Detection of a New Nitrosamine, *N*-Nitroso-*N*-methylaniline, and Other Nitrosamines in Icelandic Smoked Mutton

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Varying levels (1–48 ppb) of *N*-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR), and a previously undetected nitrosamine, *N*-nitroso-*N*-methylaniline (NMA), were detected in Icelandic smoked mutton. Traces (0.6–2.4 ppb) of *N*-nitrosothiazolidine (NThZ) and low levels (56–475 ppb) of *N*-nitrosothiazolidine-4-carboxylic acid (NTCA), the two *N*-nitroso compounds commonly detected in smoked meats, were also present in such products. Evidence presented suggests that NMA in the meat is produced by the interaction of nitrite and smoke generated by burning sheep dung, the traditional source of fuel used for smoking such products. Nitrosation of laboratory-generated sheep dung smoke produced NMA, as well as NDMA and NPYR. The identity of NDMA, NPYR, and NMA was confirmed by mass spectrometry. It is suggested that the formation of all of the above nitrosamines in Icelandic smoked mutton can be minimized by changing or modifying the method of smoking.

Of all foods, nitrite-cured meats, including fried bacon, have been investigated most thoroughly for the presence of nitrosamines, many of which are potent carcinogens in experimental animals (Preussmann and Stewart, 1984; Sen, 1986; Hotchkiss, 1987; Massey, 1988). This is not entirely unexpected because the curing and smoking process employed for the preparation of such products offers an environment that is conducive to nitrosamine formation (Pensabene and Fiddler, 1983; National Research Council/National Academy of Sciences, 1981; Gray, 1981). Research carried out during the past 15–20 years has provided a better understanding of the mechanism of formation of these compounds, and processing changes have reduced the levels of volatile nitrosamines in the above products (National Research Council/National Academy of Sciences, 1981; Sen, 1986). Recent surveys suggest that most cured meats, except fried bacon, contain only traces (low ppb levels) of volatile nitrosamines, and even that only in sporadic instances (Sen et al., 1979; Canas et al., 1986; Hotchkiss, 1987; Massey, 1988).

During the past few years, scientists have diverted their attention to the search for nonvolatile *N*-nitroso compounds and newer volatile nitrosamines in foods. This has led to the discovery of several new *N*-nitroso compounds such as *N*-nitrosothiazolidine (NThZ) and *N*-nitrosothiazolidine-4-carboxylic (NTCA) in smoked meats (Kimoto et al., 1982; Mandagere et al., 1984; Helgason et al., 1984). Icelandic smoked mutton has received attention for two reasons. First, consumption of such meats by parents has been suggested to induce a high incidence of juvenile diabetes in their male progeny (Helgason et al., 1984). Second, the reported occurrence of NTCA, sometimes at excessively high levels (up to 6.7 ppm), has been observed in Icelandic smoked mutton (Helgason et al., 1984). Other researchers (Sen et al., 1985a; Pensabene and Fiddler, 1985; Tricker et al., 1984) have also detected high levels of NTCA in smoked meats and bacon processed in Canada, the United States, and the U.K. Although little is known about the toxicity of NTCA, it has been shown to induce hyperglycemia in mice after a single intraperitoneal injection (Helgason et al., 1984).

During an investigation of the occurrence of NTCA and other *N*-nitroso compounds in Icelandic smoked mutton, we observed the presence of an unknown volatile nitrosamine that has now been identified to be *N*-nitroso-*N*-methylaniline (NMA). Since this appeared to be the first reported occurrence of NMA in any food and since it is carcinogenic to laboratory animals (Preussman and Stewart, 1984), we studied the source of the contamination and investigated its mechanism of formation.

EXPERIMENTAL SECTION

Caution. Since most nitrosamines are potent carcinogens, proper precaution should be taken while handling or working with these chemicals.

(A) Samples. Samples of Icelandic smoked mutton and other materials (see below) involved in their processing were collected directly from meat packers in Reykjavik and elsewhere in Iceland and sent by air to the Health Protection Branch Laboratories, Ottawa. The meats were analyzed both before and after cooking, which was done by boiling the meat in water for 30 min. All meat samples were cut into small pieces, homogenized with a blender, and stored at +4 or −20 °C until analyzed.

The other ingredients referred to above were nitrite salt, common salt, brine, and Kasslat F, which is an aqueous solution (pH 6–6.5) consisting of dextrose, sodium polyphosphate, lactose, spice oil, sodium ascorbate, monosodium glutamate, and ascorbic acid. Samples of nettings, sheep dung, birch logs, and beech sawdust were also collected. The first four items (nitrite to Kasslat F) were analyzed as such for volatile nitrosamines (Sen et al., 1979; 1985a), whereas the nettings were analyzed for volatile nitrosamines as such and again after deliberate nitrosation (see section E) to determine if they contained any NMA precursors. Aliquots of sheep dung, birch logs, and beech sawdust were burnt in a laboratory kiln, and the smoke generated from each was separately collected and analyzed as described below.

(B) Laboratory Generation of Smoke. A kiln was designed from a small cylindrical tin can (17 cm × 10 cm i.d.) that had an open top and a rectangular (4.5 cm × 6.5 cm) opening at the bottom side wall to allow entry of air during the kilning oper-

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ation. A circular wire-mesh screen was inserted inside the can and placed securely at the middle in a horizontal position; this served as the fuel bed. About 20–25 g of freshly dried (heated for 2–3 h at 55 °C in an oven) sheep dung was first soaked with ~5 mL of ethanol and then placed on the wire-mesh screen. The fire was lighted with a match and the material was allowed to burn for 4–5 min until the flame subsided and the sheep dung began to smoulder. At that point, the smoke was collected by placing an inverted large glass funnel on top of the can and sucking the smoke through Tygon tubing and a gas bubbler (Kontes catalog no. 657000) filled with ~50 mL of 0.1 N HCl. A water aspirator provided the suction. This was continued until the fire in the fuel faded away. If the fire died out prematurely, it was restarted after soaking with another ~2 mL of ethanol, and the whole process was repeated. For dried wood chips and sawdust, the smoke was collected as above but only a 5-g sample was used for generating the fire.

(C) Determination of Volatile Nitrosamines and of NThZ in Smoked Meats. A 20-g aliquot of a homogenized meat sample was analyzed by a low-temperature alkaline vacuum distillation method described previously (Sen et al., 1979, 1985a). *N*-Nitrosodi-*n*-propylamine (NDPA) at a level of 10 ppb was routinely added as an internal standard to each sample before analysis. If the recovery of NDPA was <75% in any sample, the analysis was repeated. Quantitation was carried out by gas–liquid chromatography–thermal energy analysis (GLC–TEA). For the analysis of the common volatile nitrosamines such as *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR), a Carbowax 20M column with added alkali (Sen et al., 1979) was used, whereas a coiled glass column packed with Carbowax 20M without any added alkali (Sen et al., 1985b) or a DB-wax megabore column (30 m × 0.53 mm i.d., 1-μm coating; J&W Scientific Inc., Folsom, CA 95630) was used for the analysis of NMA and NThZ. The operating conditions for the megabore column were as follows: carrier gas (He) flow, 8 mL/min; injector temperature, 65 °C; GLC temperature, 80 °C for 2 min, then increased to 135 °C at 6 °C/min with a hold for 5 min at 135 °C, and then increased to 180 °C at 10 °C/min (held for 10 min).

(D) Determination of *N*-Nitrosoproline (NPRO) and NTCA in Smoked Meats. The analysis was carried out as before (Sen et al., 1985a).

(E) Analysis of Nettings. A 2–3-g aliquot of nettings (cut into small pieces) was analyzed for volatile nitrosamines by the method of Sen et al. (1987). Another 2–3-g aliquot was nitrosated by incubating in the dark overnight with 100 mL of $NaNO_2$ (0.5 mg/mL or 7.2 mM) at pH 3, the mixture was made alkaline by adding 100 mL of 6 N KOH, and the contents were analyzed for NMA by the low-temperature vacuum distillation method as mentioned above.

(F) Analysis of Smoke Condensates before and after Nitrosation. A 10–25-mL aliquot of the smoke condensate collected in dilute HCl (section B) was mixed with 200 mL of 3 N KOH, and the mixture was analyzed for volatile nitrosamines as described in section A. Another 10–25-mL aliquot was nitrosated by incubating overnight with 5 mL of $NaNO_2$ solution (10 mg/mL or 0.145 M) at pH 2, the excess nitrite was destroyed by adding 200 mg of sulfamic acid, and the mixture was analyzed for volatile nitrosamines as above.

(G) High-Performance Liquid Chromatography–TEA (HPLC–TEA) of NMA. A 10–25-μL aliquot of the final extract from section C or F was analyzed by HPLC–TEA (Fine et al., 1976) using the following conditions: HPLC column, 25 cm × 4.1 mm i.d. stainless steel tubing packed with Lichrosorb Si 100 (5 μm) (Altech/Applied Science, State College, PA 16804); mobile phase, 1% acetone in *n*-hexane; flow rate, 2 mL/min; TEA furnace temperature, 550 °C; cold trap, mixture of dry ice and acetone. A Waters Associates solvent delivery system (Model 6000) and a Rheodyne injector (Model 7125; sample loop 20 or 50 μL) were used for the HPLC analysis. After 10 min of sample run the column was flushed with 40% acetone in *n*-hexane for 10 min at 2 mL/min and then equilibrated with the original mobile phase before re-use. Suitable aliquots of NMA standard were analyzed similarly.

(H) Cleanup of Sample Extracts for Mass Spectrometric (MS) Confirmation. Selected meat extracts (from section C) that were positive for NMA, NDMA, or NPYR were cleaned up on a basic alumina column (Sen et al., 1985a), except that the alumina was deactivated by adding 1.5% instead of 3% water and the column was made from 10 g (instead of 5 g) of alumina in a 1 cm i.d. column. The cleaned-up extract was concentrated to 0.5 mL, and a 1–2-μL aliquot was used for GLC–MS.

For the nitrosated smoke condensates, a much more elaborate cleanup procedure was used. It employed a two-step process involving fractionation on a basic alumina column followed by further cleanup, using a gradient elution technique on a reverse-phase C-18 extraction tube (Supelco Canada Ltd., Oakville, Ont). The alumina cleanup was similar to that used for the meat extracts, except that five separate 10-mL dichloromethane (DCM) eluates were collected instead of one 50-mL fraction. This allowed finer separations of different nitrosamines from each other and from impurities. Each DCM fraction was carefully concentrated to 1 mL with a Kuderna-Danish (K-D) concentrator, and an aliquot was analyzed by GLC–TEA or HPLC–TEA. Only the positive fractions were used for cleanup on C-18 extraction tubes as described below.

Water (2 mL) was added to each of the positive fractions, and the sample was heated in a water bath (55–65 °C) until all DCM was evaporated as described before (Sen et al., 1990). The aqueous residue was passed through a C-18 extraction tube (3 mL) and eluted with 3-mL portions of water, 5% methanol in water, 20% methanol, 50% methanol, and 75% methanol. Each of these eluates was separately extracted with DCM, the DCM extract was concentrated to 1.0 mL (using a K-D concentrator), and 2–10 μL was analyzed by GLC–TEA or HPLC–TEA. The fractions containing the nitrosamine of interest were used for GLC–MS confirmation. The details of the two-step cleanup process were reported by Sen et al. (1990).

(I) GLC–MS Confirmation. The identity of NMA in the cleaned-up extracts of nitrosated smoke was confirmed by MS using both the selected ion monitoring technique (resolution 1 in 8000) and repetitive exponential scanning (full-scan MS), whereas only the latter technique was used for the identification of NDMA and NPYR in such extracts. A VG analytical hybrid mass spectral system (Model 7070 EQ) was used for this purpose. The GLC conditions and MS parameters used were similar to those described previously (Sen et al., 1989).

For GLC–MS confirmation of NMA in smoked meat, the selected ion monitoring (SIM) technique using two fragment ions, namely, the molecular ion at m/z 136.0637 and the ion at m/z 106.0657, at a resolution of 8000 (10% valley definition) and tandem mass spectrometry (MS/MS) were used. In the MS/MS mode, the instrument was operated in the configuration EBQQ (E = electric sector, B = magnetic sector, and Q = quadrupole) (Weber et al., 1988; Sen et al., 1990). Argon (at a total analyzer pressure of 4×10^{-7} Torr) was used as the collison gas, and the collison energy was set at 19 eV. The reactions monitored were m/z 136 → 106 and m/z 136 → 77. The identity of NDMA in one sample of smoked meat was confirmed by the SIM technique using the molecular ion at m/z 74.0480.

RESULTS AND DISCUSSION

Table I presents the levels of both volatile and nonvolatile *N*-nitrosamines detected in nine samples of Icelandic smoked mutton. Of the volatile nitrosamines, traces (1–8.8 ppb) of NDMA were present in most of the samples, NPYR and NThZ being present less frequently and in lower concentrations (1–2.4 ppb) than NDMA. The unusual volatile nitrosamine detected was NMA, initially identified on the basis of coincidental retention times on both GLC–TEA and HPLC–TEA (Figures 1 and 2). Since TEA is a highly selective detector, positive results on both GLC and HPLC systems are usually considered good evidence for the presence of a particular *N*-nitroso compound (Fan et al., 1978). In addition, the identity of NMA in one sample of Icelandic smoked mutton (Table I) was confirmed by GLC–SIM-MS and GLC–MS/MS.

Table I. Levels of Volatile and Nonvolatile Nitrosamines Detected in Icelandic Smoked Mutton

sample no. and type	levels (ppb) of nitrosamines[a]				
	NDMA	NPYR	NThZ	NTCA	NMA
1, raw	8.8	1.1	2.4	426	47.6
1, cooked	2	1	2.4	130	34.7
2, raw	N[b]	N	N	475	N
2, cooked	N	N	N	201	N
3, cooked	N	N	1	274	4.7
4, cooked	N	N	1.5	390	20
5, raw	1.6	N	N	153	3
5, cooked	1.3	N	N	143	3
6, raw	1.3	N	N	188	8.6
6, cooked	1.0	trace	trace	94	3.5
7, raw	2.5	1.2	N	227	3
7, cooked	6	1.3	0.6	111	2
8, raw	4.3[c]	1.3	N	256	6.4[d]
8, cooked	6.3	1.1	trace	275	4.7
9, raw	N	N	N	56	0.8
9, cooked	1.9	0.6	N	*e*	4.5

[a] Abbreviations: NDMA = *N*-nitrosodimethylamine, NPYR = *N*-nitrosopyrrolidine, NThZ = *N*-nitrosothiazolidine, NTCA = *N*-nitrosothiazolidine-4-carboxylic acid, and NMA = *N*-nitroso-*N*-methylaniline. [b] N = negative (detection limits 0.1–1 ppb). [c] Confirmed by GLC–SIM-MS. [d] Confirmed by GLC–SIM-MS and GLC–MS/MS. [e] Not analyzed.

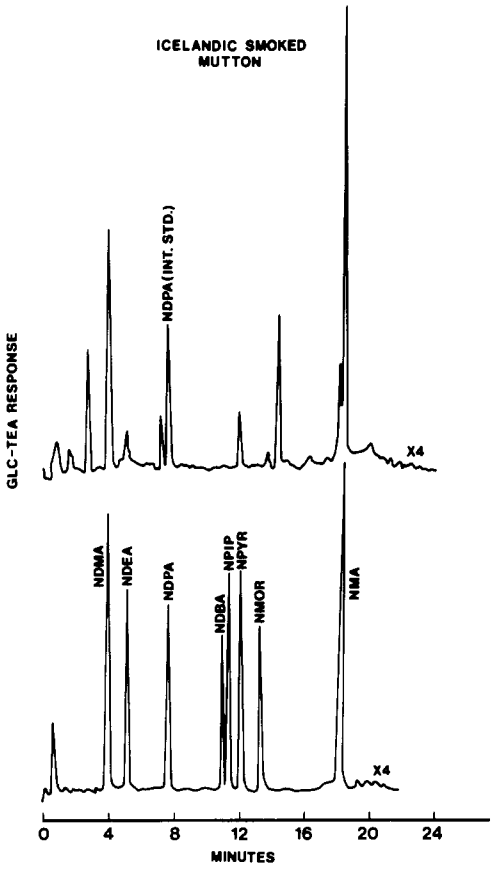

Figure 1. Chromatograms from GLC–TEA: (bottom) 200–500 pg of various *N*-nitrosamine standards; (top) 2.7 μL/1.0 mL final extract of an Icelandic smoked mutton (raw) (sample 1 in Table I). The small peak just before the NMA peak in the meat sample is due to NThZ. The analysis was carried out with the megabore column.

Its concentration in the positive mutton samples ranged between 0.8 and 47.6 ppb.

This appears to be the first reported finding of NMA in any food. It was detected in all but one sample, and except in three cases, its concentration in the smoked meats was <10 ppb.

NTCA was the main nonvolatile *N*-nitroso compound

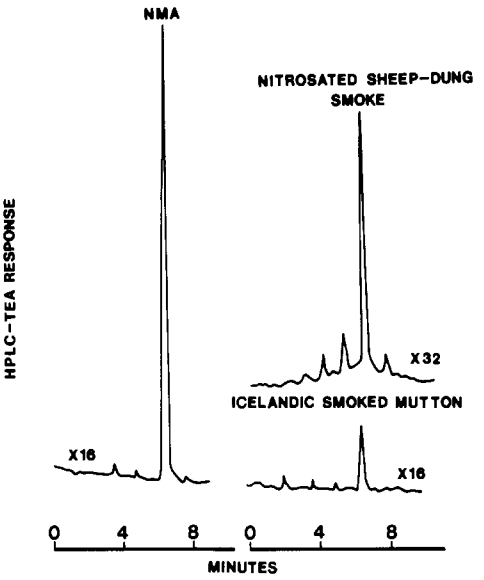

Figure 2. Chromatograms from HPLC–TEA: (left) 72-ng NMA standard; (right bottom) 20 μL/0.9 mL final extract of an Icelandic smoked mutton sample; (right top) 10 μL/1.0 mL cleaned-up extract from nitrosated sheep dung smoke.

detected in these samples. However, its levels were much lower than those reported previously for such products (Helgason et al., 1984). Low levels of NPRO (10–50 ppb) were also detected in some samples.

The methodologies used for the determination of both volatile and nonvolatile nitrosamines in meats are well established and have been shown to give good recoveries of various nitrosamines (Sen et al., 1979, 1985a). Additional studies were, however, carried out to reconfirm the accuracy of the method by analyzing Icelandic smoked mutton spiked with 10–25 ppb NMA. The recoveries of added NMA were highly satisfactory (70–90%). When such samples were analyzed with added morpholine (5 mg), no artifactual formation of NMOR was observed. Similar studies carried out previously (Sen et al., 1985a) have also demonstrated that the method for NTCA and NPRO is not prone to artifactual formation.

Since the finding of NMA in these meats was unusual, further studies were conducted to investigate its mechanism of formation. NMA might have formed due to the interaction of nitrite and amine additives in the nettings because such interactions had been previously shown to be the cause of *N*-nitrosodi-*n*-butylamine formation in cured pork products packaged in elastic rubber nettings (Sen et al., 1987). This was, however, found to be unlikely because the nettings gave negative results for NMA even after deliberate nitrosation. The other ingredients (nitrite salt, common salt, brine, and Kassalat F) also gave negative results for NMA. Four samples of raw lamb meat, each procured from different retail outlets in Ottawa, were also negative for NMA even after deliberate nitrosation.

The investigation was then concentrated on the smoking methods used. Traditionally, such meats are processed by a direct smoking process using smoke generated by burning sheep dung. The lamb carcasses are cured by immersing in brine at 6–10 °C for up to 7 days or by injecting brine. The pieces of meat are then briefly rinsed with cold water and smoked in a smokehouse for 48–72 h. The smoke-generating fire is lit in two open metal drums on the floor of the kiln that contains sheep dung (often mixed with hay) as the fuel. This is a unique process different from that used in North America, where smoke is normally produced by burning wood chips or sawdust.

Table II. *N*-Nitrosamines Detected in Unnitrosated and Nitrosated Smoke Condensates Generated by Burning Sheep Dung and Birch or Beech Wood Chips

sample no. and type	ng of nitrosamine detected per 10–25 mL of smoke condensate					
	unnitrosated smoke condensate[a]			nitrosated smoke condensate[a]		
	NDMA	NPYR	NMA	NDMA	NPYR	NMA
A (sheep dung)	364	N[b]	N	7410	1131	1200
B (sheep dung)	259	N	N	11950[c]	5344[c]	1360[c]
C (sheep dung)	*d*	*d*	*d*	2455	324	2800
D (sheep dung)	*d*	*d*	*d*	670	N	1700
D (sheep dung), without ethanol as starter fuel	*d*	*d*	*d*	1935	N	2500
E (beech)	N	N	N	N	N	N
F (beech)	N	N	N	N	N	N
G (birch)	N	N	N	N	N	N
H (birch)	N	N	N	N	N	N

[a] Except in sample D, ethanol was used as a starter. [b] N = none detected (limit 10–100 ng per 25 mL of smoke condensate). [c] Confirmed by GLC–full-scan MS. [d] Not analyzed.

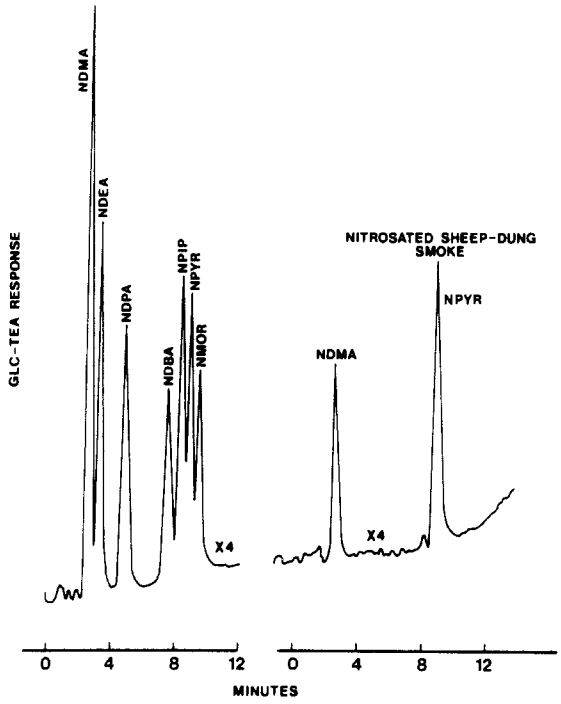

Figure 3. Chromatograms from GLC–TEA of nitrosated sheep dung smoke using the Carbowax 20M column with added alkali: (left) ca. 500 pg of each nitrosamine standard; (right) 6 μL/5.0 mL of a cleaned-up nitrosated sheep dung smoke condensate (fraction 5 after fractionation on basic alumina) showing the presence of only NDMA and NPYR (NMA eluted earlier in fraction 3 on basic alumina).

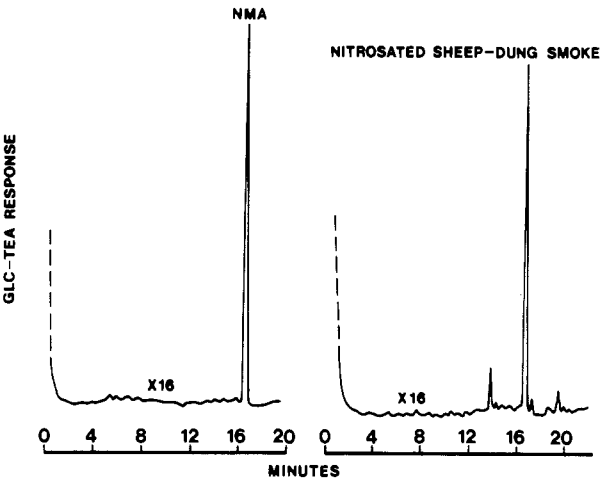

Figure 4. Chromatograms from GLC–TEA using the megabore column: (left) 2.2-ng NMA standard; (right) 2 μL/1.0 mL of the nitrosated sheep dung smoke condensate analyzed in Figure 3 after clean up on both alumina and C-18 extraction tube.

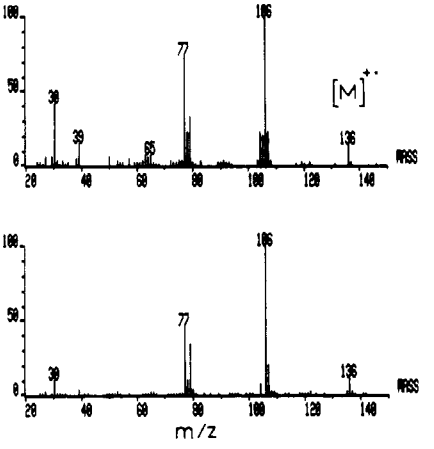

Figure 5. (Bottom) Electron impact ionization mass spectra (background-subtracted) of NMA standard. (Top) Material isolated from nitrosated sheep dung smoke condensate.

The results of the analysis of laboratory-generated smoke condensates are presented in Table II. The smoke condensates from the wood chips (beech or birch) were all negative for NDMA, NPYR, and NMA even after deliberate nitrosation, suggesting that such smokes did not contain significant levels of nitrosamines or the respective precursor amines. The unnitrosated sheep dung smoke samples contained only low levels of NDMA. The corresponding nitrosated samples contained all three nitrosamines (NDMA, NPYR, and NMA), suggesting that these smoke condensates contained the corresponding precursor amines. Typical chromatograms from these analyses are shown in Figures 2–4. The identity of NDMA, NPYR, and NMA in the cleaned-up extracts of nitrosated sheep dung smoke was confirmed by GLC full-scan MS analysis. Only the mass spectrum of the isolated NMA and that of the authentic standard are shown in Figure 5. The two spectra are very similar, showing the presence of all the important fragments at m/z 136 ($[M]^{\cdot+}$), 106 ($[M - NO]^{\cdot+}$), 77 ($[C_6H_5]^+$), and 30 ($[NO]^+$). The mass spectra of NDMA and NPYR isolated from the same sample also agreed well with those of the standards (not shown).

The findings of NDMA, NPYR, and NMA precursors in sheep dung smoke is of interest because during the prolonged period of smoking, lasting up to 72 h, interaction between nitrite and these precursors could form the above nitrosamines. It is of interest that traces of the same three nitrosamines were also detected in most Icelandic smoked mutton. Wood chips mainly consist of cellulose and other carbohydrates, whereas sheep dung

like many animal feces contain amines and various other nitrogen-containing compounds (Fruton and Simmonds, 1959). Dimethylamine has also been reported to be excreted in rat urine (Asatoor and Simenhoff, 1965). Similarly, smoke generated by burning cow dung may also produce traces of the above nitrosamines if such smoke is used for processing nitrite-treated foods. In India and many other developing nations, dried cow dung cakes are extensively used as a fuel for cooking.

Bacon and meats processed by old-fashioned smoking methods, in which smoke generated by burning wood chips or sawdusts was allowed to enter directly in the smokehouse, contained higher levels (1400–9000 ppb) of NTCA than those processed with liquid wood smoke (these contained undetectable to 328 ppb NTCA) (Sen et al., 1986). Smoke generated by the former process probably provided higher levels of formaldehyde, which is one of the precursors of NTCA, than when liquid smoke was used. The fact that Icelandic smoked mutton is also processed by a direct smoking method may explain why these products were earlier found to contain extremely high levels (up to 6760 ppb) of NTCA. It is suggested that changing the method of smoking from sheep dung smoke (direct smoking) to a liquid wood smoke spray process may reduce the levels of all four compounds, namely, NDMA, NPYR, NMA, and NTCA, in Icelandic smoked mutton. Additional research is, however, needed to determine the effectiveness and feasibility of this proposal.

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