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High-performance liquid chromatography with peroxyoxalate chemiluminescence detection of bisphenol A migrated from polycarbonate baby bottles using 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride as a label

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Abstract

A highly sensitive and selective high-performance liquid chromatographic method with peroxyoxalate chemiluminescence detection for the determination of bisphenol A at sub-ppb levels is described. Bisphenol A was derivatized with 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride and the excess unreacted reagent was removed by a simple solid-phase extraction procedure with recoveries of approximately 60%. The separation was carried out isocratically on an ODS column and the derivatized bisphenol A was detected by peroxyoxalate chemiluminescence. A mixture of bis[2-(3,6,9-trioxadecanyloxycarbonyl)-4- nitrophenyl]oxalate (0.6 m*M*) and hydrogen peroxide (25.0 m*M*) dissolved in acetonitrile was used as a chemiluminescence reagent solution with a mixture of imidazole-HNO_3 buffer (40.0 m*M*, pH 7.0): acetonitrile (17:83, v/v) as a mobile phase. The linear standard curve was obtained over the range from 0.57 (2.5) to 22.8 (100) ppb (n*M*) (r=0.996) with a detection limit of 0.38 ppb (2.8 fmol on column) at a signal-to-noise ratio of 3. The method was successfully applied to the determination of bisphenol A in hot water in contact with commercially available baby bottle samples.

Keywords: Polycarbonate bottle; Peroxyoxalate chemiluminescence detection; Bisphenol A; 4-(4,5-Diphenyl-1*H*-imidazol-2-yl) benzoyl chloride

1. Introduction

In 1993, Krishnan et al. reported that an estrogenic substance was released from polycarbonate containers during autoclaving, the substance was identified as 4,4′-isopropylidenediphenol (bisphenol A, BPA) by ^{1}H-NMR spectroscopy and mass spectrometry (MS) [1]. This compound has attracted considerable attentions owing to its endocrine disrupting effect as a xenobiotic with estrogenic action. Animal experiments have proved that BPA induces proliferation, alternation and c-fos gene expression in

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the female reproductive tract [2]. Although it binds to estrogen receptors and acts as either an agonist or antagonist [3], over one million tons of BPA are produced yearly all over the world [4]. BPA is the monomer used in the manufacture of epoxy resins for the lacquer lining of metal food cans, as a monomer of polycarbonates [5], and the monomer of plastic used in the base paste of the dental sealants [6]. Though polycarbonates are tough and durable plastics having good physical stability at high temperatures, it is possible that at higher temperatures and especially at alkaline pH, hydrolysis of polycarbonates might occur to form traces of free BPA monomer on its surface, which could thereafter migrates during subsequent contact with foods or beverages. Researches have shown that BPA can exhibit xenoestrogenic effects in vitro at very low concentrations of 6 ppb (25 n*M*) [7], thus, a highly sensitive and selective method is necessary for monitoring the level of BPA in biological and environmental samples.

Recent years, gas chromatography-MS (GC-MS), high-performance liquid chromatography (HPLC) with MS, UV or fluorescence detection methods have been developed for BPA determination[4,8,9,10,11]. Among these methods, either complicated instruments or large sample volumes are necessary for ultra trace determination. In our laboratory, we developed a fluorescent labeling reagent, 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl) [12], for derivatization of amines [13] and phenolic compounds [14] (i.e. phenol, chlorophenols and xylenols) which have been detected in ultra trace quantities by HPLC with fluorescence detection.

In this work, a sensitive HPLC-peroxyoxalate chemiluminescence (PO-CL) detection method was developed in an attempt to sensitively detect BPA at sub-ppb level which only required a small sample volume (not more than 1.0 ml). The proposed method was successfully applied to the determination of BPA derived from commercially available baby bottle samples.

2. Experimental

2.1. Materials and reagents

BPA was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). DIB-Cl was synthesized in our laboratory as reported previously [12]. Bis[2-(3,6,9-trioxadecanyloxycarbonyl)-4-nitrophenyl]oxalate (T-DPO), hydrogen peroxide, acetonitrile and triethylamine (TEA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Solid-phase extraction (SPE) cartridges packed with ODS were obtained from Daiso (Osaka, Japan). Water was passed through a pure line WL21P (Yamato Sciences, Tokyo, Japan).

Standard stock solution of BPA was prepared by dissolving the appropriate amounts in acetonitrile to give a final concentration of 50 m*M*, which was kept at 4°C in the dark. Working solutions were prepared by further diluting the stock solution in acetonitrile.

2.2. Preparation of authentic DIB-BPA sample

Authentic DIB-BPA sample was prepared as follows: To the suspension of DIB-Cl (1.00 g, 2.79 mmol) in 250 ml of acetonitrile, BPA (0.32 g, 1.39 mmol) was added under stirring. To this mixture, TEA (1.2 ml) was added and the resultant clear yellow solution was further stirred at room temperature (ca. 25°C) for 1 h to give white-yellowish precipitates. After filtration, the precipitates were washed thoroughly with cold acetonitrile and dried in vacuo. Yield: 0.85 g, 70.0%, mp: 199–201°C (Yanaco MP-53 melting point apparatus, Kyoto, Japan). Calcd. for $C_{59}H_{44}N_4O_4.H_2O$: C, 77.95; H, 5.32; N, 6.16%, found: C, 78.25; H, 5.53; N, 5.81%. FAB-MS (*m*/*z*): 873 $[M+H]^+$, (JMS-DX 303 fast-atom bombardment mass spectrometer, JEOL, Tokyo).

2.3. HPLC-PO-CL system

The HPLC system consisted of two LC-10ADvp chromatographic pumps (Shimadzu, Kyoto, Japan), a 7725 injector with a 5-μl sample loop (Rheodyne, Cotati, CA, USA), a Daisopak-SP-120-5-ODS-BP analytical column (250×4.6 mm I.D., 5 μm, Daiso, Osaka, Japan), a JASCO 825-CL chemiluminescence detector (Jasco, Tokyo, Japan), an SC-77 signal cleaner (System Instrument Company, Tokyo, Japan), and an R-111 recorder (Shimadzu). The HPLC separation was carried out isocratically using a mixture of acetonitrile–imidazole–HNO_3 buffer (40.0 m*M*, pH 7.0) (=83:17, v/v) as the mobile

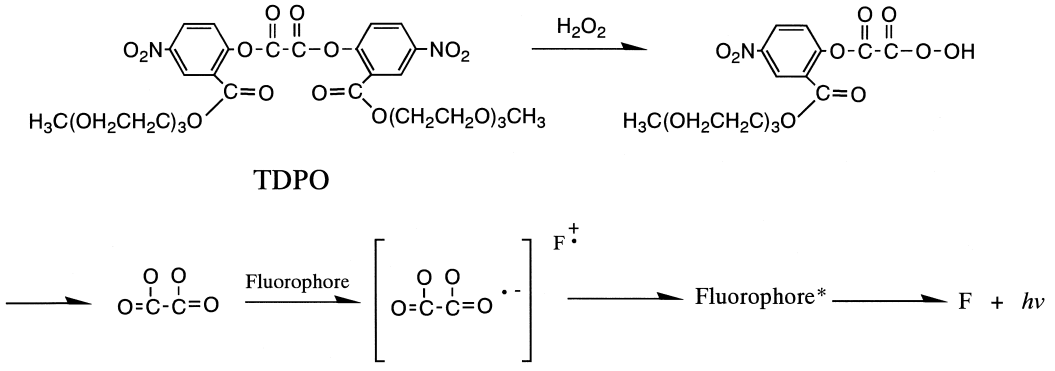

Fig. 1. Peroxyoxalate chemiluminescence reaction scheme of TDPO with hydrogen peroxide.

phase. A mixture of 0.6 m*M* TDPO and 25.0 m*M* hydrogen peroxide in acetonitrile as the CL reagent was freshly prepared prior to use. The flow-rate of both the mobile phase and CL reagent was set at 1.0 ml/min. The column effluent and CL reagent were mixed with a standard three-way joint immediately before the detector. Solvents were sonicated and degassed by an aspirator before use. The CL reaction scheme is shown in Fig. 1.

2.4. Sample pretreatment and derivatization reaction

A hundred-ml portions of boiling water were transferred into a commercially available baby bottle and it was tightly capped and kept in an oven at 95°C for 30 min [10]. After allowed to cool to room temperature, a portion (200 μl) of the migrated solution was transferred into a screw-capped mini amber-glass reaction vial and evaporated to dryness in a centrifugal evaporator (model RD-31, Yamato, Tokyo). Following reconstituting the residue in 100 μl of acetonitrile, an equal volume of 10 m*M* DIB-Cl in acetonitrile and 5 μl of 3 *M* TEA in acetonitrile were added, vortex-mixed well and allowed to react at room temperature for 20 min. The scheme for the derivatization reaction of BPA with DIB-Cl is shown in Fig. 2.

2.5. Removing the excess DIB-Cl by SPE

In order to remove the excess DIB-Cl, ODS cartridges were used. The cartridge was activated with 5 ml of acetonitrile and conditioned with 5 ml

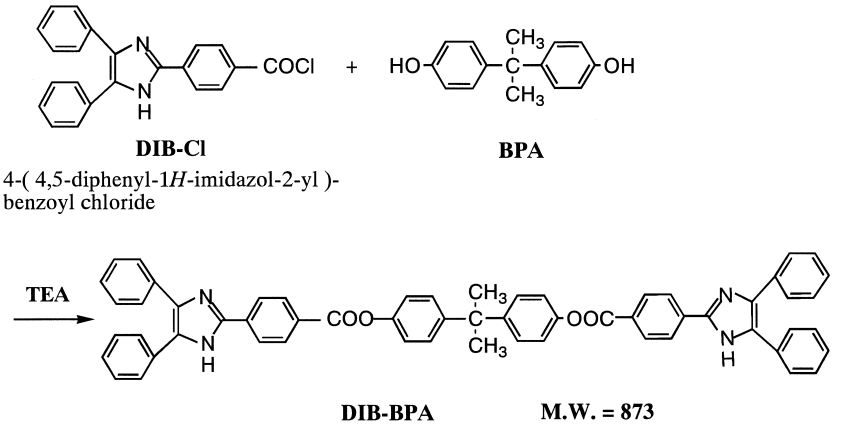

Fig. 2. Reaction scheme of bisphenol A with DIB-Cl.

of deionized water. After applying the reaction mixture, the cartridge was washed with 5 ml of acetonitrile–water (70:30, v/v) and dried under vacuum to remove the washing solution. Then, the absorbed DIB-BPA derivative was eluted with 300 μl of acetonitrile. A 5 μl portion of the eluate was injected onto the HPLC column.

2.6. Calibration curve and precision

The calibration curve of BPA was prepared in the concentration range from 0.57 (2.5) to 2.28 (10) ppb (n*M*) in spiked samples which were prepared by adding aliquots of BPA standard solutions to the solution migrated from baby bottles. Peak height was corrected by subtracting from that obtained from the migrated solution. Intra- and inter-assay variations were assessed using the sample spiked at the levels of 1.14 ppb (5 n*M*) and 2.28 ppb (10 n*M*) of BPA. Precision and accuracy are reported as percent relative standard deviation (RSD,%) and percent accuracy {[(mean observed concentration)/(spiked concentration)]×100}, respectively.

3. Results and discussion

3.1. Removal of the excess reagent

Due to the excess unreacted reagent which appeared as a broad band in the chromatogram, a baseline separation from the peak corresponds to DIB-BPA could not be obtained. Therefore, a SPE treatment was attempted to remove or reduce the reagent blank peak. An increase in acetonitrile content in the washing solution resulted in a decrease in the recovery of DIB-BPA after SPE. However, the use of 70% of aqueous acetonitrile as a washing solution resulted in chromatograms where the DIB-BPA peak could be completely separated from the reagent blank associated with a down-shifting of the base line (Fig. 3). The maximum peak height was obtained by eluting with 300 μl of acetonitrile. The recovery of DIB-BPA by using SPE treatment was approximately 60%. Although this is relatively low, yet a good baseline separation was obtained and the sensitivity could subsequently be increased substantially.

3.2. Optimization of determination condition of BPA

The parameters for determination of BPA were investigated by using the HPLC-PO-CL system described in Experimental. The effect of DIB-Cl concentration on relative chemiluminescence intensity (RCI) was studied (Fig. 4). BPA gave the maximum and constant RCI at DIB-Cl concentrations of $\geq$7.5 m*M*. A concentration of 10 m*M* DIB-Cl was selected for subsequent experiments. The RCI increased with an increase in TDPO and hydrogen peroxide concentrations with the maximum signal-to-noise (S/N) ratios and RCI obtained at concentrations of 0.6 and 25.0 m*M* of TDPO and hydrogen peroxide, respectively, therefore, these concentrations were adopted for the following experiments (Fig. 5). Imidazole was used as a catalyst to enhance CL intensity and also as a basic pH modifier [15,16]. The maximum RCI was obtained with 40.0 m*M* imidazole-HNO_3 buffer solution at pH 7.0 included in the mobile phase, whereas a flow-rate of the chemiluminescence reagent at 1.0 ml/min gave the highest signal. Since no significant increase in RCI was observed by changing the reaction coil length from 40 to 160 cm, a 40 cm coil was used.

3.3. Calibration curve, detection limit and precision

The calibration curve of standard BPA solution showed a good linearity ($r=0.996$) between the chemiluminescence intensity and concentrations of BPA from 0.57 (2.5) to 22.8 (100) ppb (n*M*). The detection limit at a S/N ratio of 3 was 0.38 ppb (2.8 fmol on column).

3.4. Validation

The working curve used for the determination of BPA migrated from baby bottle was linear over the range from 0.57 to 2.28 ppb; $y=369.50x+0.27$ ($r=0.999$), the limit of quantification (LOQ) was 0.57 ppb (4.2 fmol on column). Intra- and inter-day validation data of the proposed method are shown in Table 1. The relative standard deviation (RSD,%) of the intra-day precision study ($n=4$) were 7.8 and 3.0, and the inter-day assay ($n=3$) were 12.3 and 7.5

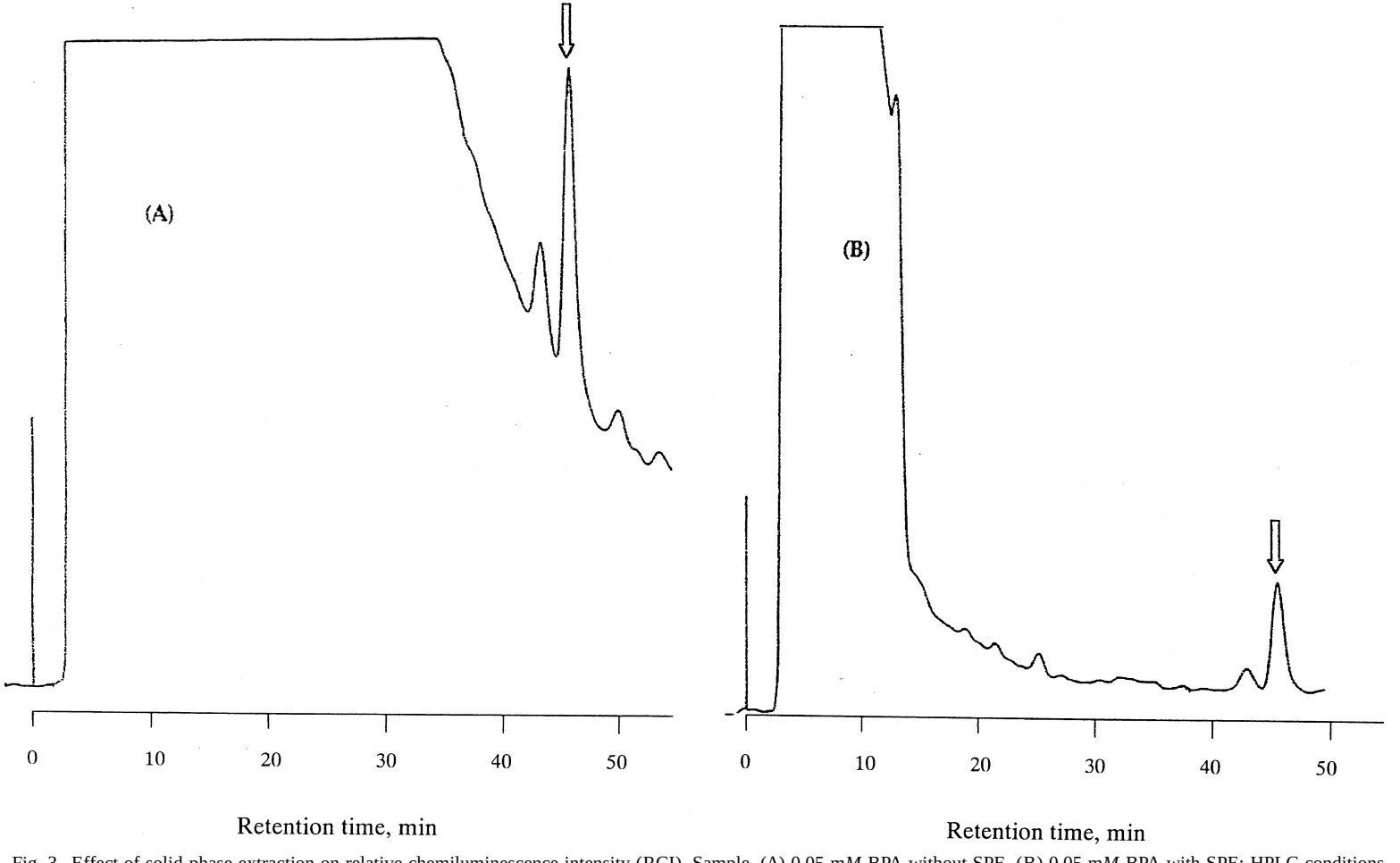

Fig. 3. Effect of solid-phase extraction on relative chemiluminescence intensity (RCI). Sample, (A) 0.05 m*M* BPA without SPE, (B) 0.05 m*M* BPA with SPE; HPLC conditions were described in the Experimental.

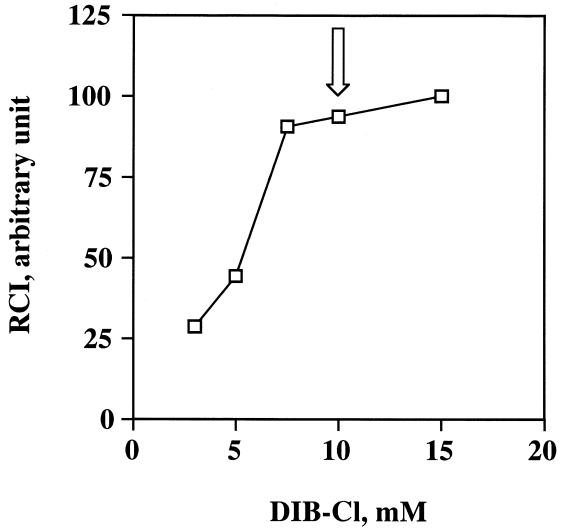

Fig. 4. Effect of DIB-Cl concentration on relative chemiluminescence intensity(RCI). Sample, 0.1 m*M* BPA; HPLC conditions were described in Experimental.

for the 1.14 and 2.28 ppb of BPA spiked samples, respectively. Since the quenching of fluorescent intensity was not observed with the elapse of time after the derivatization, the DIB-BPA derivative is stable for at least 24 h.

3.5. Identification of authentic DIB-BPA sample

BPA has two equivalent phenolic groups that react with DIB-Cl. In order to confirm whether DIB-BPA is the mono- or di-labeled fluorescent derivative, we prepared and purified an authentic DIB-BPA sample. The elemental analysis as well as the FAB-MS data suggest that the formation of a di-labeled DIB-BPA derivative is favored if BPA and DIB-Cl are allowed to react in 1:2 molar ratio. The molecular ion obtained with FAB-MS at 873 is in agreement with that calculated for the di-labeled DIB-BPA derivative. Under relatively mild conditions (i.e., TEA,

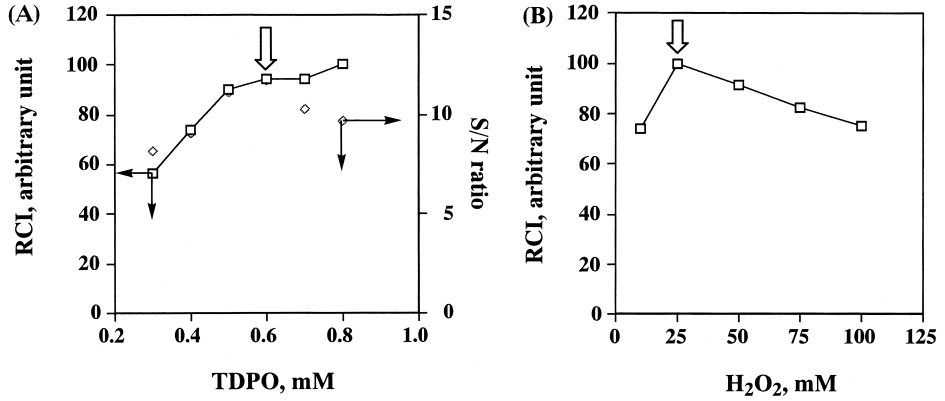

Fig. 5. Effects of the concentrations of TDPO (A) and H_2O_2 (B) on relative chemiluminescence intensity (RCI). Sample, 0.1 m*M* BPA; HPLC conditions were described in Experimental.

Table 1
Intra- and inter-day accuracy and precision of BPA in spiked migrated solution from baby bottles

BPA added (μg/l)	Intra-assay ($n=4$)			Inter-assay ($n=3$)	
	Found±SD[a] (μg/l)	Accuracy[b] (%)	RSD[c] (%)	Found±SD[a] (μg/l)	RSD[c] (%)
0	0.60±0.04	–	–	–	–
1.14	1.67±0.13	93.86	7.8	1.01±0.12	12.3
2.28	2.70±0.08	92.11	3.0	2.01±0.15	7.5

[a] SD=standard deviation.
[b] Expressed as [(mean observed concentration)/(spiked concentration)]×100.
[c] RSD=relative standard deviation.

room temperature, 1 h) the reactivity of DIB-Cl with BPA is satisfactory and a yield of 70% was obtained.

3.6. Assay of baby bottle samples

Baby bottle samples were obtained from commercial sources including two plastic bottles (sample 1 and 2) and a glass bottle (sample 3). The plastic bottles are made of polycarbonate. Migration tests for plastic and glass baby bottles were repeated for four successive times of each as shown in Table 2. For the first migration test, BPA was found to migrate at 0.59 and 0.75 ppb from sample 1 and 2 respectively, while none appeared to migrate from the glass bottle. In the second and third migration test, BPA was at concentration less than detection limit. After evaporating larger volumes (5 times as was stated in Experimental) of the migrated solutions in contact with the baby bottles, BPA concentrations were calculated to be 0.13 and 0.16 ppb respectively, which represents a five-fold decreasing compared to the first migration test of each. Next, baby bottles were washed with a brush facilitate migration by creating scratches on the inner surface prior to the fourth migration test, in order to observe if there would be any change in the concentration of BPA migrated. No significant change was observed compared with the third migration test. In case of the glass bottle, after the second migration test, no BPA could be detected even by increasing the volume of migrated solutions. The representative chromatograms for BPA derived from baby bottles (sample 2, first migration) are shown in Fig. 6. Peaks appeared at shorter retention times than BPA have not been identified. However it is possible that decomposed BPA-like compounds from polycarbonate containers might exist in the sample.

Table 2
Determination of migrated BPA from baby bottle samples

Sample	Raw Material	Migration. Sequences	Assay of BPA (ppb) (Mean± SD)[a] ($n=4$)
1	Polycarbonate	1st	0.59±0.08
		2nd	0.13±0.01
		3rd	0.14±0.01
		4th[b]	0.18±0.02
2	Polycarbonate	1st	0.75±0.09
		2nd	0.16±0.02
		3rd	Trace[c]
		4th[b]	Trace[c]
3	Glass	1st	N.D.[d]
		2nd	N.D.[d]
		3rd	N.D.[d]
		4th[b]	N.D.[d]

[a] SD = standard deviation.
[b] Baby bottles were cleaned with a brush before the 4th migration test.
[c] Less than limit of quantification.
[d] N.D. = not detected.

4. Conclusions

In this paper, we reported a highly sensitive and selective method for determination of BPA at sub-ppb level. Fluorescence di-labeling with DIB-Cl was confirmed by elemental analysis and MS and resulted in an intense CL of the derivative. A linear standard

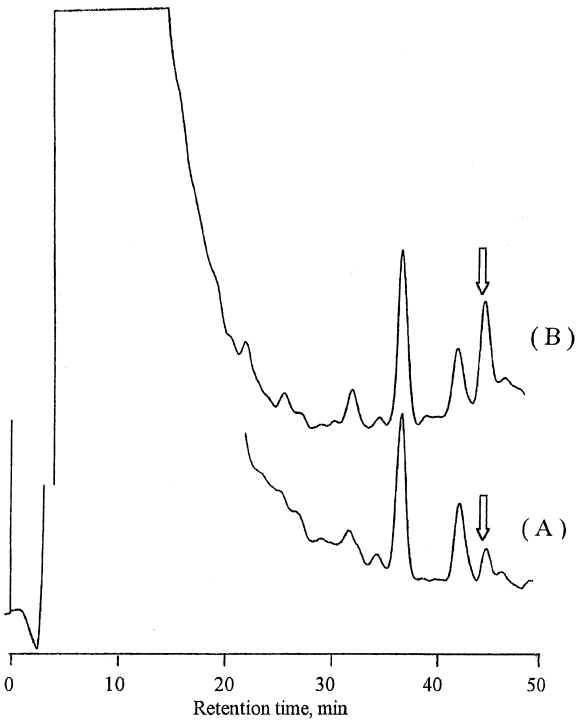

Fig. 6. Chromatograms for BPA derived from a baby bottle. Sample, (A) baby bottle sample 2, first migration, (B) baby bottle spiked 1.14 ppb BPA; HPLC conditions were described in Experimental.

curve was obtained from 0.57 (2.5) to 22.8 (100) ppb (nM) ($r = 0.996$). Compared with the fluorescence detection method we reported previously [14], the use of chemiluminescence detection in the proposed method resulted in a 40-fold increase in the sensitivity; 10~20 times more sensitive compared with HPLC-UV and fluorescence methods [9,10,11], with comparable sensitivity to that obtained by LC-MS [8]. By using this method, BPA migrated from the inner surface of polycarbonate baby bottles to hot water was detected at 0.59–0.75 ppb level during the first use. Although BPA from baby bottles seem to migrate at trace amount, it might be more harmful to newborns and infants considering the body weights compared to those of adults. Moreover, the accumulation of BPA in human tissues should not be under estimated. We suggest that polycarbonate baby bottles should be rinsed thoroughly before put into the first use.

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