Highly sensitive and selective determination of 9,10-phenanthrenequinone in airborne particulates using high-performance liquid chromatography with pre-column derivatization and fluorescence detection

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Abstract

9,10-Phenanthrenequinone (PQ), is one of the components of diesel exhaust particulates, has potent harmful effects on human health. For the determination of PQ in airborne particulates, a highly sensitive and selective high-performance liquid chromatography (HPLC) method was developed with fluorescence detection following the pre-column fluorescent derivatization. PQ was derivatized with benzaldehyde as a derivatizing reagent in the presence of ammonium acetate to give fluorescent 2-phenyl-1*H*-phenanthro [9,10-*d*] imidazole. The maximum fluorescence intensity of the derivative was observed by the reaction with 0.2 M benzaldehyde and 0.5 M ammonium acetate at 100 °C for 30 min. The HPLC separation of fluorescent derivative of PQ was performed within 20 min on an ODS column with a mixture of acetonitrile–water (55:45, v/v) as a mobile phase. Highly sensitive and selective determination of PQ was attained with the detection limit of 5 fmol (S/N = 3). By the proposed method, PQ in airborne particulates was successfully determined in the range 0.26–0.30 ng/m^3 (*n* = 3).
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1. Introduction

9,10-Phenanthrenequinone (PQ) is an atmospheric contaminant that has been found in diesel exhaust particulates (DEP) [1,2]. PQ is produced from phenanthrene by photooxidation [3]. Phenanthrene is a well-known polycyclic aromatic hydrocarbon (PAH) mainly generated by incomplete combustion of organic materials and is widely distributed in the environment: DEP [1], airborne particulates [4], precipitation [5], soil [6], etc. Therefore, PQ is concerned to be ubiquitous compound in the environment as well as phenanthrene. Recently, PQ has become the focus of attention owing to its potential harmful effects on human health. Quinone compounds, including PQ are capable of generating reactive oxygen species (ROS) in biological system [7–9], which give oxidative stress that may include enzyme inactivation, lipid peroxidation and DNA damage leading potentially to gene mutations and cancer [10–12]. In fact, PQ serves as a potent inhibitor of some enzymes, such as protein kinases [13], nitric oxide synthases [14–16] and cyclooxygenase-2 [17]. Furthermore, it has been reported that PQ disrupts progesterone secretion and may alter the reproductive functions, including pregnancy [18]. Inhalation of PQ in pollutant air has been considered to be a causative factor in several diseases, such as lung cancer, asthma and allergic inflammation [9,17,19]. However, the exposed level to PQ for humans is still uncertain. From these aspects, a sensitive and selective method for the determination of PQ is of great necessity in the field of environmental and toxicological sciences.

For the determination of PQ, several methods have been reported, which include a spectrofluorometry [20], flow-injection analysis (FIA) with photometric detection [21], high-performance liquid chromatography (HPLC) with

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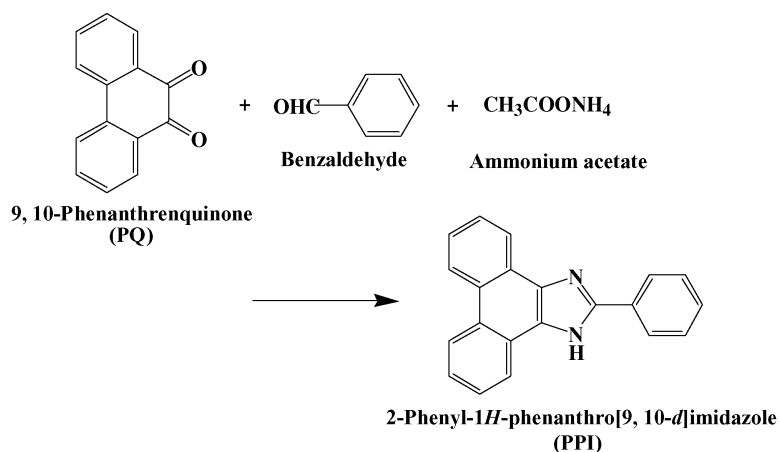*E-mail address:* n-kuro@net.nagasaki-u.ac.jp (N. Kuroda).

Fig. 1. Reaction scheme for the fluorescence derivatization of PQ with benzaldehyde and ammonium acetate.

chemiluminescence (CL) detection [22] and gas chromatography with mass spectrometry (GC–MS) [23]. However, these methods are not sufficiently sensitive for the determination of small amounts of PQ in environmental samples.

In this research, we attempted to establish a sensitive and selective method for the determination of PQ in airborne particulates. PQ shows weak fluorescence (FL), which does not provide enough sensitivity and selectivity for the trace analysis of PQ in airborne particulates that contain a large variety of fluorescent PAH and other contaminants. On the other hand, in the field of organic synthesis it has been reported that PQ reacts with benzaldehyde in the presence of ammonium acetate to produce 2-phenyl-1H-phenanthro [9,10-d] imidazole (PPI; Fig. 1) [24,25]. The resultant PPI exhibits strong FL and thus the above reaction was applied to the pre-column fluorescent derivatization of PQ followed by the reversed-phase HPLC. After optimizing the reaction conditions, the proposed method was successfully applied to the determination of PQ in airborne particulates.

2. Experimental

2.1. Material and reagents

PQ, benzaldehyde, ammonium acetate, acetic acid and methanol were obtained from Wako (Osaka, Japan). Acetonitrile of HPLC grade was obtained from Nacalai Tesque (Kyoto, Japan). Purified water was prepared by a Simpli Lab UV (Millipore, Bedford, MA, USA). The other chemicals were of analytical reagent grade.

2.2. Derivatization procedure for PQ

To a 100 μl portion of methanolic sample solution, containing PQ in a screw-capped vial, 50 μl of 0.2 M benzaldehyde in methanol and 50 μl of 0.5 M ammonium acetate in acetic acid were added. After vortex-mixed, the reaction mix-

ture was heated at 100 °C for 30 min. An aliquot of 20 μl of the reaction mixture was injected into the HPLC.

2.3. Apparatus and HPLC conditions

The HPLC system consisted of an LC-10AS (Shimadzu, Kyoto, Japan), a Shimadzu RF-10AxL fluorescence detector, a 7125 injector with a 20 μl loop (Rheodyne, Cotati, CA, USA), and a Shimadzu C-R7A integrator. Chromatographic separation was performed on a Cosmosil 5C18MS (250 mm × 4.6 mm, i.d., 5 μm; Nacalai Tesque) by an isocratic elution with a mixture of acetonitrile–water (55:45, v/v) at a flow rate of 1.0 ml/min. The detection wavelengths were an excitation at 265 nm and an emission at 390 nm. The column temperature was ambient. An agilent 1100 series fluorescence detector (Hewlett-Packard, Waldbronn, Germany) connected to a chemistation computer system was used for multi-wavelength fluorescence detection.

2.4. Preparation of authentic PPI

Authentic PPI was prepared as follows: 0.39 g of ammonium acetate (5 mmol) and 104 mg of PQ (0.5 mmol) were dissolved in 10 ml of acetic acid. To this mixture, 200 μl of 98% benzaldehyde (2 mmol) was added and heated at 100 °C for 2 h. After cooling to room temperature, the mixture was poured into cold water. The resultant precipitates were recrystallized from methanol to give brown crystals; yield: 79 mg, 54%, mp: 262 °C (Yanagimoto MP-53 melting point apparatus, Kyoto, Japan). Calculated for $C_{21}H_{14}N_2$: C, 85.69%; H, 4.79%, N, 9.52%, found: C, 84.61%, H, 4.71%, N, 9.05%. Electron impact ionization (EI)–MS (m/z): 294 [M^+] (JMS-DX 303 electron impact mass spectrometer, JEOL, Tokyo).

2.5. Airborne particulates sample

Sampling of airborne particulates was carried out at the main avenue of Nagasaki City. Airborne particulates were

collected on Q-R100 silica-fiber filters (Advantec Toyo, Tokyo, Japan) for 24 h at a flow rate of 1200 l/min by a Model No. 120 FT type high-volume air sampler (Kimoto Electro. Kogyo, Osaka, Japan). The filters collected airborne particulates were stored in a refrigerator at −20 °C until analysis. The filter (1 cm × 1 cm) was extracted ultrasonically with 4 ml of methanol for 10 min. After taking the organic layer (3 ml), the extraction was repeated again. These organic layers were combined and evaporated to dryness, and the resultant residue was dissolved in 100 μl of methanol. The reconstitute solution was treated according to the derivatization procedure.

3. Results and discussion

3.1. Optimization of fluorescent derivatization conditions

A typical chromatogram obtained with a standard solution of PQ is shown in Fig. 2. PQ derivative gave a single peak and have a retention time of 19 min under the HPLC conditions described in Section 2. The retention time of this peak was confirmed to be identical with that of authentic PPI by co-chromatography. Reaction product was stable for at least 5 days when the reaction mixture was kept in a refrigerator at 5 °C.

For the optimization of derivatization conditions, the effects of the organic solvent, reagent concentration, reaction temperature and time were investigated by using a standard PQ solution (0.1 μM). The effect of the organic solvent for dissolving PQ and benzaldehyde on relative fluorescence intensity (RFI) was examined with *N,N*-dimethylformamide, dimethyl sulfoxide, dioxane, ethyl acetate, tetrahydrofuran, acetonitrile and methanol. Among these solvents, the maximum fluorescence intensity was obtained with methanol (Fig. 3) and thus was selected as the organic solvent. The effects of benzaldehyde and ammonium acetate concentra-
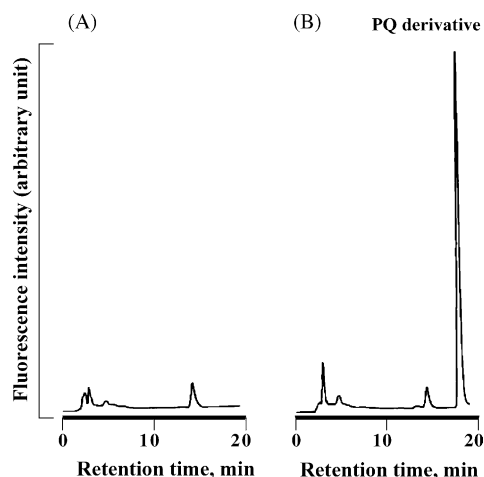

Fig. 2. Chromatograms for (A) reagent blank and (B) standard solution of PQ (5 pmol/injection).

tions on RFI are shown in Fig. 4. The maximum and constant fluorescence intensities were obtained in the presence of more than 0.1 M of benzaldehyde (Fig. 4A) and 0.3 M of ammonium acetate (Fig. 4B). Therefore, 0.2 M of benzaldehyde and 0.5 M of ammonium acetate were chosen.

The reaction temperature and time were also influenced on the fluorescence intensity (Fig. 5). The derivatization reaction was proceeded with an increase in the reaction temperature. At 100 °C, the maximum fluorescence intensity was achieved by heating for more than 15 min. As a result, 100 °C and 30 min were chosen for the reaction temperature and time, respectively.

3.2. Calibration curve, detection limit and reproducibility

The calibration curve obtained with the standard PQ showed good linear relationship ($r = 1.000$) between the concentrations and peak heights in the range from 0.01 to 20 pmol/injection (eight calibration points). The slope and
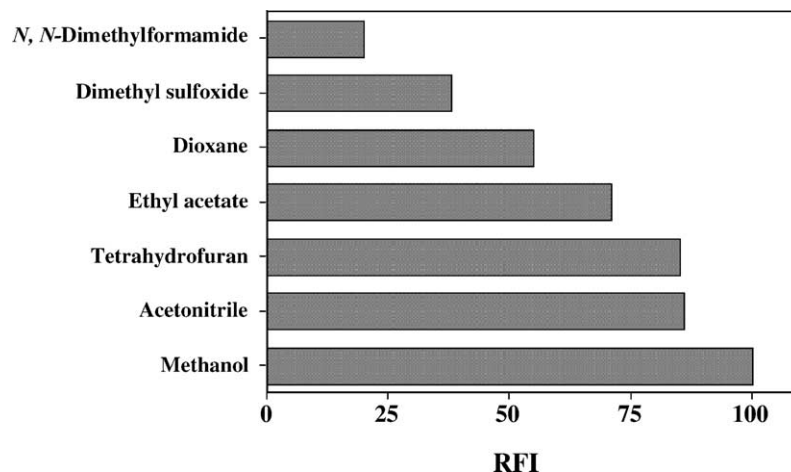

Fig. 3. Effect of organic solvent on RFI. A standard solution of PQ (0.1 μM) was treated according to the derivatization procedure except for organic solvent.
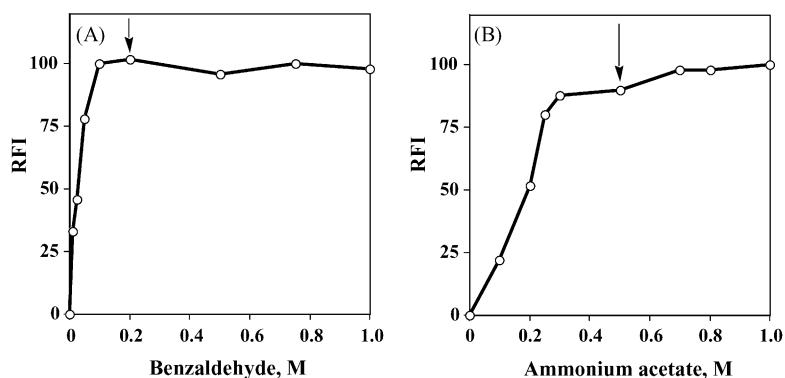
Fig. 4. Effects of (A) benzaldehyde and (B) ammonium acetate concentrations on RFI. A standard solution of PQ (0.1 μM) was treated according to the derivatization procedure except for reagent concentration.

intercept of regression equation (mean ± standard error, $n = 3$) were 11.37 ± 0.09 and -0.22 ± 0.38, respectively. The detection limit for standard PQ at a signal-to-noise (S/N) ratio of 3 was 5 fmol/injection. The sensitivity of the proposed method was 200 times higher than those of HPLC–CL [22] and GC–MS methods [23], and 40 times higher than that of FIA method [21]. The reaction yield, estimated by comparing the slopes of the calibration curves obtained with authentic PPI and the reaction mixture, was 80%. The reproducibility of the proposed method was examined using three levels (0.1, 1 and 5 pmol/injection) of standard PQ solution: the relative standard deviations (R.S.D.) for intra-day ($n = 4$) analyses were 2.3, 3.2 and 2.9%, respectively and inter-day ($n = 4$) analyses were 4.5, 4.1 and 3.1%, respectively.

3.3. Determination of PQ in airborne particulates

The proposed method was applied to the determination of PQ in airborne particulates. Collected airborne particulates on filter were used to investigate the extractability of PQ by spiking 10 μl of standard PQ in methanol. After drying, the filter was treated as in the manner for the proposed analytical procedure. To choose the extraction solvent, extraction by ultrasonication was examined with methanol, acetonitrile, ethyl acetate, dichloromethane and n-hexane. Among them,

Fig. 6. Effect of sonication time on the extractability of PQ. Airborne particulates spiked with standard PQ (50 pmol) was treated according to Section 2.

the maximum extractability was obtained with methanol and thus was selected as the extraction solvent. The effect of the sonication time on the extractability was studied by adding 4 ml of methanol to airborne particulates (Fig. 6). The constant and maximum extractabilities were achieved by a sonication time >5 min: the sonication time was set for 10 min. The number of repeated extractions was also examined to obtain a maximal extractability (Fig. 7). The maximum and

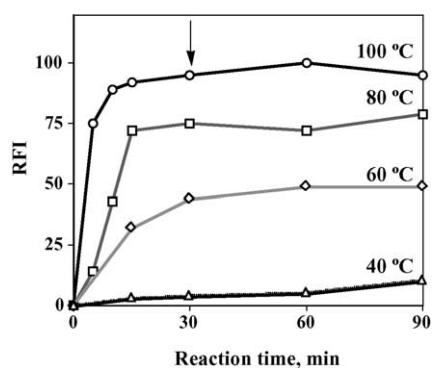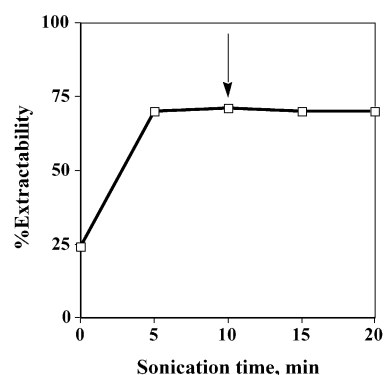

Fig. 5. Effects of reaction time and temperature on RFI. A standard solution of PQ (0.1 μM) was treated according to the derivatization procedure except for reaction time and temperature.
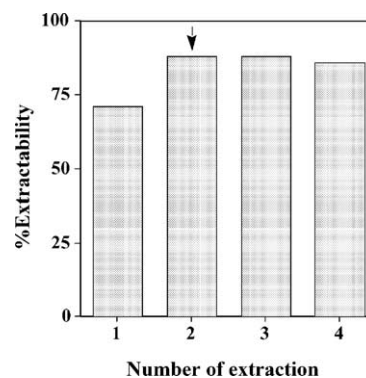

Fig. 7. Effect of the number of extraction on the extractability of PQ. Airborne particulates spiked with standard PQ (50 pmol) was treated according to Section 2.

Table 1
Precision and recovery of the method for PQ in airborne particulates

Spiked amount (pmol)	Intra-day ($n = 3$)			Inter-day ($n = 3$)		
	Found (pmol)	R.S.D.[a] (%)	Recovery[b] (%)	Found (pmol)	R.S.D.[a] (%)	Recovery[b] (%)
5.0	4.94	6.9	98.8	4.94	7.5	98.8
20	19.0	3.2	95.0	18.3	5.8	91.5
50	48.2	1.1	96.4	48.4	3.3	96.8

[a] R.S.D.: relative standard deviation.
[b] Expressed as [(found amount)/(spiked amount)] × 100.

constant extractability was attained with two- or more-fold extraction and the number of extraction was performed twice.

Fig. 8 represents the typical chromatograms obtained with the extracts from airborne particulates spiked with standard PQ. A peak corresponded to PQ derivative was detected without any interfering peak. Confirmation of the peak obtained with the extract was carried out by comparing the excitation and emission spectra with those of authentic PPI on the multi-wavelength fluorescence spectrophotometer. As shown in Fig. 9, the excitation and emission spectra obtained from the peak for extract were in good agreement with those obtained from the standard PPI solution (similarity > 99.7%).

The calibration curve of recovered PQ from airborne particulates was linear over the range from 0.02 to 20 pmol/injection with a coefficient of correlation being $r = 1.000$ (seven calibration points). The slope and intercept of regression equation (mean ± standard error, $n = 3$) were $9.51 ± 0.24$ and $−1.36 ± 0.11$, respectively. Intra- and inter-day variation data of the proposed method were evaluated by using airborne particulates spiked with PQ standard (5.0, 20 and 50 pmol). As shown in Table 1, the R.S.D.s for intra-day precision were 6.9% or better for all points measured. Inter-day precision studies also gave satisfactory reproducibilities (<7.5%). The recoveries, which were calculated by using the observed and spiked data for PQ, were more than 91.5%.

The average concentration ($n = 3$) of PQ found in airborne particulates collected at Nagasaki city was 0.28 ng/m^3. These
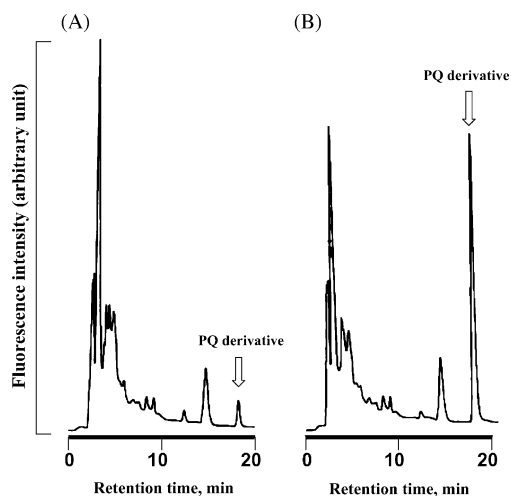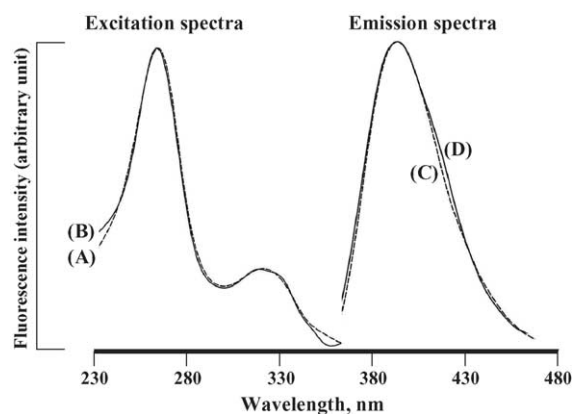

Fig. 9. Excitation and emission spectra obtained from the peak for extract from airborne particulates and that for the authentic PPI solution. Excitation spectra with the emission wavelength at 390 nm of PPI (A, dashed line) and the extract (B, solid line). Emission spectra with the excitation wavelength at 265 nm of PPI (C, dashed line) and the extract (D, solid line).

results are well in accordance with the published data [23,26]. We previously reported that the average concentration of phenanthrene in Nagasaki city was 1.58 ng/m^3 [4]. The concentration of PQ obtained with the presented method was about five times lower than that of phenanthrene.

4. Conclusion

In this paper, we reported a highly sensitive and selective HPLC method for the determination of PQ with pre-column fluorescent derivatization. The fluorescence derivatization reaction proceeded with a relatively simple procedure to give an intensely fluorescent derivative. The proposed method was successfully applied to the determination of PQ in airborne particulates with a simple extraction procedure prior to the derivatization. This method should be useful for monitoring of PQ in environmental and toxicological investigations.

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Fig. 8. Chromatograms for PQ in the extract from (A) airborne particulates and (B) airborne particulates spiked with standard PQ (50 pmol).

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