Determination of Atmospheric Nitrobenzanthrones by High-Performance Liquid Chromatography with Chemiluminescence Detection

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A method using high-performance liquid chromatography with chemiluminescence detection was developed for analyzing mutagenic nitrobenzanthrone (NBA) isomers in airborne particulates. The method was a modification of our previously described method for analyzing nitropolycyclic aromatic hydrocarbons (NPAHs). The pretreatment and reducing conditions for 1-, 2-, 3- and 10-NBAs were the same as those for NPAHs. In order to separate these NBA isomers, we used a polymeric-type ODS column (Cosmosil 5C-18MS); a mixture of 40% acetonitrile and 60% 10 mM imidazole–$HClO_4$ buffer was employed as the mobile phase at a flow rate of 1 mL/min. The isomers of 1-, 2-, 3- and 10-NBA were determined in chemiluminescence with linear calibration graphs from 0.1 to 4 pmol, from 200 to 4000 pmol, from 1 to 50 pmol and from 10 to 400 pmol, respectively. The detection limits (S/N = 3) of 1-, 2-, 3- and 10-NBA isomers were 0.02 pmol, 35 pmol, 0.3 pmol and 3 pmol, respectively. The method was used to analyze airborne particulates at a heavy traffic site in Kanazawa. 2- and 3-NBAs were detected in the extracts of the particulates, while 1-NBA and 10-NBA were not detected. The atmospheric concentrations of 2- and 3-NBAs were 1.83 pmol/m^3 and 24.7 fmol/m^3, respectively.

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Introduction

Several nitropolycyclic aromatic hydrocarbons (NPAHs), such as 1,3-, 1,6- and 1,8-dinitropyrenes (DNPs), show strong direct-acting mutagenicity by the Ames test using *Salmonella typhimurium* strains such as TA98, YG1021 and YG1024.[1-4] NPAHs were formed mainly through incomplete combustion of organic matters such as coal and petroleum and have been detected in the atmosphere, surface soil, sediments and rainwater.[5–11] Furthermore, several mutagenic NPAHs were formed in the atmosphere by the heterogeneous or homogeneous reactions of parent PAHs with NO_x and OH radicals.[12–15] NPAHs need to be monitored because they are a health risk for humans and are present throughout the environment. We previously developed a method using high-performance liquid chromatography with chemiluminescence detection to determine trace levels of NPAHs.[16] We used this method to monitor the atmospheric behavior and to determine the major contributors of NPAHs containing 1,3-, 1,6-, 1,8-DNPs, which are the strongest mutagenic NPAHs group, in some Japanese cities and in Vladivostok, Russia.[5-8,17]

A nitropolycyclic aromatic ketone, 3-nitrobenzanthrone (3-NBA, 3-nitro-7*H*-benz[*d*,*e*]anthracene-7-one) has been known as strong mutagenic and carcinogenic compound.[18] The mutagenicity of 3-NBA was comparable to that of 1,8-DNP,[18] which is the strongest mutagen according to the Ames test. 3-NBA and its metabolites showed genotoxicity in both animal and human cells.[19-22] 3-NBA was detected in diesel exhaust particulates (in the range from 0.1 to 24 pmol/mg),[18,23] in atmospheric particulates (from 1.4 to 249 fmol/m^3),[18,24,27] and in surface soil (from 4.3 to 4211 fmol/g),[25,26] which suggests that the major contributors of 3-NBA were diesel-engine vehicles, although 3-NBA was formed from the atmospheric reaction of benzanthrone (BA), with NO_2 and O_3.[18,24,27,28] On the other hand, among the 3-NBA isomers, 9- and 11-NBAs are also mutagenic[18] and 2-NBA is easily formed from the gas-phase reaction of BA and NO_3 or OH radical in the presence of NO_2 in the atmosphere.[27]

Gas chromatography with mass spectrometry (GC/MS) and HPLC with ultraviolet or fluorescence detection (HPLC/UVD, HPLC/FLD) were used for determining 3-NBA in environmental samples.[23,24,26,27] Among these methods, HPLC/FLD was highly sensitive to an amino derivative of 3-NBA.[26] A Pt/Rh-coated alumina column was able to reduce NPAHs to their amino derivatives (APAHs).[29] We previously developed the HPLC method with chemiluminescence detection by introducing an on-line clean-up, Pt/Rh reduction and concentration columns for NPAHs.[30,31] In the present study, an

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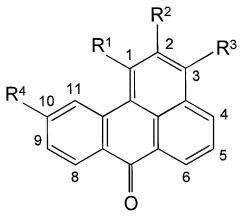
HPLC method for simultaneous determination of atmospheric 1-, 2-, 3- and 10-NBAs was developed by modifying the experimental conditions of the above method.[30,31] These NBA isomers in airborne particulates collected at a heavy traffic road in Kanazawa will be analyzed by the proposed method.

Materials and Methods

Chemicals

Figure 1 shows the structures of NBA isomers. 3-NBA was purchased from Aldrich Chemicals (Milwaukee, WI, USA), 1-, 2- and 10-NBAs were kindly provided by Professor S. Fujisawa of the Faculty of Science, Toho University. 2-Fluoro-7-nitrofluorene (FNF, an internal standard) was purchased from Aldrich Chemicals. All other chemicals used were obtained from commercial sources.[30,31]



Compound	NO_2 position*
1-NBA	R1
2-NBA	R2
3-NBA	R3
10-NBA	R4

*Other Rs are H.

Fig. 1 Structures of NBA isomers. For abbreviations, see text.

Reducer system

The reduction conditions for NBA isomers were the same as described previously.[30] The reduction system consisted of a LC-10A pump (Shimadzu, Kyoto, Japan), a SIL-10A-10A auto sample injector (Shimadzu), a SPD-10AV UV-VIS detector (Shimadzu) and a C-R7A integrator (Shimadzu). The reduction column packed with Pt/Rh-coated alumina (4.0 i.d. × 10 mm; Shimadzu) was kept at 80˚C in an HIC-6A column oven (Shimadzu) and a Cosmosil 5C18-MS ODS column (4.6 i.d. × 150 mm; Nacalai Tesque, Kyoto, Japan) was kept at 20˚C in a CTO-10AC column oven (Shimadzu). The mobile phase was 75% ethanol–0.02 M acetic acid-sodium acetate buffer (pH 5.5) and the flow rate was 0.2 mL/min.

LC/MS conditions

NBA isomers eluted from the Pt/Rh reduction column were detected on a platform LCZ mass spectrometer (Micromass, Wythenshawe, Manchester, UK). Analysis was performed using electrospray ionization (ESI) in positive ion mode. The detected ion was m/z = 246.5, which corresponded to NH_2-BA ($[M+H]^+$). The cone voltage was set at 25 V. The desolvation temperature and ESI needle potential were held at 400˚C and 3.5 kV, respectively.

Analysis system

The HPLC analysis system consisted of five LC-10A pumps (Shimadzu), a SIL-10A auto sample injector (Shimadzu), a DGU-14 degasser (Shimadzu), a CLD-10A chemiluminescence detector (Shimadzu), an SPD-10AV UV-VIS detector (Shimadzu), a SCL-10A system controller (Shimadzu) and a C-R7A integrator (Shimadzu). The clean-up column (4.6 i.d. × 150 mm), concentration column (4.6 i.d. × 30 mm), separation columns (4.6 i.d. × 250 mm), two guard columns (4.6 i.d. × 30 mm and 4.6 i.d. × 50 mm) were packed with Cosmosil 5C18-MS (Nacalai Tesque), and the reduction column (4.0 i.d. × 10 mm) was a nitroarene reaction column (Shimadzu). The reduction column was kept at 80˚C in an HIC-6A column oven (Shimadzu) and the two guard, clean-up, concentration and separation columns were all kept at 20˚C in a CTO-10AC column oven. The mobile phase for separation of NBA isomers was a mixture of acetonitrile–10 mM imidazole–$HClO_4$ buffer (4:6, v/v) at the flow rate of 1 mL/min.

After the sample solution was introduced into the HPLC system, the NBA isomers were separated from interfering substances on the clean-up column. By changing the position of the switching valve, the ABA isomers produced by passing the NBA isomers through the reduction column were concentrated on the concentration column. Then, the ABA isomers were eluted into the separation column by changing the position of the switching valve and were determined by chemiluminescence detection. All other conditions and system operation were the same as those given in our previous papers.[30,31]

Sample preparation

A high-volume air sampler (Kimoto Electric Company Ltd., Osaka, Japan) was set on a sidewalk 1 m away from a heavy traffic road in Kanazawa. Airborne particulates were collected with a 2500QAT-UP quartz fiber filter (8″ × 11″, Pallflex Products, Putnam, CT, USA) for 24 h at the flow rate of 1.3 m^3/min.

The filter was treated according to our previous paper.[31] A filter containing about 35 mg airborne particulates was cut into small pieces and placed in a flask. After addition of a FNF solution as an internal standard, NBA isomers were extracted ultrasonically twice with benzene/ethanol (3:1, v/v); the solution was then filtered with a piece of No. 6 filter paper (Toyo Roshi, Tokyo, Japan) and a 0.45 μm HLC-Disk membrane filter (Kanto Chemicals, Tokyo, Japan). The filtrate was washed with a sodium hydroxide solution, a sulfuric acid solution and water, and then evaporated to dryness. The residue was dissolved in 0.5 mL of 75% ethanol–0.02 M acetic acid-sodium acetate buffer (pH 5.5). This sample solution was filtered with a 0.45 μm membrane filter again, and an aliquot of this solution was injected into the HPLC analysis system.

Results and Discussion

Reduction of NPAHs

NPAHs have been reduced by various methods, including electrochemical, chemical and metal-catalytic reduction methods.[16,29,30,32] Recently, a combination of hydrazine and Raney nickel catalyst was used to reduce 3-NBA.[26] In the experiment, NBA isomers were reduced by Pt/Rh catalyst. This catalyst was used to reduce NPAHs to their corresponding aminopolycyclic aromatic hydrocarbons (APAHs).[30] The elution times of APAHs from the ODS column were shorter than those of the corresponding NPAHs, which suggests that the elution times of the reduced products of NBA isomers would be different from those of the original NBA isomers. Therefore, 1 nmol of each NBA (in a volume of 100 μL) was injected into the reduction systems without (A) and with (B) the reduction column before the ODS column (4.6 i.d. × 150 mm), and then the eluate was introduced into the UV detector (λ = 254 nm). Although the peaks of the impurities were also observed in each chromatogram (Fig. 2A), the retention times of the reduced compounds (Fig. 2B) were shorter than those of the NBA isomers (Fig. 2A). The peaks of un-reacted NBA isomers were not detected in the chromatograms B, which suggests that each

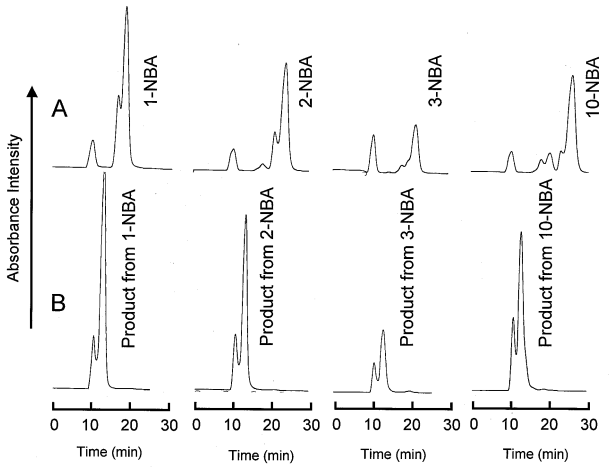


Fig. 2 Chromatograms for (A) NBA isomers and (B) their corresponding reduced products. Concentrations of each NBA: 1×10^{-5} M. Injection volume: 100 μL. UV absorption wavelength: 254 nm.

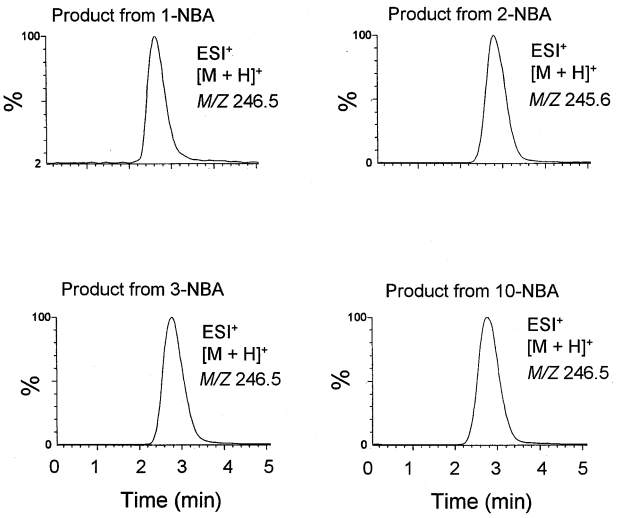


Fig. 3 Total ion chromatograms for reduced product fraction using selected ion monitoring at *m*/*z* 246.5. For HPLC/MS conditions, see text.

NBA was completely reduced by the proposed reduction system.[30]

In order to obtain information about the molecular weights of the products, each product fraction from the reduction system was introduced into a platform LCZ mass spectrometer. Figure 3 shows the total ion chromatograms of four product fractions using the selected ion monitoring at *m*/*z* 246.5. Each product was eluted in the range from 2.2 min to 3.6 min. The protonated molecular ion $[M+H]^+$ was observed for each NBA in positive ion mode, suggesting that each product was monoaminobenzanthrone (mono-ABA) (*m*/*z* = 246.5).

Separation of NBA isomers

We first examined the elution times of NBA isomers from the reduction column. The elution order was 1-NBA (15.5 – 21.5 min), 3-NBA (18.5 – 23.5 min), 2-NBA (20.0 – 27.5 min) and 10-NBA (22.5 – 29.0 min). Therefore, to analyze these NBA isomers simultaneously, we set the loading time of the analysis system was set from 15 to 30 min. This fraction contained not only FNF (internal standard) but also 2-nitrofluorene (2-NF), 1,6- and 1,8-DNPs.[31]

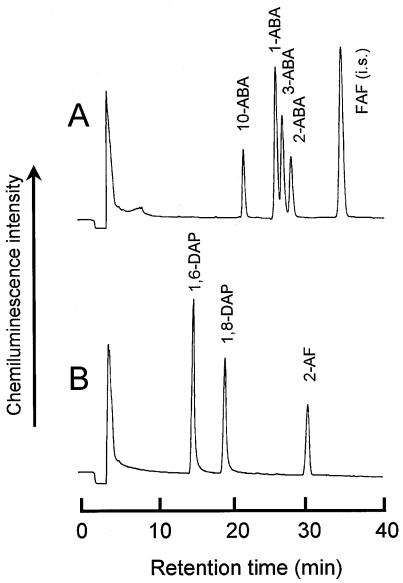


Fig. 4 Chromatograms for (A) ABA isomers and FAF and (B) 2-AF, 1,6- and 1,8-DAPs standard. For HPLC conditions, see text. Concentrations of standard NBA isomers: 2 pM (1-NBA); 2 nM (2-NBA); 25 pM (3-NBA); 200 pM (10-NBA). Injection volume: 100 μL.

The separation efficiencies of three kinds of reversed phase HPLC columns were tested by using ABA isomers. Cosmosil 5C-18MS was a monomeric-type octadecylsilyl column, and Inertsil ODS-P (4.6 i.d. × 250 mm; GL Sciences, Tokyo, Japan) was a polymeric-type octadecylsilyl column. Cosmosil 5NPE (4.6 i.d. × 250 mm; Nacalai Tesque) was a column whose stationary phase was bonded with nitrophenylethylethyl groups. It was effective for the separation of compounds that had π electrons. Table 1 shows the elution times of ABA isomers from the columns kept at 20˚C. The elution times from the Cosmosil 5C-18MS column were shorter than those from the others. When the mobile phase was 50% acetonitrile, 1- and 3-ABAs were not separated by the Cosmosil 5C-18MS column, 2- and 3-ABAs were not separated by the Cosmosil 5NPE column, and 1- and 10-ABAs were not separated by the Inertsil ODS-P column. When the acetonitrile concentration in the mobile phase was decreased to 40%, 1- and 3-ABAs were separately eluted from the Cosmosil 5C-18MS column. However, 2- and 3-ABAs, and 1- and 10-ABAs were still unresolved on the Cosmosil 5NPE and Inertsil ODS-P columns, respectively. Therefore, we selected the Cosmosil 5C-18-MS column and a mixture of 40% acetonitrile and 60% of 10 mM imidazole-$HClO_4$ buffer as the mobile phase for separation of ABA isomers. Figure 4 shows the chromatograms for (A) ABA isomers and FAF and (B) 2-AF, 1,6- and 1,8-DAPs under the above conditions. FAF, 2-AF, 1,6- and 1,8-DAPs were eluted at 34.52, 30.01, 14.94 and 19.12 min, respectively. These retention times were different from those of the ABA isomers.

Pretreatment

The benzene–ethanol extracts from the environmental samples contain various organic compounds that interfere with quantitative analysis of NBA isomers and NPAHs. In our previous method,[16] 5% sodium hydroxide and 20% sulfuric acid were used to remove acidic and basic interfering matters from the benzene–ethanol extracts containing NPAHs, because NPAHs existed in the neutral fraction. However, NBA isomers

Table 1 Elution times of ABA isomers from three columns with different mobile phases

Compound	Time/min[a]					
	Cosmosil 5C-18MS		Cosmosil 5NPE		Inertsil ODS-P	
	Mobile phase A	Mobile phase B	Mobile phase A	Mobile phase B	Mobile phase A	Mobile phase B
1-ABA	12.31	25.58	14.92	31.78	14.77	33.05
2-ABA	13.13	27.67	13.95	29.71	17.49	40.80
3-ABA	12.35	26.41	13.70	29.13	18.46	43.91
10-ABA	10.65	21.40	12.07	24.57	14.25	32.43

a. Each column (4.6 mm i.d. × 250 mm) was kept at 20°C in a column oven. Mobile phase A, 50% acetonitrile; B, 40% acetonitrile.

Table 2 Validations of the analysis system

Compound	Range/pmol	r^2	DL[a]/pmol	QL[b]/pmol	RSD,[c] %
1-NBA	0.1 – 4	0.9993	0.02	0.07	3.0
2-NBA	200 – 4000	0.9999	35	116	2.1
3-NBA	1 – 50	0.9995	0.3	1	2.7
10-NBA	10 – 400	0.9997	3	10	1.3

a. Detection limit, $S/N = 3$.
b. Quantitation limit, $S/N = 10$.
c. Relative standard deviation, $n = 3$.

are weakly basic because the oxygen atom of BA (at 7 position) has two lone pairs.[33] Therefore, we compared the recoveries of NBA isomers before and after the sodium hydroxide and sulfuric acid treatments. High recoveries of all NBA isomers were obtained (peak heights were 98 to 104% of the peak heights of the original sample), suggesting that our proposed pretreatment method was also suitable for the determination of NBA isomers.

Accuracy

The standard solution of NBA isomers (100 μL) was injected into the analysis system to validate the method using FNF as an internal standard. The detection limits ($S/N = 3$) ranged from 0.02 pmol for 1-NBA to 35 pmol for 2-NBA (Table 2). Relative standard deviations ($n = 3$) were less than 3%. The calibration curves showed good linearity ($r^2 > 0.993$) from 0.1 to 4 pmol for 1-NBA, from 200 to 4000 pmol for 2-NBA, from 1 to 50 pmol for 3-NBA and from 10 to 400 pmol for 10-NBA, respectively.

Application to airborne particulates

3-NBA is a powerful mutagen in bacteria. Its mutagenicity as determined by the Ames test using the *S. typhimurium* TA98 (208000 rev./nmol) and YG 1024 (6290000 rev./nmol) strains was comparable to that of 1,8-DNP which is the strongest mutagen in NPAHs.[18] Both 9- and 11-NBA were also mutagenic, but their mutagenicities were weaker than that of 3-NBA.[18]

Airborne particulates collected at a heavy traffic road site in Kanazawa in winter contained 2- and 3-NBAs (Fig. 5B). Their calculated atmospheric concentrations of 1.83 pmol/m^3 and 24.7 fmol/m^3, respectively. These values were about three orders of magnitude and one order of magnitude higher than the concentration of 1,8-DNP (2.6 fmol/m^3) in the same sample. The concentration of 2-NBA was significantly higher than that of 1-nitropyrene (0.3 pmol/m^3), which was the NPAH with the highest concentration detected so far at this site. This result

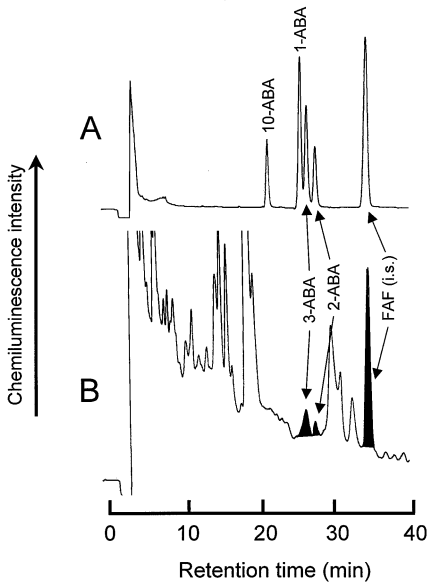


Fig. 5 Chromatograms for (A) NBA isomer standards and (B) benzene–ethanol extracts from airborne particulates collected at a heavy traffic road side in Kanazawa. Atmospheric concentrations of NBA isomers: 1.83 pmol/m^3 (2-NBA); 24.7 fmol/m^3 (3-NBA). Concentrations of standard NBA isomers and the injection volume were the same as those shown in Fig. 4.

suggests that 2-NBA was also an important contributor to the direct-acting mutagenicity in the atmosphere. The atmospheric concentration of 3-NBA in Kanazawa in the present study was almost at the same level as that in airborne particulates collected at a heavy traffic site in Tokyo.[18] 1- and 10-NBAs were not detected (Fig. 5B), even though our system was more sensitive to these compounds than to 2-NBA. Based on localization energies, the direct nitration of the carbon positions in BA was found to be in the order 3 > 4 > 1 > 9 ~ 11 > 6 > 5 ~ 2 > 8 ~ 10.[34] However, the high concentration of 2-NBA was detected in this study, which appears to be inconsistent with this order. This is because most atmospheric 2-NBA might be formed in the atmosphere rather than in burning processes, such as that in a diesel-engine.[27]

In conclusion, it can be stated that the proposed analysis method based on our previous study[30,31] is useful for the determination of atmospheric NBA isomers.

Acknowledgements

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