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Selective determination of volatile N-nitrosamines by derivatization with diethyl chlorothiophosphate and gas chromatography with flame photometric detection

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Abstract

A selective and sensitive method was developed for the determination of volatile N-nitrosamines by gas chromatography (GC). After denitrosation of N-nitrosamines with hydrobromic acid, the resulting secondary amines were converted into their N-diethylthiophosphoryl derivatives and then measured by GC using a DB-1701 capillary column with flame photometric detection. The calibration graphs for N-nitrosamines in the range 0.05–1 nmol were linear and sufficiently reproducible for quantitative determination. This method was successfully applied to cigarette smoke samples without prior clean-up. N-Nitrosamines and secondary amines were completely separated by extraction with diethyl ether containing 25% 2-propanol. Overall recoveries of N-nitrosamines added to cigarette smoke samples were 83–110%. By using this method, N-nitrosamines in these samples could be determined without any interference from coexisting substances. Analytical results for the contents of N-nitrosamines and secondary amines in mainstream and sidestream smokes of several cigarettes are presented.

1. Introduction

It is well known that N-nitrosamines are potent mutagenic and carcinogenic compounds in humans and laboratory animals, and are widely distributed in various human environments. Volatile N-nitrosamines are found in foodstuffs [1–6], drinking water [7], rubber products [8–12], drug formulations [13,14], herbicide formulations [15,16], tobacco and tobacco smoke [17–24] and indoor and outdoor environments [25–27]. These N-nitrosamines are formed by the reaction of secondary amines with nitrosating agents such as nitrite or nitrate in the human diet [4], the environment [24] and in vivo in the stomach or small intestine of experimental animals [28–31]. The toxicity of N-nitrosamines is manifested even at μg/kg levels, so that a sensitive and selective method for the determination of these nitrosamines at trace levels is essential.

In general, the determination of volatile N-nitrosamines in samples has been carried out by gas chromatography (GC). In most GC methods, N-nitrosamines are determined directly in the free form using a thermal energy analyser (TEA) [3–15,17–24], based on the detection of the chemiluminescence emitted from a reaction between released NO radicals and ozone after thermal cleavage of the N–NO bond in N-nitroso compounds. Although GC–TEA is sensitive and

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specific for N-nitroso compounds, it is very expensive. Therefore, nitrogen–phosphorus detection (NPD) has been used for the determination of N-nitrosodimethylamine in the free form [2,16]. However, the application of GC–TEA and GC–NPD methods to the free forms of N-nitrosamines requires time-consuming clean-up by alumina or silica gel column chromatography prior to GC analysis. Further, N-nitrosamines tend to be decomposed by acid or peroxide produced in the solvents during these pretreatments [2]. GC with electron-capture detection based on the conversion of N-nitrosamines into their corresponding N-nitramine analogues by pertrifluoroacetic acid oxidation has been reported [32], but this method requires purification of the derivatives by adsorption chromatography on basic alumina prior to GC analysis.

Recently, we have developed a selective and sensitive method for the determination of secondary amines by GC with flame photometric detection (FPD), in which these compounds are analysed as their N-diethylthiophosphoryl (DETP) derivatives [33,34]. On the other hand, it is well known that N-nitrosamines easily undergo cleavage at the N–NO bond in the presence of hydrobromic acid, resulting in the formation of the corresponding secondary amines [35–37]. Therefore, we investigated a selective and sensitive method for the determination of N-nitrosamines by a combination of these techniques. This method is based on the denitrosation of N-nitrosamines to secondary amines, subsequent conversion of the secondary amines into DETP derivatives and GC–FPD analysis. By using this method, the contents of N-nitrosamines in mainstream and sidestream smokes of cigarettes were studied. Further, their contents were compared with the secondary amine contents measured by a previous method [33,34].

2. Experimental

2.1. Reagents

N-Nitrosodimethylamine (NDMA) was purchased from Wako (Osaka, Japan), N-nitrosomorphorine (NMOR) from Nacalai Tesque (Kyoto, Japan) and N-nitrosodiethylamine (NDEA), N-nitrosodibutylamine (NDBA), N-nitrosopyrrolidine (NPYR) and N-nitrosopiperidine (NPIP) from Sigma (St. Louis, MO, USA). Each N-nitrosamine was dissolved in dichloromethane to make a stock solution at a concentration of 0.2 *M* and used after dilution with same solvent to the required concentration. Phenylphosphonic acid diethyl ester (PPDE) as an internal standard (I.S.) for N-nitrosamine analysis was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and stored as 0.1 *M* solution in *n*-hexane. Dimethylamine (DMA) and diethylamine (DEA) as the hydrochlorides, di-*n*-propylamine (DPA), di-*n*-butylamine (DBA), pyrrolidine (PYR), piperidine (PIP), morpholine (MOR), hexamethyleneimine (HMI), N-methylbenzylamine (NMBzA) and N-methylcyclohexylamine (NMCHA) as an I.S. for secondary amine analysis were purchased from Nacalai Tesque. N-Methylethylamine (MEA) was purchased from Fluka (Buchs, Switzerland). Each amine was dissolved in 0.05 *M* hydrochloric acid containing 50% acetonitrile to make a stock solution at a concentration of 0.2 *M* and used after dilution with 0.05 *M* hydrochloric acid to the required concentration (0.01–0.1 m*M*). Hydrobromic acid (Nacalai Tesque) was used at a concentration of 47%. Diethyl chlorothiophosphate (DECTP) (Tokyo Kasei Kogyo) was used as a 1% solution in acetonitrile after distillation. Peroxide-free diethyl ether was obtained from Dojindo Laboratories (Kumamoto, Japan) and diethyl ether containing 25% 2-propanol was used as an extraction solvent. All other chemicals were of analytical-reagent grade.

2.2. Preparation of samples

As shown in Table 1, four commercial cigarettes were selected on the basis of their tar and nicotine contents and filter or non-filter types. All cigarettes were purchased in Okayama, Japan, during the winter of 1994. The cigarettes were smoked with a laboratory-made smoking machine described by Pieraccini et al. [38]. The mainstream and sidestream smokes of cigarettes were separately bubbled in two trapping bottles

Table 1
Commercial cigarettes used in the experiments

Cigarette[a]	Content per cigarette[b]		
	Mass (mg)	Tar (mg)	Nicotine (mg)
A	1018	24	2.4
B	696	10	1.0
C	662	6	0.5
D	584	1	0.1

[a] A = non-filter cigarette; B–D = filter cigarettes.
[b] The filter is not included in these contents. Each mass represents the average of three difference measurements. Contents of tar and nicotine represent the values labelled on the product.

each containing 25 ml of 5% hydrochloric acid, and used as mainstream and sidestream smoke samples, respectively. An aliquot (0.5–2 ml) of each hydrochloric acid solution containing cigarette smoke was pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap, and then extracted twice with half the volume of diethyl ether containing 25% 2-propanol. After centrifugation at 2000 *g* for 1 min, the organic layer (upper layer) was transferred into another tube, and the combined organic layer was called the N-nitrosamine fraction. The remaining aqueous layer (lower layer) was called the amine fraction. Both fractions were used as samples for derivatization.

2.3. Derivatization procedure

N-nitrosamines were derivatized as secondary amines after denitrosation with hydrobromic acid to produce the corresponding secondary amines. After evaporation to dryness of the solvent in the N-nitrosamine fraction at 80°C under a gentle stream of nitrogen (accomplished within a few minutes), 20 μl of hydrobromic acid were added to the residue. Then the test-tube was immediately capped and heated for 5 min at 100°C. After cooling, to the reaction mixture was added 0.5 ml of distilled water and the mixture was neutralized with 2 *M* sodium hydroxide. To this solution were added 0.2 ml of 1% DECTP and 0.2 ml of 10% sodium carbonate, then the mixture was heated at 60°C for 10 min after

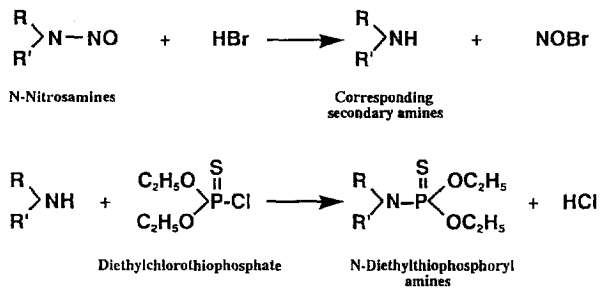

Fig. 1. Process for derivatization of N-nitrosamines.

tightly capping the tube. In order to remove the excess of reagent, the reaction mixture was incubated again at 60°C for 5 min after addition of 0.2 ml of 50 m*M* cysteic acid. The reaction mixture was extracted with 0.2–0.4 ml of *n*-hexane containing 2 nmol of PPDE (I.S.) and 1 μl of this extract was injected into the GC–FPD system. The derivatization process for N-nitrosamines is summarized in Fig. 1. Derivatization of secondary amines in the amine fraction was performed by a previously reported method [33,34].

2.4. Gas chromatography

GC analysis was carried out with a Shimadzu Model 14A gas chromatograph equipped with a flame photometric detector (P-filter). A fused-silica capillary column (15 m × 0.53 mm I.D., 1.0 μm film thickness) of cross-linked DB-1701 (J&W, Folsom, CA, USA) was used. The operating conditions were as follows: column temperature, programmed from 100 to 260°C at 10°C/min; injection and detector temperatures, 280°C; and nitrogen flow-rate, 10 ml/min. The peak heights of secondary amines or N-nitrosamines and each I.S. were measured and the peak-height ratios against the I.S. were calculated.

3. Results and discussion

The reaction conditions for denitrosation and subsequent N-diethylthiophosphorylation were investigated to establish the optimum derivatization method for N-nitrosamines. Denitrosation

has been tried in the determination of N-nitrosamines by high-performance liquid chromatography [35–37]. Although hydrobromic acid–acetic acid was used as the denitrosation reagent in that work, denitrosation with hydrobromic acid without acetic acid was found to be sufficient for our method. NDMA, NDEA, NPYR, NPIP, NMOR and NDBA were used as standard N-nitrosamines in our experiments. The denitrosation of these N-nitrosamines proceeded rapidly in a minimum excess of ca. 10^3 mol of hydrobromic acid per mole of N-nitrosamine. This reaction was completed within 5 min at 100°C. The yields of denitrosation of NDMA, NPIP, NMOR and NDBA were 92, 93, 93 and 99%, respectively. Excess hydrobromic acid in the reaction mixture was neutralized with sodium hydroxide solution before derivatization.

The derivatization of the secondary amines produced from N-nitrosamines was carried out by a previously reported method [33,34]. As described in the previous report, the N-diethylthiophosphorylation of secondary amines with DECTP proceeded rapidly and quantitatively in aqueous alkaline media. This reaction was completed within 5 min at 60°C using 2 mg of DECTP. The resulting N-diethylthiophosphoryl (DETP) amines were quantitatively extracted into *n*-hexane. The excess DECTP could be removed by reaction with cysteic acid because the DETP derivative of cysteic acid was not extracted into this solvent in alkaline media. The phosphorus-containing DETP derivatives from N-nitrosamines provided an excellent response in the flame photometric detector. These derivatives were volatile and stable, and eluted as separate symmetrical peaks although some peaks originating from reagents were observed (Fig. 2A and B).

In order to test the linearity of the calibration graphs, various amounts of N-nitrosamines ranging from 0.05 to 1 nmol, except for NDEA

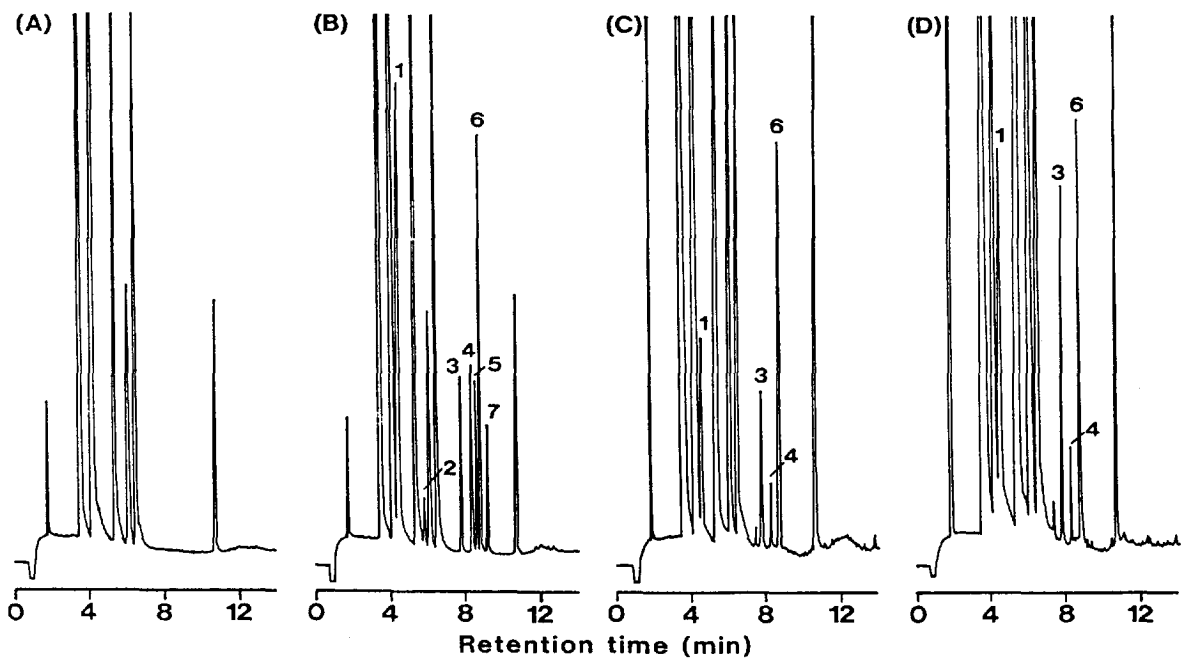

Fig. 2. Typical gas chromatograms obtained from (A) reagent blank, (B) standard N-nitrosamine (containing 0.5 nmol of each N-nitrosamine), (C) mainstream smoke and (D) sidestream smoke. GC conditions are given under Experimental. Peaks: 1 = N-nitrosodimethylamine; 2 = N-nitrosodiethylamine; 3 = N-nitrosopyrrolidine; 4 = N-nitrosopiperidine; 5 = N-nitrosomorpholine; 6 = phenylphosphonic acid diethyl ester (I.S.); 7 = N-nitrosodibutylamine.

(0.1–1 nmol), were derivatized in a mixture according to the denitrosation and subsequent N-diethylthiophosphorylation, and aliquots representing 0.25–5 pmol of each compound were injected into the GC–FPD system. In each case, a linear relationship was obtained and the reproducibility was found to be satisfactory (Table 2).

In order to demonstrate the applicability of the method to environmental samples, the contents of N-nitrosamines in cigarette smoke were analysed. Cigarette smoke was collected with a laboratory-made smoking machine [38] by bubbling in 5% hydrochloric acid. The mainstream and sidestream smoke samples could be separately collected by this apparatus. Although the pyrolytic formation of volatile N-nitrosamines during tobacco combustion has been reported [24], the formation of these amines in the collecting solution was not. The N-nitrosamines must be separated from the coexisting secondary amines in these samples before derivatization, because N-nitrosamines are analysed as the same derivatives as secondary amines. Although dichloromethane is usually used for the extraction of N-nitrosamines, sampling of the organic layer was difficult because this solvent is isolated in the lower layer. Among other solvents tested, diethyl ether proved to be sufficient for rapid extraction and evaporation, but the extraction of N-nitrosamines with this solvent was found to be incomplete. In order to solve this problem, the addition of another solvent to diethyl ether was tested. 2-Propanol proved to be the most satisfactory solvent for this purpose, and its optimum concentration was 25%. By extraction twice with diethyl ether containing 25% 2-propanol in acidic media, the N-nitrosamines were quantitatively transferred into the organic layer (N-nitrosamine fraction) and other amines containing secondary amines remained completely in the aqueous layer (amine fraction). Therefore, N-nitrosamines and secondary amines could be separately determined in both fractions.

Table 2
Linear regression data for N-nitrosamines

N-Nitrosamine[a]	Regression line[b]	Correlation coefficient (r)	Number of data (n)
NDMA	$y = 2.171x + 0.013$	0.998	15
NDEA	$y = 0.227x + 0.005$	0.997	12
NPYR	$y = 0.784x + 0.030$	0.996	15
NPIP	$y = 0.803x + 0.020$	0.998	15
NMOR	$y = 0.804x + 0.001$	0.998	15
NDBA	$y = 0.606x + 0.012$	0.995	15

[a] NDMA = N-nitrosodimethylamine; NDEA = N-nitrosodiethylamine; NPYR = N-nitrosopyrrolidine; NPIP = N-nitrosopiperidine; NMOR = N-nitrosomorphorine; NDBA = N-nitrosodibutylamine.

[b] y = peak-height ratio; x = amount of each N-nitrosamine (nmol).

As shown in Fig. 2C and D, the N-nitrosamines in mainstream and sidestream smoke samples could be detected without any interference from coexisting substances, although several unidentified peaks were observed. The detection limits for NDMA, NDEA, NPYP, NPIP, NMOR and NDBA were 0.20, 0.50, 0.15, 0.13, 0.18 and 0.20 nmol per cigarette, respectively. The presence of NDMA, NMEA, MPYR and NPIP had been reported in cigarette smoke samples [17–24], but NDMA, NPYR and NPIP were detected in the smoke samples of four commercial cigarettes tested in this study. As shown in Table 3, the overall recoveries of N-nitrosamines added to mainstream and sidestream smoke samples were 83–110%, and the reproducibility was found to be satisfactory.

The contents of N-nitrosamines and secondary amines in the mainstream and sidestream smokes of different brands of cigarettes are shown in Fig. 3. The cigarette containing higher concentration of tar and nicotine (Table 1) tended to contain higher concentrations of N-nitrosamines and secondary amines. The total contents of N-nitrosamines containing NDMA, NPYP and NPIP in sidestream smoke samples were found to be 2–6 times higher than those in mainstream smoke samples. The concentrations per cigarette of NDMA, NPYR and NPIP in sidestream smoke were 1.33–4.51 nmol (98–334 ng), 2.92–12.49 nmol (292–1249 ng) and 0.77–3.15 nmol (88–359 ng), respectively, and these values were similar to the literature values: NDMA 143–1770

Table 3
Recoveries of N-nitrosamines added to cigarette smoke samples

Sample	N-Nitrosamine[a]	Added (nmol/ml)	Amount found[b] (nmol/ml)		Recovery (%)
			Non-addition	Addition	
Mainstream smoke	NDMA	0.100	0.098 ± 0.006	0.189 ± 0.014	91
	NDEA	0.100	ND[c]	0.088 ± 0.012	88
	NPYR	0.100	0.224 ± 0.015	0.322 ± 0.015	98
	NPIP	0.100	0.082 ± 0.004	0.174 ± 0.007	92
	NMOR	0.100	ND	0.095 ± 0.004	95
	NDBA	0.100	ND	0.093 ± 0.003	93
Sidestream smoke	NDMA	0.100	0.181 ± 0.012	0.269 ± 0.025	88
	NDEA	0.100	ND	0.110 ± 0.011	110
	NPYR	0.100	0.499 ± 0.018	0.582 ± 0.043	83
	NPIP	0.100	0.127 ± 0.007	0.216 ± 0.020	89
	NMOR	0.100	ND	0.107 ± 0.010	107
	NDBA	0.100	ND	0.093 ± 0.006	93

[a] NDMA = N-nitrosodimethylamine; NDEA = N-nitrosodiethylamine; NPYR = N-nitrosopyrrolidine; NPIP = N-nitrosopiperidine; NMOR = N-nitrosomorphorine; NDBA = N-nitrosodibutylamine.
[b] Mean ± S.D. ($n = 3$).
[c] Not detectable.

ng, NPYR 28–700 ng and NPIP 5–20 ng. However, the concentrations per cigarette of NDMA, NPYR and NPIP in mainstream smoke samples were 0.85–2.63 nmol (63–195 ng), 0–5.58 nmol (0–558 ng) and 0–2.05 nmol (0–234 ng,), respectively, and these values were relatively high in comparison with the literature values: NDMA 0–97 ng, NPYR 2–65 ng and NPIP 0–14 ng. On the other hand, both smoke samples contained high concentrations of DMA, PYR and PIP, which are precursor secondary amines for the above N-nitrosamines. MOR was also detected at trace levels in these samples but the corresponding N-nitrosamine NMOR was not detectable.

The total contents of secondary amines in sidestream smoke samples were found to be 50–100 times higher than those of N-nitrosamines. On the other hand, the contents of secondary amines in mainstream smoke samples of filter-tipped cigarettes were very low or not detectable, but those of non-filtered cigarette were relatively high. These results indicate that a filter-tip is effective for trapping of secondary amines but is less effective for N-nitrosamines. Further, these results suggest that the cancer risk due to exposure to cigarette smoke increases not only in smokers but also in non-smokers, because the N-nitrosamine contents in sidestream smoke samples are significantly high in comparison with those in mainstream smoke samples and both smokers and non-smokers are exposed to sidestream smoke.

4. Conclusion

These experiments have conclusively demonstrated that N-nitrosamines can be accurately and precisely determined by GC–FPD as their N-diethylthiophosphoryl derivatives after denitrosation. This method is selective and sensitive, and cigarette smoke samples can be analysed directly without pretreatment except for separation from secondary amines by solvent extraction and without any interference from other coexisting substances. We believe that this method provides a useful tool for environmental analysis.

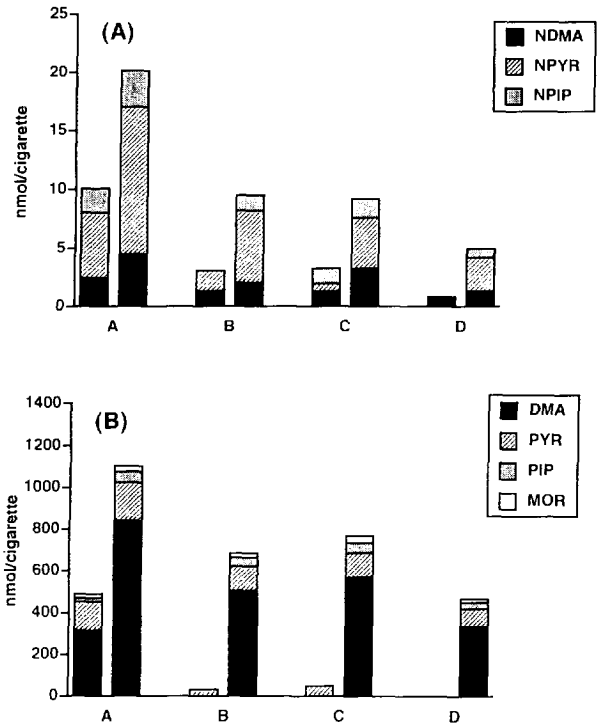

Fig. 3. Contents of (A) N-nitrosamines and (B) secondary amines in mainstream and sidestream smoke samples from four commercial cigarettes. For cigarettes A–D, see Table 1. Smoke: left column = mainstream smoke; right column = sidestream smoke. N-nitrosamines: NDMA = N-nitrosodimethylamine; NPYR = N-nitrosopyrrolidine; NPIP = N-nitrosopiperidine. Secondary amines: DMA = dimethylamine; PYR = pyrrolidine; PIP = piperidine; MOR = morpholine.

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