Gas Chromatographic Determination and Mass Spectrometric Confirmation of *N*-Nitrosodimethylamine in Smoke-Processed Marine Fish

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The gas chromatographic method for the determination of *N*-nitrosodimethylamine in smoked nitrite-treated chub fish has been modified to include other species of fish. The nitrosamine, confirmed by mass spectrometry, was isolated from samples of raw, smoked, and smoked nitrite- and/or nitrate-treated sable, salmon, and shad. Levels of *N*-nitrosodimethylamine found ranged from 4 to 26 ppb (μg/kg).

Magee and Barnes (1967) have described *N*-nitrosamines as one of the most formidable and versatile groups of carcinogens yet discovered. Their role as environmental hazards in the etiology of human cancer has caused growing apprehension among experts (*Brit. Ind. Biol. Res. Ass. Inform. Bull.*, 1968; Eisenbrand *et al.*, 1969; *Lancet*, 1968; Lijinsky and Epstein, 1970). Much of this concern is related to the widespread occurrence of nitrosamine precursors (nitrates, nitrites, and secondary amines) in foodstuffs and their possible interactions to form the *N*-nitroso compounds. The possibility that such reactions could take place in the mammalian stomach after ingestion of foods containing nitrites and secondary amines has also received attention (Sander *et al.*, 1968; Sen *et al.*, 1969). Nitrates are widely distributed in the human environment and are easily reduced to nitrites. In addition, nitrates and/or nitrites are utilized in this country as preservatives and color additives in smoked fish and meats under prescribed limitations (*Federal Register*, 1961, 1962, 1964, 1969). Only meager information is available on the environmental distribution of secondary amines. The presence of dimethylamine and diethylamine, along with some primary and tertiary types, has been noted in fish and other products, but the characterization of other secondary amines has not as yet been accomplished (Lijinsky and Epstein, 1970).

Unfortunately, the question of whether the aforementioned nitrosation reactions do occur in foods containing nitrites and secondary amines and/or in the mammalian stomach after ingestion of such products has not been sufficiently resolved to permit any definitive conclusions. The major obstacle to progress in the area has been the lack of reliable analytical techniques to provide quantitative determination as well as unequivocal identification of the *N*-nitrosamines. Such identifications are mandatory when dealing with the suspected presence of carcinogenic compounds.

Aside from two reports in the literature which are concerned with relatively high levels of *N*-nitrosodimethylamine formed under unusual conditions, substantive analytical evidence for the presence of nitrosamines in foods has not been presented. Ender *et al.* (1967) utilized various spectrophotometric procedures to characterize *N*-nitrosodimethylamine in herring meal treated with excessive levels of sodium nitrite. Duplessis *et al.* (1968) employed nuclear magnetic resonance and infrared spectrophotometry to confirm the presence of the same compound in the fruit of a solanaceous bush used as food in the Transkei region of South Africa. Ender and Ceh (1968) have also reported low levels of nitrosamines (range, 0.5 to 40 μg/kg) in smoked fish, meat, and mushrooms. However, these authors indicate that it is not possible to guarantee the exact levels and types of nitrosamines, although various methods have been used for detection. They also noted that the possibility of artifacts as well as the risk of contamination during the analysis must be considered. This is in contrast to their earlier studies on fish meal in which, as previously indicated, much larger quantities of *N*-nitrosodimethylamine were involved and confirmation was achieved by infrared spectrophotometry.

Howard *et al.* (1970) reported a gas chromatographic (glc) procedure for the determination of *N*-nitrosodimethylamine in smoked nitrite-treated chub, which had a demonstrated sensitivity of 10 ppb (μg/kg). They also indicated that mass spectrometry could be utilized to confirm the identity of the nitrosamine at levels as low as the aforementioned 10 ppb. Since publication of this work, studies have continued on other species of fish (sable, shad, and salmon) in which nitrites and nitrates are utilized as preservatives and color fixatives (*Federal Register*, 1961, 1962, 1964, 1969). These investigations were deemed necessary, since marine fish are reported to have a higher amine content than freshwater fish (Rea and Shewan, 1949) and, therefore, would present a greater potential for nitrosamine formation. This report describes the results of these studies in which *N*-nitrosodimethylamine has been isolated at levels up to 26 ppb and its identification has been confirmed by mass spectrometry in samples of raw, smoked, and smoked nitrite- and/or nitrate-treated fish. Although confirmation of identity at levels much below 10 ppb involves an inordinate effort, it was undertaken for some samples to elucidate the extent of processing effects on nitrosamine levels.

EXPERIMENTAL

Materials. The apparatus and reagents used in this study have been described in detail by Howard *et al.* (1970). The solvents, methylene chloride, methanol, and pentane, and the Celite 545 were purified and tested prior to use to assure the absence of interfering peaks.

Gas Chromatography. A Barber-Colman 5000 Series Selectra-System gas chromatograph with a modified KCl thermionic detector was employed. A 9 ft × 4 mm i.d. glass coiled column packed with 10% Carbowax 1540 + 5% KOH on 100/120 mesh Gas Chrom Q support was used as an alternative to the original column (10% Carbowax + 3% KOH on Gas Chrom P) with the same chromatographic parameters previously described (Howard *et al.*, 1970). Un-

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der the conditions used, a retention time of 14.5 min was obtained for *N*-nitrosodimethylamine.

Gas-Liquid Chromatography–Mass Spectrometry Apparatus. The instrumentation setup for the glc–mass spectrometry combination was identical to the system described by Damico and Barron (1971) except for the following features. Electron impact ionization was utilized and a three-way valve (Hoke, 305 stainless steel tubing; maximum temperature 180° C) was installed between the exit of the column and the separator so that the column effluent could be isolated from the mass spectrometer system in order to vent the solvent front and thereby prevent the solvent from entering the spectrometer. The glc column was a 9 ft × $^1/_8$ in. o.d. stainless steel column packed with 10% Carbowax 1540 + 5% KOH on 100/120 mesh Gas Chrom Q.

Analytical Procedures. During this investigation, the analytical procedure for the determination of *N*-nitrosodimethylamine in chub fish (Howard *et al.*, 1970) was modified to improve the cleanup and recovery values obtained for salmon, shad, and sable fish.

A 37.5-ml aliquot (equivalent to 25 g sample) of the filtered digest was added to a 2 l. boiling flask containing 175 ml of distilled water, 25 g of KOH, and boiling chips. Using the prescribed apparatus (Howard *et al.*, 1970), the solution was then distilled and a total distillate of 75 ml was collected in a 250-ml separatory funnel. The distillate was acidified with 10 ml of 6 *N* HCl and extracted three times (3 min shaking time for each extraction) with 75 ml portions of methylene chloride. The methylene chloride extracts were pooled in a 500 ml separatory funnel and extracted once with 75 ml of 1 *N* NaOH (3 min shaking time). The lower methylene chloride layer was filtered through 35 g of anhydrous Na_2SO_4 (held in a 60 ml coarse fritted-glass funnel and prewetted with 25 ml of methylene chloride) into a 500 ml Kuderna-Danish evaporator with a 4 ml concentrator tube attached. The Na_2SO_4 was washed with 50 ml of methylene chloride. The remainder of the procedure, including concentration, column chromatography, and gas chromatographic analysis, was as previously described (Howard *et al.*, 1970).

Preparation of Sample for Confirmatory Glc-Mass Spectrometric Analysis. A 300 ml aliquot (equivalent to 200 g of sample) of the filtered digest was transferred to a 5-l. boiling flask containing 1000 ml of distilled water, 136 g of KOH, and boiling chips. The distillation apparatus (Howard *et al.*, 1970) was connected to the boiling flask. The solution was distilled and 90 ml of distillate (principally methanol) was collected in a 500 ml separatory funnel. The funnel was removed and replaced with a 1 l. separatory funnel in which 360 ml of distillate was collected.

The 90 ml methanolic distillate was diluted with an equal volume of distilled water and the solution was shaken for 3 min with 180 ml of methylene chloride. After separation of the layers, the lower layer was passed through 50 g of anhydrous Na_2SO_4 (held in a 60 ml coarse fritted-glass funnel and prewetted with 25 ml of methylene chloride) into a 1 l. Kuderna-Danish evaporator with a 4 ml concentrator tube attached. The extraction was repeated two times and the individual methylene chloride extracts were filtered through the $NaSO_4$. Finally, the Na_2SO_4 was washed with an additional 25 ml portion of methylene chloride. Carborundum grains were placed in the concentrator tube, the distilling column was inserted, and the solvent was carefully concentrated to 1 ml as described by Howard *et al.* (1970).

The 1.0 ml concentrate and 360-ml distillate were transferred to a 5 l. boiling flask containing 61 g of KOH and boiling chips. The solution was then redistilled (as above); 120 ml of distillate was collected in a 500 ml separatory funnel. The distillate was shaken three times (3 min each time) with 120 ml aliquots of methylene chloride. The extracts were passed successively through 50 g of Na_2SO_4 into the Kuderna-Danish evaporator and evaporated to 1 ml, as described above. The 1 ml concentrate was quantitatively transferred to an acid-Celite column (Howard *et al.*, 1970) and the same procedure was followed subsequently. The methylene chloride was concentrated to a final volume of 0.5 ml, and an 8 μl aliquot was injected into the gas chromatograph, using the modified detector, to ascertain the quantity of *N*-nitrosodimethylamine present. The 0.5 ml concentrate was then carefully concentrated to not less than 100 μl, since further evaporation will result in substantial losses of *N*-nitrosodimethylamine. Prior to each confirmation the standard was injected at a concentration equivalent to that found in the sample. [Note: It was established that at least 1 μg of the *N*-nitrosodimethylamine (or a concentration of 10 ng/μl) must be present for confirmation.]

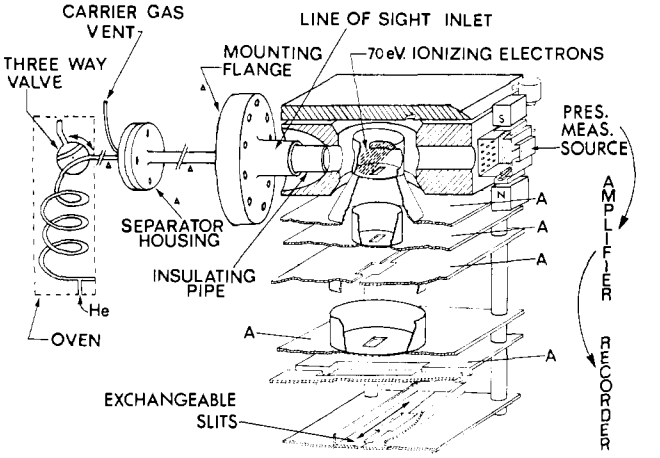

Figure 1. Instrumental setup for glc-mass spectrometric confirmation of *N*-nitrosodimethylamine in fish products. A, Ion optic components of the ion source

Because of these concentration limitations, a 5-μl aliquot (50 ng) of the sample concentrate must be injected on the column in order to obtain an identifiable mass spectrum for confirmation of *N*-nitrosodimethylamine in the fish samples. Accordingly, 5 μl of the sample solution (100 μl total) was injected on the glc column at 70° C with a carrier gas flow of 40 ml per min. The carrier gas vent (see Figure 1) was immediately capped with a silicone septum and the three-way valve was adjusted so that the column effluent was venting to atmosphere in the column compartment. After 3 min, the column was programmed to 120° C at a rate of 10° per min. The three-way valve was adjusted 6 min after the sample injection so that the column effluent was directed against the membrane and the silicone plug was then removed from the carrier gas vent. Initially, the response of the pressure measuring source was negative when the mass spectrometer was isolated from the column effluent. Sometimes, the amplifier must be recompensated during the run because the recorder pen was off scale and attenuating was unsatisfactory, since relatively high sensitivity was needed to detect the *N*-nitrosodimethylamine peak. As the peak eluted from the column, its mass spectrum was recorded at maximum glc response by magnetic scanning. (Note: A background mass spectrum was recorded just before the *N*-nitrosodimethylamine peak.) After the *N*-nitrosodimethylamine eluted from the column, the column effluent was isolated from the

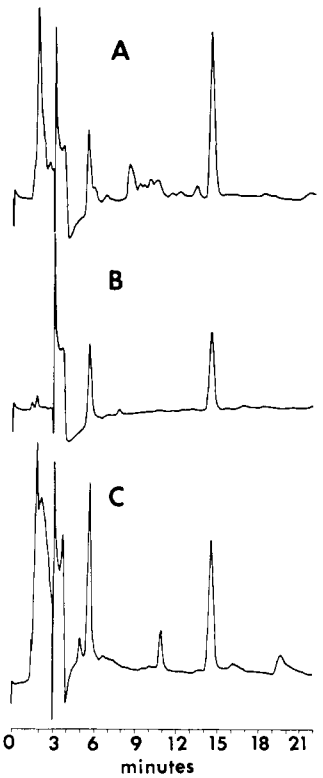

Figure 2. Gas chromatograms of *N*-nitrosodimethylamine extracted from marine fish using modified thermionic detector. A, Isolate from 400 g of smoked nitrite-treated sable; B, isolate from 200 g of smoked nitrate-treated shad; and C, isolate from 400 g of smoked nitrate-treated salmon

Table I. Recoveries of *N*-Nitrosodimethylamine Added at Levels of 10 ppb to 400 g of Fish

Species of Fish	No. of Runs	Range %	Av. %
Salmon	7	71–80	75
Shad	7	71–94	78
Sable	3	72–81	77

Table II. Results of Analysis of *N*-Nitrosodimethylamine in Fish[a]

Type of Processing	*N*-Nitrosodimethylamine Found (ppb)	
	Processing Plant 1	Processing Plant 2
Sable		
Raw	4, 4	4, 4
Smoked	9	5, 4
Smoked, nitrate-treated[b]	14, 12	...
Smoked, nitrate-treated[b]	14, 13	...
Smoked, nitrite-treated	...	8, 9
Smoked, nitrite- and nitrate-treated	...	20, 26
Salmon		
Raw	0	0
Smoked	5	0
Smoked, nitrate-treated	16, 17	...
Smoked, nitrite-treated	...	4, 6
Shad		
Raw	0	...
Smoked, nitrate-treated[b]	10	...
Smoked, nitrate-treated[b]	12	...

[a] Skins and bones removed prior to analysis. [b] Identical samples with exception of packaging.

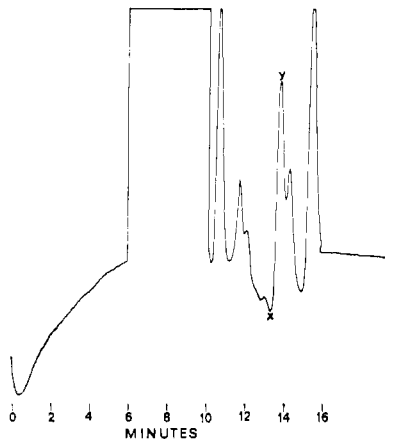

Figure 3. Gas chromatogram of an extract of smoked nitrite-treated sable fish containing *N*-nitrosodimethylamine, utilizing the pressure measuring source as the glc detector

mass spectrometer by adjusting the three-way valve to prevent contamination of the ion source by components with longer retention times than *N*-nitrosodimethylamine.

RESULTS AND DISCUSSION

The sable, salmon, and shad fish utilized in this study were obtained from two processing plants in the United States. The products included raw fish and fish smoked without prior treatment or treated with nitrate, nitrite, or both. All of the smoked fish were subjected to the so-called "hot-smoked process."

Prior to recovery runs, the individual sample lots of fish were comminuted and analyzed for *N*-nitrosodimethylamine by glc. In instances where the nitrosamine was found, the recovery values were adjusted appropriately. As shown in Table I, recoveries of *N*-nitrosodimethylamine added at levels of 10 ppb to 400 g of the sable, shad, and salmon samples ranged from 71 to 94%.

The results of the analyses of the various fish samples collected from the two processing plants are summarized in Table II.

N-Nitrosodimethylamine was found in all the sable samples analyzed at the following levels: raw, 4 ppb; smoked, 4 to 9 ppb; smoked nitrate-treated, 8 to 14 ppb; and smoked, nitrite- and nitrate-treated, 23 ppb (average value of two determinations of one lot). The nitrosamine was not detected in the raw salmon and shad. However, the compound was found in one of the smoked salmon samples at the 5 ppb level and in the smoked nitrate-treated salmon and shad products at levels ranging from 10 to 17 ppb. Representative chromatograms of extracts of smoked nitrite- or nitrate-treated sable, shad, and salmon obtained with the modified thermionic detector are shown in Figure 2.

The presence of *N*-nitrosodimethylamine was confirmed by mass spectrometry in all of the fish samples reported to contain it in Table II. The chromatogram obtained for the smoked nitrite-treated sable, utilizing the pressure measuring source as the glc detector, is presented in Figure 3. The amplifier of the pressure measuring source was recompensated 10 min after the injection of the sample in order to observe peaks with retention times in the region of the *N*-nitrosodimethylamine. This chromatogram contains extraneous peaks which are not observed in the chromatograms (Figure 2) obtained with the modified thermionic detector.

The mass spectrum obtained for the *N*-nitrosodimethylamine (designated as glc peak y, Figure 3) isolated from the smoked nitrite-treated sable is presented in Figure 4 (A).

Table III. Mass Spectral Data for *N*-Nitrosodimethylamine Isolated from Marine Fish Samples and 100 ng of *N*-Nitrosodimethylamine Standard

	Relative Intensities (%) for Samples				
m/e	A	B	C	D	E
26	4.5	3.0	...	3.4	7.5
27	17	20	1.0	20	2.5
28	45	35	...	...	...
29	...	...	...	9.3	...
30	9.0	10	1.0	13	20
38	...	8.0	...	...	10
39	12	19	...	17	15
40	9.0	10	3.1	7.6	12
41	23	17	50	42	20
42	100	100	100	100	100
43	9.0	15	62	34	50
53	...	...	...	...	7.5
54	3.4	...	...	...	2.5
55	...	40	59	43	7.5
56	6.0	...	...	2.5	...
58	...	...	...	25	...
59	1.0	0.50	...	4.2	5.0
69	14	8.0	...	14	...
70	14	10	9.4	12	5.0
74	39	47	62	40	90

[a] A, Raw sable; B, shad—smoked nitrite-treated; C, salmon—smoked nitrate-treated; D, sable—smoked nitrate-treated; and E, 100 ng of *N*-nitrosodimethylamine standard.

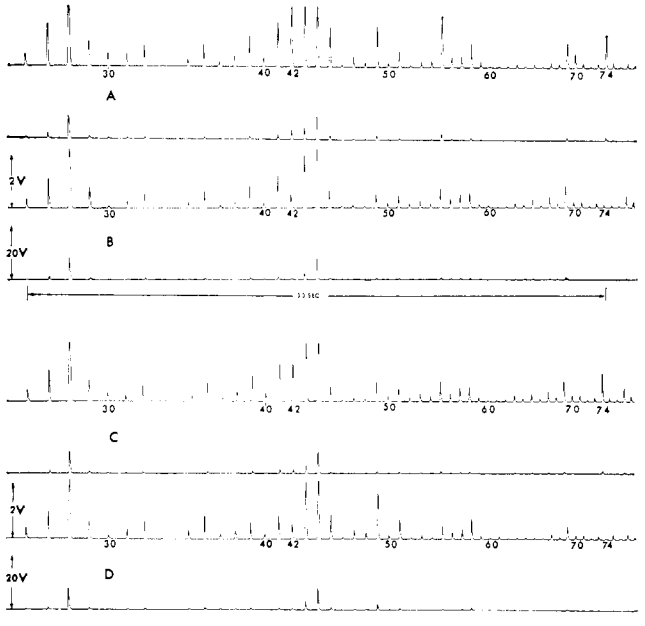

Figure 4. Mass spectra of fish samples and *N*-nitrosodimethylamine. A, *N*-Nitrosodimethylamine isolated from smoked nitrite-treated sable fish; B, background for A; C, 100 ng of *N*-nitrosodimethylamine standard; and D, background for C

The mass spectrum of the background recorded just prior to the elution of the *N*-nitrosodimethylamine peak (designated as x, Figure 3) is shown in Figure 4 (B). Spectra for 100 ng of the *N*-nitrosodimethylamine standard (C) and background (D) (residual methylene chloride and column bleed) are also given. All spectra were recorded with the same instrumental parameters, using two recorder sensitivity ranges.

A comparison of the relative intensities obtained for the *N*-nitrosodimethylamine isolated from the various fish samples and the 100-ng nitrosamine standard after compensation is presented in Table III. An examination of the data reveals that the relative intensities of the *m/e* 74 ion and the other lower mass peaks of the nitrosamine in the fish extracts show some variations from those obtained for the standard. With respect to the lower mass peaks, similar variations were observed in the spectra from successive runs for the 100-ng nitrosamine standard; however, the relative intensities of the *m/e* 74 ion varied only 10 to 17%. The variations in the *m/e* 74 ion may be attributed to the fish background which contributes to the ion current of the nitrosamine at *m/e* 42 (base peak) and, thereby, affects the relative intensity of the mass peak at *m/e* 74. Such variations were not observed in the absolute intensity of the *m/e* 74 ion as presented in Figure 4. The glc response from the pressure measuring source and the maximum peak height of the *m/e* 74 ion obtained for the nitrosamine in the standard and the fish extract, as shown in Table III, were found to be consistent. On the basis of the above data and identical glc retention times we concluded that the component is, in fact, *N*-nitrosodimethylamine.

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