The use of enzyme-linked immunosorbent assays (ELISA) for the determination of pollutants in environmental and industrial wastes

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Abstract Twelve enzyme-linked immunosorbent assays (ELISA), for the determination of surfactants [linear alkylbenzene sulfonates (LAS), alkyl ethoxylates (AE), and alkylphenol ethoxylates (APE)], endocrine disruptors [alkylphenol (AP), AP + APE, and bisphenol A (BPA)], estrogens [17beta-estradiol (E2), estrone (E1), estrogen (ES: E1 + E2 + estriol (E3)), 17alfa-ethynylestradiol (EE2)], dioxins and polychlorinated biphenyls (PCBs), were validated on environmental water and industrial wastes. The lowest quantification limits of these ELISAs were 0.05 μg/L (BPA, E2, E1, ES and EE2), 2 μg/L (AE), 3 μg/L (dioxins and PCBs), 5 μg/L (AP, AP + APE) and 20 μg/L (LAS and APE). To apply these ELISAs to environmental or industrial waste samples, simple and appropriate pre-treatment methods were also developed for each ELISA. With optimized pre-treatments, the values of ELISAs were well co-related, in all cases, to those of instrumental analytical methods such as liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and high-resolution gas chromatography mass spectrometry (HR-GC-MS), etc.
Keywords Dioxin; endocrine disruptor; estrogen; immunoassay; polychlorinated biphenyl (PCB); surfactant

Water Science & Technology **Vol 54 No 11–12 pp 1–9** © IWA Publishing 2006

Introduction

Generally, instrumental analytical methods such as high-performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LC-MS), LC-MS/MS, and GC-MS are employed for quantification of surfactants (Di Corcia *et al.*, 1994), endocrine disruptors (Fujitsuka, 1999; Basheer *et al.*, 2005), estrogens (Ferguson *et al.*, 2001; Isobe *et al.*, 2003), and persistent organic pollutants (POPs) such as dioxins (Christoffer *et al.*, 1989; Fabrellas *et al.*, 2004) and polychlorinated biphenyls (PCBs) (Shin *et al.*, 2004) in environmental and industrial wastes. Although these analytical methods are highly reliable, they have several potential drawbacks, including expensive instrumentation, large sample volume, extensive purification, and 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 such as enzyme-linked immunosorbent assay (ELISA) for these contaminants. In this study, twelve kinds of ELISAs, for quantification of surfactants [linear alkylbenzene sulfonates (LAS) (Fujita *et al.*, 1998), alkyl ethoxylates

doi: 10.2166/wst.2006.735

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(AE) (Goda *et al.*, 2005), and alkylphenol ethoxylates (APE) (Goda *et al.*, 2004)], endocrine disruptors [alkylphenol (AP), AP + APE, and bisphenol A (BPA)], estrogens [17beta-estradiol (E2), estrone (E1), estrogen (ES: E1 + E2 + estriol (E3)), 17alfa-ethynylestradiol (EE2)] (Goda *et al.*, 2000), POPs [dioxins and polychlorinated biphenyls (PCBs)], were validated. ELISAs are rapid, simple, and cost-effective analytical methods. In some instances, however, they give over- and underestimated values due to antibody cross-reactivity and matrix effects. Therefore, it is also important to develop a practical pre-treatment method for ELISA analysis to alleviate the discrepancies between ELISA and instrumental analysis results. In this paper, we describe the performance of the developed ELISAs in combination with sample pre-treatment procedures by demonstrating the assay working ranges and comparative results between ELISA and instrumental analysis on environmental and industrial waste samples.

Materials and methods

Reagents

LAS (C12), AE (C12EO7), BPA, E2, E1, EE2, and 2,3,4,7,8-pentachloro dibenzofuran were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Nonylphenol polyethoxylate (NP10EO) and nonylphenol were purchased from Kanto Chemical Industries Ltd. (Tokyo, Japan). Kanechlor 400 was supplied by GL-Sciences, Inc. (Tokyo, Japan).

ELISA assays

The developed ELISA kits used in this study, such as LAS, AE, APE, AP, AP + APE, super sensitive BPA (ssBPA), E2, E1 ES, EE2, dioxins, and PCBs were purchased from Japan EnviroChemicals Ltd. (Tokyo, Japan) or Abraxis LLC (PA, USA). All the assays were conducted according to the instruction manuals supplied with the ELISA kits (http://www.jechem.co.jp/eco/index.e.html).

Pre-treatment of samples

Surfactants. For LAS ELISA analysis, the sample was just filtered with a glass-fibre filter (pore size 1 μm), and adjusted to 10% (v/v) methanol/water by the addition of methanol. For AE ELISA, APE ELISA, and instrumental analysis, 1 L of filtered sample mixed with 10 mL acetate buffer (1 M, pH5) was passed through either C18 (500 mg, JT Baker, NJ, USA) or PS-2 (500 mg, Waters, MA, USA) SPE column, both of which had been pre-conditioned with 5 mL of methanol and 10 mL of distilled water. After the cartridge was washed 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.

Endocrine disruptors. For sea and lake water sample analysis by AP ELISA and ssBPA ELISA, the sample was filtered with a glass-fibre filter (pore size 1 μm) and passed through a NEXUS SPE column (200 mg, Varian, CA, USA) pre-conditioned with 10 mL of dichloromethane, 5 mL of methanol and 5 mL of distilled water. After the cartridge was washed 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 ssBPA and to 10% methanol +1% dimethylsulfoxide (DMSO) (v/v) for AP ELISA analysis. For GC-MS/MS analysis, the eluted solution was further evaporated and reconstituted with 2 mL of hexane. The hexane solution was passed through an aminopropyl SPE column (500 mg, Waters, MA, USA)

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pre-conditioned with 10 mL of acetone and 10 mL of hexane. After the cartridge was washed with 8 mL of dichloromethane/hexane (1:1), the AP fraction was eluted with 8 mL of dichloromethane/ethyl acetate (1:1), and then the BPA fraction was eluted with 8 mL of acetone. The eluants (AP or BPA) were evaporated and derivatized to analyse by GC-MS/MS.

Estrogens. Samples, collected from a sewage treatment plant (STP), were filtered with a glass-fibre filter (pore size 1 μm). For ELISA analysis, the estrogenic hormones were extracted from water using a Nexus SPE column. Prior to extraction, the columns had been conditioned with 5 mL of methanol and 10 mL of distilled water. The hormones were then eluted with 5 mL of dichloromethane, and blown down to dryness under a gentle stream of nitrogen gas. For ELISA analysis, the sample was reconstituted to 10% (v/v) methanol. For LC-MS/MS analysis, the extract was eluted with ethyl acetate containing 17% (v/v) methanol from the C-18 SPE column (500 mg, Waters, MA, USA), dried up and dissolved in dichloromethane/hexane (1:1). The solution was then applied to a florisil SPE column (500 mg, Waters, MA, USA) and the hormones were eluted with dichloromethane containing 5% (v/v) acetone. The eluant was further applied to a NH2 SPE and the hormones were eluted with acetone/dichloromethane (1:1). The eluant was dried and dissolved in LC-MS/MS mobile phase.

Dioxins. Twenty samples of flue gas and 10 samples each of bottom and fly ash were used in this experiment. The flue gas samples were collected with DioANA filter according to JIS K0311 (2005). The collected gas, fly ash, and bottom ash samples were treated with/without hydrochloric acid, and then extracted with accelerated solvent extraction method (ASE-200 Dionex) according to the instruction manual. The extracted samples were further purified with automated sample preparation system (SPD-600, Kyoto Electronics Manufacturing Co. Ltd., Kyoto, Japan).

PCBs. Insulating oil samples were mixed vigorously with hexane and DMSO. Then the hexane layer was removed and freshly prepared hexane was added. After repetition of this washing step, the DMSO layer was diluted with aqueous solution and extracted with hexane. After being dehydrated, the hexane layer was sulfonated with sulfuric reagent and washed with aqueous solution. The hexane layer was evaporated and reconstituted to DMSO.

Results and discussion

ELISA assay working range

The assay working ranges for the ELISAs are listed in Table 1. The lowest and highest quantification limits were defined as approximately 85% and 10% B/Bo (%), respectively. The lowest quantification limit of the LAS (20 μg/L) and AE ELISA (2 μg/L) were well below the regulatory limits, enforced by Ministry of Health, Labour and Welfare of Japan since 1 April 2004, for drinking water (200 μg/L for LAS and 20 μg/L for nonionic surfactants). Therefore, these ELISAs can be used for environmental water sample analysis without any extraction steps. The other ELISAs needed extraction steps described in the Methods section.

Comparison of ELISA with instrumental analysis results

Surfactant ELISA. LAS contents estimated with 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 Figure 1a.

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Table 1 Assay working range of each ELISA

	Assay diluent	Standard	Quantification limit (μg/L)[5]	
			Low	High
Surfactants				
LAS ELISA	10% methanol	C12 LAS[1]	20	1 000
AE ELISA	20% methanol	C12EO7[2]	2	100
APE ELISA	10% methanol	NP*10* EO[3]	20	1 000
Endocrine disruptors				
AP ELISA	10% methanol	Nonylphenol (NP)	5	500
AP + APE ELISA	10% methanol	NP	5	500
ssBPA ELISA	10% methanol	BPA	0.05	10
Estrogens				
E2 ELISA	10% methanol	E2	0.05	1
E1 ELISA	10% methanol	E1	0.05	5
EE2 ELISA	10% methanol	EE2	0.05	3
ES ELISA	10% methanol	E2	0.05	3
POPs				
Dioxins ELISA	100% DMSO	2,3,4,7,8-PeCDF[4]	3	50
PCBs ELISA	100% DMSO	Kanechlor 400	3	1 000

[1]C12 LAS represents LAS with alkyl chain length of 12
[2]C12EO7 represents alkyl ethoxylates (AE) with alkyl and ethoxy chain lengths of 12 and 7, respectively
[3]NP*10*EO represents mixture of nonylphenol ethoxylates (NPnEO) with averaged ethoxy chain length of 10
[4]PeCDF represents pentachloro dibenzofuran
[5]The lowest and highest quantification limits were defined as approximately 85% and 10% B/Bo (%), respectively

These data indicate that the LAS ELISA can be used for environmental monitoring without any extraction step, as opposed to HPLC analysis requiring 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 Figure 1b and c. The AE and APE ELISAs were found to be useful for monitoring environmental samples with the aid of a simple SPE column pre-treatment.

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 samples, as shown in Figures 2a and b). These data suggest that AP and BPA in environmental samples can be determined with these ELISAs, together with a simultaneous one-step extraction of a NEXUS column, as

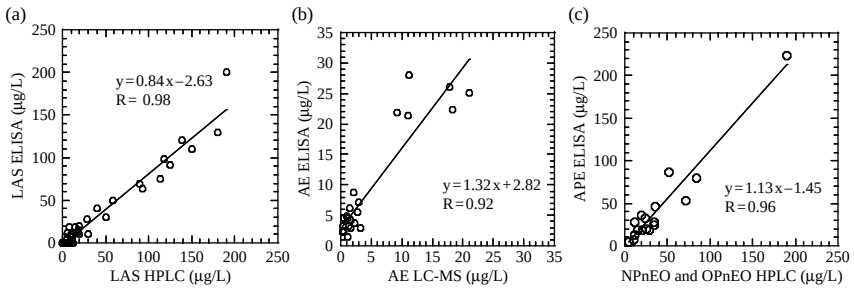

Figure 1 Comparison of analytical results between ELISA and instrumental analysis for determination of (a) LAS, (b) AE, and (c) APE in river water samples

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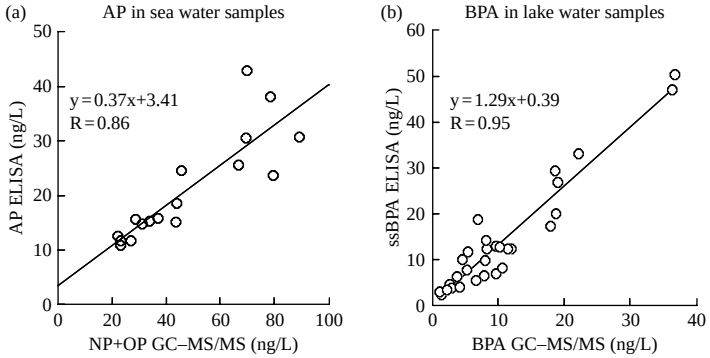

Figure 2 Comparison of analytical results between ELISA and instrumental analysis for determination of endocrine disruptors such as alkylphenol (AP) and Bisphenol A (BPA)

opposed to GC-MS further requiring purification and derivatization steps. Regarding the AP ELISA, a slope (ELISA/GC-MS) of 0.37 indicates that the AP ELISA tended to underestimate the concentration. Therefore, a conversion factor is necessary to correctly estimate the AP values as determined with GC-MS.

Estrogen ELISA. (1) Effect of the eluent from C18 SPE column in the E2 ELISA assay.

The data comparison between LC-MS/MS and E2 ELISA in STP samples is shown in Figure 3. The discrepancy of E2 ELISA values from those of LC-MS/MS was much smaller in dichloromethane eluent (slope = 1.49, R = 0.95) than in methanol eluent (slope = 2.23, R = 0.93). This result suggests that the methanol eluent extract would have contained hydrophilic matrices, causing overestimation in ELISA. It was also confirmed that both dichloromethane and methanol thoroughly eluted E1, E2, and EE2 from C18 SPE column (data not shown), thus the dichloromethane was chosen as the eluent.

(2) Comparison of E2 and EE2 ELISA kits.

Twenty-eight STP samples (10 samples from primary effluent, 13 samples from aeration tank, and 5 samples from secondary effluent) were measured for E2 with LC-MS/MS, the developed E2 ELISA (E2-JEC), and 4 commercial E2 ELISA kits 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 2. The values of 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

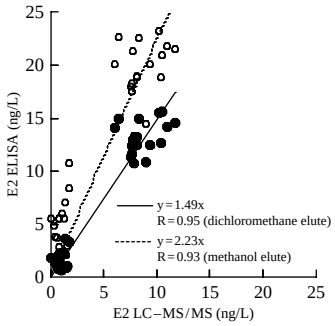

Figure 3 Comparison of analytical results between E2 ELISA and LC-MS/MS instrumental analysis for determination of E2 in STP. For E2 ELISA, the sample was extracted with SPE column (C18) and eluted with dichloromethane (●) or methanol (○)

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Table 2 Averaged value of E2 (ng/L) in a STP by LC-MS/MS and ELISA kits

Sample	N[a]	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

were apt to overestimate 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 of LC-MS/MS, respectively. These data indicate that selecting the appropriate antibody is very important for environmental analysis. Unlike commercially available kits developed for clinical assay, the antibody of E2- JEC was selected based on not only sensitivity but also the tolerance against the interfering matrices in environment, such as surfactant (data not shown).

Forty-eight STP samples (18 samples from primary effluent, 19 samples from aeration tank, and 11 samples from secondary effluent) were measured for EE2 with LC-MS/MS, the developed EE2 ELISA (EE2-JEC), and a commercially available EE2 ELISA kit from R-Biopharm (EE2-RB). EE2 was not detected in all the STP samples with both LC-MS/MS (less than 0.5 ng/L) and EE2-JEC (less than 0.2 ng/L). The EE2-RB, however, overestimated EE2 ranging from 0.8 to 3.1 ng/L in all the samples (data not shown).

Dioxins ELISA. (1) Conversion coefficients to calculate from ELISA values to TEQ value.

In order to obtain conversion coefficient from ELISA value (2,3,4,7,8-PeCDF equivalent) to TEQ value, 20 samples of flue gas and 10 samples each of fly and bottom ash samples, and a dioxin mixed sample, were determined with HR-GC-MS and ELISA. The dioxin mixture was prepared according to the typical flue gas isomer composition reported by the Ministry of the Environment, Japan. Table 2 shows the conversion coefficients in the above-mentioned samples, determined from the slopes of comparison data between ELISA and HR-GC-MS (data not shown).

(2) Comparison between HR-GC-MS and ELISA based on TEQ values.

Twenty flue gas samples and 10 each of fly and bottom samples were determined with HR-GC-MS and ELISA. The 2,3,4,7,8-PeCFD equivalent values of ELISA were converted to TEQ values with multiplying conversion coefficients in each sample matrix (shown in Table 3), and then compared with TEQ values obtained with HR-GC-MS. As shown in Figure 4, good correlations were observed in all the sample matrices with the slopes of 0.988 for flue gas, 0.996 for fly ash, and 0.997 for bottom ash, and correlation coefficients (R) of 0.976 for flue gas, 0.985 for fly ash, and 0.991 for bottom ash. These data suggest that the newly developed dioxin ELISA gives accurate TEQ values by calculating with established conversion coefficients for each matrix.

Table 3 Conversion coefficients

Sample matrices	Conversion coefficients
Pure dioxin mixture	0.222
Flue gas	0.0514
Fly ash	0.0554
Bottom ash	0.0481

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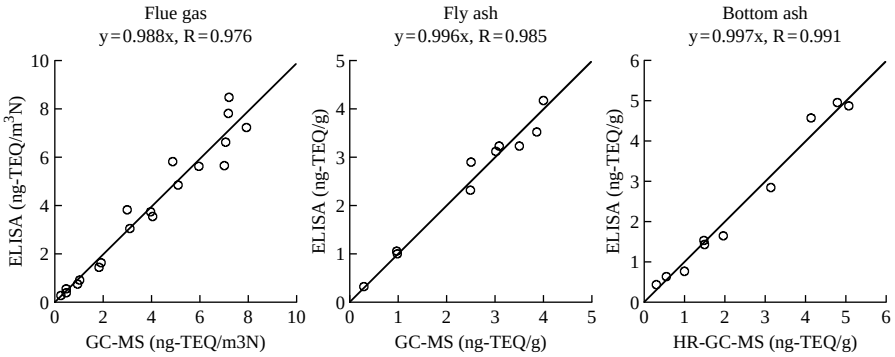

Figure 4 Comparison between HR-GC-MS and ELISA based on TEQ values

PCBs ELISA. Twenty-six insulating oils contaminated with PCBs were determined with PCBs ELISA and GC-ECD. For ELISA analysis, samples were pre-treated and assayed as described in the Methods section. On the other hand, the values of GC-ECD were obtained according to Notification No. 192 (1992) of the Ministry of Health and Welfare of Japan. As shown in Table 4 and Figure 5, the values of PCBs ELISA were well correlated to those of GC-ECD, both in lower and higher PCB concentrations based on the ELISA/GC ratios (average: 0.89, SD: 0.16, max: 1.19 and min: 0.61), slopes (1.002) and correlation coefficients (R: 0.991). These data suggest that combining Cocktail PCB ELISA and optimized pre-treatment method are a rapid, simple, and highly accurate analytical method for determination of PCBs in insulating oils.

Table 4 Comparison of PCB ELISA and GC-ECD in determination of PCBs in real insulating oils

Sample No.	GC-ECD (mg/Kg)	ELISA (mg/Kg)	ELISA/GC ratio	Dominant Kanechlor (KC)
1	1.0	1.1	1.08	KC-400
2	3.4	2.3	0.70	KC-300/400
3	12.8	8.8	0.68	KC-500
4	13.7	13.3	0.97	KC-500
5	13.2	12.9	0.98	KC-500
6	2.3	2.2	0.95	KC-300–600
7	44.6	46.4	1.04	KC-500
8	20.2	16.0	0.79	KC-400/500
9	31.0	28.1	0.91	KC-500
10	8.6	9.0	1.04	KC-300/400
11	77.2	79.1	1.02	KC-300/500
12	95.2	103.5	1.09	KC-500
13	4.2	2.9	0.69	KC-500
14	5.7	6.8	1.19	KC-500
15	15.1	11.3	0.75	KC-300/500
16	42.1	29.1	0.69	KC-300
17	1.7	1.7	1.02	KC-500
18	4.6	4.4	0.95	KC-500
19	2.5	1.8	0.73	KC-500
20	1.0	1.0	0.96	KC-500
21	2.9	1.8	0.61	KC-500
22	22.2	22.0	0.99	KC-300/400
23	7.2	5.0	0.69	KC-500
24	21.6	19.2	0.89	KC-300/400
25	20.1	17.0	0.84	KC-300/400
26	6.8	6.3	0.93	KC-500
		average	0.89	
		SD	0.16	
		MAX	1.19	
		MIN	0.61	

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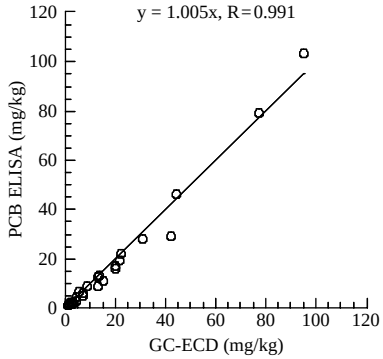

Figure 5 Comparison between ELISA and GC-ECD data

Conclusion

Twelve ELISAs, developed for detection of environmental pollutants such as surfactants, endocrine disruptors, estrogens, and POPs, were validated by comparing them with conventional analytical methods such as HPLC, LC-MS, and GC-MS. In all cases, good correlations were obtained between the values of ELISAs and those of instrumental analysis. These data indicate that ELISAs described here are very accurate, and can be used as a screening method in conjunction with simple pre-treatment methods. Contrary to conventional analytical methods, ELISA generates, in some cases, slightly over- or under-estimated values due to antibody cross-reactivity and matrix effect. ELISA, however, offers considerable advantages over conventional analytical methods because of ease of handling in operation as well as in pre-treatment steps, fast measurement, high sample turnover, and acceptable costs. With these important and attractive features, the validated ELISAs in this study can contribute to the routine monitoring of pollutants in environmental and industrial wastes.

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