4-Carbomethoxybenzaldehyde as a highly sensitive pre-column fluorescence derivatization reagent for 9,10-phenanthrenequinone

Naoya Kishikawa, Maiko Nakao, Mohamed Saleh Elgawish, Kaname Ohyama, Kenichiro Nakashima, Naotaka Kuroda*

Graduate School of Biomedical Sciences, Course of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

*Corresponding author : Naotaka Kuroda

Graduate School of Biomedical Sciences, Course of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

Tel: +81-95-819-2894; Fax: +81-95-819-2444

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

Abstract

9,10-Phenanthrenequinone (PQ) is harmful environmental pollutant that is detected in airborne particulates. The measurement of PQ in the air should be necessary to evaluate the potential adverse effects of PQ on human health. We have recently developed a determination method for PQ based on the fluorescence derivatization of PQ using benzaldehyde and ammonium acetate as a reagent. In this study, in order to obtain more sensitive and selective fluorescence derivatization reaction, we measured the fluorescence of the reaction mixture of PQ with 21 kinds of aromatic aldehydes in the presence of ammonium acetate. Among the tested aldehydes, 4-carbomethoxybenzaldehyde was found to be the best reagent in regard to fluorescence intensity and emission wavelength maximum. Based on the fluorescence derivatization with 4-carbomethoxybenzaldehyde, a highly sensitive chromatographic method was developed for the determination of PQ with the detection limit (S/N=3) of 1.2 fmol/injection.

Keywords: Phenanthrenequinone; Environmental pollutant; Fluorescence derivatization; Screening.

1. Introduction

9,10-Phenanthrenequinone (PQ, Fig. 1) is one of the environmental pollutants that is detected on the surface of airborne particulates [1,2]. It has been reported that PQ has harmful effect on living tissues and cells. For example, PQ acts as a generator of reactive oxygen species (ROS) through the redox cycle in biological system and ROS can induce several types of oxidative damage such as lipid peroxidation [3,4]. Additionally, PQ serves as an inhibitor of certain enzymes, such as nitric oxide synthase and glyceraldehyde-3-phosphate dehydrogenase, by the covalent binding to the active site of enzymes [5,6]. It has been reported that several respiratory diseases, such as asthma and inflammation, can be caused by inhalation of PQ in the air [7,8]. Therefore, it is necessary to measure the concentration of PQ in the atmospheric environment in order to evaluate the potential adverse effects of PQ on human health.

Recently, we found that PQ can be converted to strongly fluorescent lophine derivative by the reaction with benzaldehyde in the presence of ammonium acetate (Fig. 1). Based on this reaction, we developed a determination method for PQ in airborne particulates by pre-column derivatization and high-performance liquid chromatography (HPLC) with fluorescence detection [9]. Although the developed method allowed sensitive determination of PQ, the formed derivative emitted fluorescence at relatively short wavelength region and its detection was

sometimes interfered by fluorescent compounds existed in airborne particulates. On the other hand, it has been reported that the fluorescence characteristics of lophine derivatives were dependent on the substituents of 2-phenyl moiety of imidazole [10]. Therefore, in this study, in order to discover more suitable reagent for pre-column fluorescence derivatization of PQ, we examined the fluorescence characteristics of 21 kinds of aromatic aldehydes after the reaction with PQ (Fig. 2). Among the tested aromatic aldehyde, 4-carbomethoxybenzaldehyde was found to be the most suitable derivatization reagent in terms of fluorescence intensity and wavelength. In this instance, we developed an improved HPLC method for PQ using 4-carbomethoxybenzaldehyde as a derivatization reagent, and the developed method was successfully applied to determine PQ content in airborne particulates.

2. Experimental

2.1 Chemicals

The aromatic aldehydes tested in this study are shown in Fig. 2 with their structures. Benzaldehyde, 4-methoxybenzaldehyde, 1-naphthaldehyde, PQ, ammonium acetate, acetic acid and methanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-(Dihydroxyboryl)benzaldehyde, 3,4,5-trihydroxybenzaldehyde, 4-acetoxybenzaldehyde and 4-diethylaminobenzaldehyde were from Sigma-Aldrich (St. Louis, MO, USA). 4-Dimehthylaminobenzaldehyde and 4-diphenylaminobenzldehyde were from Kishida Chemicals (Tokyo, Japan) and Kokusan Chemical (Tokyo), respectively. Other aldehydes were purchased from Tokyo Chemical Industry (Tokyo). 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 Evaluation of aromatic aldehyde as derivatization reagent for PQ

To 100 µL of 100 µM PQ solution in methanol, 50 µL of 0.2 M aromatic aldehyde in methanol and 50 µL of 0.5 M ammonium acetate in acetic acid were added. After vortex-mixing, the reaction mixture was heated at 100˚C for 30 min. The reaction mixture was then diluted 20 times with methanol for the fluorescence measurement. Fluorescence spectra and intensities of the reaction mixtures were measured with an

RF-1500 fluorescence spectrophotometer (Shimadzu, Kyoto).

2.3 Pre-column fluorescence derivatization of PQ with 4-carbomethoxybenzaldehyde

To 100 µL of PQ solution in methanol, 50 µL of 0.2 M 4-carbomethoxybenzaldehyde in methanol and 50 µL of 0.5 M ammonium acetate in acetic acid were added. After vortex-mixing, the reaction mixture was heated at 100˚C for 30 min. An aliquot of 20 µL of the reaction mixture was injected into the HPLC system.

2.4 HPLC system and conditions

The HPLC system consisted of a Shimadzu LC-10AS pump, 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 x 4.6 mm, I.D., 5 µm; Nacalai Tesque) by an isocratic elution with a mixture of acetonitrile-water (75:25, v/v) at a flow rate of 1.0 mL min^{-1}. The column temperature was ambient. The fluorescence detection wavelength was set at 465 nm with 370 nm of excitation wavelength.

2.5 Synthesis of the fluorescent product derived from PQ, 4-carbomethoxybenzaldehyde and ammonium acetate

The fluorescent product, 2-(4-carbomethoxy)phenyl-1*H*-phenanthro[9,10-*d*]imidazole, was prepared as follows: 1.0 g of ammonium acetate (12.5 mmol), 312 mg of PQ (1.5 mmol) and 246 mg of

4-carbomethoxybenzaldehyde (1.5 mmol) were dissolved in 3 mL of acetic acid. The mixture was heated at 100 ºC for 9 h with stirring. After cooling to room temperature, the mixture was poured into cold water. The resultant precipitates were recrystallized from ethyl acetate to give brown crystals; yield: 318 mg, 60%, mp: >300 °C (MP-53 melting point apparatus, Yanagimoto, Kyoto). Calculated for $C_{23}H_{16}N_2O_2$: C, 78.47%; H, 4.54%, N, 7.95%, found: C, 78.10%, H, 4.61%, N, 7.81%. Electron impact ionization (EI)–MS m/z: 352 ($[M]^+$, 100%), 293 ($[M\text{-}COOCH_3]^+$, 32%), 190 (23%), 163 (10%) (JMS-700N mass spectrometer, JEOL, Tokyo).

2.6 Determination of PQ in airborne particulates

Airborne particulates were collected on a QR-100 quartz-fiber filter (Advantec Toyo, Tokyo) 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) at a main avenue of Nagasaki City. The filter (1 cm × 1 cm) was extracted ultrasonically with 4 ml of methanol for 10 min. After taking the organic fraction (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 described in Section 2.3. An aliquot of 20 µL of the reaction mixture was injected into HPLC after filtration through a 0.45-µm membrane.

3.Results and Discussion

Table 1 summarizes the relative fluorescence intensity, excitation and emission wavelength maximum obtained from the reaction mixture of PQ with 21 kinds of aromatic aldehydes in the presence of ammonium acetate. Among the tested aromatic aldehydes, 4-carboxybenzaldehyde, 4-carbomethoxybenzaldehyde, 4-formylbenzaldehyde, 4-phenylbenzaldehyde and 4-(methylthio)benzaldehyde gave more than 1.5 times stronger fluorescence as compared with benzaldehyde. However, the emission wavelength maxima of the reaction mixture of 4-formylbenzaldehyde, 4-phenylbenzaldehyde and 4-(methylthio)benzaldehyde were observed at short wavelength region as same as benzaldehyde. On the other hand, the emission wavelength maxima of the reaction mixture of 4-carboxybenzaldehyde and 4-carbomethoxybenzaldehyde were observed at longer wavelength region than that of the reaction mixture of benzaldehyde. Although the emission wavelength maxima of the reaction mixture of 4-dimethylaminobenzaldehyde, 4-diethylaminobenzaldehyde and 4-diphenylaminobenzaldehyde were also observed at long wavelength region, these aldehydes gave extremely weak fluorescence. In order to evaluate the effect of the substituents of aromatic aldehydes, we examined the relationship between the fluorescence characteristics of the reaction mixture and the Hammett constants of the substituent at the *p*-position (σ_p) of mono-substituted benzaldehyde. The significant

positive correlation (r = 0.74) was observed between the fluorescence intensities and the Hammett constants. This result suggests that the introduction of the electron-withdrawing groups including carbomethoxy group at the *p*-position of aromatic aldehyde caused an increase in the fluorescence intensity. On the other hand, there was no significant correlation between the emission wavelength maxima and the Hammett constants. Therefore, we concluded that 4-carbomethoxybenzaldehyde might be the most suitable reagent for fluorescence derivatization of PQ because it shows 1.9 times stronger fluorescence intensity as compared with benzaldehyde and the emission wavelength maximum was observed at longest wavelength region among the tested aromatic aldehydes. The strong fluorescence of the product should provides good sensitivity and the fluorescence at long wavelength region should be able to reduce the interference with the components of airborne particulates

Figure 3 shows typical HPLC chromatogram of standard PQ solution obtained after the derivatization with 4-carbomethoxybenzaldehyde. The fluorescent derivative of PQ was detected at 8.5 min on the chromatogram without any interference derived from the derivatization reagent. The calibration curve obtained with the standard PQ showed a good linear relationship (r = 0.999) between the concentrations and peak heights in the range from 0.005 to 10 pmol/injection. The detection limit for standard PQ at a signal-to-noise (S/N) ratio of 3 was 1.2 fmol/injection. The sensitivity of the proposed method was approximately 4 times higher than that of

our previous method using benzaldehyde as a derivatization reagent [9]. In addition, the sensitivity was 800, 100, 50 and 5 times higher than those of gas chromatography with mass spectrometry (GC-MS) [11], liquid chromatography with tandem mass spectrometry (LC-MS/MS) [12], HPLC with post-column fluorescence derivatization method [13] and HPLC with chemiluminescence detection method [14], respectively. The proposed method is the most sensitive among the determination methods for PQ reported so far. The reaction yield calculated by comparing the slopes of the calibration curves obtained with authentic fluorescence product and the reaction mixture was 96%. The reactivity of 4-carbomethoxybenzaldehyde to PQ was slightly higher than that of benzaldehyde (the reaction yield was 80%). The repeatability of the proposed method was examined using different concentrations (0.1, 1 and 10 pmol/injection) of standard PQ solution: the relative standard deviations (R.S.D.) for intra-day (n = 5) analyses were 2.2, 2.3 and 0.4%, respectively and for inter-day (n = 5) analyses were 8.8, 5.8 and 5.4%, respectively.

The practicability of the proposed method was demonstrated by application of the method for the determination of PQ in airborne particulates. Figure 3(C) shows the typical chromatogram obtained with the extracts from airborne particulates. It is clear from the chromatograms that PQ can be detected without any interference. Therefore, 4-carbomethoxybenzaldehyde could convert PQ to fluorescent derivative even in the presence of other components in airborne

particulates and the detection of formed derivative was not interfered with these components.

Conclusion

In this study, we evaluated 21 kinds of aromatic aldehyde for their ability as a fluorescence derivatization reagent for PQ. As a result, we discovered 4-carbomethoxybenzaldehyde as a suitable derivatization reagent for PQ in consideration of the strong fluorescence intensity and the excitation wavelength maximum at long wavelength region. By using 4-carbomethoxybenzaldehyde, we developed a sensitive method for the determination of PQ by pre-column derivatization and HPLC with fluorescence detection. The developed method provides the best sensitivity for the detection of PQ among the methods for PQ reported so far. Additionally, 4-carbomethoxybenzaldehyde should be applied to develop a sensitive determination for quinoid polycyclic aromatic hydrocarbons other than PQ such as 1,2-naphthoquinone.

References

[1] K. Bekki, H. Takigami, G. Suzuki, N. Tang and K. Hayakawa, J. Health Sci. **55** (2009) 601.

[2] G. Andreou and S. Rapsomanikis, J. Hazard. Mater. **172** (2009) 363.

[3] Y. Motoyama, K. Bekki, W.C. Sang, N. Tang, T. Kameda, A. Toriba, K. Taguchi and K. Hayakawa, J. Health Sci. **55** (2009) 845.

[4] K. Taguchi, M. Shimada, S. Fujii, D. Sumi, X. Pan, S. Yamano, T. Nishiyama, A. Hiratsuka, M. Yamamoto, A.K. Cho, J.R. Froines and Y. Kumagai, Free Radic. Biol. Med. **44** (2008) 1645.

[5] K. Taguchi, Y. Kumagai, A. Endo, M. Kikushima, Y. Ishii and N. Shimojo, J. Health Sci. **47** (2001) 571.

[6] C.E. Rodriguez, J.M. Fukuto, K. Taguchi, J. Froines and A.K. Cho, Chem. Biol. Interact. **155** (2005) 97.

[7] N. Li, M. Hao, R.F. Phalen, W.C. Hinds and A.E. Nel, Clin. Immunol. **109** (2003) 250.

[8] K. Hiyoshi, H. Takano, K.-I. Inoue, T. Ichinose, R. Yanagisawa, S. Tomura and Y. Kumagai, Clin. Exp. Allergy **35** (2005) 1243.

[9] N. Kishikawa, M. Wada, Y. Ohba, K. Nakashima and N. Kuroda, J. Chromatogr. A **1057** (2004) 83.

[10] K. Nakashima, Y. Fukuzaki, R. Nomura, R. Shimoda, Y. Nakamura, N. Kuroda, S. Akiyama and K. Irgum, Dyes pigm. **38** (1998) 127.

[11] A.K. Cho, E.D. Stefano, Y. You, C.E. Rodriguez, D.A. Schmitz, Y. Kumagai, A.H. Miguel, A. Eiguren-Fernandez, T. Kobayashi, E. Avol

and J.R. Froines, Aerosol Sci. Technol. **38** (2004) 68.

[12] O. Delhomme, M. Millet and P. Herckes, *Talanta* **74** (2008), p. 703.

[13] N. Kishikawa, H. Nakashima, K. Ohyama, K. Nakashima and N. Kuroda, Talanta **81** (2010) 1852.

[14] S. Ahmed, N. Kishikawa, K. Ohyama, T. Maki, H. Kurosaki, K. Nakashima and N. Kuroda, J. Chromatogr. A **1216** (2009) 3977.

Figure captions

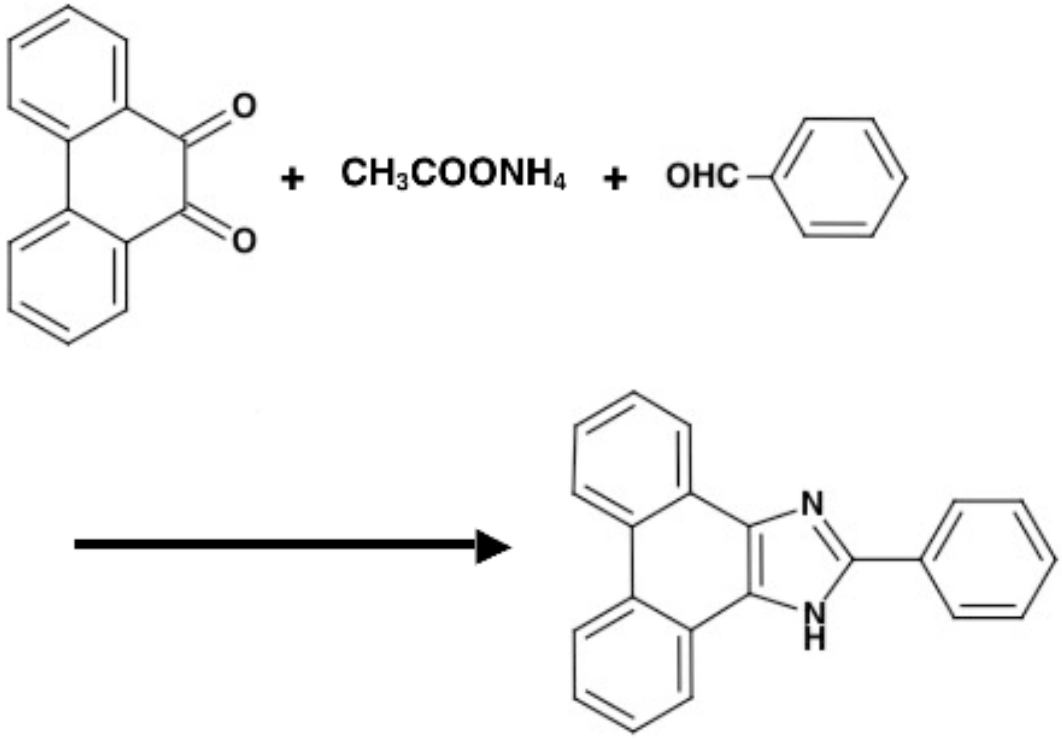

Fig. 1. Fluorescence derivatization reaction of PQ with benzaldehyde in the presence of ammonium acetate.

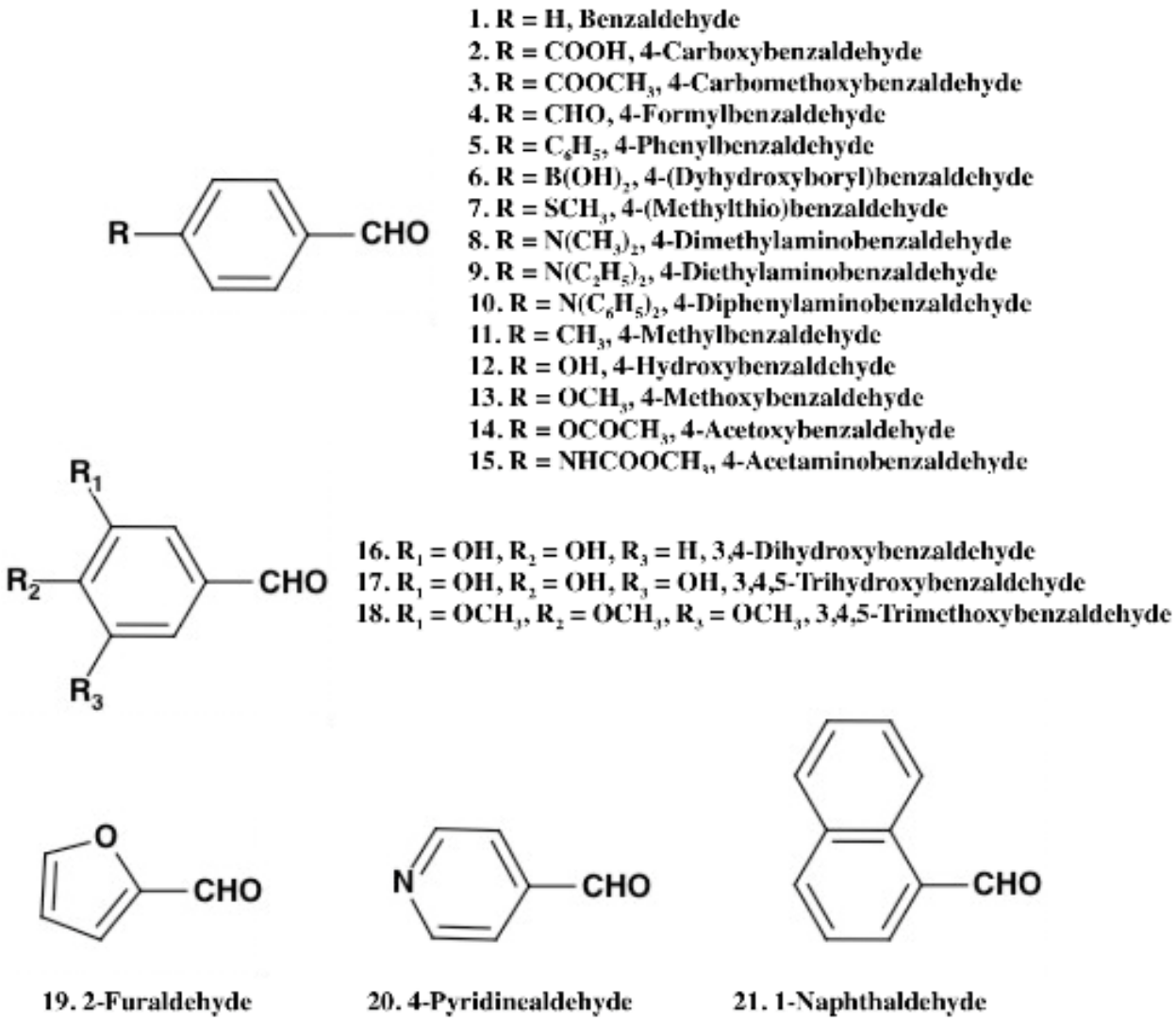

Fig. 2. Structures of aromatic aldehydes tested for the fluorescence derivatization of PQ.

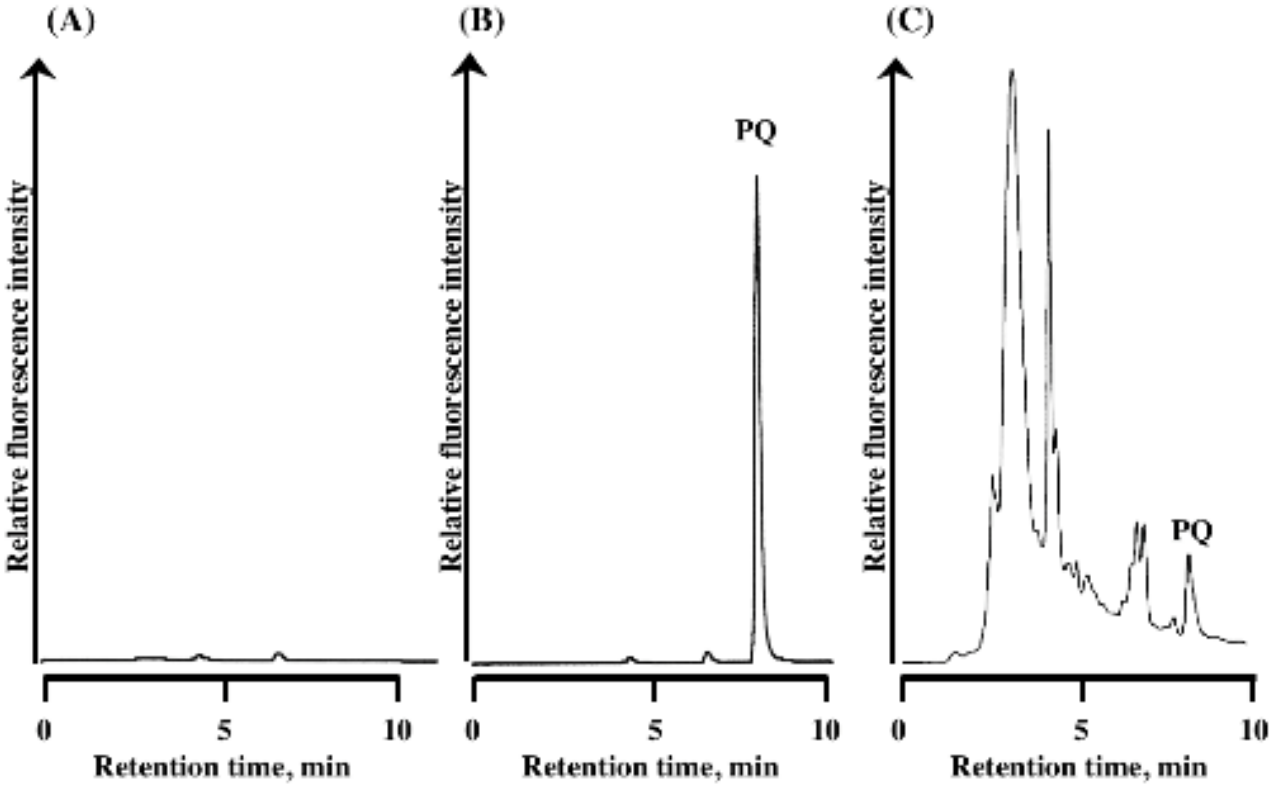

Fig. 3. Chromatograms for (A) reagent blank, (B) standard solution of 10 pmol/injection PQ and (C) PQ in the extract from airborne particulates.

Table 1 Fluorescence characteristics of the reaction mixture of PQ with aromatic aldehydes in the presence of ammonium acetate

	Aromatic aldehyde	λex	λem	RFI*
1	Benzaldehyde	265	390	100
2	4-Carboxybenzaldehyde	370	446	154
3	4-Carbomethoxybenzaldehyde	373	462	190
4	4-Formylbenzaldehyde	320	381	200
5	4-Phenylbenzaldehyde	344	400	183
6	4-(Dihydroxyboryl)benzaldehyde	317	388	146
7	4-(Methylthio)benzaldehyde	363	386	170
8	4-Dimethylaminobenzaldehyde	390	420	10
9	4-Diethylaminobenzaldehyde	400	428	5
10	4-Diphenylaminobenzaldehyde	409	432	2
11	4-Methylbenzaldehyde	310	381	125
12	4-Hydroxybenzaldehyde	322	390	85
13	4-Methoxybenzaldehyde	320	386	108
14	4-Acetoxybenzaldehyde	324	385	70
15	4-Acetaminobenzaldehyde	360	386	72
16	3,4-Dihydroxybenzaldehyde	358	393	21
17	3,4,5-Trihydroxybenzaldehyde	252	392	7
18	3,4,5-Trimethoxybenzaldehyde	358	385	68
19	2-Furaldehyde	312	382	124
20	4-Pyridinealdehyde	345	397	124
21	1-Naphthaldehyde	362	403	60

***Fluorescence intensity of the reaction mixture of PQ and benzaldehyde was taken as 100**