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In Situ Formed Organic Ion-Associate Liquid-Phase Microextraction without Centrifugation from Aqueous Solutions Using Thymol Blue and Estrogens

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Abstract: In this study, we present a method for ion-associated liquid phase (IALP) separation and concentration of analytes from an aqueous matrix into an IALP formed in situ by the charge neutralization reaction of organic cations and anions, without centrifugation. The effects of various factors on the extraction efficiency and other parameters are investigated, whereas no instrumental stirring, such as vortexing or ultrasonics, is required because the solvent (IALP) is formed in situ. The organic cation and anion used are ethylhexyloxypropylammonium and dodecyl sulfate, respectively. The developed in situ IALP microextraction method for phase separation without centrifugation is tested using the thymol blue dye and several endocrine disruptors. The tested endocrine disruptors (bisphenol A, 17β-estradiol, 17α-ethinylestradiol, and estrone) are analyzed via high-performance liquid chromatography/fluorescence detection, with respective detection limits of 0.02, 0.02, 0.02, and 0.4 μg L^{-1}, and the corresponding enrichment factor ranging from 47 to 71. This IALP microextraction method can be used to separate and concentrate environmental water samples of different matrices. The employed IALP is fast and easy to use, enables an approximately 100-fold analyte concentration, and has a high affinity for estrogens, thus holding promise for the separation, concentration, and quantitation of diverse trace analytes.

Keywords: in situ solvent formation; microextraction; ion-associate liquid phase; estrogen; without centrifugation; thymol blue

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1. Introduction

The constant influx of novel pollutants such as endocrine-disrupting chemicals and organic pollutants (POPs) into the environment due to anthropogenic activities necessitates the establishment of corresponding counteraction policies and maximum permissible limits (MPLs) [1–6]. However, MPL determination is a nontrivial task, as it relies on the accurate quantitation of analytes in environmental water and therefore often requires separation, concentration, and other challenging-to-implement pretreatment methods. Liquid-phase microextraction is a pretreatment method based on the in situ formation of a new liquid phase, often requiring centrifugation or heating unless halogenated organic materials are used. Liquid-phase microextraction encompasses homogeneous liquid–liquid extraction [7], cloud point extraction [8], dispersed liquid–liquid microextraction [9–11], and ion-associate liquid phase (IALP) microextraction [12–18]. These methods are widely used for pretreatment and concentration because of their ecofriendliness, as they generate small amounts of liquid waste and offer the advantages of high speed (several minutes), high enrichment factors (several dozen times or more), and concentration to small volumes (1 μL–1 mL).

In our pursuit of microliquid phase formation methods, we have developed a concentration procedure based on extraction to IALPs formed in situ via charge-neutralization reactions between organic cations and anions. This procedure is applicable to both uncharged hydrophobic components and charged species, with the extraction of the latter involving ion pairing with the organic cation or anion of the employed IALP. IALP extraction relies on hydrophobic interactions in the micro-ionic associates produced by supplementing aqueous samples (several tens of milliliters) with appropriate organic cation/anion combinations. It is particularly effective when it is coupled with centrifugation, able to achieve concentrations in microvolumes (several to hundreds of microliters).

In particular, in situ solvent formation obviates the need for vortexing or other agitation to reach a distribution equilibrium and offers the advantages of simplicity and rapidity. Although centrifugation is required for phase separation, alternative phase separation methods such as membrane processes have been developed [19]. The generated IALPs are diluted with organic solvents or back-extracted into acidic solutions and analyzed via atomic spectrometry [14–16] or high-performance liquid chromatography (HPLC) [13,17]. Some IALPs can be directly injected into HPLC systems [18]. Figure S1 presents an overview of previously reported microextraction procedures based on IALPs formed in situ upon the supplementation of aqueous samples with organic cations and anions followed by centrifugation.

The conversion of target analytes into easily extractable or quantifiable compounds, such as dyes and chelating species, allows the separation and concentration steps to be completed in a single vessel. The advantages of IALP extraction include (1) efficient sample concentration (several tens of milliliters) to small volumes (several to hundreds of microliters); (2) small amounts of aqueous samples, reagents, and generated waste; (3) reduced instrument contamination and rapid and easy operation due to the use of a single centrifuge tube; and (4) avoidance of hazardous organic solvents.

Moreover, IALP extraction allows various extractants to be generated by changing the organic cation/anion combination and has been applied to the analysis of ammonia [12], nitrite ions, di(2-ethylhexyl) phthalate [13], heavy metals [14,16], polycyclic aromatic hydrocarbons [17], lithium [15], and bisphenol A (BPA) [18]. In our study on BPA [18], the amounts of ethylhexyloxypropylammonium cation ($EHOPA^+$) and dodecyl sulfate anion (DS^-) added were examined, revealing that IALPs separated relatively quickly at higher amounts without centrifugation. Therefore, in this study, we identified the concentration range of $EHOPA^+/DS^-$ in which IALPs are phase-separated after only 30 min of standing and investigated the effects of various factors on the extraction efficiency and other parameters. Several microextraction methods for phase separation without centrifugation have already been reported [20–24]. However, they require stirring with equipment such as vortex or ultrasound [20], more than 12 h of standing [21], N_2 injection and filtration [22], or special devices [23]. Even when solvents are generated in situ, microwave heating may be necessary for phase separation [24]. The microextraction method proposed in this study does not require equipment to partition the target component from the aqueous phase to the solvent, such as vortexing or ultrasound, because the solvent is generated in situ. Furthermore, it does not require centrifugation for phase separation and simply uses a commonplace instrument for extraction, a volumetric flask. The proposed microextraction was validated using the thymol blue dye and several estrogens, which were chosen owing to their different hydrophobicity and variety of applied analytical methods. Thymol blue was measured using UV/Vis spectroscopy and colorimetric analysis. Meanwhile, BPA and estrogens were quantified using HPLC-fluorescence detection (FLD). FLD is more selective and sensitive than UV detection. However, the fluorescence intensity varies between instruments, and even within the same instrument, there can be variations as a result of lamp degradation. Therefore, in addition to the fluorescence intensity, we also determineddistribution and extraction efficiency.

Estrogens are environmentally harmful endocrine-disrupting substances that are discharged from sludge treatment facilities and detected in various water systems [25].

In Australia, guideline values have been established for BPA, 17β-estradiol (E2), 17α-ethinylestradiol (EE2), and estrone (E1) in drinking water (Table 1) [26]. In Japan, BPA, E2, and EE2 are designated as drinking water quality indicators and substances requiring investigation by the Ministry of the Environment, with provisional guideline values being set [27]. In the EU, E2, EE2, and E1 are on the watch list, and maximum permissible limit of detections have been set [28], although guidance values are only given for E2 [29]. No MPLs for estrogens have been set yet.

Table 1. GVs (guideline values, guidance value) and other properties of bisphenol A (BPA) and estrogens.

Analytes	**Abbreviation**	**CAS. No**	**Formula**	**M_w (Da)**	**log K_{owwin} [1], [30]**	**pK_a [2] [31–34]**	**GVs (μg L^{-1})**			**MAMDL [3] (ng L^{-1}) [28]**	**TDL [4] (ng L^{-1}) [35]**
							Japan [27]	**Australia [26]**	**EU [29]**		
Bisphenol A	BPA	80–05–7	$C_{15}H_{24}O_2$	228.29	3.64	9.6	100	200	--	--	--
17β-Estradiol	E2	50–28–2	$C_{18}H_{24}O_2$	272.39	3.94	10.23	0.08	0.175	0.001	0.4	0.1
Ethinyl estradiol	EE2	57–63–6	$C_{20}H_{24}O_2$	296.44	4.12	10.4	0.02	0.0015	--	0.035	0.1
Estrone	E1	53–16–7	$C_{18}H_{22}O_2$	270.40	3.43	10.26	--	0.03	--	0.4	0.1

[1] log K_{owwin}: logarithm of octanol-water partition coefficient estimated using KOWWIN v1.68 [30]. [2] pK_a: negative logarithm of acid dissociation constants. [3] MAMDL: maximum acceptable method detection limit. [4] Target detection limit.

Solvent and solid-phase extractions have been established as official pretreatment procedures for the analysis of these exogenous endocrine disruptors [35,36]. Solid-phase extraction is the most commonly used method for estrogen analysis, although more ecofriendly alternatives, such as microextraction, have been examined to reduce sample and reagent consumption.

2. Materials and Methods

2.1. Reagents

The 1 M $EHOPA^+$ solution was prepared by dissolving ethylhexyloxypropylamine (19 g; Tokyo Chemical Industry, Tokyo, Japan) in 2 M hydrochloric acid and adjusting the volume to 100 mL with 2 M hydrochloric acid. The 0.1 M DS^- solution was prepared by dissolving sodium dodecyl sulfate (2.9 g; Nacalai Tesque, Kyoto, Japan; biochemical research–grade) in water and adjusting the volume to 100 mL with water. The BPA standard solution was prepared by dissolving BPA (1.0 mg; Wako Pure Chemical Industries, Osaka, Japan; environmental analysis–grade) in water (100 mL). The E2, EE2, and E1 standard solutions were prepared by dissolving E2, EE2, and E1 (1.0 mg each; Wako Pure Chemical Industries, Osaka, Japan; biochemical research–grade) in acetonitrile (100 mL). Water samples were stored in brown-glass or polyethylene bottles in a cool dark place. The structures of the analytes (BPA, E2, EE2, E1), dye (TB) and organic cation source (EHOPA) are shown in Figure S2.

2.2. Equipment

All the apparatus was made of glass. The temperature of the 1 M $EHOPA^+$ and 0.1 M DS^- solutions was controlled to be 25 °C using a thermostat (Thermominder SX-10N, Taitec, Koshigaya, Japan) to avoid low temperatures, due to the temperature-sensitive nature of DS^- precluding IALP formation. pH measurements were conducted using a D–24 pH meter (Horiba, Kyoto, Japan) or a SevenCompact S220 pH/ion meter (Mettler Toledo, Switzerland). Images were taken using a Fujifilm Finepix XP70 (Tokyo, Japan) or Sony NEX–3N (Tokyo, Japan) digital camera.

The HPLC-FLD instrument (Shimadzu, Kyoto, Japan)) and column (GLSciences, Tokyo, Japan) were the same as those used in our previous BPA study [18].

2.3. IALP Formation and Separation Investigated Using TB

A 50 mL volumetric flask was used in preference to a broad-mouth centrifuge tube to facilitate the collection of the separated IALP. The IALP volume was calculated from the height of the IALP layer and throat diameter of the volumetric flask. The water sample (~40 mL) contained in the 50 mL volumetric flask was sequentially supplemented with the TB (0.1 mM, 1 mL), $EHOPA^+$ (1 M, 1.6 mL), and DS^- (1 M, 7.5 mL) solutions, and the volume was adjusted to 50 mL with pure water. The flask was shaken and left to stand. The IALP formed at the top of the flask was sampled (0.1 mL) at specific timepoints and diluted with *N*,*N*-dimethylformamide (DMF) (0.5 mL) for subsequent analysis. Its absorbance was measured at 412 nm using a quartz cell with a light path width of 2 mm and light path length of 1 cm. The IALP volume used for the distribution ratio calculation was measured with a microsyringe.

The extraction efficiency (%*E*) was defined as the ratio of the mass of solute extracted into the organic phase to the total mass of solute. This reflects the ratio of the volumes of the aqueous and organic phases (i.e., the IALP). The TB %*E* was calculated using Equation (1).

$$\%E = (\mathrm{A}_1 - \mathrm{A}_2)/\mathrm{A}_1 \tag{1}$$

where A_1 and A_2 are the IALP absorbances during the first and second extractions. D was calculated from the concentrations of TB in the organic (C_o) and aqueous (C_a) phases as

$$D = \mathrm{C}_\mathrm{o}/\mathrm{C}_\mathrm{a} \tag{2}$$

%*E* can also be expressed as

$$\%E = D/(D + \mathrm{V}_\mathrm{a}/\mathrm{V}_\mathrm{o}) \times 100 \tag{3}$$

where V_o and V_a are the volumes of organic and aqueous phases, respectively. This equation indicates that %*E* is negatively correlated with the V_a/V_o ratio.

2.4. Effects of Organic Cation/Anion Concentrations and Standing Time on IALP Volume

First, the height of the water column in the volumetric flask was determined per constant volume of water. After adding water to the female flask up to the marked line, a constant volume of water was added and the height of the water column per volume of water was calculated. For a volumetric flask with stopper No. 13, the height of the water column was approximately 0.8 cm/mL. For a volumetric flask with stopper No. 9, the height of the water column was approximately 1.2 cm/mL. The IALP volume was obtained by measuring the heights of the water column and IALP with a ruler, or by taking a photograph and analyzing the image using ImageJ (ver. 1.54h) [37].

2.5. BPA and Estrogen Analysis via HPLC-FLD Coupled with IALP Extraction

In experiments requiring centrifugation, a 50 mL glass centrifuge tube was sequentially charged with the water sample (30 mL), BPA and estrogen standards, $EHOPA^+$ solution (1 M, 1.6 mL), and sodium dodecyl sulfate solution (0.1 M, 7.5 mL) and shaken to form an ion-associate (IA) emulsion. The mixture was centrifuged (3500 rpm, 30 min) to separate the aqueous phase and IALP. The IALP containing the target components was aliquoted with a syringe (100 µL) and injected (sample loop: 20 µL) into the HPLC-FLD system for analysis.

For experiments without centrifugation, a 50 mL volumetric flask was sequentially charged with the water sample (40 mL), BPA and estrogen standards, $EHOPA^+$ solution (1 M, 1.6 mL), and sodium dodecyl sulfate solution (0.1 M, 7.5 mL) and shaken to form an IA emulsion. After 30 min of standing, the aqueous phase and IALP were separated, and the latter was aliquoted with a 100 µL syringe and injected into the HPLC-FLD system (sample loop: 20 µL). The extraction rate was calculated from the slope of the calibration curve after two extractions.

2.6. Investigation of Optimal Conditions for HPLC-FL Analysis

The optimal eluent was determined by varying the acetonitrile/KCl (0.05 M) mixing ratio (75:25, 60:40, and 45:55) at a flow rate of 1.0 mL min^{-1}. The effects of KCl concentration were also studied.

2.7. Extraction and Distribution of BPA and Estrogens to IALP

The IALP was formed in water samples containing BPA and estrogens, and was analyzed after 5, 10, 15, 20, 25, and 30 min using HPLC-FLD.

To calculate %E for BPA and estrogens, we used the slopes of the calibration curves in the first and second extractions [17]. Equation (3) was then employed to calculate the logarithm of the distribution coefficient (log D) based on the obtained %E and the volumes of IALP and the aqueous phase.

2.8. Detection Limits, Concentration Factor, and Detection Sensitivity

The detection and quantitation limits were examined at [EHOPA$^+$]/[DS$^-$] = 31 mM/12 mM and 32 mM/15 mM. Blanks were measured five times, and the detection limit was determined as the concentration equivalent to three times the standard deviation of these measurements. The enrichment factor was calculated by comparing the concentrations of analytes in the original aqueous phase (BPA; E2; EE2: 20 µg L^{-1}; and E1: 200 µg L^{-1}) and IALP.

2.9. Application to Real Samples

Real samples included (i) seawater (pH = 8.3, conductivity = 5.05 S m^{-1}) collected from Toyama Bay (Toyama Prefecture) and filtered through a glass fiber filter paper; (ii) effluent from the Hamakurozaki water purification plant (Toyama Prefecture), which had a pH of 7.49 and electrical conductivity of 0.395 mS cm^{-1}; and (iii) river water (pH = 6.8, conductivity = 18 mS m^{-1}) collected at the Jokoji Bridge of Oyabe River, which flows in Toyama Bay in Japan Sea, and filtered through a glass fiber filter paper.

A 50 mL volumetric flask was sequentially charged with the BPA/estrogen-containing water sample (~40 mL), EHOPA$^+$ solution (1 M, 1.6 mL), and DS$^-$ solution (0.1 M, 7 mL). The volume was adjusted to 50 mL using ultrapure water, and the flask was shaken to form IAs and left to stand for 30 min (1 h for effluent). The IALP layer and a fraction of the aqueous phase (1 mL in total) were transferred into a beaker where the aqueous phase was removed and the IALP was washed with ultrapure water (100 µL). The water was removed and the washed IALP was analyzed via HPLC-FLD.

2.10. Spontaneous Separation of IALP

Figure 1 presents the employed microextraction procedure. Regarding IALP preparation, a 50 mL volumetric flask was sequentially charged with the water sample (40 mL), EHOPA$^+$ solution (1 M, 1.6 mL), and DS$^-$ solution (0.1 M, 7.5 mL), and the volume was adjusted to 50 mL. The flask was shaken to form an IA emulsion and left to stand for 30 min. IALP was aliquoted with a syringe and analyzed via HPLC-FLD or UV/Vis spectrophotometery to determine %E.

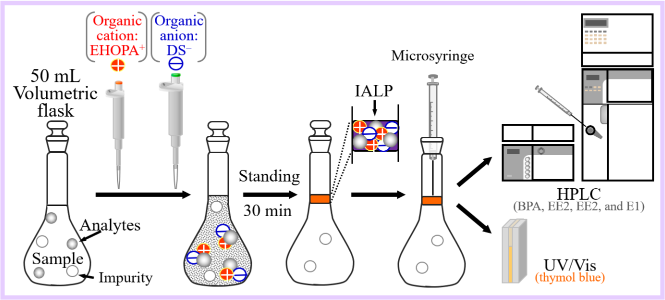


Figure 1. Overview of the employed microextraction procedure.

3. Results and Discussion

3.1. IALP Formation and Separation Investigated Using Thymol Blue (TB)

Under the employed conditions (aqueous phase with pH~2), bromocresol green, bromophenol blue, and phenol red were faintly colored (yellow), whereas methylene blue was deeply colored (blue) but not completely extracted into the IALP because of its high hydrophilicity (Table 2). In contrast, TB (Figure S2) was vividly colored at pH 2 (vermilion) and therefore chosen as a model substrate to investigate the possibility of spontaneous IALP separation without performing time-consuming HPLC measurements.

Table 2. Selected properties of the dyes examined as model substrates.

Dye	Abbreviation	CAS. No	Formula	M_w (Da)	log K_{owwin} [1], [30]	pK_a [2] (pK_{a1})	Color at pH 2
Thymol blue	TB	76–61–9	$C_{27}H_{30}O_5S$	466.60	7.21	1.65 [38]	Red–Yellow
Methylene blue	MB	61–73–4	$C_{16}H_{18}Cl_1N_3S$	319.85	0.75	0–1 [39]	Blue
Bromocresol green	BCG	76–60–8	$C_{21}H_{14}Br_4O_5S$	698.02	7.86	4.90 [38]	Yellow
Bromophenol blue	BPB	115–39–9	$C_{19}H_{10}Br_4O_5S$	669.96	6.77	4.10 [38]	Yellow
Phenol red	PR	143–74–8	$C_{19}H_{14}O_5S$	354.38	3.21	8.00 [38]	Yellow

[1] log K_{owwin}: logarithm of octanol-water partition coefficient estimated using KOWWIN v1.68 [30]. [2] pK_a: negative logarithm of acid dissociation constants.

The aqueous phase after IALP formation exhibited a pH of 1.9. The pK_a of TB is 1.65; therefore, HTB^- and H_2TB were able to mix, whereas changes in the added amount of the EHOPA-HCl solution would alter the color. The effects of the addition of EHOPA-2M HCl solution on the color of TB and pH were investigated (Figure 2). The pH of the aqueous solution was measured after the addition of the EHOPA-HCl solution to the TB solution. During the absorbance measurements, dimethylformamide (DMF) was added to the solution to match the measuring conditions of IALP. The absorbance at 412 and 416 nm exhibited slight changes upon addition of 0.2–2.0 mL of EHOPA-HCl. When the volume of EHOPA-HCl was increased from 1.2 to 2.0 mL, the pH decreased from 2.2 to 1.8. The wavelengths of the maximum absorbance for H_2TB, HTB^-, and TB^{2-} of TB in the aqueous solution are reported at 545, 430, and 600 nm, respectively [40]. Even though the pH of the aqueous solution is 1.9, the majority of TB was considered to be present in the form of HTB^- since DMF was added during the absorbance measurement.

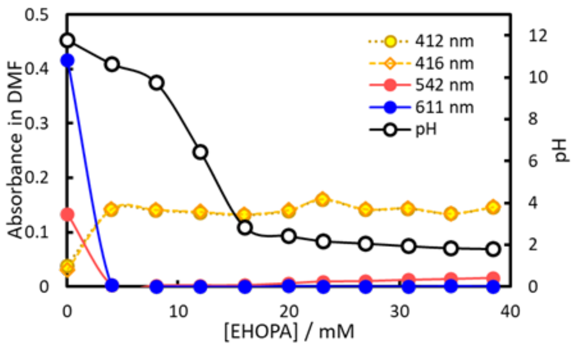
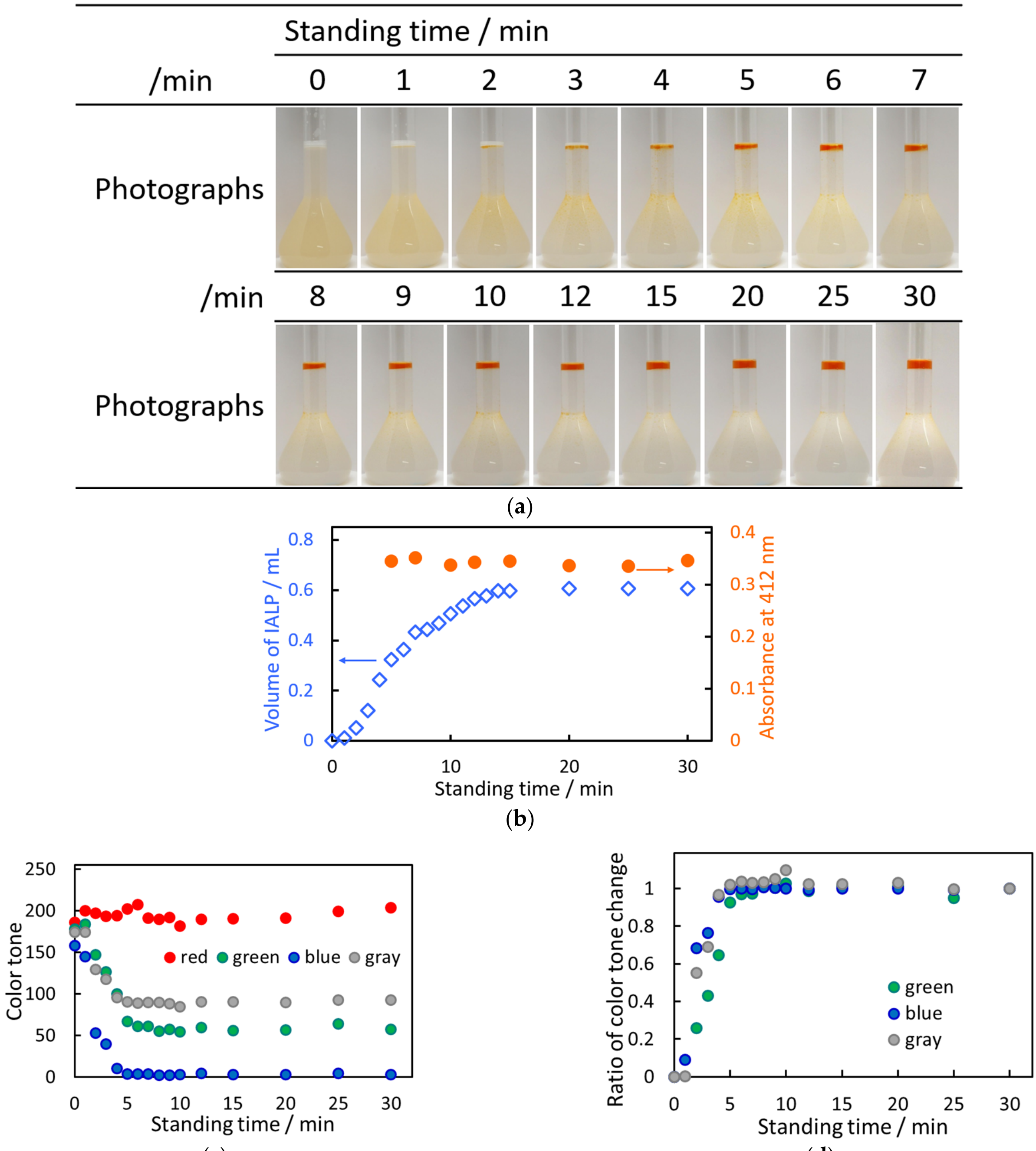


Figure 2. Effect of the concentration of EHOPA on the pH and thymol blue dye absorbance. [TB]: 50 µM.

The volume of the TB-containing IALP and its absorbance at 412 nm were measured at 5–30 min after IA formation (Figure 3b), stabilizing at ~0.6 mL and 0.35 after 15 and 5 min, respectively. TB extraction was rapid, with the concentrate (IALP) being aliquoted with a microsyringe or a similar device and analyzed (0.1 mL per measurement). Figure 3c presents the results of the analysis of the IALP portion (Figure 3a) by ImageJ [37]. Figure 3d shows the ratio of change in the green, blue, and gray tones in Figure 3c. The ratios of color tone change were calculated using Equation (4):

$$Ratio\ of\ color\ tone\ change = \frac{(CT_i - CT_{30})}{(CT_0 - CT_{30})} \quad (4)$$

where CT_0, CT_i, and CT_{30} are the color tones at 0, i, and 30 min.



(a)

(b)

(c)

(d)

Figure 3. (**a**) Photographs of the ion-associate liquid phases (IALPs) formed in 50 mL volumetric flasks at different standing times. (**b**) Effects of standing time on the IALP absorbance and volume. (**c**) Color tone (CT). (**d**) Ratio of color tone change. Conditions: [ethylhexyloxypropylammonium cation, EHOPA$^+$], 32 mM; [dodecyl sulfate anion, DS$^-$], 15 mM; [TB], 2.0 µM.

3.2. *Effects of Organic Ion Concentrations and Standing Time on IALP Volume*

Common IALP extraction methods rely on centrifugation to achieve phase separation and concentration. However, under certain conditions, the $EHOPA^+/DS^-$-based IALP separated from the aqueous phase without centrifugation, i.e., upon standing.

With the increasing concentrations of $EHOPA^+$ and DS^-, the volume of the IALP produced upon standing increased (i.e., the concentration factor decreased), and the lowest ion concentrations affording acceptable IALP separation were identified as $[EHOPA^+]$ = 32 mM and $[DS^-]$ = 15 mM (Figure 4). The separation, concentration, and HPLC-FLD analysis of BPA and estrogens were performed under slightly different conditions: $[EHOPA^+]$ = 31 mM and $[DS^-]$ = 12 mM. The added cations and anions aggregated to form IAs, IA clusters, and, finally, IALP. Given that turbidity was caused by the presence of IA clusters, the clarity of the aqueous phase was positively correlated with the extent of IALP separation.

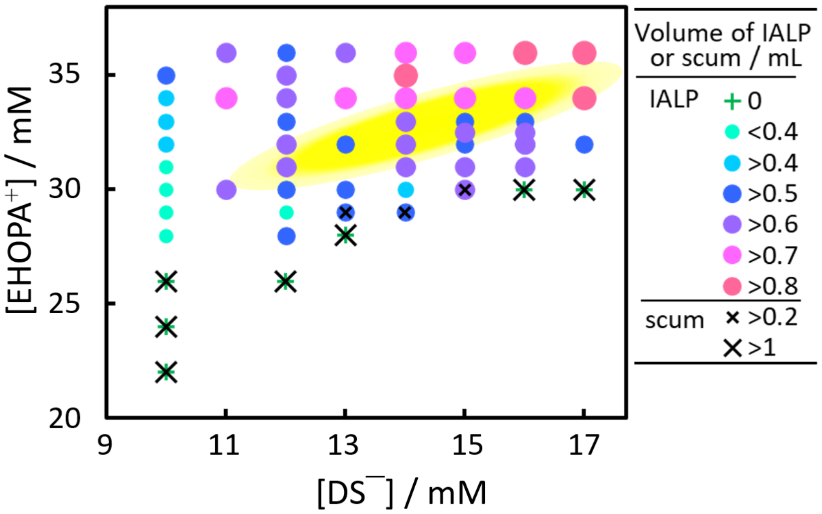


Figure 4. Effects of $[EHOPA^+]$ and $[DS^-]$ on IALP volume (mL). Conditions resulting in an aqueous phase with low turbidity are indicated by a yellow oval.

Figure 5 shows photographs of IALP formation at $[DS^-]$ = 12 and 17 mM and $[EHOPA^+]$ = 29–34 mM, revealing that the aqueous phase was the clearest at $[DS^-]$ = 12 mM and $[EHOPA^+]$ = 31 mM. Notably, scum was formed at $[DS^-]$ = 17 mM and $[EHOPA^+]$ = 30 mM, whereas the aqueous phase became clear only at $[EHOPA^+]$ = 34 mM.

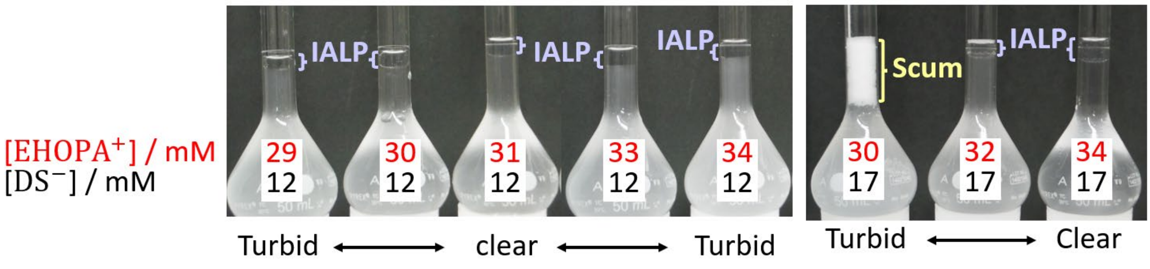


Figure 5. Photographs of IALP and aqueous phase formed at different $[EHOPA^+]$ and $[DS^-]$.

Figure 6 shows the effect of standing time on the IALP volume formed at different concentrations of $EHOPA^+$ and DS^-; at low $EHOPA^+$ and DS^- concentrations, the IALP volume is low and takes a long time to stabilize.

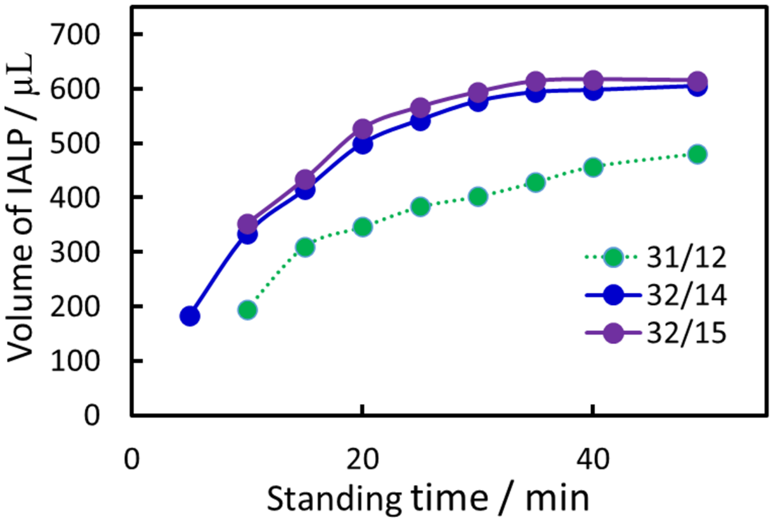


Figure 6. Effect of standing time on the volume of IALP formed at 23 °C and [EHOPA$^+$] (mM)/[DS$^-$] (mM) = 31/12 (green), 32/14 (blue), and 32/15 (purple).

3.3. pH of Aqueous Phase

The solution containing the organic cation (1 M EHOPA$^+$) and, hence, the aqueous phase after IALP formation was acidic. IALP formation was not possible when the pH of the aqueous phase after ion-associate formation approximated the pK_a of EHOPA$^+$ (9.76 [41]). Therefore, the pH of the original water sample and aqueous phase were measured after organic cation/anion addition ([EHOPA$^+$] = 32 mM, [DS$^-$] = 15 mM). For samples with an initial pH of ≤11 and ≥12, the aqueous phase after organic cation/anion addition exhibited pH values of 1.9 and ~pK_a (EHOPA$^+$), meaning that IALP formation occurred only in the former case.

3.4. Optimal HPLC-FLD Conditions

Under the HPLC conditions reported for detecting BPA [18], E2, EE2, and E1 were not separated. Therefore, the composition of the mobile phase was altered to afford the necessary conditions to separate the selected estrogens (Figure S3). Table 3 lists the HPLC-FLD conditions used for the analysis of a mixture containing BPA, E2, EE2, and E1, with the corresponding chromatogram shown in Figure 7. Hereinafter, these conditions are referred to as optimal.

Table 3. Conditions for BPA and estrogen analysis via high-performance liquid chromatography (HPLC)–fluorescence detection (FLD) coupled with IALP microextraction.

HPLC-FLD Conditions	
Injection volume	20 μL
Mobile phase	Acetonitrile:0.05 M KCl = 45:55 (*v*/*v*)
Flow rate	1.0 mL min^{-1}
Column oven	40 °C
Excitation wavelength	280 nm
Fluorescence wavelength	310 nm
[EHOPA$^+$]/[DS$^-$]	32 mM/15 mM 31 mM/12 mM for sample
Temperature	25 °C

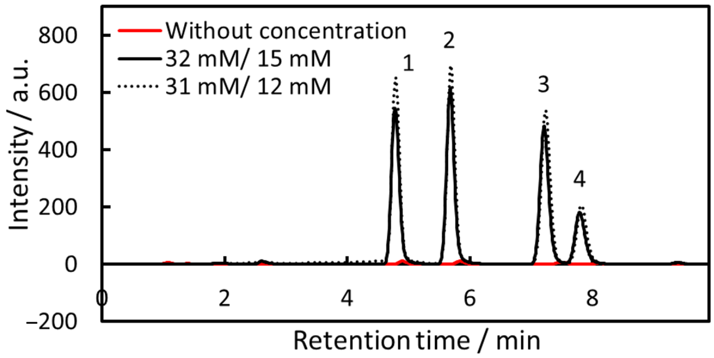


Figure 7. HPLC-FLD chromatograms of BPA–estrogen mixtures with and without concentration. 1: BPA, 2: E2, 3: EE2, 4: E1. [BPA] = [E2] = [EE2] = 20 μg L^{-1}, [E1] = 200 μg L^{-1}. Without concentration, red line; with concentration, [$EHOPA^+$] (mM)/[DS^-] (mM) = 31/12 dotted line, and 32/15 solid line.

3.5. Effects of Centrifugation

Figure 8 shows the effects of centrifugation (30 min) on the height of analyte peaks obtained under optimal conditions at different analyte concentrations.

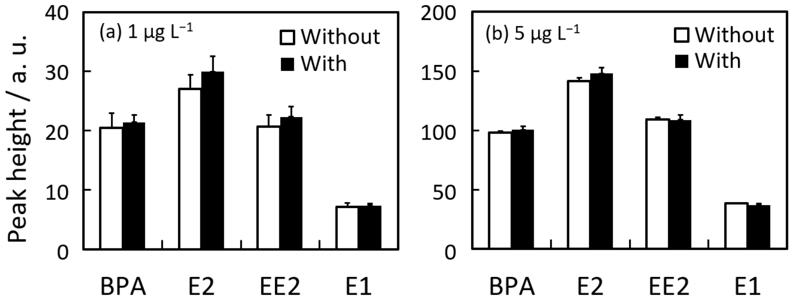


Figure 8. Effects of centrifugation on the height of analyte peaks obtained under the optimal conditions ($n = 4$) for (**a**) [BPA] = [E2] = [EE2] = 1 μg L^{-1}, [E1] = 10 μg L^{-1}, and (**b**) [BPA] = [E2] = [EE2] = 5 μg L^{-1}, [E1] = 50 μg L^{-1}.

No significant differences ($p < 0.01$, 0.05) between the results obtained with and without centrifugation were observed, indicating that the centrifugation-free separation and concentration was feasible. Such findings were corroborated by the extraction efficiencies of BPA, E2, EE2, and E1 obtained under the optimal conditions with (3500 rpm, 30 min) and without centrifugation (92%/93%, 95%/95%, 97%/97%, and 95%/96%, respectively). The detection limits (DLs) of BPA, E2, EE2, and E1 without centrifugation were determined as 0.028, 0.017, 0.045, and 0.27 μg L^{-1}, respectively (Table 4).

Table 4. Detection limits (DLs), enrichment factors, and sensitivities for BPA and estrogen analysis by HPLC-FLD coupled with IALP microextraction without centrifugation.

	DL (μg L^{-1})	**DL (μg L^{-1})**	**Enrichment Factor**	**Sensitivity**	**DL (μg L^{-1})**	**Enrichment Factor**	**Sensitivity**
Concentration	No	Concentration by IALP–ME [1]					
[$EHOPA^+$] (mM) [DS^-] (mM)	-- --		32 15			31 12	
BPA	3.04	0.028	47	109	0.020	56	151
E2	2.86	0.017	56	168	0.016	63	175
EE2	1.94	0.045	63	43	0.018	71	106
E1	31.4	0.27	57	116	0.26	64	121
No. of runs	3		5			5	

[1] IALP–ME: ion-associate liquid-phase microextraction.

3.6. Effects of Organic-Ion Addition Order

Figure 9 presents the effects of the order in which the organic ions were added under optimal conditions on the height of the analyte peaks.

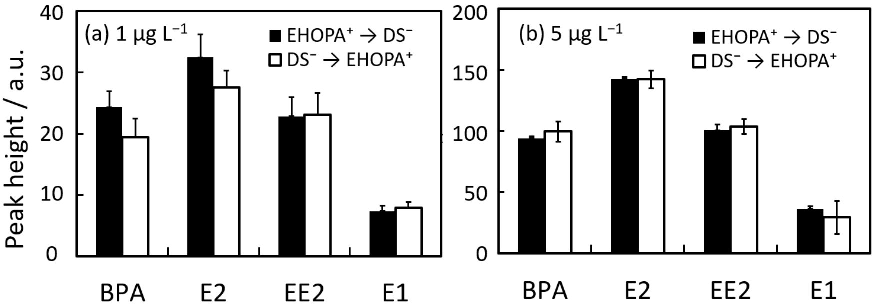


Figure 9. Effects of the addition order of organic ions on the height of analyte peaks obtained at the following under the optimal conditions: (**a**) [BPA] = [E2] = [EE2] = 1 μg L^{-1}, [E1] = 10 μg L^{-1}; (**b**) [BPA] = [E2] = [EE2] = 5 μg L^{-1}, [E1] = 50 μg L^{-1} (n = 5).

The addition order had no significant effect on the peak areas of EE2 and E1 at all tested concentrations and BPA and E2 at 5 μg L^{-1}. However, a significant difference ($p < 0.10$) was observed for BPA and E2 at 1 μg L^{-1}, in which case the peak height was slightly higher when $EHOPA^+$ was added first.

Uncharged analytes directly bind to IAs, whereas the charged ones interact with organic cations or anions to form ion pairs, which subsequently bind to IAs and are incorporated into IALP. BPA and estrogen were not charged under the employed conditions (pH 2) and were therefore expected to directly bind to IAs, in which case the addition order or organic ions should have no effect on the extraction efficiency. However, the significant effect of the addition order observed at low concentrations suggested that this assumption was wrong. Consequently, we hypothesized that BPA and estrogens act as hydrogen-bond donors to generate charged aggregates upon interacting with $EHOPA^+$ (a hydrogen-bond acceptor), and the resulting aggregates are incorporated into IALP.

The order of addition of organic ions is considered to have little effect on IALP formation, because the organic anions were added immediately after the organic cations. Based on the above results, we decided to add the organic cation first.

3.7. Effects of Standing Time on Analyte Peak Height

Figure 10 shows the effects of standing time on analyte peak height as a measure of analyte extraction efficiency. Similarly to the case of TB, the peak heights were stable in the 5–30 min range, indicating that the target substances were distributed into the ionic aggregates at the initial stage of IALP formation.

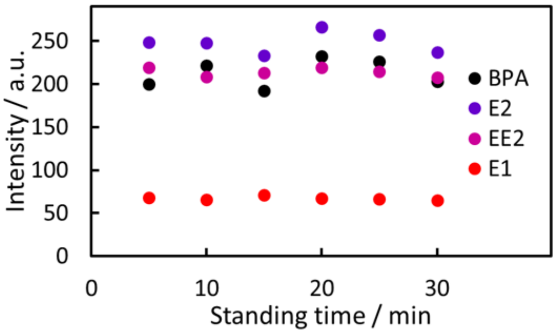


Figure 10. Effects of standing time on analyte peak height and IALP volume under the optimal conditions (n = 3) at [BPA] = [E2] = [EE2] = 10 μg L^{-1} and [E1] = 100 μg L^{-1}.

3.8. Distribution of Estrogens to Different IALPs

The TB extraction efficiencies at 15 and 30 min (%E = 95.1% and 95.5%, respectively) corresponded to the distribution ratios of log D = 3.16 and 3.2, respectively.

To evaluate the formed IALP without centrifugation, we calculated log D and %E. Figure 11 shows the relationship between the distribution of estrogens and log K_{owwin} for three IALPs: $\text{EHOPA}^+/\text{DS}^-$, benzethonium chloride (BTC)/ethylbenzene sulfonate (EBS), and BTC/toluene sulfonate (TS). The smallest log D of estrogens was observed for the $\text{EHOPA}^+/\text{DS}^-$ IALP, attributed to the high volume of IALPs formed from EHOPA-DS, which is more than 60 times higher than that of IALPs formed from BTC and EBS and BTC and TS.

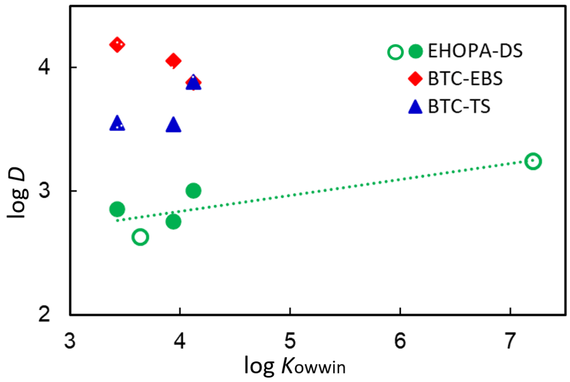


Figure 11. Relationship between log K_{owwin} [30] and log D. The green circles indicate IALP formed from EHOPA^+ and DS^-, the red diamonds indicate IALP formed from benzethonium chloride (BTC) and sodium ethylbenzenesulfonate (EBS), the blue triangles indicate IALP formed from BTC and sodium toluenesulfonate (TS), the green, red, and blue filled markers indicate estrogens (E1, E2, and EE2), and open green circles indicate BPA and thymol blue. The log D values for IALP formed from BTC and EBS and BTC and TS were obtained from [14].

3.9. Detection Limits, Concentration Factors, and Sensitivities

Table 4 presents the performances of the HPLC-FLD analyses with and without IALP extraction, revealing that IALP extraction enabled 47- to 71-fold analyte enrichment and 106- to 175-fold increases in detection sensitivity. Interestingly, the ratio $[\text{EHOPA}^+]$ (mM)/$[\text{DS}^-]$ (mM) = 31/12 resulted in slower phase separation compared to 32/15 (Figure 6). However, because of the smaller volume of IALP (Figure 4), it exhibited a higher peak intensity (Figure 7), greater enrichment factor, and lower detection limit (Table 4).

Table 5 lists the interday prescisions of the HPLC-FLD analyses with IALP extraction. BPA and E2 are in the 1% range for different days.

Table 5. Interday precision for BPA and estrogen analysis by HPLC-FLD coupled with IALP microextraction without centrifugation.

	Concentration (μg L^{-1})	**RSD_1 [1] (%)**	**RSD_2 [1] (%)**
BPA	5	1.4	1.5
E2	5	1.3	1.2
EE2	5	3.5	4.5
E1	50	3.6	5.5
No. of runs	--	4	5

[1] RSD: relative standard deviation.

Table 6 lists the detection limits of some previously reported methods. Our proposed method uses volumetric flasks and commercially available reagents, does not require vortexing or centrifugation, and achieves a one-hundred-times-higher sensitivity for BPA and estrogens compared to the reported values.

Table 6. Extraction procedure for estrogens without derivatizing via HPLC-FLD.

Sample Preparation	Analytical Method	Sample or Matrix	Extractants	Analytes	LCR (μg L^{-1})	DL (μg L^{-1})	Enrichment Factor		Reference
							Volume or Weight	Sensitivity	
IALP–ME	HPLC-FLD	water	EHOPA$^+$, DS$^-$	BPA, E2, EE2, E1	0.2–5 2–50	0.02 0.3	56–71	106–171	This work
IALP–ME	HPLC-FLD	water	EHOPA$^+$, DS$^-$	BPA	0.5–5	0.009	100	310	[18]
DLLME-SFHDES	HPLC-FLD	sewage	1-dodecanol, octanoic acid	BPA, E2	0.00505–5 0.00558–5	0.00153 0.00189	111 106		[9]
AR-VA-ISFDES-LLME	HPLC-FLD	water and beverage	sodium octanoate	BPA	0.12–240	0.03–0.1	29		[11]
ILBABS	HPLC-FLD	wastewater	1-butyl-3-methylimidazolium dicyanamide, $KNaC_4H_4O_6$	EE2	ND	ND	1000	ND	[21]
DLLME	HPLC-FLD	surface and waste water	chlorobenzene	E2 EE2	0.01–0.3 0.01–0.5	0.002 0.0065	145 178		[42]
SPE	HPLC-FLD	wastewater	C18	E2, EE2	10–200	0.0025	2500		[43]
MSPE	HPLC-FLD	tap water	MGON	E2, EE2	0.01–0.25 0.003–0.25	0.0027 0.0008	91 ± 6 119 ± 7		[44]
DPX	UV/MCR-LS/SVM	river water	styrene-divinylbenzene	E2, EE2	10–50	0.06 0.002	ND		[45]
SPE	HPLC-DAD	wastewater	CDMACD	E2 E1	0.7–300 0.04–250	0.02 0.01	81 ± 3 93 ± 2		[46]
VA-SPE	HPLC-DAD	aquaculture water	Luffa cylindrica	E2 E1	15–1000	9.8 ± 0.1 9.6 ± 0.1	4		[47]

LCR: linear calibration range; DL: detection limit; IALP–ME: ion-associate liquid-phase microextraction; EHOPA$^+$: ethylhexyloxypropylamine; DS$^-$: dodecylsulfate; HPLC: high-performance liquid chromatography; FLD: fluorescence detection; BPA: bisphenol A; E2: 17β-estradiol; EE2: ethinyl estradiol; E1: estrone; DLLME-SFHDES: dispersive liquid–liquid microextraction method based on solidification of floating hydrophobic deep eutectic solvents; AR-VA-ISFDES-LLME: acid-induced dispersive liquid–liquid microextraction based on in situ formation of hydrophobic deep eutectic solvents; ILBABS: ionic-liquid-based aqueous biphasic systems, ND: no description; SPE: solid-phase extraction; MSPE: magnetic solid-phase extraction; MGON: maghemite–graphene oxide nanoparticles; DPX: disposable pipette extraction; UV: ultraviolet; MCR-ALS: multivariate curve resolution with alternating least squares; SVM, support vector machine; CDMACD: *β*-cyclodextrin-decorated magnetic-activated carbon adsorbent; DAD: photodiode array detector; VA-SPE: vortex-assisted solid-phase extraction; UHPLC-MS/MS: ultra-high-performance liquid chromatography tandem mass spectrometry.

3.10. Application to Real Samples

Next, we applied our method to real samples. Estrogens are known to be present in sewage [9]; thus, we selected treated sewage water and river water from a sewage treatment plant or chemical factory upstream were selected as sample waters. Additionally, seawater was chosen to assess the effect of a high salt concentration and matrix interference.

3.10.1. Discharged Water from a Sewage Treatment Plant

Table 7 shows the results of recovery experiments performed on the effluent from the Hamakurozaki water purification plant. The recovery of BPA and estrogens from treated sewage water exceeded 87%. The %*E* from ultrapure water to IALP ranged from 93% to 97% (as discussed in Section 3.5), whereas the %*E* to treated sewage water showed a slight decrease, ranging from 89% to 94% (Table 7).

Table 7. Results of addition and recovery experiments performed on the effluent from the Hamakurozaki water purification plant (Toyama Prefecture).

Analyte	Added (μg L^{-1})	Detected (μg L^{-1})	RSD [1] (%)	Recovery (%)	%*E*	No. of Runs
BPA	-	0.19	2.1	-	-	5
E2	-	<0.02	-	-	-	5
EE2	-	<0.02	-	-	-	5
E1	-	<0.4	-	-	-	5
BPA	0	0.28	11	-	89	3
	5	4.4	7.3	88		3
	10	8.9	9.2	89		3
E2	0	<0.02	81	-	93	3
	5	4.7	8.3	94		3
	10	9.3	14	93		3
EE2	0	<0.02	12	-	94	3
	5	4.4	12	88		3
	10	8.7	17	87		3
E1	0	<0.4	75	-	92	3
	50	45	12	89		3
	100	87	16	87		3

[1] RSD: relative standard deviation.

3.10.2. Seawater

Figure 12 shows the calibration curves obtained for solutions of BPA and estrogens in purified water and seawater, showing that for each analyte, the calibration curve slope was slightly lower (88–92%) when seawater was used. In the case of seawater, the phase separation is faster than in pure water due to the salting-out effect, meaning that the presence of salt in seawater promotes the phase separation. Figures 3b and 10 show that the extraction %*E* did not change even after time elapsed and phase separation progressed, suggesting that the rate did not decrease due to the salting-out effect. A slight decrease in the slope was observed for both treated sewage (87–92%) and river water (90–97%).

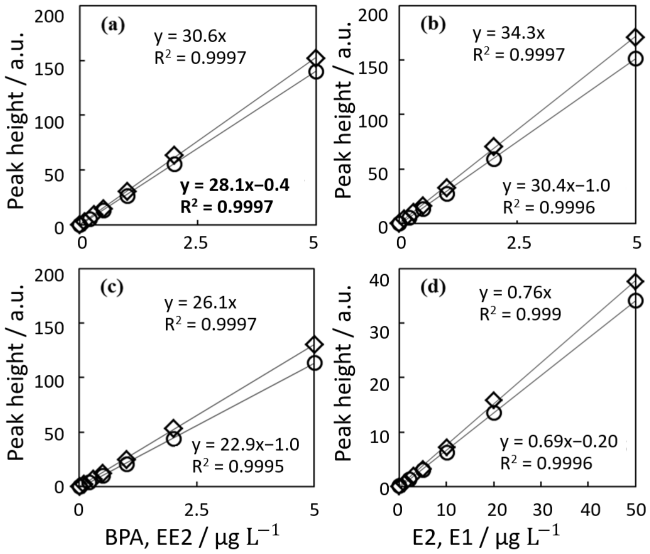


Figure 12. Calibration curves for the analyses of (**a**) BPA, (**b**) E2, (**c**) EE2, and (**d**) E1 in pure water (◇) and seawater (○). The linearity range in seawater was determined as 0–5 μg L^{-1} for BPA, E2, and EE2 and 0–50 μg L^{-1} for E1. Conditions: [EHOPA^{+}], 31 mM; [DS^{-}], 12 mM; pH, 1.9.

The recovery ranged from 78% to 92%, and no BPA, E2, EE2, or E1 were detected in nonspiked seawater (Table 8). Hence, the developed IALP extraction method was concluded to be efficient for the quantitation of BPA and estrogens in seawater.

Table 8. Results of addition and recovery experiments performed on the seawater from the Toyama Bay (Toyama Prefecture).

Analyte	Added ($\mu g\ L^{-1}$)	Detected ($\mu g\ L^{-1}$)	RSD [1] (%)	Recovery (%)	No. of Runs
BPA	0	<0.02	32	-	5
	2	1.74	3.3	87.2	5
E2	0	<0.02	15	-	5
	2	1.59	4.0	79.5	5
EE2	0	<0.02	43	-	5
	2	1.56	4.2	77.8	5
E1	0	<0.4	12	-	5
	20	18.36	5.5	91.8	5

[1] RSD: relative standard deviation.

Comparisons of the calibration curves of ultrapure water or seawater with and without the washing operation revealed that the slope of the calibration curve was smaller when the washing operation was involved. Therefore, for enrichment of BPA and estrogen by IALP ($EHOPA^+/DS^-$), the measurement should be conducted without the washing operation.

3.10.3. River Water

The proposed method was applied to the analysis of a river water sample. The BPA concentration detected in the river water was 0.02 $\mu g\ L^{-1}$, which is equal to the detection limit (0.02 $\mu g\ L^{-1}$) and below the lower limit of quantification (0.07 $\mu g\ L^{-1}$), while E2, EE2 and E1 were not detected. Such a result is reasonable because a sewage treatment plant and a chemical plant are located within 3 km upstream of the water sampling point. The slope ratios of the calibration curves prepared from ultrapure water and river water are as follows: BPA: 97%, E2: 94%, EE2: 89%, and E1: 90%.

4. Conclusions

With increasing concentrations of organic cations and anions, the IALP volume increased, and a liquid IALP was formed without centrifugation. IALP ($EHOPA^+/DS^-$) formation required an excess amount of $EHOPA^+$ relative to DS^-, whereas centrifugation had no significant effect on the results of the HPLC-FLD analysis. The position of the region where the IALP was formed in the absence of centrifugation was identified in the $[EHOPA^+]$–$[DS^-]$ space. Extraction efficiencies of >90% were obtained for BPA and estrogens without centrifugation. The separation/concentration of BPA and estrogens was coupled with their analysis by HPLC-FLD to achieve the accurate quantitation of water samples. Even though the recommended standing time was 30 min, the samples could be analyzed via HPLC after 5 min of standing.

To achieve MAMDL for estrogen, our HPLC-FLD method requires several tens of thousands of fold enrichments. Although our method increased sensitivity by about 100-fold, it was insufficient for the quantification of E2 and EE2. However, the lower detection limit for BPA was 1/5000 of the standard value, indicating that the developed method is suitable for screening BPA.

The $EHOPA^+/DS^-$ IALP does not contain any benzene rings or imidazole groups and can be used for fluorometric and absorbance determination. It is fast and easy to use (no centrifugation required), enables analyte concentration (approximately 100-fold), and has a high affinity for estrogens. The developed IALP extraction method was successfully applied to the separation and concentration of BPA and estrogens for HPLC-FLD quantitation. The above advantages render our method suitable for the separation, concentration, and quantitation of other trace analytes such as polycyclic aromatic hydrocarbons, phthalates, and alkyl phenols, upon relevant adjustments of the HPLC conditions. Since the concentration step does not require the use of a vortex or centrifuge, our method is applicable to the concentration of easily changeable constituents at the water sampling site.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations11060173/s1, Figure S1: Overview of previous microextraction of ion-associate liquid phase (IALP) formed in situ from the aqueous phase by adding organic cation and organic anion solutions; Figure S2: Chemical structure of analytes (BPA, E2, EE2, E1, and TB dye), and organic cation source (EHOPA); Figure S3: Chromatograms of BPA, E2, and EE2 mixtures without enrichment when varying the ratio of acetonitrile to aqueous phase in the mobile phase.

Author Contributions: Conceptualization, S.O. and N.H.; methodology, S.O., S.T. (Seira Takahashi), N.K. and N.H.; investigation, S.O., S.T. (Seira Takahashi), T.O. and N.H.; resources, K.S., H.K., T.O., S.T. (Shigeru Taguchi) and N.H.; writing—original draft preparation, S.O. and N.H.; writing—review and editing, S.O., K.S., H.K., S.T. (Shigeru Taguchi) and N.H.; visualization, S.O., S.T. (Seira Takahashi), N.K. and N.H.; supervision, K.S., H.K., S.T. (Shigeru Taguchi) and N.H.; project administration, N.H.; funding acquisition, N.H. All authors have read and agreed to the published version of the manuscript.

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