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High-performance liquid chromatography–fluorescence determination of dinitropyrenes in soil after column chromatographic clean-up and on-line reduction

Tetsushi Watanabe[a,*], Shigenobu Ishida[a], Makito Kishiji[a], Yoshifumi Takahashi[a], Aki Furuta[a], Terue Kasai[a], Keiji Wakabayashi[b], Teruhisa Hirayama[a]

[a]*Kyoto Pharmaceutical University, 5, Nakauchicho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan*

[b]*Cancer Prevention Division, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan*

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Abstract

In order to quantify 1,3-dinitropyrene (DNP), 1,6-DNP and 1,8-DNP in soil, we developed an efficient clean-up procedure and a sensitive determination method using fluorescence detection. DNP isomers were efficiently cleaned by three stages of fractionation, i.e., a silica gel open column chromatography using stepwise elution and two further purification steps by high-performance liquid chromatography (HPLC) using a monomeric-type octadecylsilyl (ODS) column and a polymeric-type ODS column. The recoveries of DNPs during the whole clean-up process were 94% or more. The fraction corresponding to DNPs was injected into an analytical polymeric-type ODS column for HPLC to separate DNP isomers. The effluent from the analytical ODS column was directly introduced to a catalyst column, which was packed with 5 μm alumina coated with platinum and rhodium (Pt–Rh), in order to reduce DNPs to diamino compounds, and then the fluorescence of diaminopyrenes was detected. The immediate detection of diaminopyrene isomers after on-line reduction afforded a sensitive detection of DNP isomers. The detection limits for DNPs were in the range of 0.7 to 4 pg. These developed methods were applied to four soil samples collected at parks in residential areas.

Keywords: On-line reduction; Dinitropyrenes

1. Introduction

Nitrated polycyclic aromatic hydrocarbons (NO_2-PAHs) are produced during the incomplete combustion process of organic compounds such as fossil fuels and emitted as gaseous and/or particulate matter [1]. In addition to the emission from combustion systems, NO_2-PAHs are also formed by the reaction of parent hydrocarbons with nitrogen oxides in ambient air [2]. These NO_2-PAHs have become of enormous concern because of their genotoxicity [3] and ubiquity in the atmospheric environment including diesel exhaust [4–6]. In particular, great attention has been directed to 1,3-dinitropyrene (DNP), 1,6-DNP and 1,8-DNP because these compounds are the strongest bacterial mutagens so far reported in the literature [3] and have exhibited carcinogenicity in experimental animals [7–9]. The International Agency for Research on Cancer (IARC) listed 1,6-DNP and 1,8-DNP as possible human carcinogens in IARC Monographs [10].

Recently, we reported that 1,6-DNP and 1,8-DNP

*Corresponding author.

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were identified as major mutagenic compounds in the organic extracts of soil samples collected at ordinary parks in Osaka City, Japan. Considering the strong genotoxicity of DNP isomers, it is quite important to quantify the DNP levels in our environment to assess their potential health risks. DNP isomers were detected in airborne particulate matter collected in several cities using high-volume air samplers [11–13]. However, the necessity of expensive apparatuses such as high-volume air samplers and power supply and the noise problem arising during sampling impose limitations on the collection of air samples. Determination of DNPs in surface soil seems to be a promising alternative method to monitor the compounds in our environment, because we can obtain adequate amounts of soil samples to quantify the compounds without any specific equipment or power source. However, there appear to be no reports in which the clean-up and determination methods of DNP isomers in soil samples are described. The extremely complex chemical nature of soil precludes ready detection of trace amounts of DNP isomers.

The purpose of this study is to develop the clean-up and analytical methods to determine 1,3-, 1,6- and 1,8-DNP isomers in soil. We adopted normal-phase and reversed-phase column chromatography for the clean-up and applied high-performance liquid chromatography (HPLC) combined with on-line reduction and fluorometric detection for the determination of DNP isomers. DNPs become highly fluorescent when reduced to diaminopyrenes (DAPs), although DNPs do not fluoresce [14]. Electrochemical reducer [15,16] and reducer columns packed with catalytically active metal powders [12,14,17–19] have been used in an on-line reduction of DNPs, but the reduction efficiency of the electrochemical reducer was reported to be not quantitative [15,16]. Zinc [12,17,18], cadmium–copper [12] and platinum–rhodium (Pt–Rh) [14,19] were utilized as the catalytic active metal. A cadmium–copper column has disposal problems and the lifetime of a zinc column was short [17,18]. In contrast, Pt–Rh catalyst columns have been reported to have an indefinite lifetime [14,19]. Therefore, we selected Pt–Rh as a packing material of the reducer column in this study. In this report, the clean-up method and optimum conditions for HPLC analysis of DNPs will be discussed.

2. Experimental

2.1. Reagents

1,3-, 1,6- And 1,8-DNPs were obtained from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile and methanol were purchased from Wako (Osaka, Japan). Silica gel (63–200 μm particle size) was obtained from Merck (Darmstadt, Germany). Alumina (5 μm particle size) that was coated with Pt–Rh (9:1) was obtained from Johnson Matthey (Malvern, PA, USA). The Pt–Rh concentration was about 1%. All other chemicals used were of analytical grade.

1,3-, 1,6- And 1,8-DAPs were prepared by the reduction of the corresponding DNPs using sodium hydrosulfide [13]. The structure of DAPs were confirmed by mass spectrometry.

2.2. Reducer column

A stainless steel column (50×4.0 I.D. mm) was dry-packed with a catalyst metal powder, i.e., alumina coated with Pt–Rh. The catalyst was activated by a stream of hydrogen at 350°C for 3 h at a flow-rate of 2 ml/min. The reduction ability of the catalyst was tested from ambient temperature up to 80°C eluting with methanol–water (80:20) at a flow-rate of 0.5 ml/min to 1.0 ml/min.

2.3. Sample preparation

Soil was collected from the ground of parks located in residential areas in Osaka City, Matsubara City and Uji City, Japan, spread on Petri dishes and allowed to stand to dryness for 2 days at room temperature in the dark. 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) for 10 min twice. The extracts were combined, filtered and then evaporated to dryness.

2.4. Partial purification

The DNP standards or organic extracts of soil were dissolved in 1 ml chloroform or toluene and then 0.3 ml of the solution was applied to a silica gel

column (220×10 I.D. mm). The silica gel was activated for 18 h at 160°C and then deactivated with distilled water (7.4%, w/w) prior to use. Stepwise elution was carried out with 20 ml *n*-hexane, 20 ml *n*-hexane–benzene (9:1), 20 ml *n*-hexane–benzene (2:1), 20 ml *n*-hexane–benzene (1:1) and 30 ml benzene and 20 ml chloroform. The benzene fraction was evaporated to dryness and redissolved in 0.5 ml acetonitrile–water (60:40) to be fractionated by HPLC. Benzene and chloroform were replaceable by toluene and acetone, respectively, as the solvent for the stepwise elution. The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-6A pump, a Rheodyne (Cotati, CA, USA) 7125 injector (1 ml sample loop), an ODS column, i.e., an STR ODS II column or a Wakosil-PAHs column, a Shimadzu SPD-10AV spectrophotometric detector and a Shimadzu R-11 recorder. An aliquot of the solution was injected into the STR ODS II column (5 μm particle size, 4.0×250 mm I.D., Shimadzu Techno-Research, Kyoto, Japan) for HPLC and eluted with acetonitrile–water (60:40) at a flow-rate of 0.7 ml/min. The fractions corresponding to DNPs were evaporated to dryness. The residue was dissolved in 0.5 ml acetonitrile–water (60:40) and the aliquot was injected into the Wakosil-PAHs column (5 μm particle size, 4.0×250 mm I.D., Wako) for HPLC with a mobile phase of acetonitrile–water (60:40) at a flow-rate of 0.7 ml/min. All HPLC was performed at ambient temperature, and the eluates were monitored for absorbance at 254 nm. The fractions corresponding to DNPs were evaporated to dryness. The residue was dissolved in 0.5 ml methanol–water (80:20) as sample solution for determination of DNP isomers.

2.5. HPLC analysis of 1,3-, 1,6- and 1,8-DNPs

An analytical system consisted of an Irica (Kyoto, Japan) Σ981 pump, an Irica Σ80 injector (1 ml sample loop), an ODS column, i.e., an L-column ODS column (5 μm particle size, 150×4.6 mm I.D., Chemicals Inspection and Testing Institute, Tokyo, Japan), a Cosmosil $5C_{18}$-AR-II column (5 μm particle size, 150×4.6 mm I.D., Nacalai tesque, Kyoto, Japan), the STR ODS II column, a CAPCELL PAK C_{18} UG80 column (5 μm particle size, 150×4.6 mm I.D., Shiseido, Tokyo, Japan) or a YMC-Pack ODS-A column (5 μm particle size, 250×4.6 mm I.D., YMC, Kyoto, Japan), a reducer column, a Shimadzu RF-550 fluorescence monitor and a Shimadzu R-11 recorder. The reducer column was connected in series with the analytical column for routine analysis of DNP isomers. The analytical and the reducer columns were heated by a Shimadzu CTO-6A column oven and a Yamato (Tokyo, Japan) HF-21 heating block, respectively. An aliquot of the sample solution was injected into an analytical ODS column and eluted with methanol–water (80:20) at a flow-rate of 0.5 ml/min. 1,3-, 1,6- And 1,8-DNP were detected by the fluorescence of the corresponding DAP isomers with excitation and emission wavelengths of 375 nm and 450 nm, respectively.

2.6. Recoveries of 1,3-, 1,6- and 1,8-DNPs

One ml of standard mixture of 1,3-, 1,6- and 1,8-DNP isomers (15 ng each/ml acetone) were added to 15 g of sieved soil, which was collected at Kita-ku in Osaka City, thoroughly mixed and allowed to stand at room temperature in the dark to dryness. After 2 days drying, the soil was subjected to ultrasonic extraction followed by the clean-up as described above. Then 1,3-, 1,6- and 1,8-DNPs in the purified fractions were analyzed as described above, and the recoveries of the added DNPs were determined.

2.7. Spectral measurement

UV absorption spectra and fluorescence spectra were measured with a Shimadzu SPD-M10AV photodiode array detector and a Shimadzu RF-550 fluorescence monitor, respectively.

3. Results and discussion

3.1. Partial purification

Since organic extracts of soil are an extremely complex mixture and the concentrations of DNPs are quite low, an efficient clean-up method is required for the determination of DNPs. We selected silica gel column chromatography and two further steps of HPLC for the clean-up. The recovery of DNP

Table 1
Recoveries and retention times of 1,3-, 1,6- and 1,8-DNP isomers during clean-up process

Step	Method	Compound	Amount (ng)	Retention time (min)	Recovery (%)
1	Silica gel column chromatography[a]	1,3-DNP	50	–[b]	99[c]
		1,6-DNP	44	–[b]	100[c]
		1,8-DNP	50	–[b]	100[c]
2	HPLC on STR ODS II column[d]	1,3-DNP	40	31.2	99
		1,6-DNP	35	27.8	98
		1,8-DNP	40	26.6	99
3	HPLC on Wakosil-PAHs column[d]	1,3-DNP	40	26.6	100
		1,6-DNP	35	23.8	100
		1,8-DNP	40	21.8	100

[a] 1,3-, 1,6- And 1,8-DNPs were dissolved in chloroform or toluene and applied on the top of the silica gel column.
[b] DNPs were eluted in the benzene or toluene fraction.
[c] The value represented the recovery in the benzene fraction. The recoveries of 1,3-DNP, 1,6-DNP and 1,8-DNP in the toluene fraction were 98, 99 and 100%, respectively.
[d] 1,3-, 1,6- And 1,8-DNPs were dissolved in acetonitrile–water (60:40) and injected into a column for HPLC.

isomers at each purification step was investigated using a mixture of 1,3-, 1,6- and 1,8-DNP standards. As shown in Table 1, all DNPs were found in the benzene fraction at the first purification step on the silica gel column and their recoveries were close to 100%. When toluene was used at the stepwise elution instead of benzene, three DNP isomers were eluted in the toluene fraction and their recoveries were more than 98%. Similar high recoveries were observed for all the compounds at the second and third purification steps using the monomeric-type ODS column, i.e., the STR ODS II column, and the polymeric-type ODS column, i.e., the Wakosil-PAHs column, respectively. The retention times of authentic DNPs on these columns for HPLC are also shown in Table 1. DNP isomers were eluted at 26.6–31.2 min and 21.8–26.6 min on the STR ODS II column and the Wakosil-PAHs column, respectively. Typical HPLC elution profiles of the soil extract at the second and the third purification steps are shown in Fig. 1. Peaks coinciding in positions with those of 1,3-, 1,6- and 1,8-DNPs were observed on both chromatograms. The fractions corresponding to the retention times of DNPs, i.e., 25.1–32.7 min at the second step and 20.3–28.1 min at the third step, were collected. The fraction purified by these three steps of column chromatography was finally applied to reversed-phase HPLC with on-line reduction to detect DNPs.

3.2. On-line reduction

It was reported that a ''three-way'' catalyst, which contains Pt–Rh as active ingredients and is designated to reduce hydrocarbons in automotive exhaust emission, reduced NO_2-PAHs to the corresponding amino compounds in the methanol–water mobile phase [14] and needed certain temperature and time to reach high reduction efficiency [19]. In this study, the reduction efficiency of the reducer column, which was connected in series with the L-column ODS column, was examined from room temperature to 80°C with flow-rates from 0.5 ml/min to 1.0 ml/min of methanol–water (80:20) as a mobile phase. The best response to DNPs standards was obtained when the temperature was held at 80°C and the flow-rate was 0.5 ml/min. Therefore, the reducer column was held at 80°C and eluted at the flow-rate of 0.5 ml/min in further experiments. The reduction of DNPs was confirmed by the UV absorption and fluorescence spectra. As shown in Fig. 2, the UV spectra of catalyst-reduced 1,3-, 1,6- and 1,8-DNPs were identical to those of authentic 1,3-, 1,6- and 1,8-DAPs, respectively. Fluorescence spectra of catalyst-reduced 1,3-, 1,6- and 1,8-DNPs were measured by stop–flow scanning techniques. The highest intensities of fluorescence were observed at the following wavelength pairs of excitation and emission; 1,3-DNP 375 nm/444 nm, 1,6-DNP 356 nm/

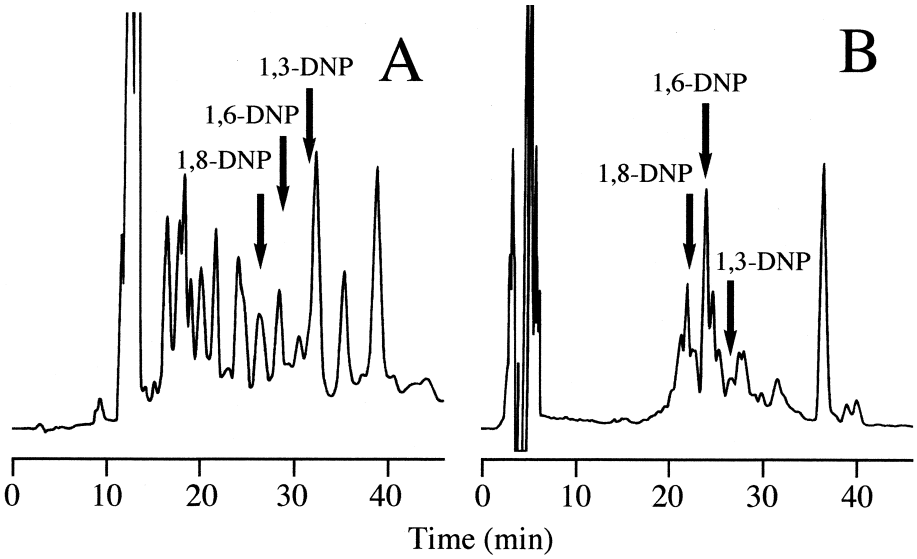

Fig. 1. HPLC profiles of soil extract at the second (A) and the third (B) purification steps. The second and the third steps were carried out on an STR ODS II column and a Wakosil-PAHs column, respectively. The eluate was monitored by the absorbance at 254 nm. The extract was obtained from soil collected from the ground at Sumiyoshi-ku in Osaka City on April 19, 1997. The retention times of authentic 1,3-, 1,6- and 1,8-DNP on the two columns are indicated by arrows.

439 nm and 1,8-DNP 368 nm/446 nm. These wavelength pairs were consistent with those of the corresponding authentic DAP isomers. These results indicate that DNP isomers were efficiently transformed to DAP isomers by the reducer column under the conditions described above and the optimum wavelength pairs are 375 nm (excitation)/450 nm (emission) for the determination of DNPs. The reducer column maintained constant reduction activity for 18 months under the conditions of almost daily use.

3.3. HPLC analysis and fluorometric detection

It is well known that 1,3-, 1,6- and 1,8-DAPs are strongly fluorescent; however, they are readily oxidized and their fluorescence levels gradually decrease in the solution [20]. In order to detect fluorescence of catalyst-reduced DNP isomers immediately after the reduction, the reducer column was disposed between the analytical column and the fluorescence detector in the HPLC system. The efficiency of the analytical columns on the separation of DNPs isomers was examined with three monomeric-type ODS columns and two polymeric-type ODS columns using methanol–water (80:20) as a mobile phase. Retention times of DNPs on each column for HPLC are shown in Table 2. On all monomeric-type ODS columns tested, 1,6-DNP and 1,8-DNP were eluted at the same retention times. On the contrary, the three DNP isomers had unique retention times on each polymeric-type ODS column, i.e., the L-column ODS column and the Cosmosil $5C_{18}$-AR-II column. The detection limits ($S/N=3$) for 1,3-DNP, 1,6-DNP and 1,8-DNP were 2, 0.7, 4 pg, respectively, when the L-column ODS column was used as the analytical column. No significant differences were observed in the peak figure and determination limits of catalyst-reduced DNPs using the L-column ODS column and the Cosmosil $5C_{18}$-AR-II column. These detection limits are better than those data obtained in previous studies using HPLC with fluorescence detection [14,17] and electrochemical detection [17].

3.4. Soil samples

We applied the clean-up method and analytical

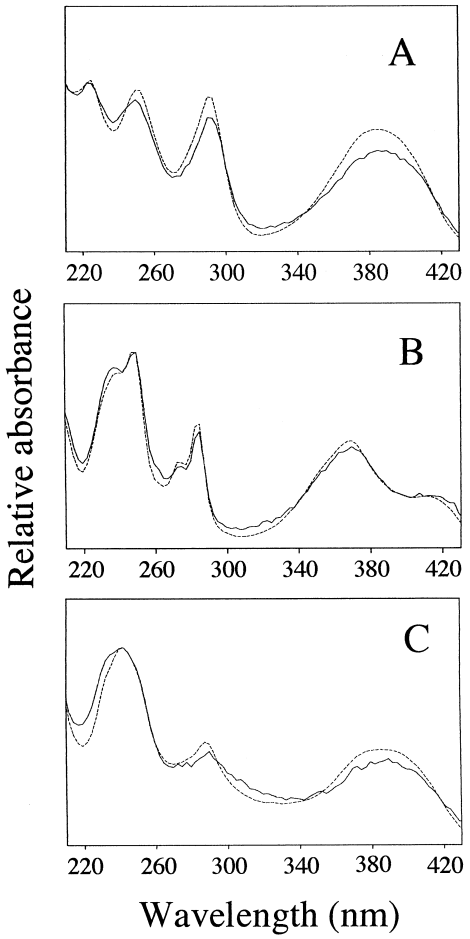

Fig. 2. UV absorbance spectra of authentic 1,3-DAP (A), 1,6-DAP (B) and 1,8-DAP (C) and the peak fractions coinciding with the retention time of catalyst-reduced 1,3-DNP (A), 1,6-DNP (B) and 1,8-DNP (C) standards on an L-column ODS column: - - -, authentic DAPs; ———, catalyst-reduced DNPs standards.

system described above to the soil samples collected at four parks in different geographic areas. All soil extracts gave peaks corresponding to the retention times of 1,3-, 1,6- and 1,8-DNPs on the chromatograms of final analysis. Fig. 3 shows typical chromatograms of a mixture of DNP standards and a purified fraction of soil extract on the L-column ODS column for HPLC. The peaks corresponding to catalyst-reduced 1,3-, 1,6- and 1,8-DNPs were clearly detected on the chromatogram of the soil extract (Fig. 3B). In order to confirm the structures of the compounds in the three peaks, their fluorescence spectra were measured by the stop–flow scanning technique. The emission and the excitation spectra of the compounds were the same as those of catalyst-reduced DNP standards, respectively, indicating that the compounds detected in the soil extract were identified as 1,3-, 1,6- and 1,8-DNP isomers.

The recoveries of DNPs in soil during the clean-up process were investigated by adding DNP standards to the soil sample, and those of 1,3-, 1,6- and 1,8-DNPs were 95%, 97% and 94%, respectively. Table 3 shows the results of quantification of DNPs in soil collected at the four parks, and the amounts are represented as the values which were corrected for the recoveries. The concentration levels of 1,3-, 1,6- and 1,8-DNPs were 0.04–2.65 ng/g, 0.07–3.07 ng/g and 0.45–3.66 ng/g, respectively.

Capillary gas chromatography with mass spectrometry [22,23] and HPLC with a chemiluminesence detector [18,19,21] has been used as a selective and sensitive analytical technique for complex mixtures such as airborne particulate matter. However, these instrumentations are not readily available in

Table 2
Retention times of catalyst-reduced 1,3-, 1,6- and 1,8-DNP isomers

HPLC column[a]	Particle size (μm)	Column size (length×I.D. mm)	Retention time (min)[b]		
			1,3-DNP	1,6-DNP	1,8-DNP
Monomeric-type					
STR ODS II	5	150×4.0	19.4	15.2	15.2
CAPCELL PAK C_{18} UG80	5	150×4.6	25.6	19.0	19.0
YMC-Pack ODS-A	5	250×4.6	44.6	33.6	33.6
Polymeric-type					
L-column	5	150×4.6	23.6	16.6	19.1
Cosmosil 5C_{18}-AR-II	5	150×4.6	22.4	15.0	18.5

[a] An HPLC column was held at 35°C.
[b] DNP isomers were eluted with methanol–water (80:20) at a flow-rate of 0.5 ml/min.

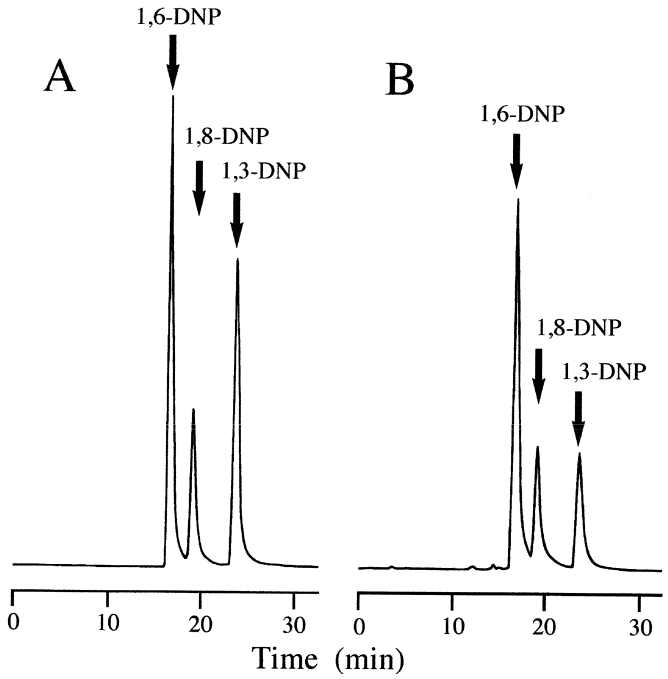

Fig. 3. HPLC profiles of DNP standards (A) and a purified fraction of soil extract (B) on an L-column ODS column. The extract was obtained from soil collected from the ground at Sumiyoshi-ku in Osaka City on April 19, 1997, and purified by the clean-up procedure. The eluate was monitored by the fluorescence with excitation and emission wavelengths of 375 nm and 450 nm, respectively. The peak fractions corresponding to the retention times of catalyst reduced 1,3-, 1,6- and 1,8-DNP standards on the column are indicated by arrows.

most laboratories. The major advantages of the methods developed in this study are (1) expensive equipment and laborious work are not necessary, (2) recoveries of DNPs during clean-up are quite high and (3) sensitivities are high enough to determine trace amounts of DNPs in soil. It was reported that DNP isomers were detected by the chemiluminescence of the corresponding diamino compounds using bis(2,4,6-trichlorophenyl)oxalate and hydrogen peroxide as reagents, and the sensitivity of HPLC with chemiluminescence detection was higher than that of fluorescence detection [24]. When the available mass of soil sample is extremely limited, chemiluminescence detection of DNP isomers may

Table 3
Concentration of 1,3-, 1,6- and 1,8-DNP isomers in soil

Sampling site	Sampling date	Concentration (ng/g soil)[a]		
		1,3-DNP	1,6-DNP	1,8-DNP
Osaka City-1 (Kita-ku)	1997 Jan. 30	0.72±0.02	0.94±0.02	0.99±0.06
Osaka City-2 (Sumiyoshi-ku)	1997 Apr. 19	2.65±0.13	3.07±0.16	3.66±0.17
Matsubara City	1997 Apr. 19	0.04±0.01	0.07±0.01	0.45±0.04
Uji City	1998 Feb. 4	1.32±0.06	2.05±0.12	2.64±0.18

[a] The organic extracts were obtained from 15 g of soil (<250 μm) by ultrasonic extractor. Values were corrected for recoveries during the clean-up process and represented as the mean±SD. (n=3).

be effective. We are now analyzing DNPs isomers in soil over an extensive area to clarify the contamination levels by these compounds.

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