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Determination of 3-nitrobenzanthrone in surface soil by normal-phase high-performance liquid chromatography with fluorescence detection

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Abstract

A sensitive method for determining 3-nitrobenzanthrone in surface soil was developed. 3-Nitrobenzanthrone was reduced to 3-aminobenzanthrone by refluxing at 60 °C with hydrazine and Raney nickel for 20 min, and 3-aminobenzanthrone was determined by normal-phase high-performance liquid chromatography (HPLC) with fluorescence detection. We used a cyanopropyl stationary phase and an *n*-hexane–ethyl acetate (3:1, v/v) mobile phase, since 3-aminobenzanthrone exhibits fluorescence in a low-polarity solvent such as *n*-hexane or ethyl acetate, but not in a polar solvent such as water or methanol. The calibration graph showed good linearity (r^2>0.9999) in the range of 0.002–2 ng, and the detection limit was 0.002 ng ($S/N=3$). 3-Nitrobenzanthrone in extracts from surface soil collected in the Chubu area (central area) of Japan was determined after clean-up using silica gel chromatography and high-performance liquid chromatography on a pyrenylethyl stationary phase. The concentration of 3-nitrobenzanthrone in surface soil was determined in the range of 1.2–1020 pg/g soil.

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Keywords: Surface soil; 3-Nitrobenzanthrone; Hydrazine; Raney nickel

1. Introduction

Nitroarenes are produced by the incomplete combustion of organic compounds such as fossil fuels [1–3] and are also produced from parent arenes and nitrogen oxides through atmospheric transformation [4–6]. Nitroarenes are widespread environmental pollutants [7–9] and some are probably carcinogenic to humans [10].

3-Nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one, 3-NBA, Fig. 1) was recently detected in airborne particulate matter [11] and might originate from both incomplete combustion of fossil fuels and an atmospheric reaction, since 3-NBA was detected in diesel exhaust particulate matter [11] and was easily produced from benzanthrone and nitrogen oxides under atmospheric conditions [11,12]. 3-NBA is highly mutagenic in the Ames *Salmonella* assay (6 290 000 revertants/nmol in *Salmonella*

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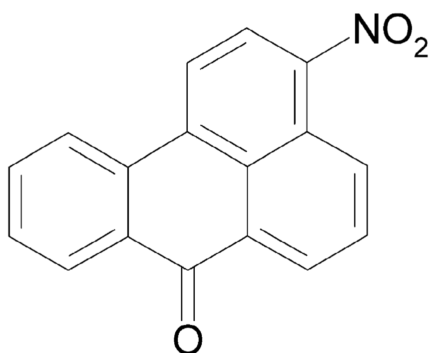

Fig. 1. Structure of 3-NBA.

typhimurium YG1024), and its potency is comparable to that of 1,8-dinitropyrene, which is the direct mutagen with the strongest activity (4 780 000 revertants/nmol in YG1024) that has been reported thus far in the literature [11]. 3-NBA induced micronuclei in mouse peripheral blood reticulocytes [11] and in a human B-lymphoblastoid cell line [13]. 3-NBA also bound covalently to DNA after metabolic activation, forming multiple DNA adducts in vitro and in vivo, all of which were products derived from reductive metabolites [14,15]. These facts indicate that 3-NBA in the environment may have an adverse effect on human health. To help estimate the potential risk of 3-NBA to human health, it is important to clarify the levels of 3-NBA in the environment.

Polycyclic aromatic compounds in soil have been studied by high-performance liquid chromatography (HPLC) [8,16–18], gas chromatography (GC) [18–20] and capillary electrophoresis (CE) [21]. Among these methods, HPLC coupled with fluorescence detection is highly sensitive and selective to polycyclic aromatic compounds. Although nitroarenes have no fluorescence, their reduced products, aminoarenes, have strong fluorescence. Therefore HPLC with fluorescence detection have been reported as highly sensitive and selective methods for the determination of nitroarenes [8].

In this study, we developed an analytical method to quantify trace amounts of 3-NBA, where 3-NBA is reduced to 3-aminobenzanthrone (3-ABA) with hydrazine and Raney nickel and then detected by normal-phase HPLC with fluorescence detection. This method was applied to surface soil after clean-up using silica gel chromatography and HPLC on a pyrenylethyl stationary phase.

2. Experimental

Caution: 3-NBA is a strong mutagen and therefore should be handled with care.

2.1. Chemicals

3-NBA was synthesized as described elsewhere [22]. 3-ABA was synthesized as follows: 5 g of tin chloride (Wako Pure Chemical Industries, Osaka, Japan) and 100 ml of concentrated hydrogen chloride (Nacalai Tesque, Kyoto, Japan) were added to 100 ml of methanol containing 0.01 g 3-NBA. After 30 min, the reacted solution was alkalized with sodium hydroxide, and 3-ABA was extracted with ethyl acetate. The extract was evaporated to dryness, and 3-ABA (red crystal) was obtained. The structure of 3-ABA was confirmed by its electron ionization mass spectra using a Shimadzu (Kyoto, Japan) GCMS-QP5050A GC–MS at 70 eV. A base peak of $m/z=245$ was observed. The melting point of 3-ABA measured by a Yanagimoto hot stage apparatus was 235–237 °C

Methanol, acetonitrile and tetrahydrofuran (THF) were purchased from Wako Pure Chemical Industries. Hydrazine monohydrate and Raney nickel were obtained from Nacalai Tesque. Ethyl acetate and *n*-hexane were from Kanto Chemicals (Tokyo, Japan). All other reagents were of analytical grade.

2.2. Sampling and extraction

Surface soil was collected at six sampling points in the Chubu area (central area) of Japan. The soil was dried in Petri dishes for 2 days at room temperature. The dried soil was screened through a 60-mesh sieve (250 μm) to remove large gravel and trash. The sieved soil (15 g) was extracted ultrasonically with methanol (200 ml) twice for 10 min each time. The extract was filtered through an Advantec Toyo (Tokyo, Japan) No. 5C filter paper, and the filtrate was evaporated to dryness. The residues were dissolved in 1 ml of chloroform.

2.3. Clean-up

Three extracted solutions (0.3 ml each) per sample were applied to three silica gel (7734 silica gel 60, 70–230 mesh, Merck, Darmstadt, Germany) columns (12× 300 mm I.D.), and the materials were then eluted successively with 20 ml *n*-hexane, 20 ml *n*-hexane–benzene (9:1, v/v), 20 ml *n*-hexane–benzene (2:1, v/v), 20 ml *n*-hexane–benzene (1:1, v/v) and 30 ml benzene. 3-NBA was eluted with 30 ml chloroform. This chloroform fraction was evaporated to dryness and the residue was dissolved in 0.5 ml of methanol. Next, 0.4 ml of this solution was subjected to HPLC using a Nacalai Tesque Cosmosil 5PYE (4.6× 150 mm, I.D.) as a stationary phase and acetone as a mobile phase. The HPLC system consisted of a Shimadzu LC-10AS pump (flow-rate, 1 ml/min), a Rheodyne (Cotati, CA, USA) 7125 sample injector (loop, 1 ml), a Shimadzu CTO-$10A_{VP}$ column oven (temperature, 30 °C) and a Shimadzu SPD-10AV UV detector (wavelength, 400 nm). Since 3-NBA was eluted at a retention time of 5.4 min, effluent from 4.9 to 5.9 min was collected, and this fraction was evaporated to dryness. The residue was dissolved in 1 ml of THF.

2.4. Reduction

Raney nickel (5 mg) and hydrazine (0.1 ml) were added to the sample solution (1 ml) in a 30-ml Kjeldahl flask. Then a 15-cm Allihn condenser was connected to this flask, the mixture was refluxed in an oil bath (oil bath: NWB-240, Nissin, Tokyo, Japan; silicone oil: KF-54, Shinetsu, Tokyo, Japan) for 20 min at 60 °C. The solution was then transferred to a 1.5-ml polyethylene tube and centrifuged at 10 000 rpm for 1 min using an Iwaki (Tokyo, Japan) CFA-12 centrifuge. The supernatant (10 μl) was applied to an HPLC system.

2.5. HPLC system for 3-aminobenzanthrone

The HPLC system consisted of a Shimadzu LC-$10AT_{VP}$ pump, a Rheodyne 7125 sample injector with a 1-ml loop and a Jasco (Tokyo, Japan) FP-1520S fluorescence detector. A Tosoh (Tokyo, Japan) TSKgel CN-80Ts (4.6× 250 mm, I.D.) cyanopropyl column and *n*-hexane–ethyl acetate (3:1, v/v) were used as a stationary phase and a mobile phase, respectively. The flow-rate of the mobile phase was 0.7 ml/min. The detection excitation and emission wavelengths were 490 and 560 nm, respectively.

3. Results and discussion

3.1. Chromatographic conditions for 3-aminobenzanthrone

To quantify trace amounts of 3-NBA in the environmental samples, we decided to use fluorescence detection. Although 3-NBA was not fluorescent, the corresponding amino derivative, 3-ABA, was fluorescent under certain conditions. Fig. 2 shows fluorescence excitation and emission spectra of 3-ABA in *n*-hexane–ethyl acetate (3:1, v/v). The fluorescence spectra showed an emission maximum at 560 nm with an excitation maximum at 490 nm. We used these wavelengths in the following experiment.

Fig. 3 shows the fluorescence intensity of 3-ABA in various solvents. Strong fluorescence was observed in low-polarity solvents such as ethyl acetate, THF and *n*-hexane. In contrast, no fluorescence was

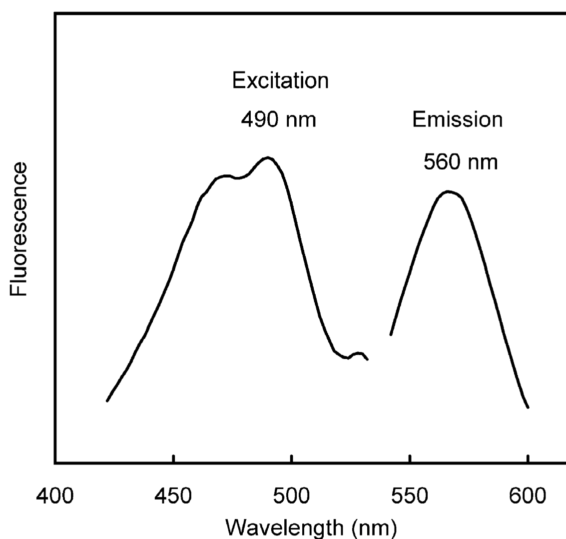

Fig. 2. Fluorescence excitation and emission spectra of 3-ABA in *n*-hexane–ethyl acetate (3:1, v/v).

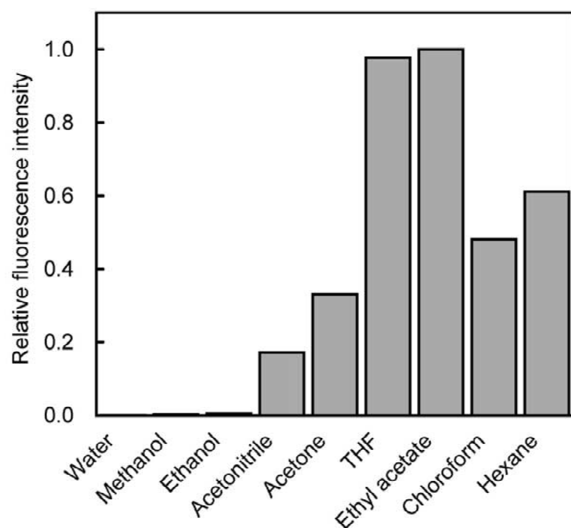

Fig. 3. Fluorescence intensity of 3-ABA in various solvents.

observed in methanol and water, and weak fluorescence was detected in acetonitrile. Therefore, we used low-polarity solvents such as *n*-hexane and ethyl acetate as a mobile phase in normal-phase HPLC. When a cyanopropyl column (Tosoh TSKgel CN-80Ts, 4.6× 250 mm, I.D.) was used as a stationary phase, a mobile phase of *n*-hexane–ethyl acetate (3:1, v/v) at a flow-rate of 0.7 ml/min gave a moderate retention time for 3-ABA (17.7 min). Thus, these stationary and mobile phases were used for the analysis of 3-ABA. THF was used as a sample solvent because 3-ABA in THF also showed high fluorescence intensity (Fig. 3).

3.2. Reduction of 3-nitrobenzanthrone

We decided to reduce 3-NBA with hydrazine and Raney nickel catalyst because reduction with hydrazine and Raney nickel has been reported to be selective to aromatic nitro-groups [23,24]. To identify optimum conditions for the reduction of 3-NBA, we investigated the effects of the reflux temperature and reflux time. The effect of reflux temperature on the fluorescence intensity of 3-ABA was investigated in the range of 15–75 °C for a fixed time of 20 min (Fig. 4A). Fluorescence increased with increasing temperature until 60 °C and then slightly decreased at 75 °C. This slight decrease may be related to the

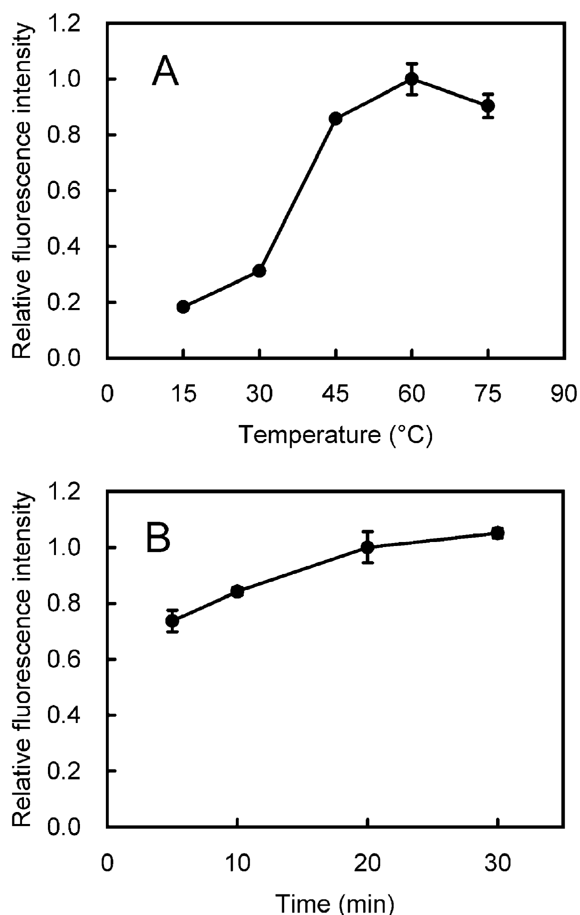

Fig. 4. The effects of reflux temperature (A) and time (B) on the reduction of 3-NBA to 3-ABA. The symbols and bars represent means and SD ($n=2$), respectively.

degradation of the product at this temperature. The influence of reflux time on fluorescence intensity of 3-ABA was investigated in the range of 5–30 min at a fixed reflux temperature of 60 °C (Fig. 4B). Fluorescence intensity increased with increasing reaction time from 5 to 30 min, however the increase in fluorescence intensity between 20 and 30 min was quite mild. Therefore, we decided to reduce 3-NBA at 60 °C for 20 min. The reduction efficiency at optimized conditions was 87.2% when 25 ng/ml 3-NBA/THF solution (1 ml) was used.

A chromatogram of a standard solution of 3-NBA under our optimized chromatographic and reduction conditions is shown in Fig. 5. A large peak was observed at a retention time of 17.7 min. This peak

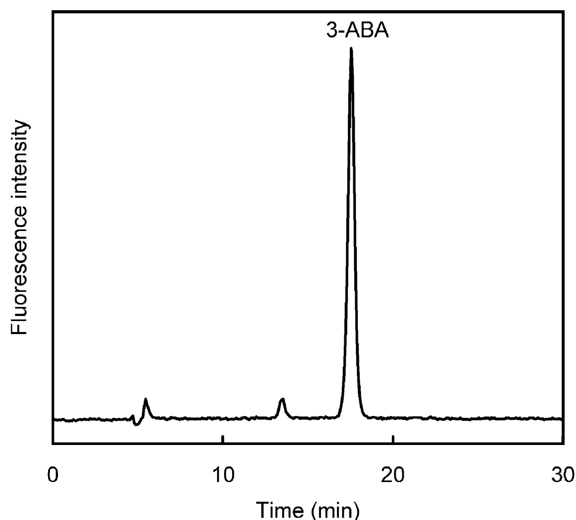

Fig. 5. Chromatogram of 3-NBA after reduction with Raney nickel and hydrazine.

was identified to be 3-ABA based on its retention time and fluorescence spectra (data not shown). Another smaller peak was observed around 13.5 min. This peak was not identified, but this may be another product from 3-NBA, because this peak was proportional to 3-NBA concentration.

3.3. Calibration graph and detection limit

Under the optimum conditions, a calibration graph showed good linearity ($r^2>0.9999$) in the range of 0.002–2 ng (0.2–200 μg/l). The detection limit calculated by the ratio of signal to noise ($S/N=3$) was 0.002 ng (0.2 μg/l). The reproducibility of this method was examined for 0.2 ng (20 μg/l). The relative standard deviation ($n=5$) was lower than 5%.

3.4. Determination of 3-nitrobenzanthrone in surface soil

3-NBA was ultrasonically extracted from surface soil. Methanol was used as an extraction solvent, because methanol is low toxicity and was high extraction efficiency of mutagen [25]. Since extracts from surface soil are very complex mixtures, efficient clean-up is needed before HPLC analysis. We used silica gel chromatography and HPLC on a pyrenylethyl stationary phase. In silica gel chromatography, 3-NBA was eluted in a chloroform fraction. In HPLC on a pyrenylethyl stationary phase (Cosmosil 5PYE, 4.6× 150 mm I.D.), 3-NBA was eluted at 5.4 min when acetone was used as a mobile phase at a flow-rate of 1 ml/min. We collected effluent with retention times of 4.9–5.9 min. Typical HPLC (UV at 400 nm) chromatograms of extract from surface soil and authentic 3-NBA are shown in Fig. 6. Large peaks were observed at retention times of 2–4 min for extract from surface soil, whereas lower absorbance was observed at 4.9–5.9 min, indicating that most of the components that showed absorbance at 400 nm were eluted before 4 min. To examine the recovery of 3-NBA during clean-up, 30 and 4 ng of 3-NBA was applied to the silica gel column and the HPLC, respectively. The recoveries in silica gel chromatography and HPLC on a pyrenylethyl stationary phase were 99 and 96%, respectively.

A typical chromatogram of extract from surface soil after clean-up and reduction is shown in Fig. 7. The peak of 3-ABA was observed at 17.7 min, and there was no interference peak. We determined 3-NBA from the peak area of 3-ABA by an external standard calibration. Therefore, concerns for quantitative handling of samples were required. The 3-NBA concentrations in surface soil samples collected at six sampling sites in the Chubu area of Japan are

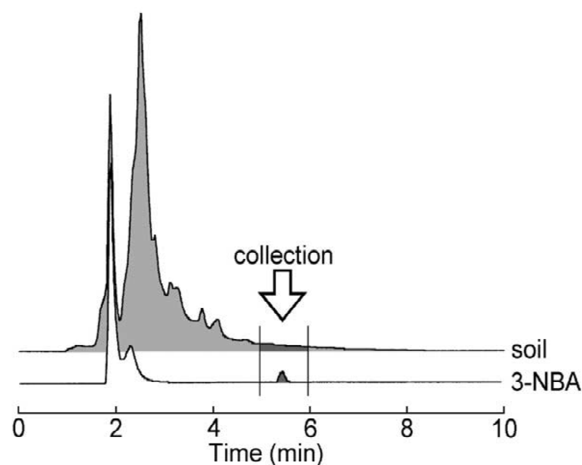

Fig. 6. UV chromatograms of extract from surface soil and a standard solution of 3-NBA. The eluate was monitored at an absorbance of 400 nm.

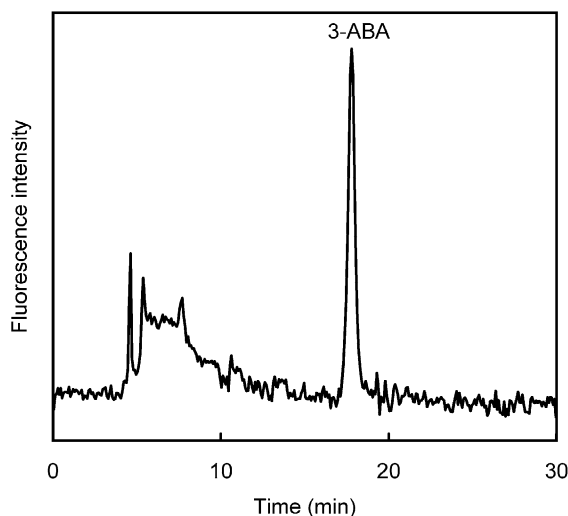

Fig. 7. Chromatogram of extract from surface soil.

listed in Table 1. 3-NBA was detected in the range of 1.2–1020 pg/g soil. This is the first detection of 3-NBA in surface soil.

We previously determined strong mutagenic 1,3-, 1,6- and 1,8-dinitropyrenes in the extracts from surface soil collected in three areas, i.e. the Kanto, Chubu, and Kinki, which are districts of megalopolis in Japan [26]. In the Chubu area, we analyzed soil samples collected at three sites. The concentration ranges of 1,3-, 1,6- and 1,8-dinitropyrenes in these three samples were 12–3270, 16–5587 and 13–6809 pg/g of soil, respectively. These samples were different from those examined in this study. The range of 3-NBA was found to be the same level as those of the dinitropyrenes.

A HPLC method with peroxyoxalate-chemiluminescence detection has been used as a sensitive and selective method for the dinitropyrenes in the environmental samples [27–29]. This chemiluminescence method required acetonitrile–imidazole buffer as a mobile phase. 3-ABA had no fluorescence in the solution containing water. Since chemiluminescence is obtained from the reaction of fluorophore and chemiluminescence reagents, this chemiluminescence method is not available for the determination of 3-ABA. The advantages of our proposed method are: (1) sensitivity and selectivity are high enough; (2) recovery during clean-up process is quite high; and (3) special equipment and technique are not necessary. This method appeared to be very useful for the determination of 3-NBA in surface soil.

Table 1
Concentration of 3-NBA in surface soil

Sampling location	Concentration (pg/g soil)
Nagoya (site A)	1020±160
Nagoya (site T)	200±27
Hekinan	65±14
Handa (site S)	51±9
Handa (site N)	18±3
Gifu	1.2±0.2

Values represent mean±SD ($n=3$).

Acknowledgements

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