

# Development of ELISAs for quantification of surfactants, endocrine disruptors and estrogens, and their application for environmental and biological sample analysis

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## Biographical Sketches of Authors

Masato Hirobe is a biochemical scientist and has served as a researcher of department of research and development, Japan EnviroChemicals. Since 2000, he has been developing the ELISA kits for monitoring environmental pollutants such as surfactants, endocrine disruptors and estrogens by generating monoclonal antibodies. Fernando Rubio is biochemical scientist and president of Abraxis, LCC. Fernando has developed immunoassays for analytes of clinical and environmental significance since 1976. His current interests are the development of immunochemical products to serve the agricultural, environmental, food safety and clinical markets. Masanori Fujita is a professor of department of environmental engineering, Osaka University. His laboratory covers various kinds of research areas such as 1) waste and waste water treatment, 2) bioremediation, 3) environmental monitoring and assessment and 4) recycling and reclaiming of waste and waste water by combining biological and engineering technology. Hiroaki Shiraishi is a head of analytical quality assurance section, environmental chemistry division, National Institute for Environmental Studies (NIES) since 1997. Since 2001, he has also served as a head of exposure assessment section of research center for environmental risk, and as a team leader of chemical, bioassay & dynamics research team of endocrine disruptors & dioxin research project of NIES.

## Abstract

Ten kinds of enzyme-linked immunosorbent assay (ELISA) systems were developed for the quantitative analysis of surfactants [linear alkylbenzene sulfonates (LAS), alkyl ethoxylates (AE), and alkylphenol ethoxylates (APE)], endocrine disruptors [alkylphenol (AP), AP+APE, and bisphenol A (BPA)] and estrogens [17beta-estradiol (E2), estrone (E1), estrogen (ES: E1+E2+estriol (E3)), 17alpha-ethynylestradiol (EE2)]. The lowest quantification limits of these ELISAs were 20 µg/L (LAS, AE and APE), 5 µg/L (AP, AP+APE), 0.05 µg/L (BPA) and 0.05 µg/L (E2, E1, ES and EE2), when the following standards were used: LAS (alkyl chain length of 12), nonylphenol ethoxylate (average-ethoxy chain length of 10), AE (alkyl and ethoxy chain lengths were 12 and 7), nonylphenol (NP), NP, BPA, E2, E1, E2 and EE2, respectively. The specificity of each ELISA was confirmed by testing several compounds, which have structural resemblance to the compounds of interest. These ELISAs were also validated by comparing them with instrumental analytical methods such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) with environmental and biological samples. Good correlations were observed between the ELISAs and instrumental analytical methods in all cases.

## Introduction

For quantification of surfactants, endocrine disruptors and estrogens in environmental samples, instrumental analysis such as HPLC, LC-MS and LC-MS/MS are generally employed. These analytical methods are highly reliable, however, they have several potential drawbacks including expensive instrumentation, large sample volume, extensive purification, utilization of large amount of solvents, and need technical expertise in operation. Due to these shortcomings, the analysis of a large number of samples may be both cost and time prohibitive. Therefore there is a strong need for rapid, simple, and cost-effective methods for quantitative analysis of these contaminants such as enzyme-linked immunosorbent assay (ELISA). In this study, ten kinds of ELISAs were developed for quantification of surfactants [linear alkylbenzene sulfonates (LAS), alkyl ethoxylates (AE), alkylphenol ethoxylates (APE)], endocrine disruptors [alkylphenol (AP), AP+APE and bisphenol A (BPA)] and estrogens [17beta-estradiol (E2), estrone (E1), estrogen (ES: E1+E2+estriol (E3)), and 17alpha-ethynylestradiol (EE2)]. ELISA is a rapid, simple

and cost-effective analytical method. In some instances ELISA tends to overestimate the target because of the cross-reactivity against matrices in some samples. Therefore, it is also important to develop a practical pretreatment method for ELISA analysis to alleviate the discrepancies between ELISA and instrumental analysis results. In this paper, we described the performance of the developed ELISAs and sample pretreatment procedures by demonstrating the assay working ranges, cross-reactivity patterns, and comparative results obtained by ELISA and instrumental analysis in environmental and biological samples.

## **Materials and Methods**

### *ELISA analysis*

The developed ELISA kits used in this study such as LAS, AE, APE, AP, AP+APE, super sensitive BPA (ssBPA), E2, E1 ES and EE2 were purchased from Japan EnviroChemicals Ltd. (Tokyo, Japan) or Abraxis LCC (PA, USA). All the assays were conducted according to the instruction manuals supplied with the ELISA kits. The assay procedures were similar for all kits except for the ssBPA ELISA serum assay. Briefly, filtered or further extracted sample (as described below) and calibration standards were adjusted to 10 % (v/v) methanol/water. One hundred micro liters of the adjusted sample and the same volume of diluted enzyme tracer solution were mixed in a separate uncoated well. One hundred micro liters aliquots were then applied to duplicate wells of the antibody coated plate and then incubated for 60 min at room temperature. After washing the plate three with 300  $\mu$ L of Dulbecco's buffered saline (PBS) containing 0.05 % (v/v) Tween 20 (PBST), 100  $\mu$ L of freshly prepared substrate (TMB) was added and the plate was incubated for 30 min at room temperature to develop color and the reaction was stopped by the addition of 100  $\mu$ L of phosphoric acid (0.5 M). The color was determined at 450 nm using a microplate reader and the absorbance values of each test sample were converted to concentrations with the aid of a commercial ELISA software package (Deltagraph) using a four-parameter logistic equation for curve fitting. On the other hand, for serum assay by the ssBPA ELISA, the sample pretreatment and the ELISA assay were conducted under cooling condition (2-8 °C) to prevent evaporation of acetone except for the washing and coloring steps. The serum sample that was treated with acetone as described below and the calibration standards were adjusted to 20 % (v/v) acetone/water and 60  $\mu$ L of the adjusted sample was combined with the same volume of diluted enzyme tracer solution and mixed in a separate uncoated well. Fifty micro liters aliquots were then applied to duplicate wells on the antibody coated plate and incubated for 60 min under cooling condition (2-8 °C). After removing the plate from cooling conditions, the plate was washed with 300  $\mu$ L of (three times). Then, 100  $\mu$ L of a freshly prepared substrate (TMB) was added and the plate was incubated for 30 min at room temperature to develop color and the reaction was stopped by the addition of 100  $\mu$ L of phosphoric acid (0.5 M). The color was measured as mentioned above.

### *Sample pretreatment*

#### 1. Pretreatment of surfactant samples

For LAS ELISA analysis, the sample was just filtered with a glass-fiber filter (pore size 1  $\mu$ m), and adjusted to 10 % (v/v) methanol/water by the addition of methanol. For AE, APE ELISA and instrumental analysis, the filtered sample that was adjusted to pH5 with acetate buffer (1M, pH5) was passed through a C18 or PS-2 cartridge preconditioned with 5mL of methanol and 10 mL of distilled water. After washing the cartridge with 5 mL of distilled water, the analyte was eluted with 10 mL of methanol. The eluted solution was then evaporated and reconstituted to 10 % (v/v) methanol for ELISA or mobile phase solvent for instrumental analysis.

#### 2. Pretreatment of endocrine disruptor samples

For sea and lake water sample analysis by AP and ssBPA ELISA, the sample was filtered with a glass-fiber filter (pore size 1  $\mu$ m) and passed through a SPE column (Nexus) preconditioned with 10 mL of dichloromethane, 5mL of methanol and 5 mL of distilled water. After washing the cartridge with 5 mL of distilled water and 50 % (v/v) aqueous methanol, the cartridge was dried for 45 min, and then the analytes (AP and BPA) were eluted with 6 mL of dichloromethane. The eluted solution was evaporated and reconstituted to 10 % (v/v) methanol/water for AP and ssBPA ELISA analysis. On the other hand, for GC-MS/MS analysis, the eluted solution was further evaporated and reconstituted with 2mL of hexane. The hexane solution was passed through an aminopropyl (NH<sub>2</sub>) SPE column preconditioned with 10 mL of acetone and 10 mL of hexane. After washing the cartridge with 8 mL of dichloromethane/hexane (1:1), the AP fraction was eluted with 8mL of dichloromethane/ethyl acetate (1:1), and

then the BPA fraction was eluted with 8mL of acetone. The eluants (AP or BPA) were evaporated and derivatized to analyze by GC-MS/MS. For human serum sample analysis by ssBPA ELISA, the serum sample was treated with acetone under cooling condition (2-8 °C) to prevent evaporation of acetone. Thirty micro liters of acetone was added to 20 µL of serum sample, and then the solution was mixed vigorously with a vortex mixer (for 30-60 sec). After centrifuging the sample at 12000 rpm for 3 min, 30 µL of supernatant was diluted with 60 µL of distilled water to obtain a 20% (v/v) acetone solution and analyzed by the ssBPA ELISA. On the other hand, for human serum sample analysis by LC-MS, the treated sample [20 % (v/v) acetone solution] was diluted with distilled water to give a final concentration of 5 % (v/v) acetone. The solution was further purified with a SPE column (Nexus) as described above (same as the environmental sample treatment procedure for ssBPA ELISA).

### 3. Pretreatment of estrogen samples

Samples, which were collected from a sewage treatment plant (STP), were filtered with a glass-fiber filter (pore size 1 µm). For ELISA analysis, the estrogenic hormones were extracted from water using a C18 SPE column. Prior to extraction, the columns were conditioned with 5mL of methanol and 10 mL of distilled water. The hormones were then eluted with 5ml of dichloromethane, blown down to dryness under a gentle stream of nitrogen gas. For ELISA analysis (except for influent analysis) by the E1 ELISA, the sample was reconstituted to 10% (v/v) methanol (Method A). On the other hand, for influent analysis by the E1 ELISA, the sample was reconstituted to 1mL of methanol and passed through an aminopropyl (NH<sub>2</sub>) SPE column preconditioned with 5mL of methanol. The sample passed through the column was collected in a glass tube, and the remaining E1 was washed out from the column with 5 mL of methanol and collected in the same glass tube. The methanol solution was evaporated and reconstituted to 10 % (v/v) methanol (Method B). For LC-MS/MS analysis, the extract was eluted with ethyl acetate containing 17 % (v/v) methanol from the C-18 SPE column, dried up and dissolved in dichloromethane/hexane (1:1). The solution was then applied to a florisil SPE column and the hormones were eluted with dichloromethane containing 5 % (v/v) acetone. The eluant was further applied to a NH<sub>2</sub> SPE column and the hormones were eluted with acetone/dichloromethane (1:1). The eluant was dried and dissolved in LC-MS/MS mobile phase.

## Results and Discussion

### ELISA Working Assay Range

The assay working ranges for the ELISAs developed in this study are listed in Table 1. The lowest and highest quantification limits were defined as approximately 85 % and 10 % B/Bo (%), respectively.

Table 1 Assay working range of each ELISA

	assay diluent	standard	quantification limit ( µg/L ) <sup>4)</sup>	
			low	high
For surfactants				
LAS ELISA	10% methanol	C12 LAS <sup>1)</sup>	20	1000
AE ELISA	10% methanol	C12EO7 <sup>2)</sup>	20	1000
APE ELISA	10% methanol	NP10 EO <sup>3)</sup>	20	1000
For endocrine disruptors				
AP ELISA	10% methanol	Nonylphenol (NP)	5	500
AP+APE	10% methanol	Nonylphenol (NP)	5	500
ssBPA ELISA (for environment sample)	10% methanol	BPA	0.05	10
ssBPA ELISA (for serum sample)	20% acetone	BPA	0.2	50
For estrogens				
E2 ELISA	10% methanol	E2	0.05	1
E1 ELISA	10% methanol	E1	0.05	5
ES ELISA (E1+E2+E3)	10% methanol	E2	0.05	3
EE2 ELISA	10% methanol	EE2	0.05	3

1) C12 LAS represents LAS whose alkyl chain length is 12.

2) C12EO7 represents alkyl ethoxylates (AE) whose alkyl and ethoxy chain lengths are 12 and 7, respectively.

3) NP10EO represents mixture of nonylphenol ethoxylates (NPnEO) whose averaged ethoxy chain length is 10.

4) The lowest and highest quantification limits were defined as approximately 85% and 10% B/Bo(%), respectively.

## Cross-reactivity patterns for each ELISA

### 1. Surfactants and AP ELISA

Cross-reactivity patterns of the LAS, AE, APE, AP and AP+APE ELISAs against the various surfactants and their related compounds are shown in Table 2. The LAS ELISA reacted with LAS exclusively and did not react with non-LAS surfactants more than 0.1%. This result suggests that the LAS ELISA can be used for the analysis of environmental samples containing significant levels of these other compounds. The AE ELISA specifically reacted with AE and reacted moderately with alkylether sulfate (AES) (31 %), sodium dodecyl sulfate (SDS) (16 %) and sodium laurate (10 %). Therefore, the AE ELISA may give a slight overestimation on determination of AE because of the cross-reactivity against those non-AE surfactants.

The APE ELISA reacted with NP10EO (nonylphenol ethoxylates mixture whose averaged ethoxy chain length was 10) (100%) and their biodegradation products such as NP7.5EO (100 %), NP5EO (80 %), NP2EO (simple nonylphenol diethoxylate) (40 %), NP1EO (simple nonylphenol monoethoxylate) (20 %), OP10EO (octylphenol ethoxylates mixture whose averaged ethoxy chain length was 10) (230 %), NP1EC (nonylphenoxy acetic acid) (200 %) and NP2EC (nonylphenol monoethoxy acetic acid) (270 %), except for nonylphenol (NP) (2.1 %) and octylphenol (OP) (4.0 %). On the other hand, AP+APE ELISA reacted with APE and their biodegradation products including AP ranging from 100 to 423%. In contrast, the APE ELISA reacted with NP (100%) and OP (96%) specifically and slightly reacted with the other APE-related compounds (ranging from 0.5 to 4.9%). Furthermore, all these ELISAs did not react with non-APE surfactants. These data indicate that the APE ELISA and AP+APE ELISA can measure most of the APE and their biodegradation products at the same time, while the AP ELISA can determine NP and OP specifically.

Table 2 Cross-reactivity patterns for surfactants, AP and AP+APE ELISA

compounds	cross-reactivity (%)				
	LAS ELISA	AE ELISA	APE ELISA	AP+APE ELISA	AP ELISA
APE and their degradation products					
nonylphenol ethoxylates (NPnEO)					
NP10EO <sup>1)</sup>	<0.1	<1	<b>100</b>	100	4.9
NP7.5EO <sup>1)</sup>			100	112	4.5
NP5EO <sup>1)</sup>			80	140	3.2
NP2EO <sup>2)</sup>			40	175	2.1
NP1EO <sup>2)</sup>			20	127	1.2
octylphenol ethoxylates (OPnEO)					
OP10EO <sup>1)</sup>			230	156	2.9
nonylphenoxy carboxylic acid					
NP3EC <sup>2)</sup>				423	3.8
NP2EC <sup>2)</sup>			270	423	1.5
NP1EC <sup>2)</sup>			200	273	0.5
nonylphenol (NP)			2.1	<b>100</b>	<b>100</b>
octylphenol (OP)			4.0	187	96
nonionic surfactants					
alkyl ethoxylates (AE, C12EO7)	<0.1	<b>100</b>	<0.2		
polyethylene glycol (EO10)	<0.1	<1	<0.2		
anionic surfactants					
linear alkylbenzene sulfonates (LAS)	<b>100</b>	<1	<0.2	0.4	<0.1
sodium dodecyl sulfate (SDS)	<0.1	16	<0.2	0.3	<0.1
alkylether sulfate (AES)	<0.1	31	<0.2	<0.1	<0.1
sodium laurate (soap)	0.1	10	<0.2	<0.1	<0.1

1) The number of NP10EO, NP7.5EO, NP5EO and OP10EO represent the averaged ethoxy chain lengths of each chemicals.

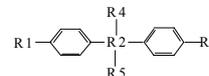
2) NP1EO, NP2EO, NP1EC, NP2EC and NP3EC represent a simple compound.



## 2. ssBPA ELISA

As shown in Table 3, the ssBPA ELISA reacted with BPA specifically and reacted moderately with bisphenol B (15.6 %) and bisphenol E (6.0 %). The amount of production of compounds A and B, however, is small (1/1000 to 11/10000 as compared to BPA), therefore, these compounds will not interfere with BPA readings in field and biological samples.

Table 3 @ross-Reactivity pattern of ssBPA ELISA



Compounds	R1	R2	R3	R4	R5	Cross-Reactivity (%)
<b>Bisphenol A (BPA)</b>	<b>OH</b>	<b>C</b>	<b>OH</b>	<b>CH3</b>	<b>CH3</b>	<b>100</b>
Bisphenol B (BPB)	OH	C	OH	CH3	C2H5	15.6
Bisphenol E (BPE)	OH	C	OH	H	CH3	6.0
Bis(p-hydroxyphenyl)methane	OH	C	OH	H	H	1.8
2,2'-Bis(4-hydroxyphenyl)-1-propanol	OH	C	OH	CH3	CH2OH	1.7
BPA Diacetate	OOCCH3	C	OOCCH3	CH3	CH3	0.2
1,2-Bis(4-hydroxyphenyl)-2-propanol	OH	CH2C	OH	OH	CH3	0.4
4,4'-Bis(p-hydroxyphenyl) pentanoic acid	OH	C	OH	CH3	C2H4COOH	<0.1
4,4'-dihydroxydiphenyl ether	OH	O	OH	-	-	0.2
p, p'-dihydroxybenzophenone	OH	C	OH	-	O	<0.1
Bisphenol S (BPS)	OH	SO2	OH	-	-	0.2
Bis[4-(2-hydroxyethoxy)phenyl]sulfone	O(CH2)2OH	SO2	O(CH2)2OH	-	-	<0.1
BPA Dimethacrylate		C		CH3	CH3	0.7
BPA Diglycidyl Ether		C		CH3	CH3	<0.1
BPX-33		C		CH3	CH3	<0.1

## 3. Estrogen ELISAs

The E2 ELISA showed high specificity to E2 and moderately reactive to EE2 (50 %), 16keto-E2 (16 %), and E2-3-glucuronide (16 %). The E1 ELISA was specific to E1 and E1-3-sulfate (E1-3S) (100 %), and moderately reactive to E1-3-glucuronide (E1-3G) (31 %). Therefore, the sample should be treated to remove E1-3S and E1-3G prior to E1 analysis. ES ELISA reacted with E2 (100 %), E1 (87 %) and E3 (55 %), respectively. This data indicates that the ES ELISA can determine the major part of estrogens all at once.

The EE2 ELISA reacted exclusively with EE2.

Table 4 Cross-reactivity patterns of estrogen ELISA

Compounds	cross-reactivity (%)			
	E2 ELISA	E1 ELISA	ES ELISA	EE2 ELISA
estrone (E1)	1.3	<b>100</b>	<b>87.0</b>	<0.2
2-methoxy E1	<0.4	0.4	<0.03	<0.2
E1-3-glucuronide(E1-3G)	-	31	-	-
E1-3-sulfate(E1-3S)	1.0	100	<0.03	<0.2
17β-estradiol (E2)	<b>100</b>	0.7	<b>100</b>	<0.2
16-keto E2(16k-E2)	16	0.2	118	<0.2
2-methoxy E2	2.0	-	0.2	<0.2
E2-17-glucuronide	<0.4	<0.1	5.0	<0.2
E2-3-glucuronide(E2-3G)	16	0.3	<0.03	<0.2
E2-3-sulfate-17-glucuronide	<0.4	-	0.5	<0.2
estriol(E3)	0.6	<0.1	<b>55</b>	<0.2
16-epi-E3	0.5	<0.1	129	<0.2
E3-16-glucuronide	<0.4	<0.1	48	<0.2
17α-Ethynylestradiol (EE2)	50	<0.1	1.0	<b>100</b>

## Comparison of ELISA with instrumental analysis results

### 1. Surfactant ELISA

LAS contents estimated by ELISA were highly correlated with HPLC results at both lower and higher LAS concentrations, with a correlation coefficient of 0.98, a slope of 0.84 and an intercept value close to  $-2.63$  as shown in Fig. 1 a). These data indicate that the LAS ELISA can be used for environmental monitoring without any extraction steps as opposed to HPLC analysis which needs an extraction step. The values of AE and APE ELISA were well correlated with those of LC-MS and HPLC with a correlation coefficient of 0.92 and 0.96, a slope of 1.32 and 1.13, and an intercept value of 2.82 and  $-1.45$  as shown in Fig. 1 b) and c). The AE and APE ELISA were found to be useful on monitoring AE and APE in environmental samples with the aid of a simple SPE column pretreatment

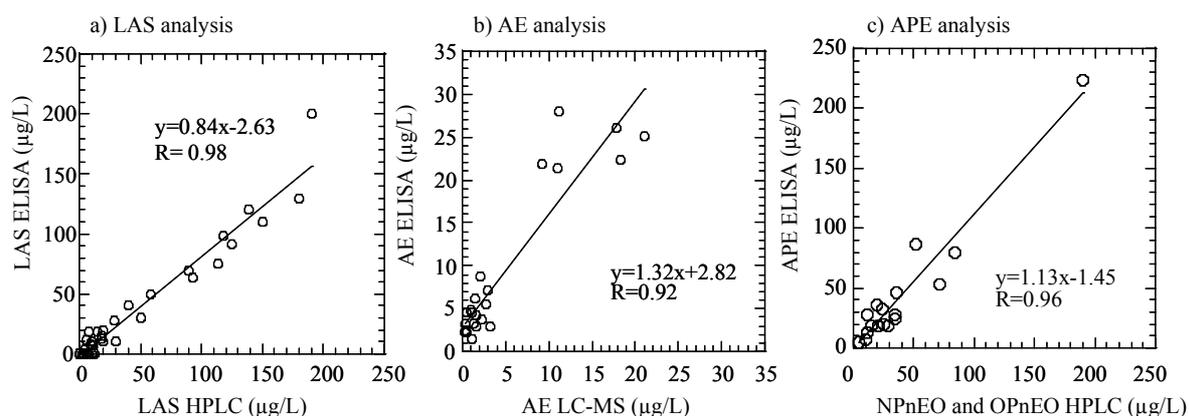


Fig. 1 Comparison of analytical results between ELISA and instrumental analysis for determination of a) LAS, b) AE, and c) APE in river water samples. For LAS ELISA analysis, the sample was only filtered. On the other hand, for AE, APE ELISA and instrumental analysis, the sample was extracted with SPE column as described elsewhere. Non-spiked samples were used for LAS and APE analysis, while some AE spiked samples were used for AE analysis.

### 2. Endocrine disruptor ELISA

The values obtained with the AP and ssBPA ELISAs were well correlated to those of GC-MS with a correlation coefficient of 0.86 (AP) and 0.95 (BPA), respectively in sea and lake water sample analysis (Fig. 2 a, 3 b)). These data suggest that AP and BPA in environmental samples can be determined by these ELISAs with a simultaneous one-step extraction by means of a SPE column (Nexus) as opposed to GC-MS which needs further purification and derivatization steps. Regarding the APELISA, a slope (ELISA/GC-MS) of 0.37 indicates that the APELISA tended to underestimate the target, therefore some kind of compensation factor seems to be needed to correctly estimate the AP values as determined by GC-MS.

For human serum sample analysis, a good correlation was observed between the ssBPA ELISA and LC-MS results with a correlation coefficient of 0.99 and a slope of 1.05 in human serum samples spiked with BPA ranging from 1 to 25 ng/mL (Fig. 2 c). These data suggest that BPA in serum samples can be measured by the ssBPA ELISA with the simple pretreatment step with acetone.

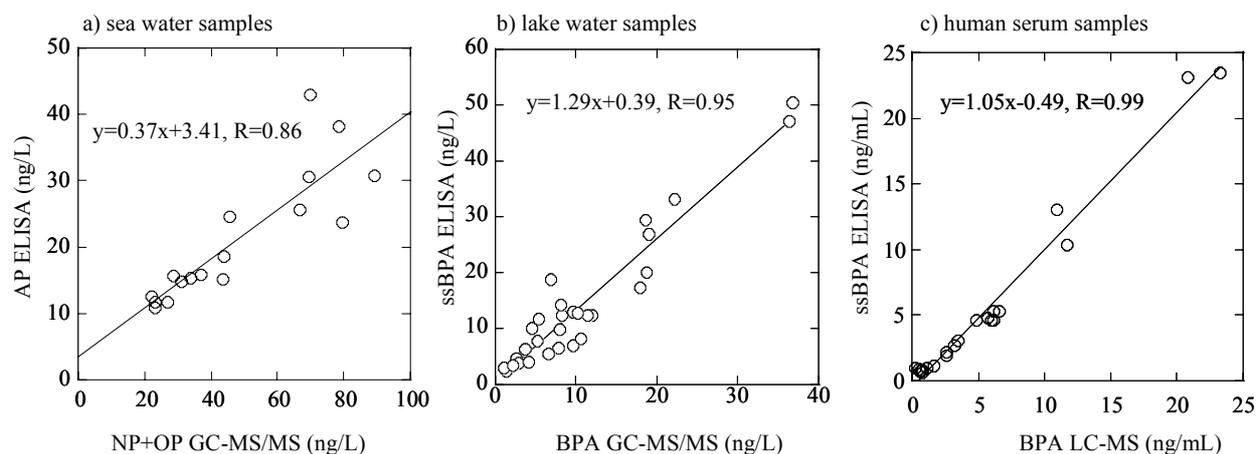


Fig. 2 Comparison of analytical results between ELISA and instrumental analysis for determination of endocrine disruptors. For sea and lake water sample analysis by AP and ssBPA ELISA, the sample was extracted with SPE column (Nexus), while for those by GC-MS/MS, the extracted sample was further purified with  $\text{NH}_2$  SPE column and then derivatized. For human serum sample analysis by ssBPA ELISA, the sample was just treated by acetone, however, for analysis by LC-MS, the treated sample was further purified with Nexus SPE column. For environmental sample analysis, non-spiked sea and lake water samples were used, while for human serum sample analysis, BPA spiked samples (ranging from 1 to 25 ng/mL) were used.

### 3. Estrogen ELISA

#### 1) Effect of the eluent from C18 SPE column in the E2 ELISA assay

The recovery test for E2 or EE2 indicates that other commercial kits overestimated their concentration in the presence of 10-100 mg/L of nonionic surfactants. Whereas developed ELISAs in this study for environmental analysis showed more resistance against nonionic surfactant up to 1000 mg/L, however, 1000 mg/L of the anionic surfactant LAS, caused a positive error in all ELISAs (data not shown). These results indicate that surfactants in the concentrated extract from the environmental samples interfere with the immunochemical reactions in the ELISAs and that the anionic surfactant is one of the causes of overestimation. Therefore, SPE method was modified to remove LAS from the extract. It was confirmed that LAS was not eluted from C18 SPE column by replacing the eluent from methanol to dichloromethane (data not shown).

The data comparison between LC-MS/MS and E2 ELISA in sewage treatment plant (STP) samples are shown in Fig. 3. The values obtained with the E2 ELISA using the dichloromethane eluent showed less discrepancy from those of LC-MS/MS (slope=1.49,  $R=0.95$ ) than the values of the E2 ELISA in methanol eluent (slope=2.23,  $R=0.93$ ). This result suggests that the methanol eluent extract might have contained hydrophilic matrices such as anionic surfactants that caused overestimation in ELISA. It was also confirmed that E1, E2 and EE2 adsorbed by C18 SPE column were eluted with both dichloromethane and methanol thoroughly (data not shown), thus the dichloromethane was chosen as the eluent from C18 SPE columns for environmental sample analysis by ELISA.

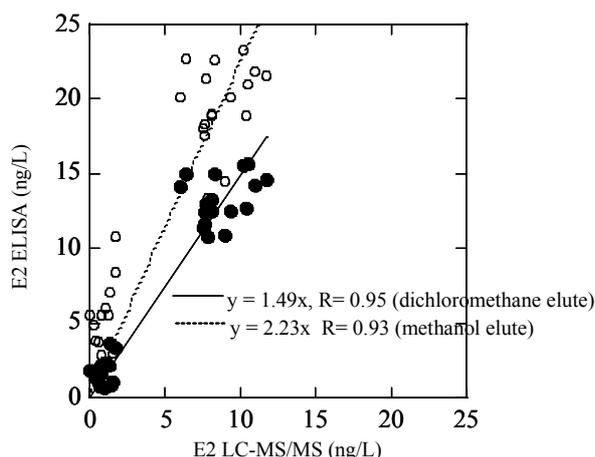


Fig. 3 Comparison of analytical results between E2 ELISA and LC-MS/MS instrumental analysis for determination of E2 in sewage water samples. For E2 ELISA, the sample was extracted with SPE column (C18) and eluted with dichloromethane (●) or methanol (○). On the other hand, for LC-MS/MS analysis, the analyte was eluted with ethyl acetate:MeOH(5:1) from C18 SPE column and further purified with Florisil and NH<sub>2</sub> SPE column.

## 2) Comparison of E2 and EE2 ELISA kits

E2 in the STP using 28 samples (10 samples from primary effluent, 13 samples of aeration tank and 5 samples of secondary effluent) were measured by LC-MS/MS, the developed E2 ELISA (E2-JEC) and 4 commercial E2 ELISA kits obtained from Assay Designs (E2-AD), Cayman Chemical (E2-CC), Neogen (E2-NG), and R-Biopharm (E2-RB). The E2 values of the same sampling points are averaged and listed in Table 5. The values obtained using the E2-JEC were well approximated to those of LC-MS/MS in the primary effluent and aeration tanks, and only 2 times higher than those of LC-MS/MS in the secondary effluent. On the other hand, the values of commercially available ELISA kits were apt to overestimate, that is, from 2 to 5 (E2-AD), from 5 to 13 (E2-CC), from 4 to 16 (E2-NG), from 16 to 65 (E2-RB) times higher than those by LC-MS/MS, respectively.

EE2 in the 48 WWTP samples (18 samples from primary effluent, 19 samples of aeration tank and 11 samples of secondary effluent) were measured by LC-MS/MS, the developed EE2 ELISA (EE2-JEC) and a commercially available EE2 ELISA kit obtained from R-Biopharm (EE2-RB). The comparison of the EE2 data was illustrated in Table 6. In all samples of STP, EE2 was not detected both by LC-MS/MS (less than 0.5 ng/L) and by the EE2-JEC (less than 0.2 ng/L), however, the EE2-RB overestimated EE2 in all samples.

Table 5 Averaged value of E2 in a STP by LC-MS/MS and ELISA kits

Sample	N <sup>a)</sup>	E2 (ng/L)					
		LC-MS/MS	E2-JEC	E2-AD	E2-CC	E2-NG	E2-RB
Primary effluent	10	8.4	12.5	21.1	81.4	97.6	202.2
Aeration Tank	13	1.9	2.1	4.4	9.4	7.8	31.6
Secondary effluent	5	1.2	2.6	5.3	15.1	18.8	75.9

a) N=number of samples

The average E2 values of 10, 13 and 15 samples of primary effluent, aeration tank and secondary effluent are listed. For ELISA analysis, the samples were treated with Method A.

Table 6 Averaged value of EE2 in a STP by LC-MS/MS and ELISA kits

Sample	N <sup>a)</sup>	EE2 (ng/L)		
		LC-MS/MS	EE2-JEC	EE2-RB
Primary effluent	18	<0.5	<0.2	3.1
Aeration Tank	19	<0.5	<0.1	1.0
Secondary effluent	11	<0.5	<0.1	0.8

a) N=number of samples

The average EE2 values of 18, 19 and 11 samples of primary effluent, aeration tank and secondary effluent are listed.

### 3) Effect of an NH<sub>2</sub> SPE column in the E1 ELISA assay

As shown in Table 4, the E1 ELISA cross-reacted with E1 as well as E1-3S (100%) and E1-3G (31 %), therefore, E1-3S and E1-3G needs to be removed prior to E1 analysis if a sample contains these E1 conjugates. In our previous study, E1-3S and E1-3G could be removed with an NH<sub>2</sub> column treatment (E1-3S and E1-3G were retained in a column but E1 was not). In effluent sample analysis, the values of E1 ELISA were well approximated to those obtained by LC-MS/MS with a slope of 1.36 and a correlation coefficient of 0.99 with Method A pretreatment. On the other hand, in influent sample analysis, the values obtained with the E1 ELISA overestimated the target with a slope of 2.63 by using Method A pretreatment, however, the overestimation of E1 ELISA was alleviated by using Method B pretreatment. These data indicates that cross-reactants in the E1 ELISA (E1-3S and 3G) which might be present in influent samples were removed with an NH<sub>2</sub> column treatment. Therefore, Method B is required for influent samples prior to analysis in the E1 ELISA, while in the other case, Method A (much easier one) is suitable for pretreatment.

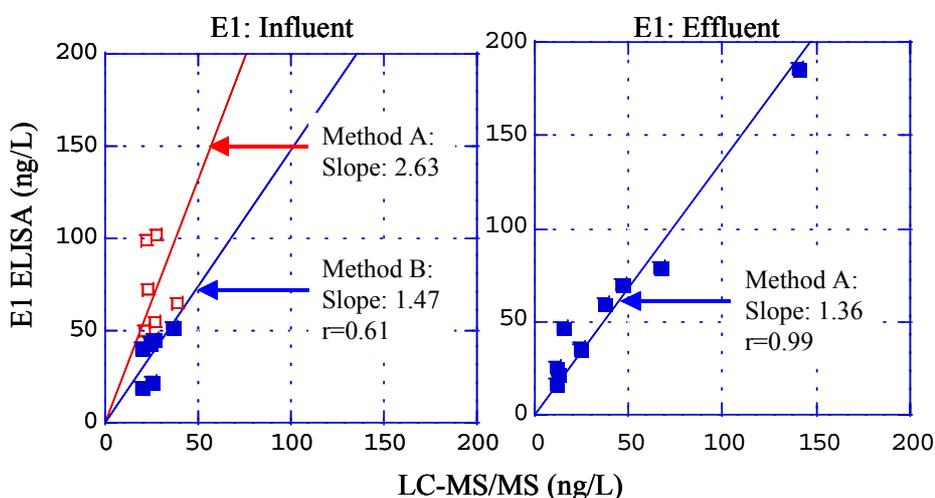


Fig. 4 Effect of an NH<sub>2</sub> SPE column for the E1 ELISA assay. Filtered influent and effluent water samples from sewage treatment plant (STP) were applied to C18 solid phase extraction (SPE) column. For ELISA analysis, the analyte was eluted with dichloromethane (Method A) or further purified by passing through an aminopropyl (NH<sub>2</sub>) SPE column (Method B). On the other hand, for LC-MS/MS analysis, the analyte was eluted with ethyl acetate:MeOH(5:1) from C18 SPE column and further purified with Florisil and NH<sub>2</sub> SPE column.

### Conclusion

The overestimation caused by matrices in environmental samples considered to be an inherent problem with some ELISA can be eliminated when a proper clean up method is adopted for environmental analysis. The appropriate choice of antibody and proper sample pretreatment, significantly reduced overestimation and the data obtained with ELISAs were comparable to that from instrument analysis. Antibodies that have tolerance against surfactants, which

is one of the matrices that might affect ELISA values were selected for the estrogen ELISAs (E2, E1 and EE2 ELISA) in this study (data not shown). For endocrine disruptor and estrogen ELISAs, dichloromethane was selected as the solvent for SPE, eluting as much target compound as possible while minimizing elution of substances that might block the immunochemical reaction.

Contrary to conventional analytical methods (GC-MS, LC-MS/MS), some ELISA may over- or under- estimated values because of their cross reactivity or matrices effect, however, ELISA offers considerable advantages over conventional analytical procedure because of the ease of handling (no special skills necessary), the ease of pretreatment procedures (unnecessary for LAS ELISA), relatively fast measurement, high sample turnover, low quantification limit and acceptable costs. With these important and attractive features, the developed ELISAs in this study can contribute to the routine monitoring of environmental pollutants.