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# Highly Sensitive Time-Resolved Fluorometric Determination for Alkylphenols by High Performance Liquid Chromatography Using $\beta$ -Diketonate Europium Chelate

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## Abstract

A time-resolved fluorescence detection system by HPLC for alkylphenols has been developed using a europium chelate fluorescent labeling reagent, 4-(1,1,1,2,2-pentafluoro-3,5-dioxo-pentyl)-[1,11'-biphenyl]-4'-sulfonyl-chloride (PDB-SCI). The labeling reaction was on to phenolic hydroxyl group of alkylphenols, and the derivatized alkylphenols were separated on a HPLC column. After separation, EuCl<sub>3</sub>, TOPO (tri-n-octylphosphine oxide), and Triton X-100 were introduced to the eluent, and the fluorescence of the europium chelate was measured with the time-resolved phosphorescence detector. The detection limit for 4-nonylphenol in the time-resolved fluorometric HPLC method was 4 to 65 times lower than that of the commonly used LC-MS method. The detection limits for 4-nonylphenol in the river water sample was 0.99 ng/L. The average recovery from river water samples was 64.8%. The precision of the method ranged from 2.5 to 5.9%. Using this method, the concentration of 4-nonylphenol in a river water sample was 4.8 ng/L.

*key words:* alkylphenols; time-resolved fluorescence; HPLC; Eu chelate; river water

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## Introduction

Nonylphenol polyethoxylates (NPEO) are important nonionic surfactants which have been used commercially for many years as emulsifiers and solubilizers in pharmaceutical [1] and agrochemical formulations, [2] cosmetics [3] and various biotechnological processes [4] due to their favorable physicochemical characteristics. The main source of nonylphenol in the environment has been recognized as a biodegradation product of NPEO [5], and nonylphenol has been reported to cause a number of estrogenic responses in a variety of aquatic organisms. [7]

Gas chromatography/mass spectrometry (GC/MS) [8,9] and, more recently, liquid chromatography/mass spectrometry (LC/MS) [10-13] have been reported for the environmental nonylphenol.

Even these methods are quite sensitive, still higher sensitivity has been required for one environmental monitoring system.

Fluorescent europium chelates exhibit large Stokes shifts (~290 nm) with no overlap between the excitation and emission spectra. These chelates have a very narrow (10-nm bandwidth) emission spectra at 615 nm. In addition, their long fluorescence lifetimes (600-1000  $\mu$ s) allow use of microsecond time-resolved fluorescence measurements, which further reduces the observed background signals. [14-16] We have constructed the time-resolved fluorometric detection system for HPLC by using the fluorescent  $\beta$ -diketonate europium (Eu) chelate label, and the time-resolved fluorometric HPLC method was applied to the analysis of estrogens. [15,16]

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In this paper, the description is on the analysis of 4-nonylphenol in a river water sample by using a  $\beta$ -diketonate Eu chelate label, 4-(1,1,1,2,2-pentafluoro-3,5-dioxo-pentyl)-[1,11'-biphenyl]-4'-sulfonyl-chloride (PDB-S-Cl)\*, and time-resolved fluorometric HPLC system. The detection limit for alkylphenols was compared with that of the commonly used LC/MS method. [10,11,13]

## Materials and Methods

### Time-resolved fluorometric HPLC

Chromatography was performed with a Shimadzu (Kyoto, Japan) LC-10 AT<sub>VP</sub> HPLC pump, a Rheodyne (Cotati, CA, USA) Model 7725 I syringe-loading sample injector valve (10- $\mu$ l loop). The time-resolved phosphorescence detector<sup>®</sup> (TRPD) equipped with 16  $\mu$ l flow cell (Tosoh, Tokyo, Japan) was used for measuring the fluorescence of the Eu chelates in HPLC. The operation and the data recording were performed with a PC computer, which was connected to TRPD via a RS-232 C cable. The TRPD and the computer software for time-resolved fluorescence measurement was Hamamatsu Photonics K.K. (Hamamatsu, Japan) made and designed for only analysis. Teflon tubings of 0.25 mm I.D. were generally used for all the flow circuit connections. The measurement conditions were as follows: excitation at 350 nm, emission at 615 nm, the excitation gate time of 0.01 ms, the delay time of 0.1 ms, and the signal gate time of 1.8 ms, and the excitation interval of 2 ms.

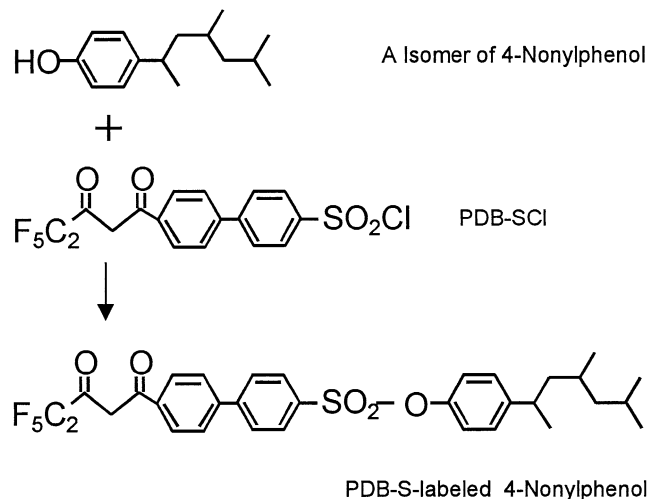
### Reagents and chemicals

A  $\beta$ -diketone europium chelate reagent, PDB-S-Cl was synthesized and purified as described previously.[16] Organic solvents and distilled water of HPLC grade (Kanto Chemicals Co., Tokyo, Japan) were used without further purification. Alkylphenols, bisphenol-A, *t*-butylphenol, 4-nonylphenol, and 4-octylphenol were purchased from Wako Pure Chemicals Industries (Osaka, Japan). The stock solutions of each alkylphenol (1000  $\mu$ g/ml) were prepared in acetonitrile. The stock solutions were stored in screw-capped centrifuge tubes at -20 in the dark. A Sep-Pak C<sub>18</sub> cartridge was purchased from Waters (Massachusetts, USA), and was preconditioned with 20 ml of acetonitrile, 5 ml of distilled water, 5 ml of ethylacetate, and 5 ml of distilled water prior to use. A Sep-Pak NH<sub>2</sub> cartridge (Waters) was also with 10 ml of methanol, 5 ml of ethylacetate, 10 ml of methanol, and 5 ml of ethylacetate prior to use. All other chemicals were of analytical reagent grade.

### Prederivatization of alkylphenols

The prederivatization of 4-nonylphenol with PDB-S-Cl was shown in Figure 1. The derivatization of the alkylphenols with

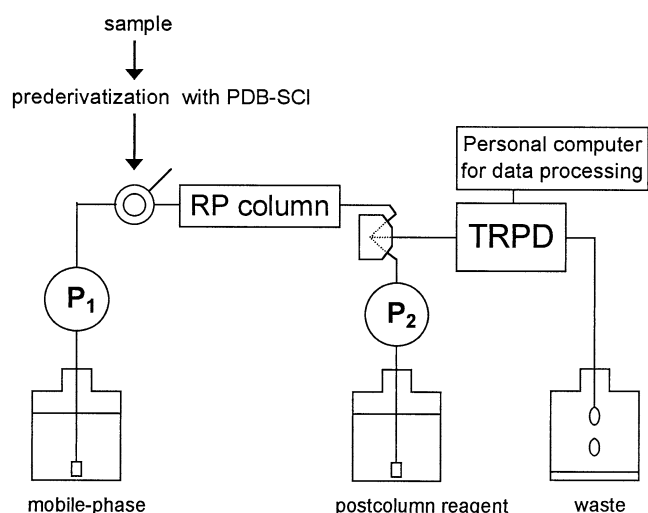
PDB-S-Cl was performed as follows. To an alkylphenol solution (22  $\mu$ l) was added 20 mM carbonate buffer of pH 10 (8  $\mu$ l) and 12.5  $\mu$ g/ml PDB-S-Cl (5  $\mu$ l). The solution was incubated for 30 min at 70 in the dark, and 10% HCl (5  $\mu$ l) was added to the reaction mixture.



**Figure 1.** Derivatization of 4-nonylphenol with PDB-S-Cl

### Analysis of prederivatized alkylphenols by time-resolved fluorometric HPLC

The PDB-S derivatives (10  $\mu$ l) were chromatographed on a reversed-phase column, YMC-Pak TMS (150 $\times$ 4.6 mm I.D., particle size 5  $\mu$ m, YMC, Kyoto, Japan), by isocratic elution with acetonitrile-water (70:30, v/v) containing 0.05% of trifluoroacetic acid as the mobile phase. The flow rate of the mobile phase was set at



**Figure 2.** Schematic representation of the post-column Eu derivatization HPLC system

P<sub>1</sub>, Shimadzu HPLC Pump (LC-10 AT<sub>VP</sub>); P<sub>2</sub>, Shimadzu HPLC Pump (LC-10 AT<sub>VP</sub>); RP column, TMS (4.6 mm I.D. $\times$ 150 mm, YMC); TRPD, time-resolved phosphorescence detector<sup>®</sup> (Hamamatsu Photonics K.K.).

\*PDB-S-Cl (CDPP): same compound as used previously [16], but the nomenclature is changed to indicate the reactive group, sulfonyl chloride group.

0.6 ml/min and the column temperature was ambient ( $24\pm 3$  °C).

The postcolumn derivatization of  $\text{Eu}^{3+}$  in HPLC was used as described previously. [16] The eluent from the LC column was mixed with a solution containing 0.2 mM  $\text{EuCl}_3$ , 0.2 mM TOPO, and 1% Triton X-100 in distilled water by the mixing tee (GL Sciences, Tokyo, Japan), delivered by a Shimadzu LC-10 ATVP HPLC pump. The flow rate was at 0.7 ml/min. The generated fluorescence was measured with the TRPD. The HPLC system is shown in Figure 2.

#### Sampling of the river water

The river water samples were taken from Tone River (Inashiki-

gun, Ibaraki, Japan) in August 2001. The samples were kept at 4 °C and analyzed within 48 h in order to keep the microbiological degradation to a minimum. No preservative was added.

#### Clean-up of the river samples

The clean-up procedure [16], which was described previously, was applied to 4-nonylphenol analysis as shown in Figure 3. The sample water was first filtered through a 48-mm Whatmann (Maidstone, UK) GF/C filter with a pore size of 1.2  $\mu\text{m}$ , and to 500 ml of the filtrate was concentrated by means of Figure 3. The resulted residue was dissolved into 0.2 ml of acetonitrile, and was processed for PDB-SCI labeling.

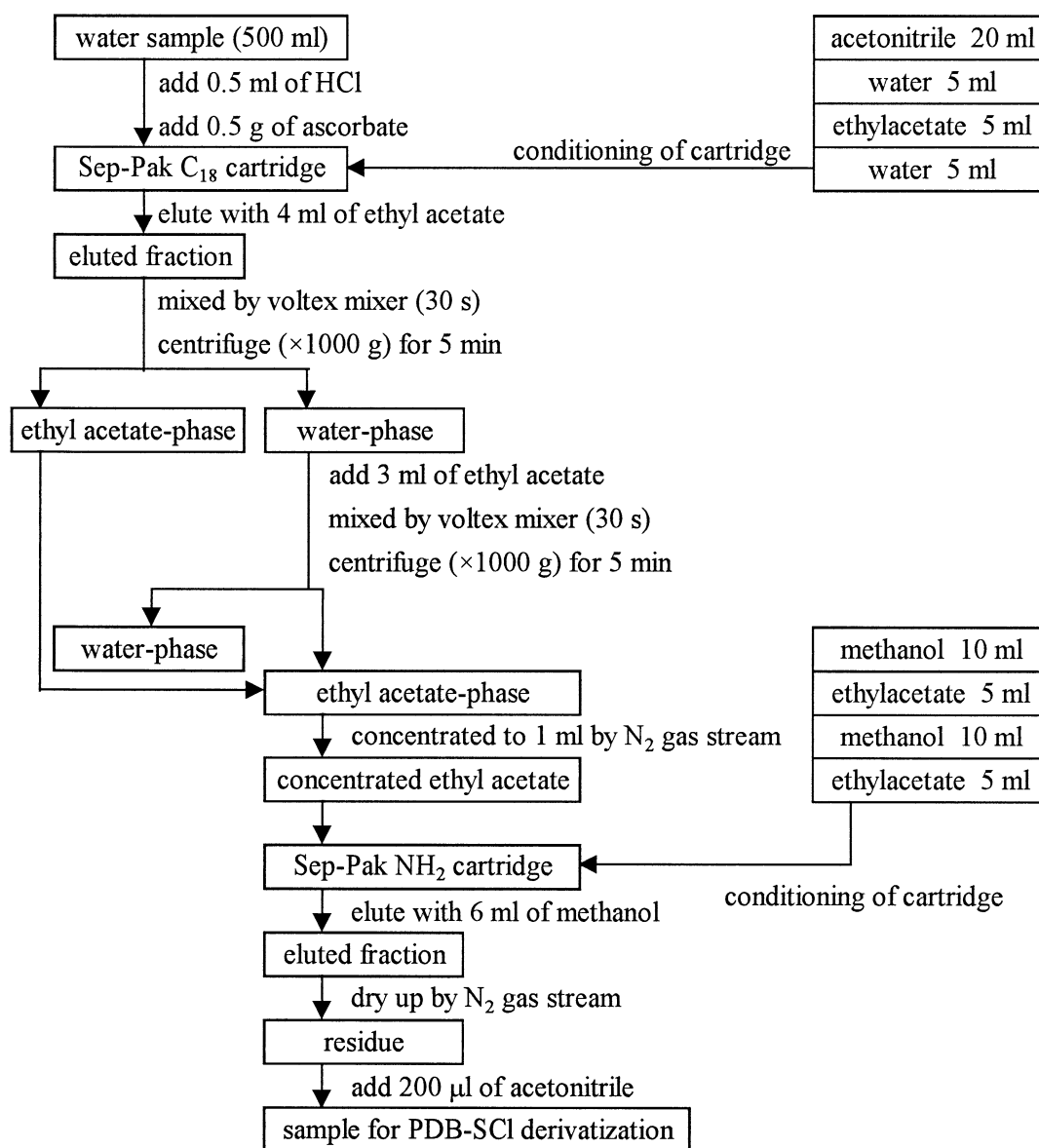


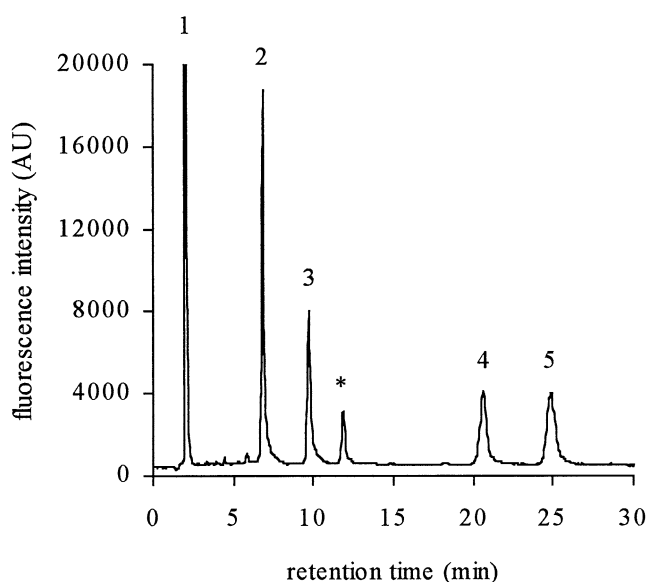
Figure 3. Water sample preparation method using solid-phase extraction

## Results and Discussion

### Time-resolved fluorometric HPLC profile of the PDB-S-derivatized alkylphenols

The HPLC profile of the PDB-S-derivatized bisphenol-A (1.38 ng), the PDB-S-derivatized *t*-butylphenol (1.38 ng), the PDB-S-derivatized 4-octylphenol (1.38 ng), and the PDB-S-derivatized 4-nonylphenol (1.38 ng) are shown in Figure 4. The peak at 2.0 min is the hydrolyzed form of PDB-SCI (PDB-SO<sub>3</sub><sup>-</sup>). The PDB-S-derivatized bisphenol-A, *t*-butylphenol, 4-octylphenol, and 4-nonylphenol were eluted at 6.8 min, 9.7 min, 20.7 min, and 24.9 min, respectively.

The PDB-S derivatives of the alkylphenols in the final reaction mixture were stable, and still gave constant time-resolved fluorescence intensities after standing for at least 24 h in the dark at room temperature.



**Figure 4.** Time-resolved fluorometric HPLC chromatogram of PDB-S-derivatized alkylphenols  
Peaks and amounts (ng per injection volume 10  $\mu$ l); 1, reagent blank (PDB-SO<sub>3</sub><sup>-</sup>); 2, bisphenol-A (1.38 ng); 3, *t*-butylphenol (1.38 ng); 4, 4-octylphenol (1.38 ng); 5, 4-nonylphenol (1.38 ng); asterisk, impurity from POB-SCI.

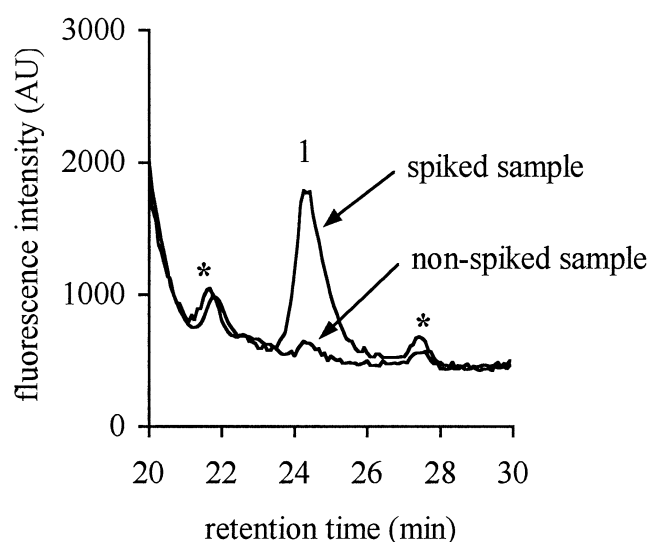
### Calibration curves and detection limits for alkylphenols in pure water sample

In time-resolved fluorometric HPLC, good linearity of the calibration curves was obtained in the concentration range from 5 ng/ml to 500 ng/ml (bisphenol-A,  $y = 72.9x$ ,  $r^2 = 1.000$ ; *t*-butylphenol,  $y = 30.0x$ ,  $r^2 = 1.000$ ; 4-octylphenol,  $y = 18.9x$ ,  $r^2 = 1.000$ ; 4-nonylphenol,  $y = 18.6x$ ,  $r^2 = 1.000$ ). The detection limits for alkylphenols were determined to be 0.49 ng/ml for bisphenol-A, 1.20 ng/ml for *t*-butylphenol, 1.57 ng/ml for 4-octylphenol,

and 1.60 ng/ml for 4-nonylphenol in the pure water sample. The detection limits, which were obtained from the peak height of the PDB-S-derivatized alkylphenols, were defined as the concentration of 3 SD of the baseline signal. The fluorescence intensity of the peak area of the alkylphenols (each 250 ng/ml) were 72,691 for bisphenol A, 41,894 for *t*-butylphenol, 41,589 for 4-octylphenol, and 42,007 for 4-nonylphenol. The fluorescence intensity of the PDB-S-derivatized bisphenol-A was approximately 1.7 times compared to those of the PDB-S-derivatized *t*-butylphenol, 4-octylphenol, and 4-nonylphenol. It was suggested that the two phenolic hydroxyl groups of bisphenol A were derivatized with PDB-SCI, since bisphenol-A has two phenolic hydroxyl groups in the molecule. The relative standard deviations of the peak intensities of the alkylphenols at 75 ng/ml for 5-times measurement were 1.8 to 2.5%.

### Recovery test and detection limit for 4-nonylphenol in river water sample

The standard addition and recovery measurement were carried out by using 500 ml of the river water sample according to the clean-up procedure described in Materials and Methods. After solid-phase extraction and labeling reaction, the solution was analyzed. The recovery was calculated from the difference of the intensities of added and non-added samples. The chromatograms of the spiked or non-spiked sample are shown in Figure 5. The average recovery ( $n=3$ ) of 4-nonylphenol (33.6 ng/L) in river water samples was 64.8%, and the method precision was 5.9%. The re-



**Figure 5.** HPLC chromatograms of the concentrated river water samples with or without spiking of 4-nonylphenol  
River water samples (500 ml) spiked with or without 4-nonylphenol were extracted as described in the text. 1, 4-nonylphenol; asterisk, peaks from river water materials.

covery of 4-nonylphenol from the river water sample was low when the clean-up procedure for estrogens was applied to the clean-up for 4-nonylphenol. Therefore, the internal standard was needed for the correction of the recovered value of 4-nonylphenol. The lower recovery of 4-nonylphenol in the clean-up procedure was due to the steps using Sep-Pak NH<sub>2</sub> cartridge.

The peaks from the river water sample did not interfere with the peak for 4-nonylphenol, and the baseline signals at the retention time for 4-nonylphenol were low as well as the signals in the measurement of the 4-nonylphenol standard (Figure 3). Most of the peaks from the river water sample were eluted within 20 min. Therefore, the highly sensitive detection of 4-nonylphenol was also possible in the river water sample. In the present method, 500 ml of the river water sample was concentrated to 200  $\mu$ l with the concentration factor of 2500, the detection limit for 4-nonylphenol in the river water sample was 0.99 ng/L.

#### Measurement of 4-nonylphenol in the original river water

In the present method, the amounts of 4-nonylphenol in the river water sample were determined by means of standard addition. The water sample was concentrated according to the procedure described in Materials and Methods, and the prepared samples were introduced into the HPLC system after addition of different concentrations of standard 4-nonylphenol. The linear correlation coefficients were more than 0.998 in the analysis of the river water sample. The line was extrapolated to the *x*-axis, and the concentration of the intersecting point with the axis was read. It was suggested that 4.8 ng/L of 4-nonylphenol was dissolved in the original

river water. On the other hand, the signals for other alkyl phenols did not be detected in the river water.

#### Comparison of the detection limits for 4-nonylphenol with other methods

In the measurement of the pure water sample, the detection limit for 4-nonylphenol in the time-resolved fluorometric HPLC was 30.9 times lower than that in a micro HPLC with UV detection (data not shown). Careri et al. [17] reported on the determination of alkylphenols including 4-nonylphenol by HPLC with UV detection using a semi-microcolumn (250 $\times$ 2.1 mm I.D.). The detection limit for nonylphenol in a groundwater was 120 ng/ml, which was 48.6 times higher than that of the present method. Thus, the detection limit for alkylphenol in the present method was approximately 30 to 50 times lower than those of the commonly used micro HPLC methods.

The detection limit for 4-nonylphenol in the present method was also compared to those of MS methods in Table 1. The detection limits of 4-nonylphenol in reference 11, 13, and 10 are 160 ng/L, 1000 ng/L, and 10000 ng/L, respectively, where however the water samples were concentrated by solid-phase extraction prior to the analysis. Therefore, the detection limits for 4-nonylphenol corresponding to the concentration of the measured solution would be 160 ng/ml for reference 11, 60 ng/ml for reference 13, and 10 ng/ml for reference 10. In the present method, the detection limit for 4-nonylphenol in the measured solution was 2.47 ng/ml. The detection limit of 4-nonylphenol in the present methods is approximately 4 to 65 times lower than those of the methods. [10,11,13]

Table 1. Comparison of the detection limits for 4-nonylphenol with other methods

method	detection limit <sup>a</sup> (ng/L)	concentration factor	detection limit <sup>b</sup> (ng/ml)	analyte	reference
LC-MS	160	1,000	160	river water	11
LC-MS	1,000	60	60	wastewater	13
semi-microcolumn LC-MS	10,000	on-line extraction (injection volume;100 $\mu$ l)	10	river water	10
this work	0.99	2,500	2.47	river water	

<sup>a</sup> The concentration corresponding to the original water sample.

<sup>b</sup> The concentration in the measured solution.

#### Conclusion

The time-resolved fluorometric HPLC method for alkylphenols including bisphenol-A, *t*-butylphenol, 4-octylphenol and 4-nonylphenol was more sensitive than the commonly used LC/MS methods. The system was applied to the measurement of 4-nonylphenol in river water sample, and the detection limit was 0.99 ng/L. The previously developed clean-up procedure for estrogens [16] was applied to that of 4-nonylphenol, and the materials from the river water did not interfere with the 4-nonylphenol analysis.

Therefore, the present method allows the determination of 4-nonylphenol in river water.

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