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DIRECT GAS CHROMATOGRAPHIC ANALYSIS OF CYCLIC N-NITROSAMINES

S. RAY*

Fisheries and Environment Canada, Fisheries and Marine Service, Resource Branch, Bedford Institute of Oceanography, Dartmouth, N.S. B2Y 4A2 (Canada)

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SUMMARY

A rapid gas chromatographic technique has been developed for quantitative estimation of cyclic N-nitrosamines. Cumbersome clean-up procedures are unnecessary and quantitative estimation can be done by injecting hexane extract without further pretreatment. The detection limit for the procedures is *ca.* 0.5 ng.

INTRODUCTION

The N-nitrosamines have recently received much attention since the discovery of their carcinogenic and mutagenic properties[1]. The toxicity[2] of nitrosamines has been reviewed. They are present in food for human consumption and have been found in tobacco smoke[3], beverages[4], bacon[5,6], cheese[5], fresh, smoked and salted fish[5–9], fish meal[10] for animal feed, etc. The reaction of sodium nitrite, used as a preservative, with secondary amines already present seems to be imperative and speculated to cause the formation of N-nitrosamines. Dimethyl- and diethylamine along with some primary and tertiary types have been reported in fish[11]. Methyl-, dimethyl- and trimethylamines have been found in salmon[12]. Ender and Ceh[13] reported the formation of dimethyl- and diethylnitrosamine from corresponding primary amines when heated with nitrite. Scanlan *et al.*[14] have shown the formation of nitrosamines from tertiary amines and quaternary ammonium compounds. Iyengar *et al.*[15] in a survey of fish products have found dimethyl- and diethyl amines in fresh, salted and smoked fish and postulated that trace natural amounts of formaldehyde in fish may catalyse the reaction between nitrites and amines of the fish, forming nitrosamines. Lijinski and Epstein[11] proposed that the primary amines, cadaverine and putrescine, may cyclize upon heating to form piperidine and pyrrolidine and subsequently react with nitrite to form nitrosamines. Bills *et al.*[16] demonstrated the production of nitrosopyrrolidine from such polyamines as spermidine and putrescine when heated in the presence of sodium nitrite. In a subsequent work[17] they obtained nitrosopyrrolidine and nitrosoproline from ornithine, nitrosopiperidine from cadaverine and lysine and nitrosopipecolic acid from lysine and suggested that the formation can even take place at the storage temperature of food. Takagi *et al.*[18] have confirmed the

* Present address: Biological Station, St. Andrews, N.B. EOG 2XO, Canada.

presence of putrescine, cadaverine and tyramine in spoiled squid and octopus. The nitrates are also widely distributed in nature and are easily reduced to nitrites by bacterial action. Fong and Chan[7] have demonstrated that nitrate-reducing *Stephalococcus aureus* and halobacteria isolated from fish were responsible for nitrite formation.

The necessity for qualitative as well as quantitative analysis has resulted in a variety of thin-layer and/or gas–liquid chromatography[4], polarography[19], gas chromatography–mass spectrometry (GC–MS)[6,9,15,20] and high-speed liquid[21] and plasma chromatographic[22] methods. Various chromatographic detectors[23] for the analysis of volatile N-nitrosamines have been compared and evaluated. All of these methods need an extensive clean-up operation, solvent extraction and in some cases derivatization[8] prior to GC.

Most of the work published so far has been done with dialkyl nitrosamines. The cyclic analogues are yet to be studied. Several of them have proved carcinogenic[24,25] to laboratory animals and have been found in food[5,6,8].

We now report a rapid and sensitive method for separation and quantitative estimation of cyclic N-nitrosamines by GC. The structural formulae for the nitrosamines studied are given in Fig. 1; stereochemistry is not shown.

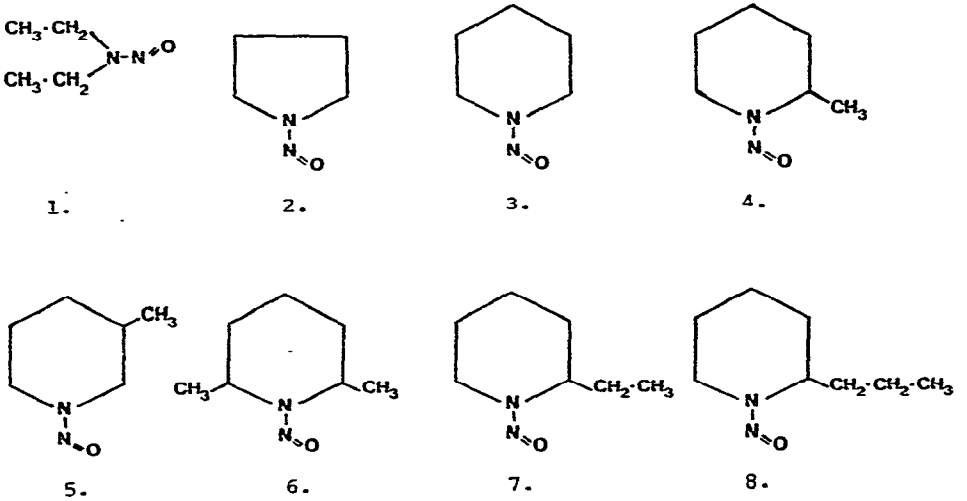

Fig. 1. 1 = Diethylnitrosamine (DEN); 2 = N-nitrosopyrrolidine (NPYR); 3 = N-nitrosopiperidine (NPIP); 4 = N-nitroso-2-methylpiperidine (2-MNP); 5 = N-nitroso-3-methylpiperidine (3-MNP); 6 = N-nitroso-*cis*-2,6-dimethylpiperidine (2,6-DMNP); 7 = N-nitroso-2-ethylpiperidine (2-ENP); 8 = N-nitroso-2-propylpiperidine (2-PNP).

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 5700 gas chromatograph equipped with a hydrogen flame ionisation detector was used in this study. The injection port was heated on-column. The recorder used was a Hewlett-Packard Model 7123A with a 1 mV full scale at a 0.5 in./min chart speed. Injections were made with a Hamilton 10-μl

syringe (701N). The columns used were 6 ft. × ⅛ in. with 10% SE-30 or 8 ft. × ⅛ in with 10% OV-17 on Gas-Chrom Q (80–100 mesh). The following GC flow-rates were used for isothermal operation: nitrogen, 30 ml/min; hydrogen 30 ml/min; air 240 ml/min.

Reagents

Starting amines were obtained commercially and checked for their purity by GC and nuclear magnetic resonance (NMR). The N-nitrosodiethylamine and N-nitrosopyrrolidine were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.) and Aldrich (Milwaukee, Wisc., U.S.A.), respectively and were used as received.

All nitrosamines were made by the following general procedure. The amine (1 mole) was dissolved in 1 mole of H_2SO_4 (9 *N*) or HCl (3 *N*) and cooled. $NaNO_2$ (97%, 1.1 mole) dissolved in a minimal quantity of water was slowly added to the reaction mixture while maintaining the temperature and constantly stirring. After the addition was complete, the mixture was maintained at the reaction temperature for an hour, and then allowed to attain room temperature. The nitrosamine, which separated in the top layer as an oil, was extracted with ether and the ethereal solution was washed with 30% KOH solution and dried over anhydrous $MgSO_4$. The yellow oil obtained after evaporation of the ether was subsequently distilled under vacuum. The reaction temperatures and boiling points are given in Table I. Primary standard solutions were prepared in distilled hexane. As nitrosamines are light-sensitive, the stock standards were stored in a refrigerator wrapped in aluminum foil. No deterioration of standards were observed under these conditions over a period of several months except for N-nitrosopyrrolodine, fresh samples of which had to be prepared quite frequently.

TABLE I

REACTION CONDITIONS FOR PREPARATION OF NITROSAMINES

Compound	*Reaction temp. (°C)*	*B.p. (°C)*
N-nitrosopiperidine	—5–0	108–109/20 mm
N-nitroso-2-methylpiperidine	0–5	109–110/20 mm
N-nitroso-3-methylpiperidine	0–5	81–82/3 mm
N-nitroso-*cis*-2,6-dimethylpiperidine	75	89–90/2 mm
N-nitroso-2-ethylpiperidine	0–5	82–84/2 mm
N-nitroso-2-propylpiperidine	0–5	100–102/3.5 mm

RESULTS AND DISCUSSION

Table II shows the compounds studied and their retention times under isothermal conditions in two different stationary phases. The chromatographic separation of components are shown in Figs. 2 and 3. The OV-17 column being longer and slightly more polar than SE-30 retains the components much longer and requires a higher elution temperature. The order of elution in both columns is exactly the same. The expectation that the higher polarity of the OV-17 stationary phase would help in obtaining better separation of the N-nitroso-2- and -3-methylpiperidine peaks was however not achieved.

TABLE II

COMPARISON OF RETENTION TIME ON TWO DIFFERENT STATIONARY PHASES

Compound	*Formula*	*Retention time (min)*	
		SE-30	*OV-17*
N-nitrosodiethylamine	$C_4H_{10}N_2O$	0.50	1.01
N-nitrosopyrrolidine	$C_4H_8N_2O$	2.03	3.50
N-nitrosopiperidine	$C_5H_{10}N_2O$	2.39	4.08
N-nitroso-2-methylpiperidine	$C_6H_{12}N_2O$	3.50	5.23
N-nitroso-3-methylpiperidine	$C_6H_{12}N_2O$	3.35	5.02
N-nitroso-*cis*-2,6-dimethylpiperidine	$C_7H_{14}N_2O$	5.20	6.46
N-nitroso-2-ethylpiperidine	$C_7H_{14}N_2O$	6.02	7.51
N-nitroso-2-propylpiperidine	$C_8H_{16}N_2O$	9.25	11.42

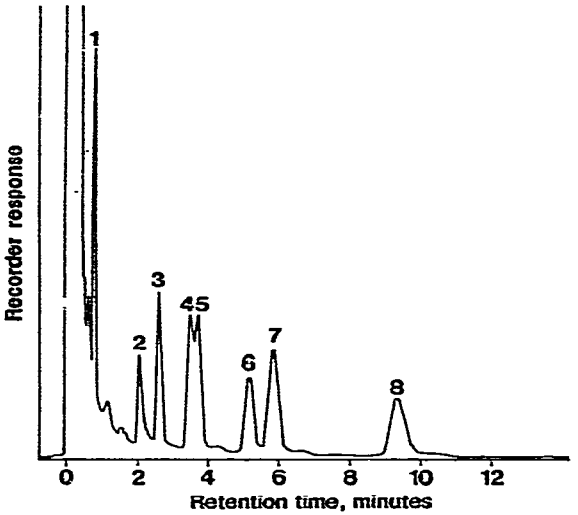

Fig. 2. Separation of nitrosamines on 10% SE-30 (6 ft. × 1/8 in.). Peaks: 1 = DEN; 2 = NPYR; 3 = NPIP; 4 = 2-MNP; 5 = 3-MNP; 6 = 2,6-DMNP; 7 = 2-ENP; 8 = 2-PNP. GC conditions: column temperature, 130°; inlet temperature, 150°; detector temperature, 250°.

Factors affecting separation

The elution order of the compounds is obviously affected by factors other than the number of carbon atoms and/or boiling points of the components, and it is possibly influenced by the three-dimensional geometry or the stereochemical arrangement of the molecules.

In the pair, nitrosodiethylamine and nitrosopyrrolidine, a study of the molecular model reveals that the cyclic nitrosamine is far smaller in size than the straight-chain compound and hence it is possibly retained much more strongly by the stationary phase and thus produces a large difference in retention time.

In case of 2-, 3-methyl nitrosamines, the starting amines have the alkyl groups in equatorial conformation. However, on nitrosation, 3-methylpiperidine retains its stereochemistry while the α-alkyl groups exhibits a strong preference for the axial[26] position. This causes a slight reduction in the size of the molecule and is reflected in a corresponding increase in the retention time of the N-nitroso-2-methylpiperidine.

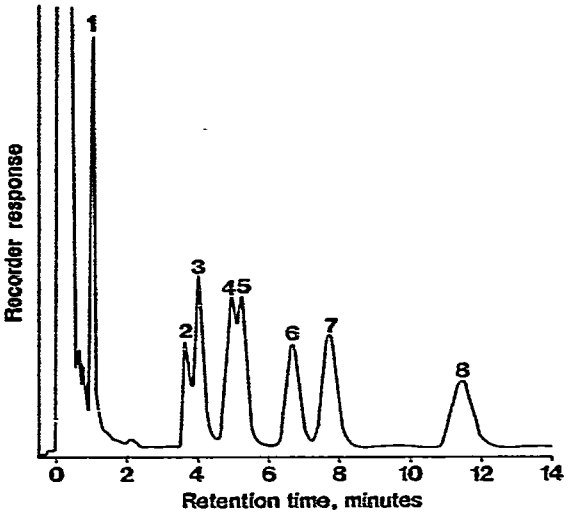

Fig. 3. Separation of nitrosamines on 10% OV-17 (8 ft. × 1/8 in.). Peaks: 1 = DEN; 2 = NPYR; 3 = NPIP; 4 = 2-MNP; 5 = 3-MNP; 6 = 2,6-DMNP; 7 = 2-ENP; 8 = 2-PNP. GC conditions: column temperature, 150°; inlet temperature, 200°; detector temperature, 250°.

In the pair, 2,6-dimethyl and 2-ethyl nitrosopiperidine, the alkyl groups in both compounds have axial[26] orientation. However, N-nitrosoethylpiperidine is possibly still smaller due to the general type of stereochemical interaction known as $A^{(1,3)}$ strain[27]. This is reflected in the larger retention time for the nitroso-2-ethylpiperidine.

Linearity of response and quantitative results

A series of standard solutions of the nitroso compounds were made in hexane. A 1-μl sample of each concentration was injected into the gas chromatograph and calibration plots for individual N-nitroso compounds were made by plotting the average peak heights of seven injections of standard solutions against nanogram of

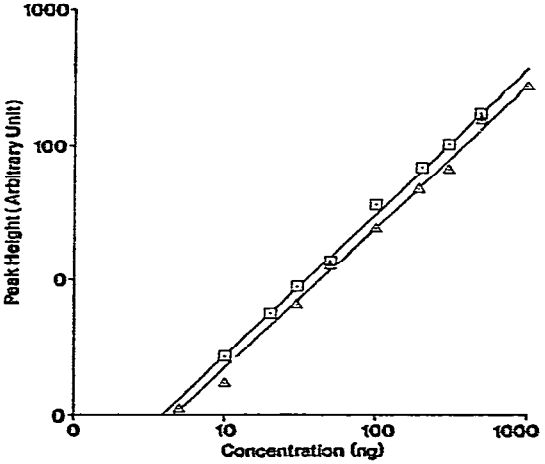

Fig. 4. Calibration plot: △, NPIP; ⊡, 2-MNP.

TABLE III

PRECISION OF DETERMINATION OF NITROSAMINES

Compound	*Peak height*	*Standard deviation (n = 7)*
N-nitrosopiperidine	89.7	2.5
N-nitroso-2-methylpiperidine	65.4	1.3
N-nitroso-2-propylpiperidine	31.0	0.6

the nitrosamines. The plots were linear up to four orders of magnitude, as studied under the conditions of the experiments (Fig. 4). Within each group the coefficient of variation was of the order of 2%.

For routine analysis of a number of samples by GC, it is only necessary to check the calibration on a daily basis; it is consistent from day to day. A typical variation observed over a period of several weeks being within $\pm$ 1% of the mean.

Precision and accuracy

The precision was determined by replicate analysis of freshly prepared calibration standards. The means and standard deviations for some of the compounds are given in Table III. The results show that the standard deviation for all compounds is $\leqslant \pm 2\%$ and permits excellent reliability.

Detection limit

All compounds studied could be detected to a *ca.* 0.5 ng level at a signal-to-noise ratio of 3:1.

Recovery experiments

Satisfactory results have been obtained from analysis of spiked fish samples.

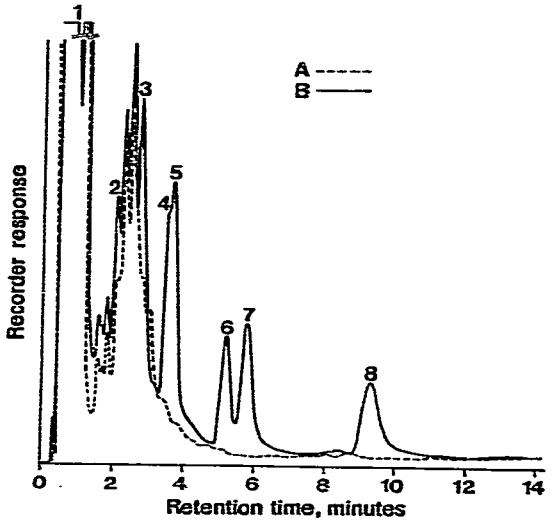

Fig. 5. Gas chromatogram of (A) control fish extract and (B) spiked fish extract with hexane (without any pretreatment). 2 = NPYR; 3 = NPIP; 4 = 2-MNP; 5 = 3-MNP; 6 = 2,6-DMNP; 7 = 2-ENP; 8 = 2-PNP.

A 5-g sample of fresh fish muscle tissue was spiked with the six nitrosopiperidines at 20-ng/kg level and extracted with two 25-ml portions of hexane with about 1 g of anhydrous sodium sulphate. The extract was reduced in volume to approximately 0.2 ml and aliquots were injected into the SE-30 column without further pretreatment. The chromatograms for the control and spiked extracts are shown in Fig. 5. Overall recoveries fall within the range of 65–95%.

CONCLUSIONS

The application of direct gas–liquid chromatographic analysis for a number of cyclic nitrosamines has been demonstrated. The method developed is shown to be of acceptable precision over a wide range and capable of complete chromatographic analysis in approximately 10 min.

It is also proved satisfactory in actual recovery experiments with standardized formulation. Interfering peaks from the fish extract in the beginning of the chromatogram do not affect the actual chromatogen, apart from nitrosopyrrolidine.

It should, perhaps, be pointed out that the above analytical procedure by itself cannot give unequivocal results, relying as it does on chromatographic retention times for identification of compounds, even on two different columns. Where positive identifications are to be obtained, confirmation by other methods (*viz.* mass spectrometry or IR) is desirable.

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