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Determination of 1-Nitropyrene Retained in Leaves of Roadside Trees

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In many areas ambient air contains mutagenic compounds derived from combustion emissions, e.g., polycyclic aromatic hydrocarbons and their nitro derivatives (Pitts et al. 1977; Tokiwa et al. 1983; Nakagawa et al. 1983; Lunde and Bjorseth 1977; Wang et al. 1980; Morita et al. 1983). Such mutagenic compounds are suspected to be a contributing factor in causing human lung cancer, especially in urban areas (Wynder and Hoffmann 1965; Menck et al. 1974; Walker et al. 1982). Their concentrations in air are conventionally determined using particulate matter obtained by high- or low-volume air samplers.

Plants, however, are known to absorb nitrogen oxides and sulfur oxides in ambient air through stomatal respiration or transpiration (Rogers et al. 1979; Natori and Totsuka 1980; Hayakawa 1987), and because their leaves are also thought to retain mutagenic compounds such as nitroarenes, they actually function as a "natural air sampler" for monitoring air pollution with mutagens. In fact, conifer needles have been found to retain some chlorinated hydrocarbons (Reischl et al. 1987). Moreover, we previously showed that extracts from leaves of woody plants growing on roadsides exhibited mutagenicity toward *Salmonella typhimurium* YG 1021 and YG 1024 strains which are both sensitive to nitroarenes (Suzuki et al. 1992). Therefore, we attempted to determine 1-nitropyrene (1-NP), one of the most prevalent nitroarenes, retained in leaves of woody plants, although the determination was presumed to be difficult on account of paucity of 1-NP and of many interfering substances derived from leaves. In the present paper we describe a method to determine the amount of 1-NP in leaves, as well as its concentration in azalea leaves growing under various traffic conditions.

MATERIALS AND METHODS

1-NP was purchased from Tokyo Kasei Co., Japan; sodium hydro-

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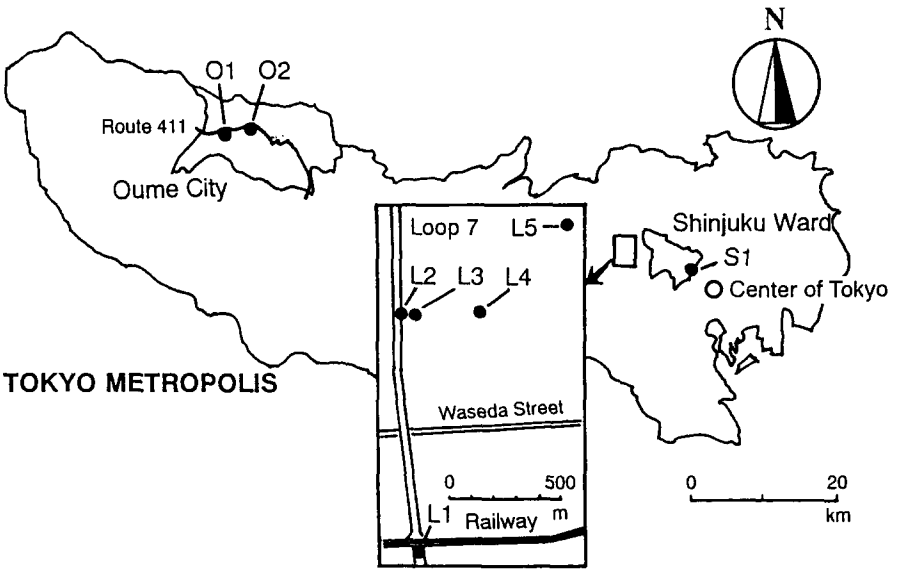

Figure 1. Sampling locations of azalea leaves. S1, roadside of Sotobori Street; L1, median strip of Loop 7; L2, roadside of Loop 7; L3, a square 70 m away from Loop 7; L4, a garden 360 m away from Loop 7; L5, a square 700 m away from Loop 7; O1, roadside of Route 411; O2, river bank of Tama river at Mitake ravine.

sulfide used as a reductant for 1-NP was from Katayama Chemical Co., Japan; n-hexane, benzene and ethyl acetate were from Kanto Chemical Co., Japan. All other chemicals were high quality commercial grade reagents.

As shown in Fig. 1, azalea (*Rhododendron oomurasaki*) leaves were collected from five sites located along Loop 7, a loop highway that runs through the Tokyo metropolitan area (49,000 cars/12 hr daytime), one site along Sotobori Street, a principal road in metropolitan Tokyo (30,000 cars/12 hr daytime), and two sites along Route 411, a road running through a mountain resort area located about 55 km west from the center of Tokyo.

Fresh leaves collected (c.a. 10 g) were extracted with ethyl acetate (10 mL/g) by means of ultrasonication for 20 min without washing or drying. Leaves and other insoluble materials were then removed by decantation and filtration through a membrane filter (TM4P1, Advantec Toyo Co., Japan, 0.2 μm pore size). The resultant solution was evaporated to about 20 mL, 0.5 g of silica gel (MERCK Kieselgel 60) was added, and the suspension was evaporated to dryness under reduced pressure at less than 45 °C. This mixture was placed at the top of an aluminum foil-covered, silica gel-packed column (2 cm i.d. x 25 cm, 25 g of silica gel) and eluted with 100 mL of cyclohexane/benzene (1/2, v/v). The 1-NP in the ethyl acetate extract

was eluted from this fraction without elution of 1-aminopyrene (1-AP). The obtained eluate was subsequently evaporated to a few mL, added to a small centrifuge tube and dried in a stream of nitrogen, after which ethanol (0.5 mL) was added and dissolved by ultrasonication. Next, an aqueous solution of sodium hydrosulfide (10%, 0.5 mL) was added to the ethanol solution and 1-NP was reduced to 1-AP by refluxing for 90 min at 90 °C (Tanabe et al. 1986). After the termination of the reaction, 2 mL of aqueous NaOH (0.15 N) was added and 1-AP was extracted three times using 1 mL of benzene. The extracts were combined, evaporated to dryness, and dissolved in 1 mL of methanol for later HPLC analysis.

The HPLC employed system (LC-9A, Shimadzu Co., Japan) was equipped with a fluorescence detector (RF-550, Shimadzu). A 10 μL aliquot of sample solution was injected onto a column (Lichrospher 100 RP-18, MERCK, 4.0 mm i.d. x 250 mm) and eluted with acetonitrile (70% v/v) in a 50 mM Tris-HCl buffer (pH 6.5) at 1 mL/min. The effluent was monitored by fluorescopy using an excitation of 281 nm and emission of 427 nm.

RESULTS AND DISCUSSION

Ultrasonication extraction of leaves in ethyl acetate gave a green solution, indicating chlorophyll was extracted from them. The ethyl acetate extract was then subjected to silica gel chromatography to remove chlorophyll and other compounds that interfere with determining the concentration of 1-NP. Because our method used fluorescence of 1-AP produced by reduction of 1-NP with sodium hydrosulfide, it was necessary to first separate 1-NP from the 1-AP that may be present in the ethyl acetate extract. By performing silica gel chromatography with various eluents, 1-NP was found to be eluted without elution of 1-AP using 100 mL of cyclohexane/benzene (1/2, v/v). Figure 2 shows fluorescence HPLC results from the cyclohexane/benzene-elutable fraction (EtAc-CB) obtained from an azalea leaves with and without reduction. The large one peak from the EtAc-CB fraction with reduction coincides with that pure 1-AP, which was obtained from 1-NP by the same reduction treatment, but no such peak is present in the corresponding fraction without reduction. Coelution of genuine 1-AP added to the EtAc-CB fraction after reduction was also confirmed. These results clearly indicate that it is possible to determine 1-NP in the ethyl acetate extract by the method comprised of the clean-up by cyclohexane/benzene elution on the silica gel column and followed by the reduction with sodium hydrosulfide.

On the other hand, it is well known that many polycyclic aromatic compounds including 1-NP tend to decompose when exposed to light while on various absorbents. We prevented such photo decomposition

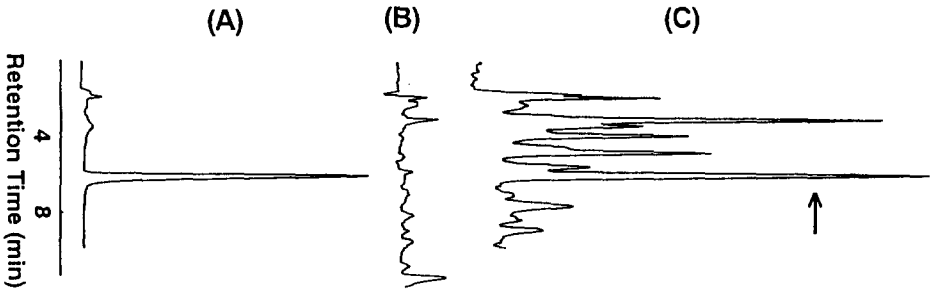

Figure 2. HPLC-fluorescence chromatograms of extracts obtained from azalea leaves. (A) Pure 1-AP; (B) EtAc-CB fraction without reduction; (C) EtAc-CB fraction with reduction. HPLC conditions: eluent, acetonitrile/50 mM Tris-HCl buffer (pH 6.5) 70/30 (v/v); column, MERCK Lichrospher RP-18 (4.0 mm i.d. x 250 mm); detection, fluorescopy at an excitation of 281 nm and emission of 427 nm.

during silica gel chromatography for clean-up by shielding the column from light using aluminum foil. Consequently, the recovery of 1-NP in the chromatography was determined to be 95.6% (n=2). Table 1 summarizes our method's overall recovery of 1-NP after it was added to azalea leaves, i.e., adsorbed by the leaves using an ethanol solution. The results were obviously good, hence we concluded it provides an valid method to determine the amount of 1-NP retained in azalea leaves.

Table 1. Recovery of 1-NP from plant leaves.

1-NP (ng/g leaves)		Recovery[b]
Added[a]	Found[b]	(%)
0.5	0.46	91.5
2.0	1.39	69.6
5.0	4.39	87.9

[a] 7.5 g of azalea leaves were used.
[b] Values are the mean of two experiments.

The newly developed method was applied to the azalea leaves growing at the roadsides or the median strip of streets in Tokyo urban district and at the roadsides in the suburbs. The results are shown in Table 2. It is noteworthy that 1-NP was detected in all azalea leaves examined. The largest concentration of 1-NP was observed in the leaves collected at site L1, the median strip of Loop 7 at Koenji, which had the largest traffic volume (49,000 cars/12 hr daytime) among the sampling points. The smallest concentration was at site 02, a river bank of Mitake ravine where was a mountain resort located

about 55 km west of the center of Tokyo. Even in the same site of Loop 7, the amount of 1-NP in the azalea leaves collected at roadside was 20 % smaller than that at the median strip, reflecting the fact that exposure to vehicle exhaust at the roadside was lower than that at the median strip. Moreover, site S1, Sotobori Street at Ichigaya site with 30,000 cars/12 hr had significantly smaller amount of 1-NP than that at L2, Loop 7 at Koenji site ($p < 0.05$). Even if compared in terms of 1-NP level per unit traffic volume, the level at L2 was higher than that at S1. This result seems to occur due to the fact that more diesel-engine vehicles passed by site L2 than S1, because diesel-engine vehicles are known to exhaust larger amounts of 1-NP than gasoline-engine vehicles (Morita et al. 1983).

Table 2. The amount of 1-NP retained in azalea leaves growing under various traffic conditions.

Sample location		1-NP content[a] (ng /g leaves)
Urban		
	L1: Median strip of Loop 7	2.50 ± 0.13
	L2: Roadside of Loop 7	1.99 ± 0.13
	L3: Square 1[b]	0.37 ± 0.03
	L4: Garden[c]	0.21 ± 0.01
	L5: Square 2[d]	0.17 ± 0.02
	S1: Roadside of Sotobori street	0.83 ± 0.21
Suburban		
	O1: Roadside of Route 411	0.23 ± 0.03
	O2: River bank at Mitake ravine	0.14 ± 0.02

[a] Values are the mean ± SD from three experiments.
[b] 70 m away from Loop 7.
[c] 360 m away from Loop 7.
[d] 700 m away from Loop 7.

On the other hand, the 1-NP contents of the azalea leaves in a square 70 m away from Loop 7 decreased to one-sixth value of the median strip, and further to one-tenth in a garden 360 m away from Loop 7. These results indicate that the exposure level of plant leaves to 1-NP steeply decreased with distance from a heavy traffic road. However, the 1-NP contents in the garden was similar values for the roadside of Route 411 in the suburbs. This indicates that ambient air at roadside even in suburbs, and in urban area even at residence area is polluted with mutagenic 1-NP derived from vehicle exhausts. These results are consistent with previously reported result of mutagenesis assay (Suzuki et al. 1992).

As mentioned above, we attempted for the first time to determine 1-NP in plant leaves, and revealed that all leaves of woody plants growing on roadside or garden in urban area and suburbs retained more or less mutagenic 1-NP. The results in this study as well as in the previous mutagenesis study are warning that dwellers not only in urban area but also in roadside in suburbs must be unavoidably exposed to mutagenic nitroarenes during daily life. These results also demonstrate that plant leaves is an available natural air samplers and that the extract poses important information for estimating air pollution.

REFERENCES

Hayakawa M (1987) Sorption of nitrogen dioxide by woody plants. Nagoya-shi Kohgai Kenkyu-sho Hou 17: 7-16

Lunde G, Bjorseth A (1977) Polycyclic aromatic hydrocarbons in long-range transported aerosols, Nature (London) 268:518-519

Menk HR, Casagrande JT, Henderson BE (1974) Industrial air pollution: possible effect on lung cancer. Science 183:210-212

Morita K, Fukamachi K, Tokiwa H (1983) Studies on airborne nitro compounds in air. Ⅲ. Determination of 1-nitropyrene in airborne particulate matter and automobile emissions. Jpn J Toxicol Environ Health 29:199-205

Nakagawa R, Kitamori S, Horikawa K, Nakashima K, Tokiwa H (1983) Identification of dinitropyrenes in diesel-exhaust particles: Their probable presence as the major mutagens. Mutation Res 124:210-211

Natori T, Totsuka T (1980) Effects of short or long term fumigation with NO_2 on plant's factors controlling NO_2 sorption rate. J Jpn Soc Air Pollut 15:329-333

Pitts JN, Grosjean D, Mischke TM, Simmon VF, Poole D (1977) Mutagenic activity of airborne particulate organic pollutants. Toxicol Lett 1:65-70

Reischl A, Reissinger M, Hutzinger O (1987) Occurrence and distribution of atmospheric organic micropollutants conifer needles. Chemosphere 16:2647-2652

Rogers HH, Jeffries HE, Witherspoon AM (1979) Measuring air pollutant uptake by plants: nitrogen dioxide. J Environ Qual 8:551-557

Suzuki J, Kuwayama K, Suzuki S (1992) Mutagenicity assay for nitroarenes of air pollutants held in leaves of woody plants. Mutation Res 271:89-96

Tanabe K, Matsushita H, Ching-Tang KUO, Imamiya S (1986) Determination of carcinogenic nitroarenes in airborne particulates by high performance liquid chromatography. Jpn Soc Air Pollut 21:535-544

Tokiwa H, Kitamori S, Nakagawa R, Matamala L (1983) Demonstration of a powerful mutagenic dinitropyrene in air borne particu-

late matter. Mutation Res 121:107–116
Walker RD, Connor TH, MacDonald EJ, Trieff NM, Legator MS, MacKenzie KW Jr, Dobbins JG (1982) Correlation of mutagenic assessment of Houston air particulate extracts in relation to lung cancer mortality rates. Environ Res 28:303–312
Wang CY, Lee MS, King SM, Warner PO (1980) Evidence for nitro aromatics as direct acting mutagens of airborne particulates. Chemosphere 9:83–88
Wynder EL, Hoffmann D (1965) Some laboratory and epidemiological aspects of air pollution carcinogenesis. J Air Pollut Control Ass 15:155–159