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Sensitive assay system for nitrosamines utilizing high-performance liquid chromatography with peroxyoxalate cheiniluminescence detection

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ABSTRACT

A high-performance liquid chromatographic system in combination with postcolumn chemiluminescence detection for the determination of nitrosamines was developed. The stability of the chemiluminogenic reagent solution was investigated using a conventional high-performance liquid chromatograph with a W-Vis spectrophotometric detector, and the optimum conditions for chemiluminescence intensity were also established with a single reagent pump postcohnnn detector. The sample was first denitrosated with hydrobromic acid-acetic acid to produce the corresponding secondary amines, which were then subjected to reaction with dansyl chloride to form dansyl derivatives. The'reaction mixtures were separated on a Perkin-Elmer HS3 C_{18} reversed-phase column with acetonitrile-water (63.5: 36.5, v/v) as mobile phase, with 3.0 mmol/l of imidaxole added as a catalyst for chemiluminescence and with the pH adjusted to 6.2 with oxalic acid to give a better separation, followed by detection with a postcolumn chemiluminescence detector using bis(2-nitrophenyl) oxalate and hydrogen peroxide as chemihrminogenic reagents. The sensitivity of this method was more than 120 times greater than that of fluorescence detection and four orders of magnitude greater than that of W-Vis spectrophotometric detection. The detection limits with this procedure at a signal-to-noise ratio of 4 were between 0.31 and 1.20 pg and the relative standard deviations were between 2.8% and 6.7% for six nitrosamines. The linearity of the calibration graphs and the correlation coefficients were good.

INTRODUCTION

N-Nitrosamines are now known to be widely distributed in the human environment and can also be formed in the human body. Owing to their potential carcinogenic properties, great interest has been focused on the development of methods for the determination of nitrosamines at trace levels. High-performance liquid chromatography (HPLC) is a useful method for the trace determination of the nitroso compounds, and several methods for the detection and determination of these compounds have been reported [1–7]. The thermal energy analyser is a highly selective detector for gas chromatography [3], and its use in combination with HPLC has been described [4], but the results showed that it cannot be operated with a reversed-phase mobile phase or inorganic buffer solutions, and the sensitivity was cu. 100 times lower than those obtained with other methods. On the other hand, we have previously developed methods for the determination of nitrosamines by HPLC with precolumn fluorescence derivatization [5–7] and obtained good results. In subsequent work, a fluorescence detection method for the determination of nitrosamines [6], based on denitrosation with hydrobromic acid-acetic acid to produce secondary amines, followed by reaction with dansyl chloride to form dansyl derivatives, was studied further. However, peroxyoxalate chemiluminescence has been shown to provide a

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highly sensitive detection method [B-lo], and the sensitivity for secondary amines was much higher than that of fluorescence detection by using dansyl chloride [11]. In this paper, we present some improved results obtained by HPLC combined with a sensitive postcolumn bis(2-nitrophenyl) oxalate-hydrogen peroxide chemiluminescence detection method for the determination of six nitrosamines.

EXPERIMENTAL

Reagents

Nitrosodimethylamine (NDMA), nitrosopyrrolidine (NPy), nitrosodiethylamine (NDEA), nitrosopiperidine (NPip), nitrosodipropylamine (NDPA) and nitrosodibutylamine (NDBA) were prepared and purified by conventional procedures [12] and were identified by mass spectrometry. Bis(2-nitrophenyl) oxalate (2-NPO) was synthesized [13], further purified by washing with chloroform and recrystallization from ethyl acetate and identified by mass spectrometry. Hydrogen peroxide (30%), acetone, acetonitrile, dichloromethane, hydrobromic acid, acetic anhydride, ethyl acetate, imidazole, oxalic acid, sodium hydrogencarbonate and hydrochloric acid (all of analytical-reagent grade) were obtained from Beijing Chemical Works (Beijing, China), and were used as received Dansyl chloride (puriss. grade) were obtained from Fluka. Redistilled water was used throughout.

Nitrosamine stock solution. Each nitrosamine was dissolved in dichloromethane and made up to a concentration of $1 \cdot 10^{-4}$ mol/l with the same solvent.

Dansyl chloride solution. A $1 \cdot 10^{-5}$ mol/l dansyl chloride solution was prepared by dissolving dansyl chloride in acetone.

2-NPO stock solution. This solution was prepared by dissolving 2-NPO in ethyl acetate to give a concentration of 10 mmol/l.

Hydrogen peroxide stock solution. This solution contained 300 mmol/l of hydrogen peroxide in ethyl acetate.

Hydrobromic acid-acetic anhydride solution. A 1: *4 (v/v)* solution was prepared by dissolving 1 ml of hydrobromic acid in 4 ml of acetic anhydride.

Sodium hydrogencarbonate solution. A solution of 0.25 mol/l $NaHCO_3$ was prepared in redistilled water.

Hydrochloric acid. Solutions of 0.01 and 0.1 mol/l in redistilled water were prepared.

Instrumentation and chromatographic conditions

A schematic diagram of the system is shown in Fig. 1. It consisted of a Model 114M eluent-delivery pump (Beckman), a Model E-120-S-2 reagent-delivery pump for the chemiluminogenic reagent solution (Eldex), a C_{18} (3 μm) analytical column (83 mm x 4.6 mm I.D.) (Perkin-Elmer), a Model 7125 injection valve with 20-μl loops (Rheodyne), a mixing device and chemiluminescence detector made in our laboratory, a detector consisting of a 40-μl quartz worm pipe micro flow cell, a photomultiplier tube (GDB-52QD), a high-voltage supply, a weak signal amplifier and a recorder were used.

The mobile phase was acetonitrile-water (63.5:36.5, v/v) with a catalyst (imidazole) added (3.0 mmol/l) and the pH was adjusted to 6.2 with oxalic acid; the flow-rate was 0.5 ml/min. The postcolumn chemiluminogenic reagent solution contained 3.0 mmol/l of 2-NPO and

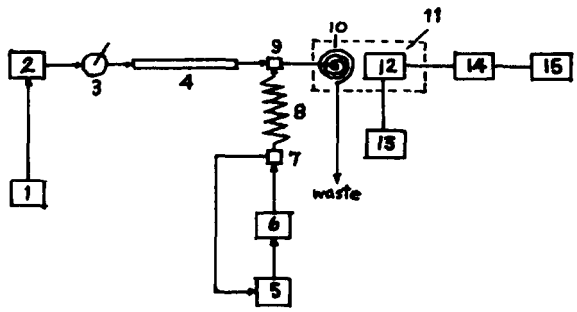

Fig. 1. Schematic diagram of the liquid chromatographic system with postcolumn chemiluminescence detection for the determination of nitrosamines. 1 = Mobile phase container; 2 = LC pump; 3 = injection valve; 4 = HPLC column; 5 = chemiluminogenic reagent solution container; 6 = postcolumn reaction pump; 7 = by-pass device (by-pass ratio = 1: 10); 8 = coil (1.5 m × 0.1 mm I.D.); 9 = mixer; 10 = flow cell (40 μl); 11 = dark box; 12 = photomultiplier tube (GDB-52QD); 13 = high-voltage supply; 14 = weak signal amplifier; 15 = recorder.

10.0 mmol/l of hydrogen peroxide in acetone–ethyl acetate binary solvents and the flow-rate was 100 μl/min.

Preparation of dansyl derivatives of nitrosamines

A portion of a mixed standard solution of nitrosamines containing 100 pmol of each of the species was placed in a graduated test-tube fitted with a stopper and evaporated to dryness in a stream of nitrogen. To the residue was added 20 μl of hydrobromic acid-acetic anhydride solution for denitrosation and the mixture was allowed to stand in the dark for 10 min at 70°C, then evaporated to dryness in a stream of nitrogen. To the residue 100 μl of 0.25 mol/l $NaHCO_3$ solution and 100 μl of dansyl chloride solution were added, the mixture was allowed to stand for 30 min at 40°C and then diluted with mobile phase to 1.0 ml. For HPLC assay, a blank experiment was required.

Recovery test

A synthetic standard mixture containing 100 pmol of each nitrosamine was added to 100 ml of redistilled water and the pH was adjusted to 7.0. The solution was passed through a mini activated carbon (100-120 mesh) column (20 mm x 1.2 mm I.D.) [4] by a sweep pump at a flow-rate of 0.5 ml/min. After elution with acetone, the eluate (0.5 ml) was collected in a graduated test-tube fitted with a stopper, evaporated to dryness in a stream of nitrogen, and then subjected to the proposed method as described above.

RESULTS AND DISCUSSION

Stability of chemiluminogenic reagent solution

In order to investigate the stability of 2-NPO in the presence of hydrogen peroxide, the residual 2-NPO was determined using reversed-phase liquid chromatography with UV-Vis spectrophotometric detection. The 2-NPO was separated using a Shimpack ODS (5 μm) column (150 mm x 6 mm I.D.) with acetonitrile-water (86: 14, v/v) as mobile phase at a flow-rate of 1.0 ml/min with detection at 298 nm. The percentage of residual 2-NPO was calculated from the peak heights at the same retention time on traces obtained for chemiluminogenic reagent solutions stored for different periods of time.

The effects of organic solvents on the stability of 2-NPO in the presence of hydrogen peroxide were examined and the results are shown in Fig. 2.

2-NPO decomposed to a significant extent in anhydrous solvents such as absolute methanol and acetonitrile, but it was much more stable in ethyl acetate, acetone and ethyl acetate-acetone. Hence the latter solvents were suitable for use in reversed-phase liquid chromatographic chemiluminescence detection. Moreover, ethyl acetate was suitable for the preparation of the 2-NPO and hydrogen peroxide stock solutions because the 2-NPO was more stable than in other solvents, and to improve the mutual solubility of the reversed-phase mobile phase and the chemiluminogenic reagent solution acetone was used as the diluent of the stock solution. When the concentrations of 2-NPO and hydrogen peroxide in the chemiluminogenic reagent solution were 3.0 and 10.0 mmol/l, respectively, the proportions of ethyl acetate and acetone in the mixture were 33:67 (v/v). However, the results showed that the stability of 2-NPO in the presence of hydrogen peroxide was hardly affected by variations in the proportions of the binary

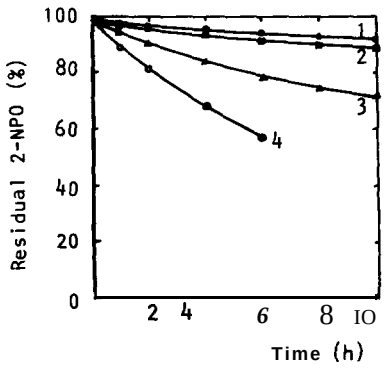

Fig. 2. Effects of organic solvents on the stability of 2-NPO in the presence of hydrogen peroxide. The concentrations of 2-NPO and hydrogen peroxide were‘3.0 and 20.0 mmolll, respectively. Solvents: 1 = ethyl acetate; 2 = acetone; 3 = acetonitrile; 4 = methanol.

solvent components. The stability of 2-NPO in the mixed solvents lay between those of ethyl acetate and acetone, as shown in Fig. 2.

The effect of the concentration of hydrogen peroxide on the stability of 2-NPO was studied and the results are shown in Fig. 3.

The decomposition of 2-NPO was accelerated with increase in the concentration of hydrogen peroxide in the chemiluminogenic reagent solution. On the other hand, the hydrolysis of 2-NPO also increased rapidly with increase in the concentration of water, which might result in a gradual decrease in the chemiluminescence intensity because the hydrogen peroxide is commercially available as a 30% aqueous solution and hence an increase in hydrogen peroxide concentration is accompanied by an increase in water concentration. For this reason the concentration of hydrogen peroxide was usually kept below 20.0 mmol/l, and the chemiluminogenic reagent solution was prepared with a stock solution just before use to prevent the decomposition of 2-NPO.

Optimization of chemiluminescence conditions

In order to examine the maximum chemiluminescence intensity of dansyl derivatives of nitrosamines under the optimum conditions, the effects of variations in the concentrations of hydrogen peroxide and 2-NPO and the flow-rate of the chemiluminogenic reagent solution on the relative chemiluminescence intensity, *i.e.*, relative peak heights in the chromatogram, for a test sample were examined. As the test sample 500 fmol of dansylnitropyrrolidine was selected.

The effects of the concentration of 2-NPO on the relative chemiluminescence intensity are shown in Fig. 4. The relative chemiluminescence intensity increased with increasing concentration of 2-NPO, and reached a nearly constant value at *ca.* 3.0 mmol/l.

Fig. 5 shows the effects of the concentration of hydrogen peroxide on the relative chemiluminescence intensity of the test sample. The relative chemiluminescence intensity increased with increasing concentration of hydrogen peroxide, and reached a nearly constant value at a concentration of 10.0 mmol/l. Therefore, the concentration of 2-NPO was fixed at 3.0 mmol/l and that of hydrogen peroxide at 10.0 mmol/l.

These conditions were adopted for chemiluminescence detection at a fixed flow-rate. Fig. 6 shows the common effects of the concentration of 2-NPO and the flow-rate of the chemiluminogenic reagent solution on the relative chemiluminescence intensity of the test sample. When the flow-rate was increased from 40 to 150 μl/

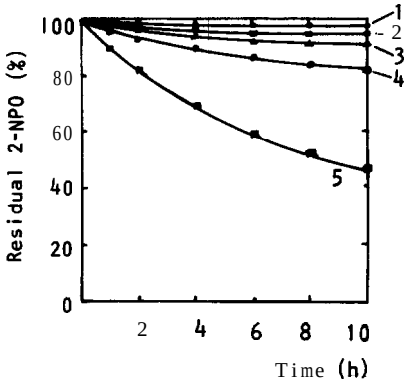

Fig. 3. Effect of the concentration of hydrogen peroxide on the stability of 2-NPO in ethyl acetate-acetone binary solvents. The concentration of 2-NPO was fixed at 3.0 mmol/l. Hydrogen peroxide concentrations: 1 = 5.0; 2 = 10.0; 3 = 20.0; 4 = 50.0; 5 = 100.0 mmol/l.

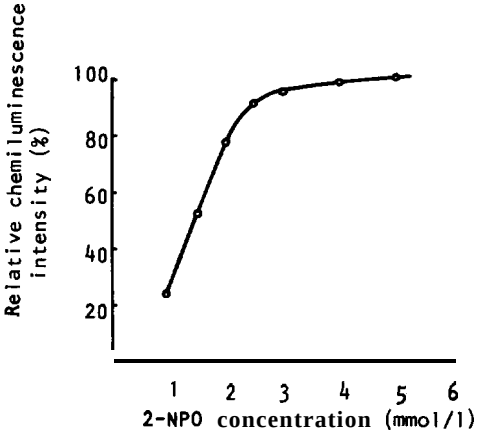

Fig. 4. Effect of the concentration of 2-NPO in the chemiluminogenic reagent solution on the relative chemiluminescence intensity. The concentration of hydrogen peroxide was fixed at 20.0 mmol/l and the flow-rate at 100 μl/min.

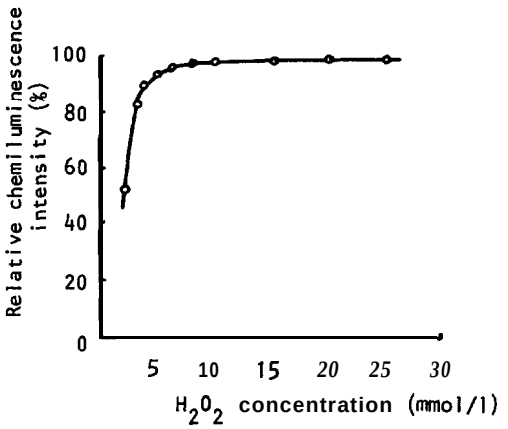

Fig. 5. Effect of the concentration of hydrogen peroxide in the chemiluminogenic reagent solution on the relative chemiluminescence intensity. The concentration of 2-NPO was fixed at 3.0 mmol/l and the flow-rate at 100 μl/min.

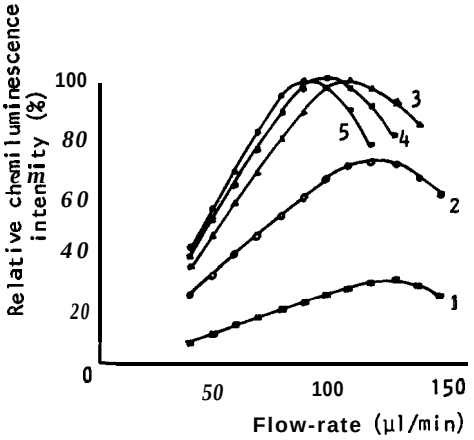

Fig. 6. Simultaneous effects of the concentration of 2-NPO and the flow-rate of the chemiluminogenic reagent solution on the relative chemiluminescence intensity. The concentration of hydrogen peroxide was fixed at 10.0 mmol/l. Concentrations of 2-NPO: 1 = 1.0; 2 = 2.0; 3 = 3.0; 4 = 4.0; 5 = 5.0 mmol/l.

min, the relative chemiluminescence intensity increased to a maximum and then decreased gradually at a fixed concentration of 2-NPO. When the concentration of 2-NPO was increased, the maximum of the relative chemiluminescence intensity shifted toward the direction of lower flow-rates.

On the basis of these results, the optimum chemiluminescence conditions were established

TABLE 1

RESULTS OF CALIBRATION, DETECTION LIMITS AND PRECISION

Parameter	NDMA	NPy	NDEA	NPip	NDPA	NDBA
Linear range (pmol)	0.02-20	0.02-20	0.02-20	0.02-20	0.02-20	0.02-20
Detection limits:						
fmol	6.9	6.5	8.6	8.0	7.4	9.4
g	$3.1 \cdot 10^{-13}$	$4.6 \cdot 10^{-13}$	$6.3 \cdot 10^{-13}$	$6.8 \cdot 10^{-13}$	$7.5 \cdot 10^{-13}$	$1.2 \cdot 10^{-12}$
Intercept	0.6	0.4	0.6	0.7	0.6	0
Slope	21.43	22.8	17.16	17	20.16	14.64
Correlation coefficient	0.9993	0.9991	0.9994	0.9998	0.9992	0.999
R.S.D. (%) (n = 7)[a]	4.3	3.4	2.8	5.5	4.0	6.7

[a] 0.2 pmol of each nitrosamine.

TABLE II

RECOVERIES FROM A WATER SAMPLE

Parameter	NDMA	NPY	NDEA	NPip	NDPA	NDBA
Amount added (pmol)	100.0	100.0	100.0	100.0	100.0	100.0
Amount recovered (pmol)	73.4	103.3	loo.3	97.0	100.8	93.9
Recovery (%)	73.4	103.3	loo.3	97.0	100.8	93.9

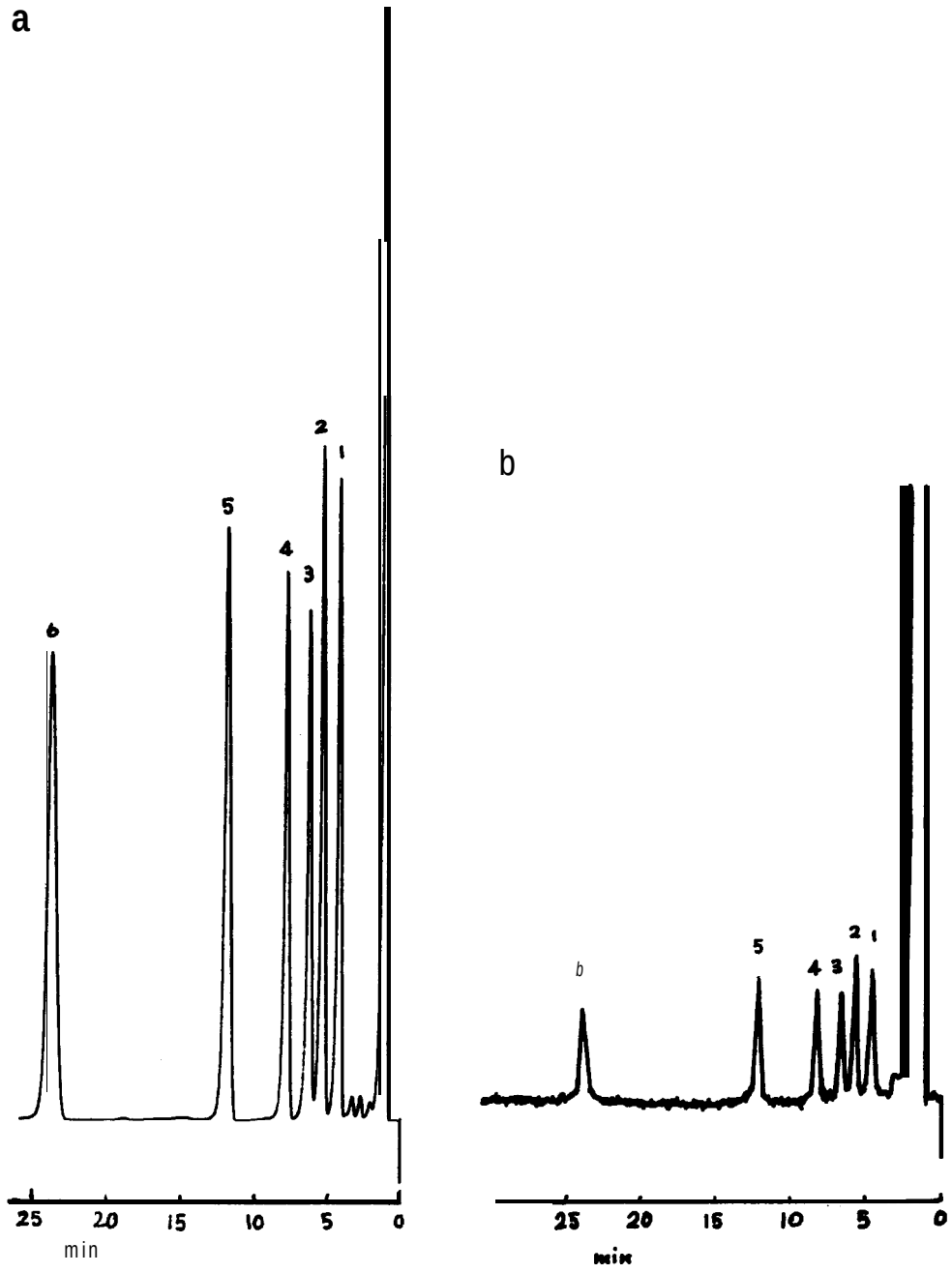

Fig. 7. Separation of standard mixtures of six nitrosamines containing (a) 500 and (b) 20 fmol/l on a Perkin-Elmer HS3 C_{18} column. Mobile phase, acetonitrile-water (63.5: 36.5, v/v) containing 3.0 mmol/l of imidaxole and the pH adjusted to 6.2 with oxalic acid; flow-rate, 0.5 ml/min; detection, postcolumn chemiluminescence with 2-NPO-hydrogen peroxide system; concentrations of 2-NPO and hydrogen peroxide in the chemiluminogenic reagent solution, 3.0 and 10.0 mmol/l, respectively, in ethyl acetate-acetone (33: 67, v/v), at a flow-rate of 100 μl/min. Peaks: 1 = dansylnitrosodimethylamine; 2 = dansylmtrosopyrrolidme; 3 = dansylnitrosodiethylamine; 4 = dansylnitrosopiperidine; 5 = dansylnitrosodipropylamine; 6 = dansylnitrosodibutylamine.

as follows: concentration of chemiluminogenic reagent solution, 3.0 mmol/l for 2-NPO and 10.0 mmol/l for hydrogen peroxide, in ethyl acetate–acetone (33:67, v/v), delivered at a flow-rate of 100 μl/min.

Fig. 7 shows two chromatograms obtained for mixtures of dansylated nitrosamines, containing (a) 500 and (b) 20 fmol of each.

Calibration, detection limits and precision

The relationship between the peak height and the amounts of six dansylnitrosamines was evaluated over the range 0.02-20 pmol. In order to verify the linearity of the chemiluminescence intensity under the optimum conditions at the working concentration of each nitrosamine, a series of working standard solutions containing different concentrations were derivatized and injected into the HPLC system with peroxyoxalate chemiluminescence detection. Linear least-squares regression was used to calculate the intercept, slope and correlation coefficient. The detection limits of the six dansyl nitrosamines were 0.31-1.20 pg (6.5-9.4 fmol) under the optimum conditions at a signal-to-noise ratio of 4. A solution containing 200 fmol of each nitrosamine was injected seven times to evaluate the repeatability of the assay system. The results are given in Table I.

The recoveries of six nitrosamines from a redistilled water sample were examined by measuring the peak height of the chemiluminescence intensity according to the above procedures. Table II shows the recoveries obtained by spiking samples with six nitrosamines at 100 pmol.

The results show that the sensitivity of the proposed method is good, the detection limits are better than those reported for other methods [1,2,4–7] and the precision is satisfactory. It is expected to have wide applications in environmental analysis and biochemical and fundamental research.

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