MS/MS. The overall analytical scheme for WWTP influent and effluent is summarized in Figure 1.

First, a 500 ml wastewater sample was filtered through a 1-μm pore size glass fiber filter. Residue on the filter was extracted by supersonic extraction with 5 ml of methanol. The methanol extract was then added to the filtrate. A volume of 0.5 ml of 20% acetic acid, 2 ml of 0.5 mol/l ion pair coupling (IPC) solution and 40 ng of each internal standard [i.e., estrone-2,4-*d2* (E1-*d2*), 17β-estradiol-16,16,17-*d3* (E2-*d3*), 17α-ethynylestradiol-2,4,16,16-*d4* (EE2-*d4*), estriol-2,4-*d2* (E3-*d2*), and sodium 17β-estradiol-2,4,16,16-*d4* 3-sulphate (E2-S-*d4*)] were added. After the mixing, the solution was passed through an Oasis HLB cartridge. Flow rate was maintained at 15 ml/min. The Oasis HLB cartridge was conditioned with methanol and purified water prior to extraction. The Oasis HLB cartridge was centrifuged with a gentle stream of nitrogen gas until it was dried completely. Then estrogen was eluted from the Oasis HLB cartridge with 6 ml of methanol. The eluent was blown dry with a gentle stream of nitrogen gas. The dry residue was dissolved in 1 ml of hexane/dichloromethane (1:1, v/v) with supersonic extraction, and cleaned-up with Sep-Pak Plus Florisil. The cartridge was washed with 10 ml of hexane/dichloromethane (1:1, v/v). Estrogens were eluted from a Sep-Pak Plus Florisil cartridge with 6 ml of acetone and 6 ml of 0.5% NH_4OH/acetone (v/v). The eluent was collected and concentrated to just dryness under a gentle stream of nitrogen gas. The dry residue was dissolved in 1 ml of methanol with supersonic extraction, and cleaned-up with Sep-Pak Plus NH_2. Free (unconjugated) estrogen was eluted with 5 ml of methanol, and conjugate estrogens were eluted

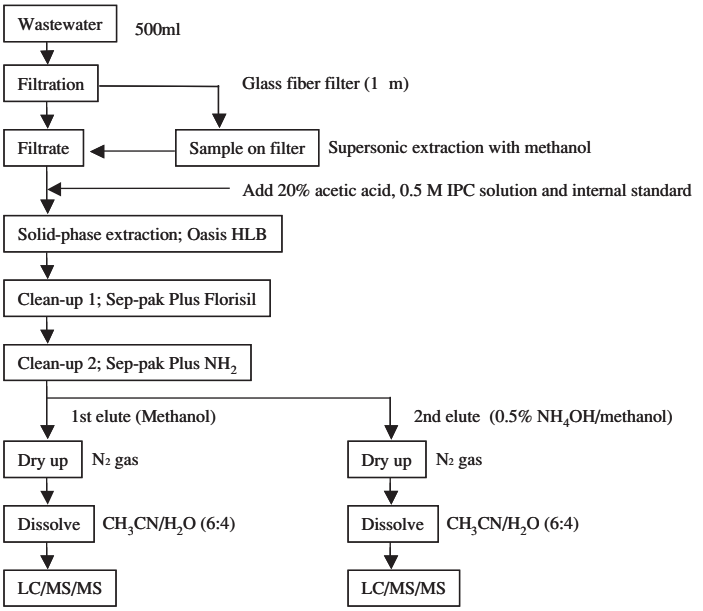

Figure 1 Flow diagram of analytical method

with 6 ml of 0.5% NH_4OH/methanol (v/v). The collected eluent was blown down to dryness with a gentle stream of nitrogen. The dry residue was dissolved in 1 ml of acetonitrile/H_2O (6:4, v/v), then analyzed by LC/MS/MS (operating conditions as in Table 1).

Field survey of estrogen in wastewater

The field surveys were conducted at twenty WWTPs where treated and untreated wastewater samples were collected for the analysis of estrogens and their conjugates (i.e., E1, E2, EE2, E3, E1-S, E2-S, E3-S, E1-G, E2-G, E3-G, E2-S&G and E2-diS). The capacities of

Table 1 Analytical conditions of LC/MS/MS

HPLC	Type of HPLC	Agilent 1100		
	Column	Agilent Zorbax Extend-C18, 2.1$\phi \times$ 150 mm, 40°C		
	Eluent	Acetonitrile: 1mM NH_4OH = 6:4, 0.14 ml/min		
	Sample size	10 µl		
MS/MS	Type of MS/MS	TSQ API-2		
	Ionization	AP-ESI, negative		
	Collision gas	Argon		
	Measurement ion (collision energy)	E1	269, 145	(50eV)
		E1-*d2*	271, 147	(50eV)
		E2	271, 145	(45eV)
		E2-*d3*	274, 145	(45eV)
		EE2	295, 145	(45eV)
		EE2-*d4*	299, 147	(45eV)
		E3	287, 171	(45eV)
		E3-*d2*	289, 173	(45eV)
		E1-S	349, 269	(35eV)
		E2-S	351, 271	(35eV)
		E2-S-*d4*	355, 275	(35eV)
		E3-S	367, 287	(35eV)
		E1-G	445, 269	(35eV)
		E2-G	447, 271	(35eV)
		E3-G	463, 287	(35eV)
		E2-S&G	527, 351	(35eV)
		E2-diS	431, 351	(35eV)

these WWTPs range from 12,000 to 680,000 m^3/day. Thirteen of them apply a conventional activated sludge process. Three WWTPs employ an anaerobic-oxic activated sludge process (A/O process). Other WWTPs adapted various combined processes: i.e., a conventional activated sludge process with rapid filtration; a conventional activated sludge process with rapid filtration and carbon adsorption; or an anaerobic-anoxic-oxic process (A_2/O process) with rapid filtration and step aeration. Grab samples were collected at these WWTP sites; 1g L-ascorbic acid was added to 1 l of sample to prevent oxidation. Samples were collected in 1 l glass bottles, refrigerated, and transported to the laboratory within a day.

Results and discussion

Analytical conditions for the LC/MS/MS method

We examined the HPLC and MS/MS conditions for optimal analysis of the estrogens and their conjugates. The LC column used was an Agilent Zorbax Extend-C18. In operating the MS/MS with electrospray ionization, better sensitivity was obtained for the estrogens and their conjugates if they were analyzed in a negative mode rather than in a positive mode. Table 1 lists MS/MS collision energies optimized for each compound. Figure 2 shows the chromatograms of the standard solutions containing 100 µg/l of each target compound and 40 µg/l of its internal standard. Calibration curves were constructed for the quantification of the estrogens and their conjugates. A linear regression analysis was performed on the standard solution using the ratio of standard area to internal standard area as follows; E1-*d2* for E1, E2-*d3* for E2, EE2-*d4* for EE2, E3-*d2* for E3, and E2-S-*d4* for E1-S, E2-S, E3-S, E1-G, E2-G, E3-G, E2-S&G and E2-diS. Linearity of the calibration curve obtained from the analysis of 0.5, 1.0, 2.0, 5.0 and 10 µg/l of each analyte was high (r^2 >0.99) for all the standard curves.

Detection limits and recovery efficiencies of the LC/MS/MS method

Detection Limits and Recovery Efficiency of the developed analytical method were examined using the standard solutions. The concentrations of the standard solutions, measured concentration (mean), standard deviation (σ), and detection limit for each compound are presented in Table 2. The detection limit was defined as three times the standard deviation (3σ) of the measurements divided by the concentration of the standard solution. When a wastewater sample required concentration more than 500 times, the detection limit for each estrogen and its conjugates was estimated (Table 2). The recovery efficiency of the analytical method was evaluated by spiking 20 ng of each compound to 500 ml of test samples; i.e., purified water, secondary settling tank effluent, and WWTP influent.

The recovery efficiencies for the estrogens with this method are presented in Table 3. The calculated recovery efficiencies for all the target compounds from purified water were higher than 75%. The recoveries from the secondary effluent and WWTP influent were higher than 94% for the free (unconjugated) estrogens (i.e., E1, E2, EE2, and E3), while they were less than 50% for the conjugated estrogens (i.e., E1-S, E2-S, E3-S, E1-G, E2-G, E3-G, E2-S&G, and E2-diS). Especially, the recoveries from the WWTP influent were calculated to be less than 15%.

Field surveys on estrogens in wastewaters

The developed analytical method was applied to the field survey at the selected municipal WWTPs. The measured concentrations of the target compounds in wastewater are shown in Figure 3. In the WWTP influent, we found: 10–57 ng/l (median, 24 ng/l) of E1; ND – 21 ng/l (median, 5.7 ng/l) of E2; 27–220 ng/l (median, 110 ng/l) of E3; 12–170 ng/l (median, 42 ng/l) of E1-S; 26–410 ng/l (median, 110 ng/l) of E2-S; 6.5–79 ng/l (median, 22 ng/l) of E3-S; ND – 88 ng/l (median, 11 ng/l) of E1-G; 5.3–100 ng/l (median, 18 ng/l) of E2-G;

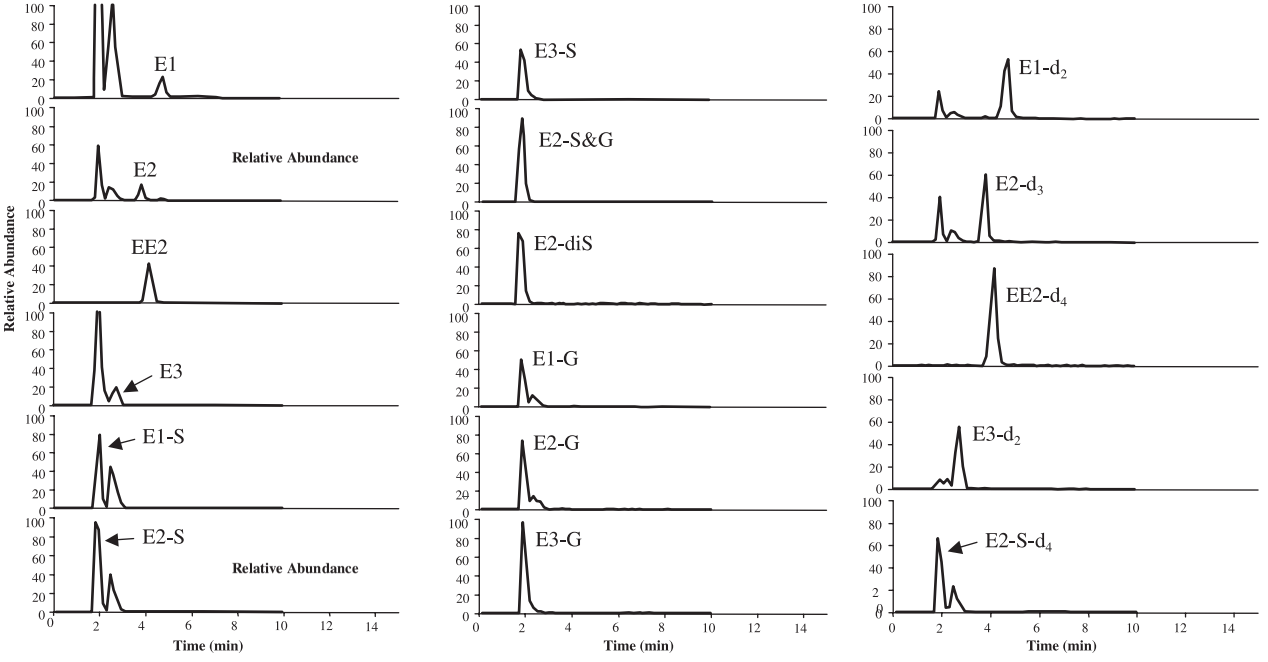

Figure 2 Chromatograms of E1, E2, EE2, E3, E1-S, E2-S, E3-S, E2-S&G, E2-diS, E1-G, E2-G, E3-G, E1-*d2*, E2-*d3*, EE2-*d4*, E3-*d2* and E2-S-*d4*

Table 2 Concentrations, standard deviations, and detection limits (ng/l) for selected estrogens

	Concentration of standard solution	Measurement (average)	σ	Detection limit for sample
E1	500	340	140	0.8
E2	500	410	80	0.5
EE2	500	770	190	1.2
E3	500	580	240	1.4
E1-S	500	490	10	0.1
E2-S	500	480	40	0.2
E3-S	500	510	40	0.2
E1-G	500	820	220	1.3
E2-G	500	570	70	0.4
E3-G	500	510	90	0.5
E2-S&G	500	400	30	0.2
E2-diS	500	540	50	0.3

Table 3 Recoveries (%) of estrogens from purified water samples through the analytical procedure

	Purified water	Secondary settling tank effluent	WWTP influent
E1	100	103	110
E2	106	100	104
EE2	94	95	94
E3	100	97	101
E1-S	98	49	10
E2-S	95	51	9.5
E3-S	93	42	12
E1-G	78	32	15
E2-G	80	22	8.5
E3-G	75	18	11
E2-S&G	104	23	7.5
E2-diS	102	81	6.5

4.1–73 ng/l (median, 22 ng/l) of E3-G; 0.8–38 ng/l (median, 5.5 ng/l) of E2-S&G; and 21–670 ng/l (median, 77 ng/l) of E2-diS.

In the secondary effluent, we observed: ND – 180 ng/l (median, 12 ng/l) of E1; ND – 11 ng/l (median, ND) of E2; ND – 5.8 ng/l (median, 1.5 ng/l) of E3; 7.5–34 ng/l (median, 13 ng/l) of E1-S; 27–94 ng/l (median, 52 ng/l) of E2-S; 37–160 ng/l (median, 69 ng/l) of E3-S; 34–140 ng/l (median, 74 ng/l) of E1-G; 47–210 ng/l (median, 91 ng/l) of E2-G; 37–150 ng/l (median, 72 ng/l) of E3-G; 3.7–17 ng/l (median, 8.9 ng/l) of E2-S&G; and

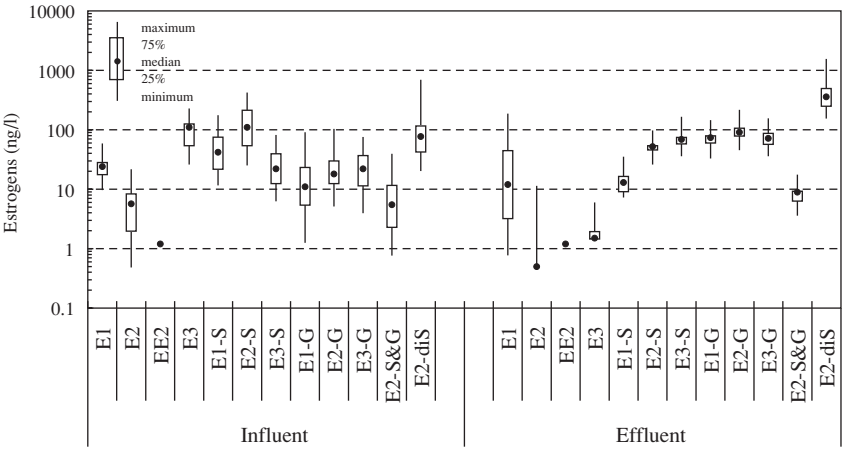

Figure 3 Concentrations of selected estrogens detected in wastewater samples from twenty WWTPs (ng/l)

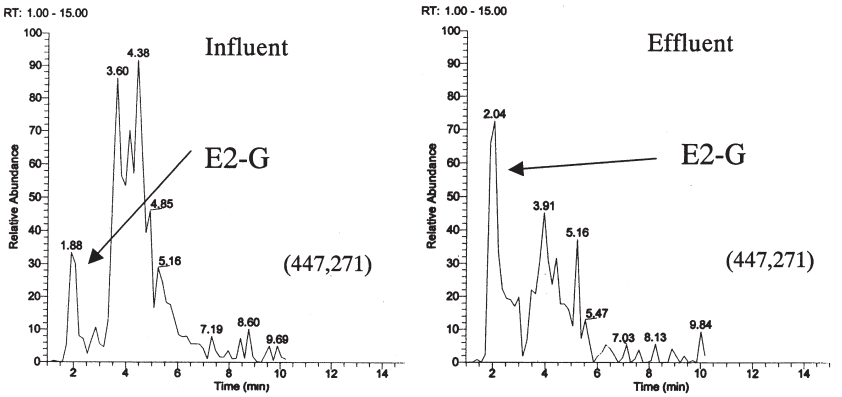

Figure 4 The chromatogram of daughter ions at $m = 271$ for E2-G of wastewater sample. Figures in parentheses are parent and daughter mass numbers

160–1,500 ng/l (median, 360 ng/l) of E2-diS. EE2 was not detected in any of the samples analyzed (including WWTP influent and secondary effluent).

The concentrations of E1, E2, and E3 were the same levels as those reported in the literature (Tanaka *et al*., 2003; MLIT, 2001b; Huang *et al*., 2001; Komori *et al*., 2001; Ternes *et al*., 1999a; Belfroid *et al*., 1999). Belfroid *et al*. (1999) reported that hormone-glucuronides exist generally below their detection limits in the effluent of WWTPs. However, the concentrations of the conjugated estrogens that we measured were higher than those of the unconjugated (free) estrogens in spite of the lower recovery ratios. We can observe clear peaks of E2-G in both the influent and the effluent samples as shown in Figure 4, demonstrating the daughter mass chromatograms at m = 271. Although the other chromatograms are not shown, we confirmed the existence of all the conjugates measured in this study. Therefore, we confirmed the existence of E2-G in wastewater.

We calculated the removal efficiency of estrogens in WWTPs by considering the difference in the recoveries between influent and secondary effluent. Reductions of E2 and E3 (free, unconjugated estrogens) in the WWTPs were very good, at nearly 100% and 99% (calculated using median values), respectively. Reduction of E1 is 45%, which was considerably less than those of E2 and E3. This observation is consistent with Ternes *et al*. (1999b), which reported that the degradation rate of E1 is less than that of E2.

The total recovered amounts of conjugated estrogens in the influent and the effluent were 3,600 ng/l and 1,800 ng/l, respectively. We found that the conjugated estrogens exist at higher concentrations in both the influent and the secondary effluent than stated in the other studies (Ternes *et al*., 1999a; Belfroid *et al*., 1999). Especially, conjugated estrogens still remained at high concentrations even in the secondary effluent.

Conclusions

An improved analytical procedure was developed that allows routine analysis of estrogens and their conjugates (i.e., E1, E2, EE2, E3, E1-S, E2-S, E3-S, E1-G, E2-G, E3-G, E2-S&G and E2-diS) in wastewater samples. The detection limits of this method ranged from 0.1 to 1.4 ng/l. The recovery efficiencies of all the estrogens from purified water were higher than 75%. The recoveries from the effluent of the secondary settling tank and WWTP influent were higher than 94% for the free estrogens, but were less than 50% for the conjugated estrogens. Recoveries of conjugated estrogens from WWTP influent were below 15%.

The developed analytical method was applied to wastewater samples collected from twenty WWTPs. The concentrations (median) of estrogens and their conjugates in the WWTP influent ranged from non-detection (ND) to as high as >100 ng/L. In the influent

samples, the concentrations of E1, E2 and E3 were the same levels as those previous reported. Belfroid *et al.* (1999) reported that hormone-glucuronides exist generally below their detection limits in the effluent of WWTPs. However, the concentrations of conjugated estrogens that we measured were higher than those of the free estrogens.

The reduction of free estrogens in the WWTPs was very good, with approximately 100% and 99% for E2 and E3, respectively, while removal efficiency for E1 (45%) was less significant than for E2 and E3, suggesting that the degradation rate of E1 was smaller than that of E2 in the wastewater treatment processes. The total recovered amounts of conjugated estrogens in the influent and the effluent were 3,600 ng/l and 1,800 ng/l, respectively. We found that conjugated estrogens exist at higher concentrations in the influent, and particularly in the secondary effluent, than stated in the other studies.

In summary, the LC/MS/MS method by Komori *et al.* (2002) for the analysis of estrogens and related substances was refined, and was successfully applied to the evaluation of the selected WWTPs in Japan. The obtained results suggest that this LC/MS/MS method is very useful when determining levels of estrogens in wastewater samples.

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