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Notes

Benzo[*c*]fluorene in Urban Air: HPLC Determination and Mutagenic Contribution Relative to Benzo[*a*]pyrene

Hiroshi MORISAKI,* Shiho NAKAMURA,* Ning TANG,* Akira TORIBA,* and Kazuichi HAYAKAWA*,†**

**Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan*
***Institute of Natural and Environmental Technology, Kanazawa University, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan*

Benzo[*c*]fluorene (BcFE) concentrations in benzene/ethanol extracts of airborne particulates were determined by high-performance liquid chromatography (HPLC) with fluorescence detection. HPLC conditions were as follows: columns, two ZORBAX Eclipse PAH (4.6 i.d. × 250 mm, 3.5 μm) and one Inertsil ODS-P (4.6 i.d. × 250 mm, 5 μm) in series; mobile phase, acetonitrile–water (98:2, v/v), 0.3 mL/min; detection wavelengths, excitation 309 nm, emission 354 nm. Particulate-phase BcFE concentrations in the atmosphere varied seasonally (winter > summer). The concentrations were 11000 ± 6100 pg m^{-3} (winter) and 40 ± 12 pg m^{-3} (summer) in Beijing, China, and 13 ± 5.0 pg m^{-3} (winter) and 2.7 ± 0.52 pg m^{-3} (summer), in Kanazawa, Japan. In both cities, the particulate-phase BcFE concentration in the atmosphere was lower than that of benzo[*a*]pyrene (BaP) by a factor of 0.03 – 0.43. However, the mutagenic contribution of particulate-phase BcFE in the atmosphere in winter calculated from the mutagenicity relative potency factor was greater than that of BaP.

Keywords Benzo[*c*]fluorene, health effect, HPLC, mutagenicity, $PM_{2.5}$

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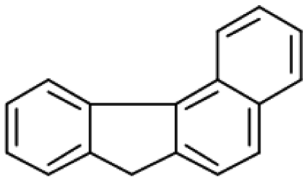
Introduction

Since the report of high atmospheric concentrations of $PM_{2.5}$ (particulates size ≤ 2.5 μm), in Beijing, China, in January, 2013, concern over the adverse health effects of $PM_{2.5}$ in humans has increased worldwide. The combustion of fuels such as coal, oil, and biomass is a major source of $PM_{2.5}$. $PM_{2.5}$ originated from the combustion of materials contains polycyclic aromatic hydrocarbons (PAHs) and nitro polycyclic aromatic hydrocarbons (NPAHs).[1-3] PAHs and NPAHs can be transported all over the world from the countries in which they are emitted.[4-6] Many PAHs and NPAHs exhibit carcinogenic, mutagenic,[7,8] or endocrine-disrupting activity.[9] Benzo[*a*]pyrene (BaP), for example, is classified as a Group 1 compound (carcinogenic to humans) by the International Agency for Research on Cancer and is used as an indicator of environmental pollution.[10-12]

The PAHs dibenzo[*a*,*l*]pyrene (DBalP) and benz[*j*]-aceanthrylene (BjAC) were recently classified as Group 2A (probably carcinogenic to humans) and Group 2B (possibly carcinogenic to humans) compounds, respectively.[13] Although benzo[*c*]fluorene (BcFE) (Fig. 1) is still in Group 3 with respect to its carcinogenicity (not classifiable as to its carcinogenicity to humans), BcFE is genotoxic.[14-17] The United States Environmental Protection Agency (EPA) reported that the relative potency factors (RPFs) of BcFE, BjAC, and dibenz[*a*,*h*]anthracene (DBA) with respect to the mutagenicity of BaP (which has a value of 1) are 20, 60, and 10, respectively.[18] The RPFs provide a measure of the mutagenicity of a given PAH relative to that of BaP. These data demonstrate that in order to estimate the health effects of BcFE exposure, it is necessary to first determine its concentration in the atmosphere.

We initially focused on determining the atmospheric concentration and relative mutagenic contribution of BcFE. A gas chromatography–mass spectrometry (GC-MS) method was recently reported for determining BcFE in atmospheric samples.[19,20] However, the peak of the low concentration detected by GC-MS was effected by the background. Although BcFE is a fluorescent compound, the use of high-performance liquid chromatography with fluorescence detection (HPLC-FLD) for the quantification of BcFE has not been reported. Therefore, the purposes of this study were to (1) develop an HPLC-FLD method for BcFE; (2) quantify BcFE in urban air; and (3) estimate the mutagenic contribution of BcFE relative to that of BaP.



MW: 216.29

Fig. 1 The structure of BcFE.

† To whom correspondence should be addressed.
E-mail: hayakawa@p.kanazawa-u.ac.jp

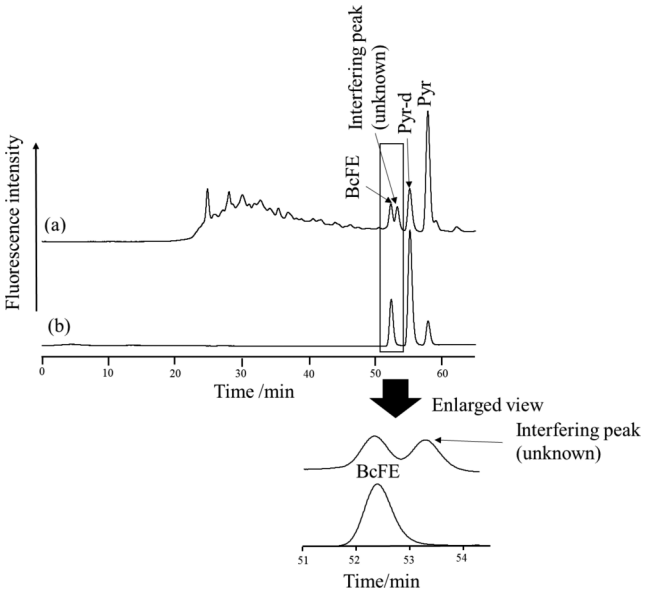


Fig. 2 HPLC chromatograms of (a) an extract of airborne particulates collected in Beijing, China, in winter 2010 and (b) standard solution.

Experimental

Reagents

BcFE (Fig. 1) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Deuterated pyrene (Pyr-d_{10}), an internal standard, was purchased from Wako Pure Chemicals (Osaka, Japan). EPA610 PAH Mix, a mixture of naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene (Flu), pyrene (Pyr), benz[*a*]anthracene (BaA), chresene (Chr), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), BaP, DBA, benzo[*ghi*]perylene (BghiPe), and indeno[1,2,3-*cd*]pyrene (IDP), was purchased from Supelco (Bellefonte, PA, USA). Other reagents were analytical grade.

Analytical conditions

A Shimadzu LC10 Series (Kyoto, Japan) HPLC system was used, which consisted of two pumps, an autoinjector, a degasser, a column oven, a fluorescence detector (excitation 309 nm, Emission 354 nm), a system controller, and an integrator (detailed information is described in Supporting Information). The columns tested included Inertsil ODS-P (4.6 i.d. × 250 mm, 5 µm, GL Sciences Inc., Tokyo, Japan), Cosmosil 5C_{18}-AR-II (4.6 i.d. × 250 mm, 5 µm, Nakalai Tesque, Kyoto, Japan), and ZORBAX Eclipse PAH (4.6 i.d. × 250 mm, 3.5 µm, Agilent Technologies, Santa Clara, CA, USA). An Inertsil ODS-P guard column (4.6 i.d. × 10 mm, 5 µm, GL Sciences Inc.) was used.

Airborne particulate samples

Samples of total suspended particulate (TSP) matter were collected in Beijing, China (Fig. S1, Supporting Information), every day between January 18 – February 1 and August 2 – 16, 2010. TSP samples were also collected in Kanazawa, Japan (Fig. S2, Supporting Information), every day between February 10 – 24 and August 3 – 16, 2010. All TSP samples were collected using a high-volume air sampler equipped with quartz fiber filters at a flow rate of 1.0 m^3 min^{-1}.

Sample pretreatment

A portion of the filter (2 – 180 cm^2) containing TSP matter was placed in a glass flask, to which internal standard (Pyr-d_{10}) solution was added. The benzene/ethanol extracts were added with dimethyl sulfoxide (DMSO), and then the mixture was evaporated. The resulting residue (DMSO) was dissolved in 900 µL acetonitrile, and an aliquot of the solution was analyzed by HPLC-FLD. Additional detailed information is provided in Supporting Information.

Table 1 The final HPLC conditions

Column	Two Zorbax Eclipse PAH (4.6 i.d. × 250 mm, 3.5 µm) + one Inersil ODS-P (4.6 i.d. × 250 mm, 5.0 µm)× 1
Guard column	Inersil ODS-P × (4.0 i.d. × 10 mm, 5.0 µm)× 1
Mobile phase	Acetonitrile/water (98/2)
Flow rate	0.3 mL min^{-1}
Oven temperature	20°C
Detection wavelength	Ex = 309 nm, Em = 354 nm (0 – 53.9 min) Ex = 331 nm, Em = 392 nm (53.9 – 65.0 min)

Results and Discussion

HPLC analysis of BcFE in urban airborne particulates

The purpose of this work was not to determine the optimum HPLC conditions for the analysis of BcFE in airborne particulates, but rather to accurately quantify BcFE. A mixture of BcFE standard and EPA610 PAH Mix was injected into the HPLC and analyzed at excitation and emission detection wavelengths of 309 and 354 nm, respectively (Fig. 2). When PAHs were separated on an Inertsil ODS-P column with acetonitrile–water in gradient elution mode (standard conditions for PAH analysis in our previous study),[21] the retention time of BcFE (37.6 min) was almost the same as that of Pyr (Fig. S3, Supporting Information). BcFE and Pyr could not be separated on the other two columns examined (Cosmosil 5C_{18}-AR-II and ZORBAX Eclipse PAH). To increase the number of theoretical plates, two columns were connected. Although two Eclipse columns or two ODS-P columns separated BcFE from Pyr-d_{10} with resolution (R_s) values of 2.3 and 2.6, the BcFE peak could not be completely separated from an unknown interfering peak in the Beijing airborne particulate samples. By combining two Eclipse columns and one ODS-P column in series, the BcFE peak could be separated from not only the Pyr-d_{10} peak but also from the interfering peak (R_s = 1.00) with a mobile phase consisting of acetonitrile–water (98:2, v/v) at a flow rate of 0.3 mL min^{-1} (Table 1).

After injecting the airborne particulate extract, we then collected the fraction eluting at the same retention time as the BcFE standard. The fluorescence spectrum of the solution measured at an excitation wavelength of 309 nm showed the same pattern of that of BcFE standard solution, with maximum fluorescence at 354 nm (Fig. S4, Supporting Information). This result suggested that the fluorescent compound in the fraction was BcFE. After the solution was evaporated to dryness and the residue was dissolved in toluene, an aliquot was analyzed by GC-MS. The total mass chromatogram showed two peaks (a: Rt = 11.20 min; b: Rt = 11.34 min). Peak a (m/z = 192), which was not fluorescent, did not show the same mass spectrum as BcFE. Peak b (m/z = 216), however, did show the same mass spectrum as BcFE. The retention time of BcFE in the proposed method was different from the retention times of BaFE (66.00 min) and BbFE (66.25 min). Thus, peak b was conclusively shown to be BcFE, indicating that the proposed

Table 2 Atmospheric concentrations of particulate-phase PAHs[a] in Beijing, China, and Kanazawa, Japan

PAH	Beijing[b]		Kanazawa[b]	
	Winter	Summer	Winter	Summer
Flu	46000 ± 28000	550 ± 140	160 ± 72	57 ± 22
Pyr	87000 ± 53000	900 ± 270	280 ± 130	100 ± 47
BcFE	11000 ± 6100	40 ± 12	13 ± 5.0	2.7 ± 0.52
BaA	39000 ± 26000	410 ± 130	94 ± 41	45 ± 9.5
Chr	30000 ± 22000	660 ± 170	220 ± 92	93 ± 24
BbF	43000 ± 29000	1300 ± 290	200 ± 82	140 ± 19
BkF	12000 ± 91000	540 ± 140	86 ± 37	55 ± 5.2
BaP	27000 ± 20000	960 ± 320	93 ± 49	99 ± 18
BghiPe	43000 ± 30000	2100 ± 660	280 ± 150	200 ± 20
IDP	18000 ± 13000	1200 ± 430	150 ± 66	100 ± 13
Total	360000 ± 23000	8500 ± 2100	1600 ± 710	890 ± 170

a. Each PAH concentration is mean ± S.D. (pg m^{-3}).
b. Airborne particulates were collected in Beijing every day during 18 January – 1 February and 2 – 16 August, 2010, and in Kanazawa every day during 10 – 24 February and 3 – 16 August, 2010.

Table 3 Atmospheric BaP_{eq} concentrations of PAHs in Beijing, China, and Kanazawa, Japan

PAH	RPF[18]	BaP_{eq} concentration[a]			
		Beijing		Kanazawa	
		Winter	Summer	Winter	Summer
Flu	0.08	3.7 (1.3)[b]	0.04 (1.4)	0.013 (2.2)	0.005 (1.5)
Pyr	0	0	0	0	0
BcFE	20	**215.5 (73.6)**	**0.79 (25.9)**	**0.254 (44.0)**	**0.053 (18.1)**
BaA	0.2	7.7 (2.6)	0.08 (2.7)	0.019 (3.3)	0.009 (3.1)
Chr	0.1	3.0 (1.0)	0.07 (2.2)	0.022 (3.9)	0.009 (3.1)
BbF	0.8	34.4 (11.7)	1.01 (33.0)	0.163 (28.2)	0.111 (37.6)
BkF	0	0	0	0	0
BaP	1	26.9 (9.2)	0.96 (31.4)	0.093 (16.1)	0.099 (33.6)
BghiPe	0.009	0.4 (0.1)	0.02 (0.6)	0.003 (0.4)	0.002 (0.6)
IDP	0.07	1.2 (0.4)	0.08 (2.7)	0.011 (1.9)	0.007 (2.4)
Total		292.8 (100)	3.05 (100)	0.58 (100)	0.29 (100)

a. Each BaP_{eq} concentration is mean ± S.D. (ng m^{-3}), calculated by BaP_{eq} = concentration × RPF.
b. Each parenthesis means % of the total BaP_{eq} PAH concentration.

HPLC-FLD method can be used to quantify BcFE in airborne particulate extracts.

Validation

Although the conditions described above may not have been optimum, we nevertheless validated the proposed method because quantification data are necessary for risk assessment. The calibration curve for BcFE (0.05 – 50 ng mL^{-1}) using Pyr-d_{10} as an internal standard was linear, with a good correlation coefficient (R^2 = 0.9992) (Fig. S5, Supporting Information). The limit of detection (LOD = 3.3σ) and the limit of quantity (LOQ = 10σ) were 1.2 pg/injection and 3.7 pg/injection, respectively. The LOD of the proposed HPLC-FLD method is approximately 26 times lower than that of the GC-MS method.[19]

The proposed method was used to quantify BcFE in airborne particulates collected in winter in Beijing, China. The difference between the concentration of BcFE as determined using the standard addition method (16.5 ng mL^{-1}) and the concentration determined using the internal standard method (15.7 ng mL^{-1}) was small enough (4.7%) to enable quantification.[22]

Concentration of BcFE in urban air

We determined the concentration of BcFE in airborne particulates collected in Beijing, China, and Kanazawa, Japan (Fig. 2). In a previous study,[21] we quantified other PAHs using the HPLC-FLD method. As shown in Table 2, the concentration of particulate-phase BcFE was lower than the concentrations of the other 4-ring PAHs (Flu, Pyr, BaA, and Chr). The BcFE concentration was higher in Beijing than in Kanazawa and was higher in winter than in summer. These trends observed for BcFE were similar to those observed for the other PAHs.

We also previously reported that 4-ring PAHs such as Pyr and BaA are present not only in the particulate phase but also in the gas phase in the atmosphere. The concentrations of BcFE in both the gas and particulate phases in 11 cities in Japan were recently reported.[20] Considering the relatively high vapor pressure of BcFE,[20] the total (gas phase + particulate phase) BcFE concentration in the atmosphere might be higher than the concentrations shown in Table 2.

Assessment of the health risks of BcFE exposure

The RPF was used to assess the health risks associated with BcFE exposure.[18] In this report, the BaP equivalent mutagenicity (BaP_{eq}) of each PAH was calculated from the concentrations shown in Table 2 using the following equation:

$$BaP_{eq} = \text{Concentration} \times \text{RPF} \quad (1)$$

According to Eq. (1), the BaP_{eq} of BcFE, as calculated from the Table 2 data, was in the range 0.053 to 220 ng m^{-3}, corresponding to 18 to 74% of the total PAHs (Table 3). BcFE exhibited a greater contribution to toxicity than BaP, by a factor of 8.0 in winter and 0.83 in summer in Beijing and 2.7 in winter and 0.54 in summer in Kanazawa.

The BaP_{eq} concentration of BcFE determined in this study, which might often be higher than the concentration of BaP, suggests that the toxic contribution of BcFE is significant. We previously reported high atmospheric concentrations of PAHs and NPAHs in several cities in Asia and Africa, including Beijing, Shenyang, Hanoi, and Cairo.[21,24,25] Properly assessing the health risks of PAH and NPAH exposure thus necessitates the determination of not only the concentration of BcFE but also the concentrations of DBalP and BjAC.

Conclusions

An HPLC-FLD method was developed for quantifying atmospheric BcFE. BcFE was separated on two ZORBAX Eclipse PAH (4.6 i.d. × 250 mm, 3.5 μm) columns and one Inertsil ODS-P (4.6 i.d. × 250 mm, 5 μm) column connected in series, using an acetonitrile/water mobile phase and fluorescence detection at excitation and emission wavelengths of 309 and 354 nm, respectively. The LOD and LOQ were 1.2 pg/injection and 3.7 pg/injection, respectively. The particulate-phase BcFE concentrations in the atmosphere were 11000 ± 6100 pg m^{-3} (winter) and 40 ± 12 pg m^{-3} (summer) in Beijing, China, and 13 ± 5.0 pg m^{-3} (winter) and 2.7 ± 0.52 pg m^{-3} (summer) in Kanazawa, Japan. Although the concentration of BcFE was lower than that of BaP, the mutagenic contribution of BcFE was greater than that of BaP.

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Supporting Information

Detailed analytical conditions, sampling conditions pretreatment, low data, error among the filters used for the extraction, results of the recovery test, accuracy and precision data, chromatogram of PAH standard mixtures by standard conditions for PAH analysis in our previous study, fluorescence spectra of (a) BcFE standard and (b) Beijing sample and calibration curves for BcFE are described. This material is available free of charge on the Web at http://www.jsac.or.ip/analsci/.

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