

Determination of Diamino- and Aminopyrenes by High Performance Liquid Chromatography with Chemiluminescence Detection

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A high performance liquid chromatographic (HPLC) method with chemiluminescence (CL) detection was developed for the sensitive determination of 1,3-diaminopyrene (1,3-DAP), 1,6-DAP, 1,8-DAP and 1-aminopyrene (1-AP). The HPLC conditions were as follows: column, Cosmosil 5C18 (4.6 mm i.d.×250 mm); mobile phase, 10 mM imidazole-perchloric acid buffer (pH 7.6)-acetonitrile (1:1, v/v); CL reagent, 0.02 mM bis(2,4,6-trichlorophenyl)oxalate (TCPO) and 15 mM hydrogen peroxide in acetonitrile. The oxidative degradation of DAPs and AP in the presence of metals was prevented by adding ascorbic acid to sample solutions. The calibration curves were straight over 2 orders of magnitude for all analytes, and their detection limits (as S/N was 3) were in the sub-fmol range. Dinitro- and nitropyrenes in sooty emissions of diesel- and gasoline-engine cars could be determined by this HPLC after reductive conversion into DAPs and AP, respectively, by refluxing the samples in the presence of sodium hydrosulfide.

Keywords Diaminopyrene, aminopyrene, dinitropyrene, nitropyrene, high performance liquid chromatography, peroxyoxalate-chemiluminescence detection, car emission soot

Nitrated polycyclic aromatic hydrocarbons (NPAHs) have attracted much attention because of their potent mutagenic activity.¹ They are formed as undesirable by-products when various organic materials react with atmospheric nitrogen during combustion^{2,3} and are found in many environmental samples.⁴⁻⁶ Dinitropyrenes (DNPs) are the most mutagenic and carcinogenic of these NPAHs.⁷⁻⁹

Both gas chromatography (GC)¹⁰ and GC/mass spectrometry⁶ have been reported as sensitive determination methods for DNPs. However, tedious procedures are required for cleaning and concentrating samples. On the other hand, a high performance liquid chromatographic (HPLC) method with fluorescence (FL) detection has also been reported¹¹, with which DNPs were determined after a reductive conversion of DNPs into diaminopyrenes (DAPs). Although the FL detection was so selective that cleaning procedures became simpler, the sensitivity was not sufficiently high to detect them at trace levels in the environment.

Recently, peroxyoxalate-chemiluminescence (PO-CL) detection has been developed as a highly sensitive and selective detection method for HPLC.^{12,13} In our preliminary experiment, PO-CL detection was much more sensitive for DAPs and 1-AP than was FL detection. The observed peak heights, however, were significantly smaller than those from the extrapolated calibration curves when the injection amounts were less than pmol. This phenomenon disturbed the trace analysis. We found that DAPs and 1-AP were very unstable in the presence of metals and that this reaction was the main reason for the above phenomenon. The problem was

solved by adding an antioxidant, such as ascorbic acid (AA), enabling the DAPs and 1-AP to be determined at trace levels. The purpose of this report is to propose an HPLC method with PO-CL detection for 1,3-, 1,6-, 1,8-DAPs and 1-AP whose sensitivities are much higher than those with FL detection.

Experimental

Chemicals and solutions

1,3-, 1,6- and 1,8-DAPs and 1-AP were prepared according to a report by Hashimoto *et al.*¹⁴ All other chemicals were of special grade.

All solutions of DAPs and 1-AP were prepared just before use in the dark as follows. Each compound was dissolved in acetonitrile, then mixed and added to an aqueous AA solution. By diluting these solutions appropriately with acetonitrile, standard solutions for HPLC were prepared. The final concentration of AA in the standard solutions was 10 mM.

The mobile phase was prepared by mixing acetonitrile and a 10 mM imidazole-perchloric acid buffer (pH 7.6) (1:1, v/v). The solution was then filtered (pore size 0.4 μ m). The chemiluminescence reagent solution was prepared by dissolving bis(2,4,6-trichlorophenyl)oxalate (TCPO) and hydrogen peroxide in acetonitrile just before use. The final concentrations of TCPO and hydrogen peroxide in the reagent solution were 0.02 mM and 15 mM, respectively. The solution was kept in an ice-water bath during the experiment.

HPLC system

The HPLC system consisted of two Shimadzu (Kyoto, Japan) LC-6A pumps, a Rheodyne (Cotati, CA, USA) Model 7125 loop injector (loop volume of 20 μ l) and an Atto (Tokyo, Japan) AC-2220 chemiluminescence detector (spiral type cell of 60 μ l). A JASCO (Tokyo, Japan) 820-FP (Ex 375 nm, Em 445 nm) intelligent spectrofluorometer was used when necessary. The analytical column was a Nakalai Tesque (Kyoto, Japan) Cosmosil 5C18 (spherical end-capped ODS, 5 μ m, 4.6 mm i.d. \times 250 mm). The flow rates of the mobile phase and the PO-CL reagent solution were 1.0 ml/min. Other details of the system were the same as those reported in our previous work.¹⁵

Stability study of DAPs

The effect of light on the DAPs and 1-AP was studied as follows. Each compound was dissolved in 10 ml of acetonitrile at a concentration of 1×10^{-7} M in a 15 ml glass vial. The vial was exposed to daylight for 2 h at $18 \pm 2^\circ$ C. The fluorescence intensity of the solution was measured at Ex 375 nm and Em 445 nm using a Hitachi (Tokyo, Japan) 650-10S fluorescence spectrophotometer.

The effect of metals on the DAPs and 1-AP was determined by preparing a 1×10^{-7} M solution of each compound in 15 ml glass vials using a 10 mM imidazole-perchloric acid buffer (pH 4.0)-acetonitrile (1:1, v/v). After adding stainless-steel tubing (1/16 in o.d. \times 300 mm, coiled) or metal powder (100 mg), the mixture was incubated in the dark for 30 min or 2 h at $18 \pm 2^\circ$ C. The fluorescence intensity of the solution was measured.

The stabilizing effect of reductants such as AA, sodium hydrosulfide, sodium sulfide, sodium formate and sodium hydrosulfite was studied as follows. The 1,3-DAP (1×10^{-7} M) solution was added with each reductant (10 mM), and then treated in the same manner as used in the test of stainless-steel tubing. After incubation, the fluorescence intensity of the solution was measured.

Pretreatment of sooty emissions

Sooty emissions (25 mg) obtained from a diesel-turbo-engine car (1986 model) and a gasoline-engine car (1984 model) were extracted with 25 ml of benzene-ethanol (3:1, v/v), homogenized ultrasonically and then filtered. After evaporating, the residue was redissolved in 1 ml of ethanol and refluxed with 1 ml of 7% (w/v) sodium hydrosulfide for 90 min.¹¹ After adding 4 ml of 0.15 M sodium hydroxide, the compounds of interest were extracted into benzene (3 ml). The benzene solution was evaporated and redissolved in 1 ml of acetonitrile containing 10 mM AA. Twenty microliters of the solution was injected into the HPLC system.

Results and Discussion

Degradation and stabilization of DAPs

As stated initially, it was found that peak areas of the

three DAPs and 1-AP were much smaller than those of the extrapolated calibration curves, especially at lower concentrations. We considered oxidation, catalyzed by metals or light, as a possible reason. The fluorescence intensities of the four solutions after 2 h exposure to daylight (I_{+hv}) were all smaller than those of control solutions which were kept in the dark (I_{-hv}). Their relative values (I_{+hv}/I_{-hv}) were in the range from 0.06 (1,3-DAP) to 0.54 (1-AP). This result suggested that the DAPs and AP were unstable in the presence of light. However, the peak heights were still smaller than expected, even after preparing all standard solutions in the dark.

Next, the effect of stainless-steel tubing was studied. The fluorescence intensities of the four solutions after 30 min of incubation in the presence of stainless-steel tubing (I_{+s}) were all smaller than those of the control solutions in the absence of stainless-steel tubing (I_{-s}). Their relative values (I_{+s}/I_{-s}) were in the range from 0.01 (1,3-DAP) to 0.88 (1-AP). In order to examine the metal specificity, 1,3-DAP was incubated with several metal powders. For all metals tested, the fluorescence intensities (I_{+m}) of the solutions were smaller than that of control solution without any metal (I_{-m}). Their relative values (I_{+m}/I_{-m}) were as follows: molybdenum*, 0.00; copper, 0.01; manganese*, 0.04; iron*, 0.07; zinc, 0.60; silicon*, 0.74; chromium*, 0.83; nickel*, 0.86 (* indicated metals are contained in SUS 316TP). The values were significantly small in the presence of stainless-steel component metals such as molybdenum, manganese and iron. This result suggested that several metals contained in the stainless-steel tubing decompose the DAPs and 1-AP in the HPLC system. Metal-catalyzed oxidation might be considered as a possible mechanism, considering that aromatic amines are oxidized in the presence of several metal catalyses.¹⁶ The detailed mechanism is now discussed.

In order to prevent an oxidative reaction from occurring, several reductants were added to the test solution before adding stainless-steel tubing. 1,3-DAP was used as the substrate, since it was shown to be the most unstable to light and metals. A positive effect was observed for AA, sodium formate, sodium hydrosulfide and sodium sulfide. Among these reductants, AA had the strongest effect. Upon the addition of AA, the fluorescence intensity of the test solution did not decrease in the presence of stainless-steel tubing. A similar tendency was observed with the other two DAPs. AA also prevented the compounds from decomposing under exposure to daylight.

On the basis of the above-mentioned results, AA was used in the present method. When AA was added to the mobile phase, the emission of CL was completely inhibited. It was necessary to remove AA from the elution bands of DAPs and 1-AP. Therefore, AA was added to the sample solution before injection, since the elution of AA was much earlier than those of above-mentioned analytes on a reversed-phase column. The effect of AA is shown in Fig. 1. In the absence of AA,

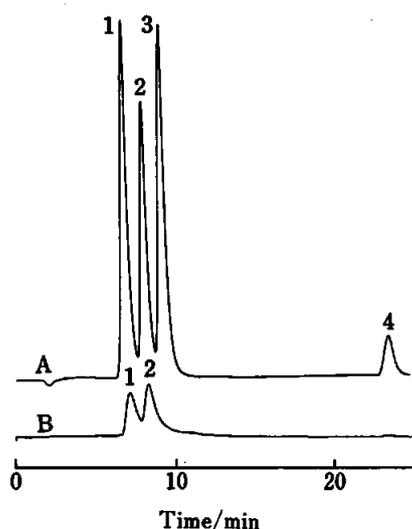


Fig. 1 Typical chromatograms of DAPs and 1-AP using chemiluminescence detection (A) in the presence and (B) in the absence of 10 mM ascorbic acid in a sample solution. Peaks: 1, 1,6-DAP; 2, 1,8-DAP; 3, 1,3-DAP; 4, 1-AP. Injection amounts: each 200 fmol.

the 1,3-DAP peak was not detected and the other peaks were very small, as mentioned previously (chromatogram B). With increasing AA concentration, the 1,3-DAP peak became detectable and all of the peak heights were maximum and constant at AA concentrations ranging from 10 to 50 mM (chromatogram A). The addition of excess AA, over 50 mM, however, gave a large negative peak at around 2 min, whose tailing interfered with any sensitive determination of the DAPs. Therefore, the concentration of AA was set at 10 mM in the sample solution in the following experiment. At 10 mM AA, the peak heights of all analytes were constant for at least 8 h.

Mobile phase

The three DAPs and 1-AP were separated on several commercially available ODS columns. A Cosmosil 5C18 column was used in this work, since the resolution factors were all over 1 for all of the peak pairs. According to previous reports that the PO-CL intensity is strong when acetonitrile is used as an organic solvent^{15,17}, and that imidazole is the best catalyst for the PO-CL reaction^{18,19}, the mobile phase was prepared by mixing acetonitrile and an imidazole-perchloric acid buffer. The three DAPs and 1-AP were separated within 25 min under the present conditions.

Postcolumn conditions

The time taken for the PO-CL intensity to reach a maximum (T_{max}) is affected by several factors, such as the pH and reagent concentration.¹⁹ The peaks for DAPs and 1-AP were not observed when the pH of the imidazole buffer was lower than 5. The peak areas of the three DAPs increased continuously up to pH 7.6,

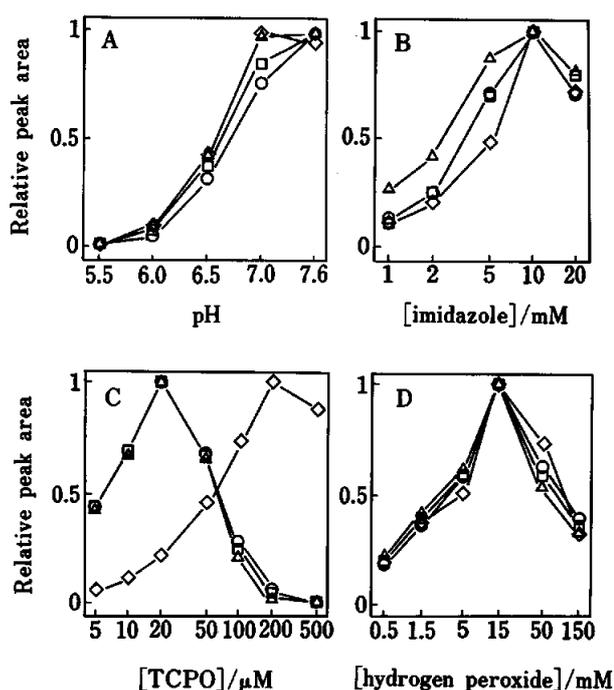


Fig. 2 Detector response of DAPs and 1-AP vs. (A) pH, (B) imidazole concentration, (C) TCPO concentration and (D) hydrogen peroxide concentration. Symbols: ○, 1,6-DAP; □, 1,8-DAP; △, 1,3-DAP; ◇, 1-AP. Other conditions are described in the text.

while 1-AP showed the largest peak area at pH 7.0 (Fig. 2A); pH 7.6 was used as optimum because a silica-based ODS column was not available at a pH of over 7.6. The peak areas increased with increasing imidazole concentration and the maximum peak heights were observed at 10 mM for all analytes (Fig. 2B).

The optimum concentrations of TCPO were 0.02 mM for three DAPs and 0.2 mM for 1-AP; 0.02 mM was selected for a sensitive determination of the DAPs (Fig. 2C). As for the concentration of hydrogen peroxide, the maximum peak areas were observed at 15 mM for all analytes (Fig. 2D). Similar curves of the four factors were also observed when the signal-to-noise (S/N) ratios were used instead of the peak areas.

Detection limits and calibration curves

The detection limits (S/N ratio was 3) of the three DAPs by PO-CL detection were all in the sub-fmol ranges, as listed in Table 1. These values were 1/70–

Table 1 Detection limits (fmol) for DAPs and 1-AP with chemiluminescence and fluorescence detections

	Detection limit			
	1,3-DAP	1,6-DAP	1,8-DAP	1-AP
CL	0.25	0.25	0.35	1.5
FL	15	8	25	25

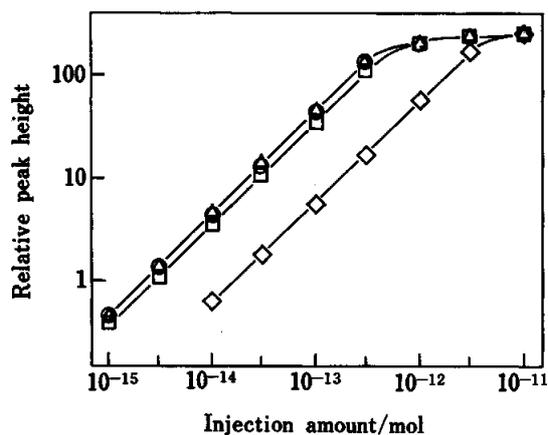


Fig. 3 Calibration curves of DAPs and 1-AP. The symbols are the same as in Fig. 2.

1/30 of those by FL detection. The FL detection limits obtained in this work, which are listed in the table, were almost at the same levels as previously reported.¹¹ The detection limit of 1-AP (1.5 fmol) was larger than those of the DAPs under these conditions. When 0.2 mM TCPO was used, the detection limit of 1-AP was in the sub-fmol range.

The calibration curves of three DAPs showed good linearity over two orders, with an injection of less than 3×10^{-13} mol. Similarly, the curve was also straight for 1-AP with an injection of less than 3×10^{-12} mol. Their correlation coefficients were 0.9996–1.0000 (Fig. 3). In a previous report concerning the HPLC method of DAPs with FL detection¹¹, the linearity of the calibration curve of 1,3-DAP seemed to be poor (the slope was larger than 1) at the lower concentration. This could be due to the instability of 1,3-DAP in the HPLC system. Linearity was observed even at lower concentration in our work.

All four curves became saturated as the relative peak heights reached over 100, as shown in Fig. 3. One possible reason for this may be saturation of the photomultiplier detector.

Analysis of sooty emissions

Figure 4 shows chromatograms obtained with sooty emissions from diesel- and gasoline-engine cars. Four peaks of 1,3-DAP, 1,6-DAP, 1,8-DAP and 1-AP were detected in both samples, but were not detected without reduction with sodium hydrosulfide. The peaks therefore were ascribable to the DNP and 1-NP originally contained in sooty emissions. From the chromatogram of a diesel-car sample, their concentrations were calculated to be: 1,3-DNP, 0.9×10^{-13} mol/mg; 1,6-DNP, 1.3×10^{-13} mol/mg; 1,8-DNP, 1.5×10^{-13} mol/mg; 1-NP, 1.1×10^{-11} mol/mg. The concentrations in a gasoline-car sample were as follows: 1,3-DNP, 1.4×10^{-14} mol/mg; 1,6-DNP, 1.8×10^{-14} mol/mg; 1,8-DNP, 2.0×10^{-14} mol/mg; 1-NP, 3.7×10^{-14} mol/mg. The concentrations of all four compounds in the latter sample were much lower than in the former and their peaks were not shown with FL detection. Another important result was that there were no interfering peaks in both chromatograms. Several unknown peaks were observed when a postcolumn electrochemical reduction was used.²⁰ This is due to the cleaning effect of benzene-ethanol extraction in the present method. These results indicate that the combination of preliminary reduction and this HPLC-CL detection method is quite useful for the sensitive and selective determination of DNPs in environmental samples.

References

1. H. S. Rosenkranz and R. Mermelstein, *Mutat. Res.*, **114**,

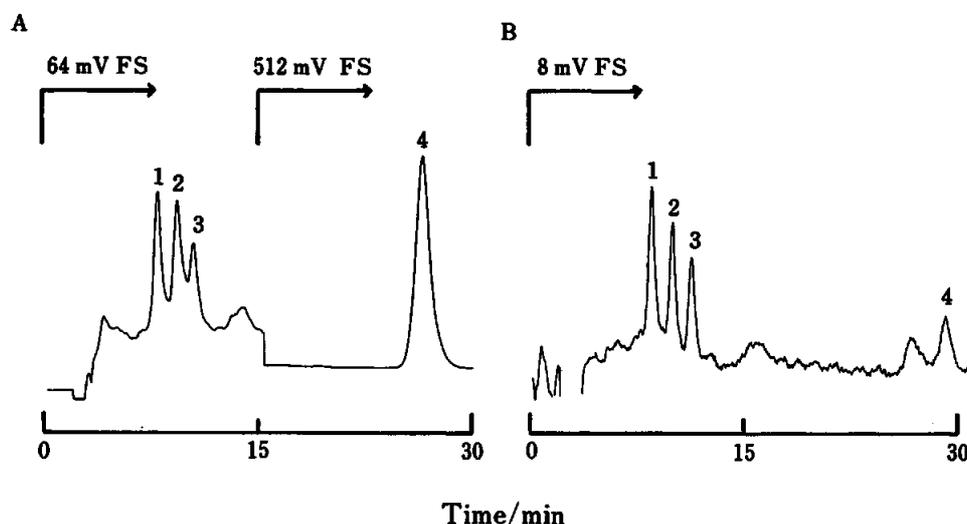


Fig. 4 Chromatograms obtained with sooty emissions from (A) diesel- and (B) gasoline-engine cars. The peaks are the same as in Fig. 1.

- 217 (1983).
2. J. A. Yergey, T. H. Risby and S. S. Lestz, *Anal. Chem.*, **54**, 354 (1982).
 3. H. Tokiwa, R. Nagakawa, K. Morita and K. Ohnishi, *Mutat. Res.*, **85**, 195 (1981).
 4. X. B. Xu, J. P. Nachtman, Z. J. Jin, E. T. Wei, S. M. Rappaport and A. L. Burlingame, *Anal. Chim. Acta*, **136**, 163 (1982).
 5. T. Nelsen, *Anal. Chem.*, **55**, 286 (1983).
 6. D. Schuetzle, T. L. Riley, T. J. Praster, T. M. Harvey and D. F. Hunt, *Anal. Chem.*, **54**, 265 (1982).
 7. H. S. Rosenkranz, E. C. McCoy, D. Sanders, M. Butler, D. Kiriazides and R. Mermelstein, *Science* [Washington, D.C.], **209**, 1039 (1980).
 8. G. Klopman, D. A. Tonucci, M. Holloway and H. S. Rosenkranz, *Mutat. Res.*, **126**, 139 (1984).
 9. K. Imada, L. K. Tay, M-S. Lee, C. Y. Wang, N. Ito and C. M. King, *Proc. Int. Conf. Carcinog. Mutagen. N-Substituted Aryl Compd.*, 3rd, p. 187, 1987.
 10. R. M. Cambell and M. L. Lee, *Anal. Chem.*, **56**, 1026 (1984).
 11. K. Tanabe, H. Matsushita, C-T. Kuo and S. Imamiya, *Taikiosen-gakkaishi*, **21**, 535 (1986).
 12. K. W. Sigvardson, J. M. Kennish and J. W. Birks, *Anal. Chem.*, **56**, 1096 (1984).
 13. K. Hayakawa, K. Hasegawa, N. Imaizumi, O. S. Wong and M. Miyazaki, *J. Chromatogr.*, **464**, 343 (1989).
 14. Y. Hashimoto and K. Shudo, *Chem. Pharm. Bull.*, **32**, 1992 (1984).
 15. N. Imaizumi, K. Hayakawa, M. Miyazaki and K. Imai, *Analyst* [London], **114**, 161 (1989).
 16. "Aminosan Kagoubutsu no Kinzoku Syokubaiharano (Metal-catalyzed Oxidation Reactions of Amino Compounds, in Japanese)" in "Shin-Jikkenkagaku-koza", ed. the Chemical Society of Japan, No. 15, Maruzen, Tokyo, 1976.
 17. S. Kobayashi and K. Imai, *Anal. Chem.*, **52**, 424 (1980).
 18. K. Imai, A. Nishitani, Y. Tsukamoto, W-H. Wang, S. Kanda, K. Hayakawa and M. Miyazaki, *Biomed. Chromatogr.*, **4**, 100 (1990).
 19. N. Hanaoka, R. S. Givens, R. L. Schowen and T. Kuwana, *Anal. Chem.*, **60**, 2193 (1988).
 20. N. Imaizumi, K. Hayakawa, Y. Suzuki and M. Miyazaki, *Biomed. Chromatogr.*, **4**, 108 (1990).

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