Determination and Occurrence of 2-(Hydroxymethyl)-*N*-nitrosothiazolidine in Fried Bacon and Other Cured Meat Products

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A sensitive method for the determination of 2-(hydroxymethyl)-*N*-nitrosothiazolidine (HMNThZ), a nonvolatile *N*-nitroso compound, in fried bacon and miscellaneous cured meats has been developed. The method consists of extraction of the sample with acetonitrile/glacial acetic acid (100:1) in the presence of small amounts of ascorbyl palmitate and sulfamic acid (both are N-nitrosation inhibitors), removal of fats and lipids by liquid–liquid partitioning with isooctane, cleanup on basic alumina containing 6% water, and analysis of the cleaned-up extract by high-performance liquid chromatography–thermal energy analyzer (HPLC–TEA). The detection limit of the method is about 1–2 ppb. Recoveries of HMNThZ added to fried bacon at 20–50 ppb levels ranged between 80 and 95%. Of various samples analyzed, 12/16 fried bacon (mean, 5.3 ppb; range, negative to 13 ppb), 0/7 raw bacon, and 1/9 miscellaneous cured meats (a smoked ham, 2.8 ppb) gave positive results. Confirmation was done by gas–liquid chromatography–thermal energy analyzer (GLC–TEA) analysis after trimethylsilylation, and also by GLC–TEA, HPLC–TEA, and GLC–mass spectrometric analyses after derivatization of HMNThZ to its *O*-methyl ether.

Recent studies have shown that fairly high levels of *N*-nitrosothiazolidine-4-carboxylic acid (NTCA) and traces of *N*-nitrosothiazolidine (NThZ) may be present in a wide variety of smoked meat products including bacon (Helgason et al., 1984; Ikins et al., 1986; Pensabene and Fiddler, 1983; Sen et al., 1985, 1986; Tricker et al., 1984). A combination of formaldehyde in the smoke and nitrite additive used for curing is responsible for the formation of these compounds. The interaction between formaldehyde and cysteine or cysteamine is known to produce thiazolidine-4-carboxylic acid and thiazolidine, respectively, which can be nitrosated easily to produce NTCA and NThZ (Pensabene and Fiddler, 1985; Ikins et al., 1986; Sen et al., 1986). Further research has also established that NTCA present in raw smoked bacon can undergo heat-induced decarboxylation during frying to produce NThZ (Sen et al., 1985; Ikins et al., 1986).

Formaldehyde, however, is not the only aldehyde that can react with cysteine or cysteamine. Other aldehydes such as glycolaldehyde, acetaldehyde, glyoxal, and D-glucose react with the above amines to produce various nitrosatable thiazolidine derivatives (Umano and Shibamoto, 1984). Since many of these carbonyl compounds are present in foods, especially smoked foods, there is a likelihood of formation of various thiazolidine derivatives in such products. In fact, Massey et al. (1985) recently detected ppm levels of 2-(hydroxymethyl)-*N*-nitrosothiazolidine-4-carboxylic acid (HMNTCA) in smoked bacon. The reaction between glycolaldehyde and cysteine is believed to produce the thiazolidine precursor of this nitrosamino acid. The above-mentioned workers, however, did not analyze fried bacon for the corresponding decarboxylation product, namely, 2-(hydroxymethyl)-*N*-nitrosothiazolidine (HMNThZ), which could form during frying due to decarboxylation of HMNTCA. The present study was, therefore, initiated to determine whether HMNThZ does indeed occur in smoked fried bacon or other smoked meats. The paper describes a sensitive method for the determination of HMNThZ in such products and presents some data on the concentrations of this compound in smoked bacon (both raw and fried) and other smoked meats. Although not much is known about the carcinogenicity of HMNTCA and HMNThZ, the latter is reported to be mutagenic (Umano and Shibamoto, 1984).

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MATERIALS AND METHODS

Materials. Cysteamine hydrochloride and glycolaldehyde were purchased from Sigma Chemical Co., St. Louis, MO. Glass-distilled dichloromethane (DCM) was purchased from American Burdick and Jackson Co., Muskegon, MI, and other glass-distilled solvents such as acetone, acetonitrile, isooctane, toluene, and ethyl acetate were obtained from Caledon Laboratories, Georgetown, Ontario. Basic alumina for column chromatography was purchased from ICN Biomedicals, Inc., K & K Laboratories, Plainview, NY. Methyl iodide and sodium hydride (~80% dispersion in oil) were obtained from Aldrich Chemical Co., Milwaukee, WI, and BDH Chemical Co., Toronto, Ontario, respectively. All other reagents used were of analytical grade.

HMNThZ standard was synthesized by reaction of cysteamine hydrochloride, glycolaldehyde, and nitrite as described by Umano and Shibamoto, (1984). The crude product was cleaned up on a basic alumina column (3% water content) to remove *N*-nitrosothiazolidine, which formed as an impurity. The column was loaded with the crude product and washed with DCM to remove NThZ. HMNThZ was then eluted with ethyl acetate containing 10% ethanol. The excess solvent was removed by flash evaporation using a rotary evaporator. The oily residue was transferred into a screw-cap vial and stored at −20 °C until used. The purity of the compound was checked by high-performance liquid chromatography–thermal energy analyzer (HPLC–TEA) and by combined gas–liquid chromatography–mass spectrometry (GLC–MS). The mass spectrum of the compound was quite clean and contained the relevant fragment ions conforming to the structure of HMNThZ (Figure 1). The molecular ion, at m/z 148, was very weak. The fragment ions at m/z 118, 88, and 60 were attributed to $(M - NO)^+$, $(M - NO - CH_2O)^+$, and $(C_2H_4S)^+$, respectively.

Caution. Since most nitrosamines are carcinogenic, proper precautions should be taken while handling or working with the compound.

Samples. Smoked bacons and miscellaneous smoked meats were purchased from local retail outlets. The bacon samples were fried and homogenized as described previously (Sen et al., 1985). The samples were stored at 4 °C and analyzed within 1 week.

High-Performance Liquid Chromatography–Thermal Energy Analyzer. A TEA detector, Model 502 (Thermedics Inc., Woburn, MA), was used as a detector for the HPLC analysis. It

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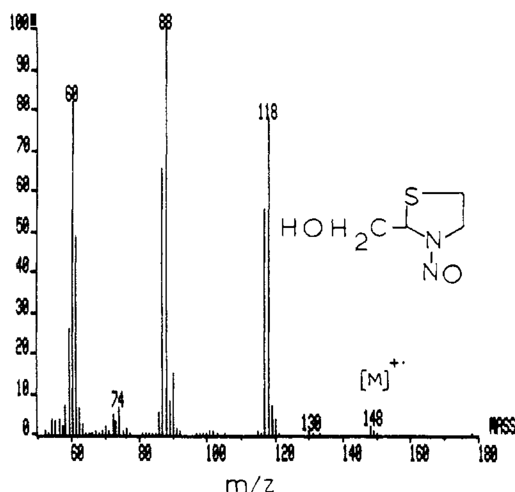

Figure 1. Electron-impact ionization mass spectrum of HMNThZ.

was operated as described previously (Fine et al., 1976a; Sen et al., 1988). A stainless steel column (25 cm × 4.1 mm (i.d.)) packed with Lichrosorb Si 100 (5 μm) (Alltech/Applied Science, State College, PA) was used for HPLC separation. Two Waters Associates Pumps (Model 6,000) and a Waters Associates solvent programmer (Model 660) were used for mobile-phase delivery. Mobile phase flow rate was 2 mL/min. For preliminary screening of the underivatized sample extract for HMNThZ, the HPLC separation was carried out in the isocratic mode with 20% acetone in *n*-hexane as the mobile phase. For HPLC–TEA analyses of the *O*-methyl ether derivative of HMNThZ and of the derivatized extract, solvent programming was employed. Mobile phase and programming conditions were as follows: 5% acetone in *n*-hexane initially and then gradually increased to 20% acetone in *n*-hexane over 15 min with the linear programming curve (curve 6) of the solvent programmer.

Gas–Liquid Chromatography–Thermal Energy Analyzer (GLC–TEA). GLC–TEA analysis was carried out as reported previously (Fine et al., 1975), but instead of using a cold trap, a CTR gas stream filter (Thermedics Inc.) was used. Two different columns were used depending on the derivative of HMNThZ analyzed: (a) 10 ft × $^1/_4$ in. coiled glass column packed with 3% OV-225 on 80–100-mesh Chromosorb WHP. The column was temperature programmed from 100 to 220 °C (held for 2 min at 100 °C) at 10 °C/min. This was used for the analysis of trimethylsilyl (TMS) derivative of HMNThZ. Carrier gas (Ar) flow was 30 mL/min; and injection temperature, 220 °C. (b) 30-m megabore column (0.52-mm i.d.), coated with DB-Wax, film thickness 1 μm (J&W Scientific Inc., Rancho Cordova, CA). Column temperature was programmed from 120 to 220 °C at the rate of 4 °C/min. Carrier gas (Ar) flow was 8 mL/min; and injector temperature, 60 °C. The above conditions were used for the analysis of HMNThZ as its *O*-methyl ether derivative. The transfer line (between GLC and TEA) temperature in both cases was 300 °C.

Mass Spectrometric Confirmation. A VG analytical hybrid mass spectrometer (Model 7070 EQ) attached to a Varian (Model Vista 6000) gas chromatograph was used for GC–MS confirmation, as described previously (Kushwaha and Sen, 1988; Weber et al., 1988). Both selected ion monitoring under high resolution (6000) and complete mass spectra were used for confirmation. A fused silica column of 30 m × 0.22 mm (i.d.) DB-Wax (film thickness, 0.25 μm) (J&W Scientific) was used for the GC separation. Temperature programming conditions for the analysis of HMNThZ as its *O*-methyl ether: 60 °C for 1 min and then heated to 200 °C at the rate of 4 °C/min.

Determination of HMNThZ. A 20-g aliquot of a homogenized sample was mixed with 0.3 g of ascorbyl palmitate, 0.5 g of sulfamic acid, 2 mL of glacial acetic acid, and 10 mL of acetonitrile. After 5 min, the mixture was extracted for 2 min with 100 mL of acetonitrile on a Sorval Omni-Mixer (Du Pont Instruments, Newtown, CT). About 50 g of anhydrous sodium sulfate (granular) was added to the mixture and the blending continued for an additional 1 min. The supernatant was carefully decanted and filtered through a Whatman No. 1 filter paper already containing ~20 g of granular anhydrous sodium sulfate. The filtrate was collected in a 500-mL separatory funnel. The residue in the mixer was extracted further with two 100-mL portions of acetonitrile, and each time the extract filtered and collected as above. The residue was discarded.

The combined acetonitrile extracts were partitioned with two 40-mL portions of isooctane (to remove fats and lipids), and the isooctane layers were discarded. The main extract was transferred to a 1-L round-bottom evaporating flask and the solution concentrated to ~2 mL (avoiding going to dryness) on a rotary evaporator (water bath ~50 °C). About 50 mL of toluene was added to the residue and the mixture concentrated to ~2 mL as above. The procedure was repeated twice, each time after addition of 50 mL of toluene. This was done to remove acetonitrile, which distilled off in the form of an azeotropic mixture with toluene. Thus, the components in the final extract was transferred to a nonpolar solvent—a condition needed for the alumina cleanup step described below.

The concentrated residue (in ~2 mL of toluene) was diluted with 10 mL of *n*-pentane, and the mixture was filtered through a coarse sintered glass funnel containing an ~1-cm layer of granular anhydrous sodium sulfate. The filtrate was directly applied to a column (2 cm × 15 cm) containing 10 g of basic alumina deactivated by adding 6% water and allowed to pass through at ~2 mL/min. The residue in the evaporating flask was rinsed with four 10-mL portions of *n*-pentane, and the rinsings were filtered and passed through the column as above. The effluent from the column was discarded. The sintered glass funnel was removed from the top of the column, and the column was successively eluted with 50-mL portions of (a) dichloromethane and (b) ethyl acetate containing 10% ethanol. HMNThZ, if present, eluted in fraction b and was first concentrated to 5 mL on a rotary evaporator. The extract was then quantitatively transferred into a graduated test tube and the solution blown down with a gentle stream of nitrogen to 1.0 mL (kept partially immersed in a ~20 °C water bath).

A 20–50-μL aliquot of the above solution was analyzed by HPLC–TEA in the isocratic mode as described above. If the result was positive for HMNThZ, the sample was concentrated to ~0.1 mL by blowing down with nitrogen as above and then derivatized by heating with *N*,*O*-bis(trimethylsilyl)acetamide (0.25 mL) as reported previously (Sen et al., 1982). The mixture was diluted to 1 mL with anhydrous acetone and a 5-μL aliquot analyzed by GLC–TEA under the conditions described above. A 2-μL aliquot of this solution was also used for GLC–MS confirmation.

Preparation of the *O*-Methyl Ether Derivative of HMNThZ. Five 20-g aliquots of fried bacon were separately extracted and cleaned up as above. The 1-mL concentrated eluates (fraction b) from the alumina columns were combined and then divided into two approximately equal portions. Each was derivatized as described below:

The solution was evaporated to ~0.1 mL by blowing down with nitrogen and then rediluted to ~1 mL with anhydrous ethyl acetate (dried over Drierite). The solution was mixed well and again evaporated to ~0.1 mL as above. About 30 mg of sodium hydride and 0.5 mL each of methyl iodide and anhydrous ethyl acetate were added to the above concentrated extract; the test tube was stoppered and mixed well for 1 min on a Vortex mixer. After 30 min the sample was mixed again for 30 s. After being allowed to stand overnight in the dark, the sample was mixed carefully with 5 mL of water (added dropwise at the beginning) and the mixture extracted with two 30-mL portions of DCM. The combined DCM extract was dried by passing through anhydrous sodium sulfate and concentrated to ~1 mL on a Kuderna-Danish concentrator (Sen and Seaman, 1981). Finally, the two derivatized extracts were combined and concentrated to 1.0 mL under a gentle stream of nitrogen. Suitable aliquots were analyzed by HPLC–TEA (10–40 μL injected), GLC–TEA (2 μL), and GLC–MS (2 μL) under the conditions described above. Aliquots of HMNThZ standard (1 and 10 μg) were also derivatized and analyzed as above for identification purposes.

RESULTS AND DISCUSSION

The overall method using HPLC–TEA for detection worked fairly well for the determination of HMNThZ in

Table I. Levels of HMNThZ Detected in Various Smoked Meat Products

	sample (producer)	level detected, ppb
	Bacon	
1	raw (A)	N[a]
	fried	1.4
2	raw (double-smoked) (B)	N
	fried	1.9
3	raw (C)	N
	fried	9.2
4	raw (A)	N
	fried	3.2
5	raw (D)	N
	fried	11.9
6	raw (G)	N
	fried	10.2
7	raw (G)	N
	fried	4.5
8	fried (C)	N
9	fried (E)	N
10	fried (F)	5.3
11	fried (C)	8.2
12	fried (D)	N
13	fried (D)	N
14	fried (C)	13
15	fried (D)	4.3
16	fried (D)	12
	Miscellaneous Cured Meats (9 Samples)	
	Black Forest ham, cooked ham, Old Vienna sausage, Polsk kielbasa sausage, smoked meat, skillet strips	2.8 (a smoke ham)

[a] N = negative (detection limit 1–2 ppb).

smoked meats including bacon. The percentage recoveries of HMNThZ added to fried bacon and smoked meats at 20–50 ppb levels ranged between 80 and 95, with a minimum detection limit of about 1–2 ppb. In a previous study (Sen et al., 1988) it was demonstrated that initial treatment of the sample with ascorbyl palmitate, sulfamic acid, and acetic acid effectively inhibited artifactual formation of nitrosamines from easily nitrosatable amines such as morpholine, methylphenylamine, and dibenzylamine. Hence, these chemicals were incorporated in the extracting medium. The extraction and cleanup procedures were adapted from two of our earlier methods developed for the determination of nonvolatile *N*-nitroso compounds in foods (Sen et al., 1982, 1988) whereas that for the defatting procedure was based on a technique reported by Fine et al. (1976b).

In total, 16 samples of fried bacon, 7 of raw bacon and 9 of miscellaneous smoked meats were analyzed (Table I). Of these, 12 samples of fried bacon and 1 ham contained detectable levels of HMNThZ. The average level of HMNThZ in fried bacon was 5.3 (range, negative to 13 ppb), which is slightly lower than that for *N*-nitrosopyrrolidine (mean, 10 ppb) but similar to that for NThZ (mean, 6.3 ppb) commonly detected in this product (Sen, 1986; Sen et al., 1985). None of the other smoked meats analyzed including the raw bacons contained any detectable levels of HMNThZ.

The identity of HMNThZ in 5 samples of fried bacon (No. 3, 5, 11, 14, and 15 in Table I) was confirmed by GLC–TEA analysis of the TMS derivative. Some typical chromatograms from HPLC–TEA and GLC–TEA analyses are shown in Figures 2 and 3, respectively.

Although the silylation technique offered a simple and rapid technique for confirming the initial HPLC–TEA findings, there were some difficulties in confirming the results by GLC–MS. Because of the presence of many interfering materials in these extracts, the selected ion monitoring technique was not suitable. Therefore, MS

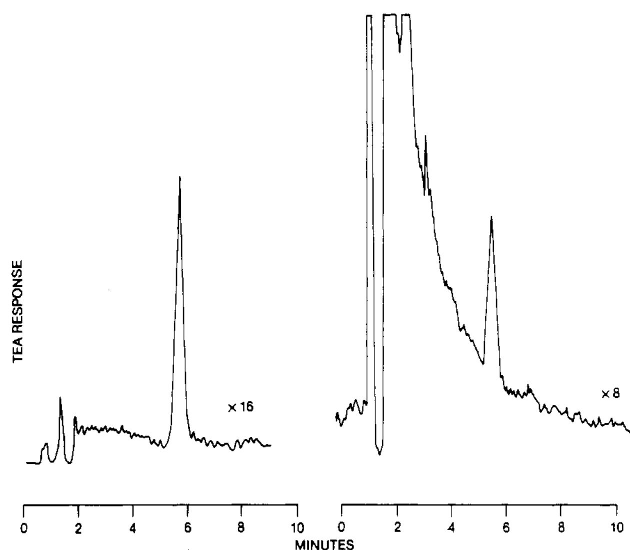

Figure 2. Chromatograms from HPLC–TEA analysis of HMNThZ standard (30 ng) (left) and of a fried bacon (no. 3 in Table I) sample (right). Analysis was carried out with the isocratic solvent system (see the Experimental Section).

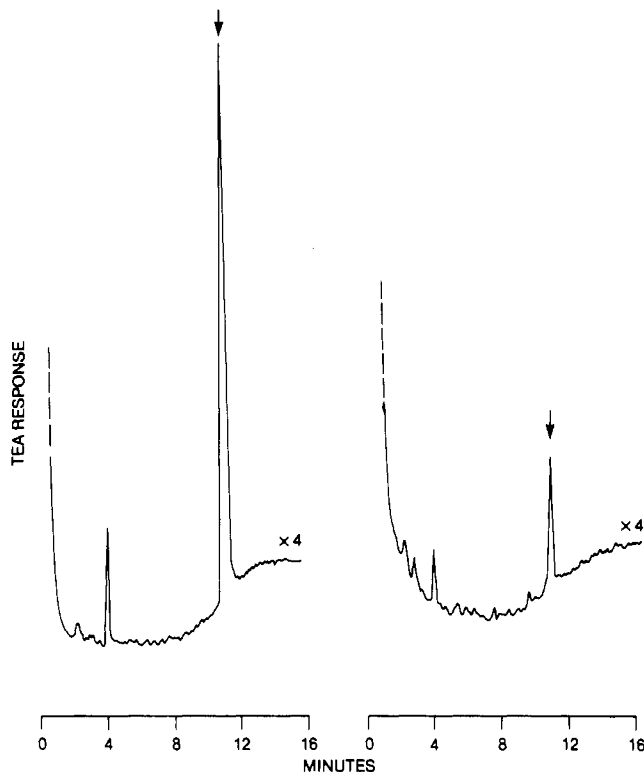

Figure 3. Chromatograms from GLC–TEA analysis of HMNThZ standard (3 ng) (left) and of the same fried bacon extract (no. 3 in Table I) (right). Both analyzed after trimethylsilylation.

confirmation in the case of two silylated fried bacon extracts was carried out by repetitive exponential scanning (1.1 s/decade) in the mass range 25–250. The reconstructed mass spectrum from the derivatized sample extracts contained some of the characteristic fragment ions of HMNThZ (trimethylsilyl derivative). However, the confirmation was not conclusive. Also, due to the instability of this derivative, further attempts to clean up the derivatized extracts were unsuccessful.

In view of the above difficulties, we prepared the O-methylated derivative. This produced a stable compound amenable to further cleanup as well as to analysis by the all three techniques, namely, HPLC–TEA, GLC–TEA, and

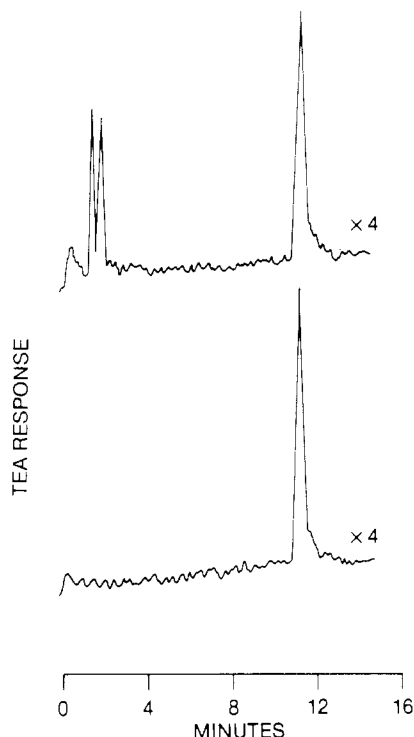

Figure 4. Chromatograms from GLC–TEA analysis of *O*-methyl ether derivatives of HMNThZ standard (bottom) (2 ng) and of fried bacon extract (no. 16 in Table I) (top).

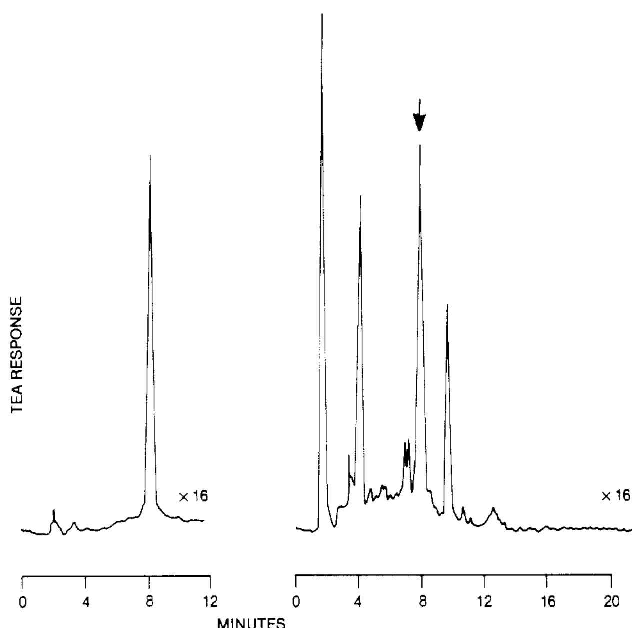

Figure 5. HPLC–TEA analysis of the *O*-methyl ether derivative of HMNThZ standard (50 ng) (left) and derivatized extract of a fried bacon (no. 16 in Table I) (right). The arrow indicates the derivative peak produced from the sample extract. The underivatized HMNThZ elutes at 17.5 min under these conditions.

GLC–MS. HPLC–TEA and GLC–TEA chromatograms are shown in Figures 4 and 5, respectively. First, the MS confirmation was carried out in the repetitive exponential scanning mode. The reconstructed mass spectrum of the sample (no. 16 in Table I) contained all the characteristic fragment ions observed in that of HMNThZ *O*-methyl ether standard (Figure 6). These were at m/z 162, 132, 117, 87, and 60 corresponding most likely to $M^{\bullet+}$, $(M - NO)^+$, $[M - (CH_2OCH_3)]^+$, $[M - (CH_2OCH_3) - NO]^+$, and $(C_2H_4S)^+$, respectively. Furthermore, the identity of

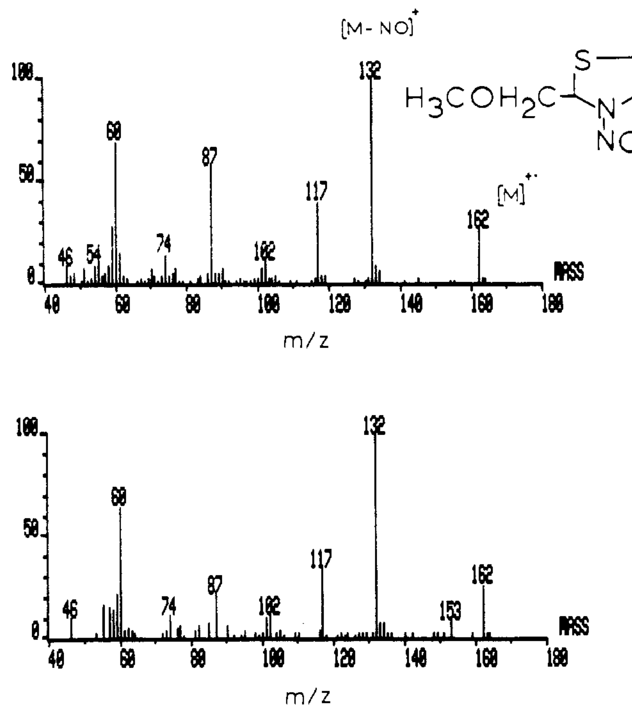

Figure 6. Electron-impact ionization mass spectra of *O*-methyl ether derivative of HMNThZ (top) and of derivatized fried bacon extract (no. 16 in Table I) (bottom).

HMNThZ *O*-methyl ether prepared from the sample was unequivocally confirmed by high-resolution (6,000) selected ion monitoring for three fragment ions (m/z 162.0463, 132.0483, 117.0123). The relative ratios of these three ions in the unknown and the standard were also very close.

Thus, the O-methylation technique offers a reliable confirmation procedure for hydroxylated *N*-nitrosamines in difficult to analyze complex samples such as fried bacon. In the past, we have successfully used the technique for MS confirmation of 3-hydroxy-*N*-nitrosopyrrolidine in fried bacon (Sen et al., 1977) and of *N*-nitrosodiethanolamine in cutting fluids (Fan et al., 1977).

It can be concluded from this study that traces of HMNThZ occur in some fried bacon but the mechanism of its formation is unclear. To our knowledge, this is the first reported occurrence of HMNThZ in fried bacon. Since none was detected in raw bacon, HMNThZ appears to be produced during frying. By analogy to the formation of NThZ from NTCA it might be theorized that HMNThZ is formed by heat-induced decarboxylation of HMNTCA. Due to the unavailability of HMNTCA standard, we were unable to investigate this possibility. Further research along these lines is highly desirable. Additional research is also needed to determine the toxicological significance of the finding.

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Registry No. HMNThZ, 92134-93-5; HMNThZ *O*-methyl ether, 120144-89-0.

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Effect of Certain Plant Phenolics on Nitrosamine Formation[1]

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Phenols are known to modify the nitrosation of amines, as catalysts or inhibitors, depending on their structure, reaction conditions, pH, and nitrite concentration. In the present work, the roles of catechol (CA), 4-hydroxychavicol (HC), eugenol (EU), and methyleugenol (MEU) on the nitrosation of model amines, viz. pyrrolidine (PYR), piperidine (PIP), and morpholine (MOR), were studied. The modifying effects of these phenolics were compared to that of ascorbic acid (AA). It was observed that HC and CA were excellent inhibitors of the nitrosation reaction while EU was less effective. MEU was a poor inhibitor.

N-Nitroso compounds (NOC) are known to be strong carcinogens in various animals including primates (Preussmann and Stewart, 1984). Human exposure to these compounds can be by ingestion or inhalation of preformed NOC or by endogenous nitrosation. It has been established unequivocally that NOC are formed in the body from precursors present in normal diet (Bartsch et al., 1984). Essentially they are formed by the reaction of amines, especially secondary and tertiary amines, or amino group containing compounds, such as dialkylamines, alkylarylamines, piperazine, pyrrolidine, etc., with nitrite. While amines are present in foods, wine, tobacco products, drugs, and other environmental chemicals such as pesticides, sources of nitrite vary (Fine, 1979; Shephard et al., 1987; Tannenbaum, 1979).

N-Nitrosation is influenced by many factors such as pH, amounts of precursors, basicity of the nitrosatable amines, and presence of catalysts and inhibitors (Challis, 1981). Simple phenols and polyphenolic compounds can decrease or increase the rate of N-nitrosation reactions depending on their structure and reaction conditions, especially pH (Douglass et al., 1978; Pignatelli et al., 1980; Mirvish, 1981; Challis, 1981). Certain polyphenolic compounds react with nitrite to give C-nitroso derivatives, which can act as catalysts of nitrosation (Pignatelli et al., 1982, 1984). However, phenols and polyphenolic compounds that are readily oxidized inhibit nitrosation reactions (Stich et al.,

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