Identification and Quantitation of 3-Hydroxy-N-Nitrosopyrrolidine in Fried Bacon[1]

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Many N-Nitrosamines are potent carcinogens and several occur sporadically at trace levels in certain foods, notably cured meats (CROSBY and SAWYER 1976) (SCANLAN 1975). N-Nitrosopyrrolidine (NPYR) is found fairly consistently in fried bacon at the ppb level. One might then suspect that 3-hydroxy-N-nitrosopyrrolidine (HNPYR) might accompany NPYR in a cured meat such as fried bacon (SCANLAN 1975). Although analytical methodology for the trace analysis of the volatile N-nitrosamines in foods is well developed, the non-volatile N-nitrosamines (such as HNPYR) have received less attention because of a lack of suitable methods. Notable progress in this area has been reported by SEN et al (1976), and by EISENBRAND et al (1976) and KUSHNIR et al (1975) for nitrosamino acids. Recently, GRAY et al (1976) mentioned the formation of HNPYR in a model system but stated that they could not isolate the compound from cooked bacon. In a previous publication LEE et al (1977) we described the formation of HNPYR from the heat induced decarboxylation of 4-hydroxy-N-nitrosoproline (HNPRO) and mentioned our identification of HNPYR in fried bacon. The present work reports a new method for the trace analysis of HNPYR. The title compound was identified and quantitated in several samples of commercial fried bacon.

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APPARATUS AND TECHNIQUES

For the identification study 6.80 kg of one brand of raw bacon was obtained from a local market. For recovery studies six replicate analyses were performed on fried nitrite-free bacon and fried-out fat which had been spiked with 10 ppb HNPYR. The nitrite-free bacon was manufactured in our pilot plant using a pickle which contained only NaCl and sucrose (3:1). Subsequent analysis showed this bacon to be free from HNPYR after frying. Quantitation studies utilized 0.453 kg packages of five commercial brands of raw bacon. Bacon was fried 7-9 minutes at 177° to a well-done crispness. Usually 0.453 kg of raw bacon yielded approximately 100 g of fried bacon and 250 g of fried-out fat. The bacon and fried-out fat were extracted separately. The fried bacon was ground in a Waring Blendor with 250 ml dist. water:methanol (3:2). Following centrifugation at 13,200 g for 20 min, the extract was placed into a separatory funnel to remove unsolidified lipid.

Fried-out fat was placed in a separatory funnel with 500 ml of the water:methanol extracting solvent. Following several minutes of intermittent shaking the solvent extract was drained off. The bacon and fried-out fat extracts for the identification study were separately carried through identical clean-up prodecures until the extracts were pooled after elution from the alumina column (see below).

The water:methanol extracts were adjusted to approximately pH 2.0 with sulfuric acid and residual nitrite was inactivated by making the extracts 0.011 M in ammonium sulfamate. After 30 min, the extracts were adjusted to pH 8, saturated with anhydrous sodium sulfate and then subjected to continuous liquid-liquid extraction with 50 ml of dichloromethane (DCM) for 12 hours. The DCM extract was dried with sodium sulfate and concentrated to a volume of 3 ml on a Kuderna-Danish (K-D) apparatus fitted with a Snyder column. The concentrate from the KD was further concentrated under a nitrogen stream to 1 ml. Quantitation and recovery studies were conducted without further purification or derivatization; clean-up on an alumina column and derivatization was necessary in the identification study.

For cleanup 1 ml of concentrate was diluted with 25 ml of diethyl ether and placed on an activated alumina column (15 cm x 14 mm i.d.). Elution of the HNPYR was achieved by 50 ml each of a series of diethyl ether:

ethanol mixtures; starting with 19:1 then 9:1 and ending with 4:1. The initial 25 ml of eluate was discarded; the HNPYR started to elute when 40 ml of the elution series had passed through the column and all had been eluted by 80 ml. HNPYR samples from the alumina column were blown to dryness under a nitrogen stream and dried in a desiccator over P_2O_5. Samples were silylated in Reacti-vials with Minivert valves (Pierce Chemical Co.). After the vial was put under vacuum for 10 min, one hundred µl of N-methyl-N-TMS-trifluoroacetamide (MSTFA) was added to form the trimethylsilyl derivative of HNPYR (TMS-HNPYR).

Authentic TMS-HNPYR was synthesized by first nitrosating 3-hydroxypyrrolidine (LIJINSKY et al 1970) followed by purification by TLC on silica gel G using ethyl ether:methanol (3:1) as the mobile phase. Following purification the 3-hydroxypyrrolidine was silylated as with the bacon samples.

A thermal energy analyzer (TEA), model TEA-502 from Thermo Electron Corp. was used as a sensitive and specific detector for N-nitrosamines (FINE and ROUNBEHLER 1975). The TEA was interfaced to a Varian series 1400 gas chromatograph (GC/TEA). The GC column for identification studies was 0.75 mm i.d. x 152 meters stainless steel wall coated with SF-96 and operated isothermally at 140° C with a flow rate of 15 ml/min He. The SF-96 column was also used under the same oven conditions in a Finnigan 1015C gas chromatograph/mass spectrometer (GC/MS); data was acquired and processed by a System 250 data system from System Industries.

The column used for quantitation and recovery (GC/TEA) was stainless steel, 91.4 cm x 3.18 mm o.d., packed with 10% SP2340 on 100/120 mesh Supelcoport (Supelco Inc.). Operating conditions were 50 ml/min He at 200° isothermal.

It was important to check for the possibility of HNPYR being generated by the analytical procedure, either by 1) nitrosation of 3-hydroxypyrrolidine (HPYR), or 2) decarboxylation of HNPRO. The first possibility was checked by preparing several model systems containing the water:methanol extracting solvent to which HPYR and sodium nitrite had been added. Levels added corresponded to 25 ppm HPYR and 50 ppm nitrite in 100 g of fried bacon. Each of the model systems was carried through the analytical procedure and checked for the absence of HNPYR by GC/TEA.

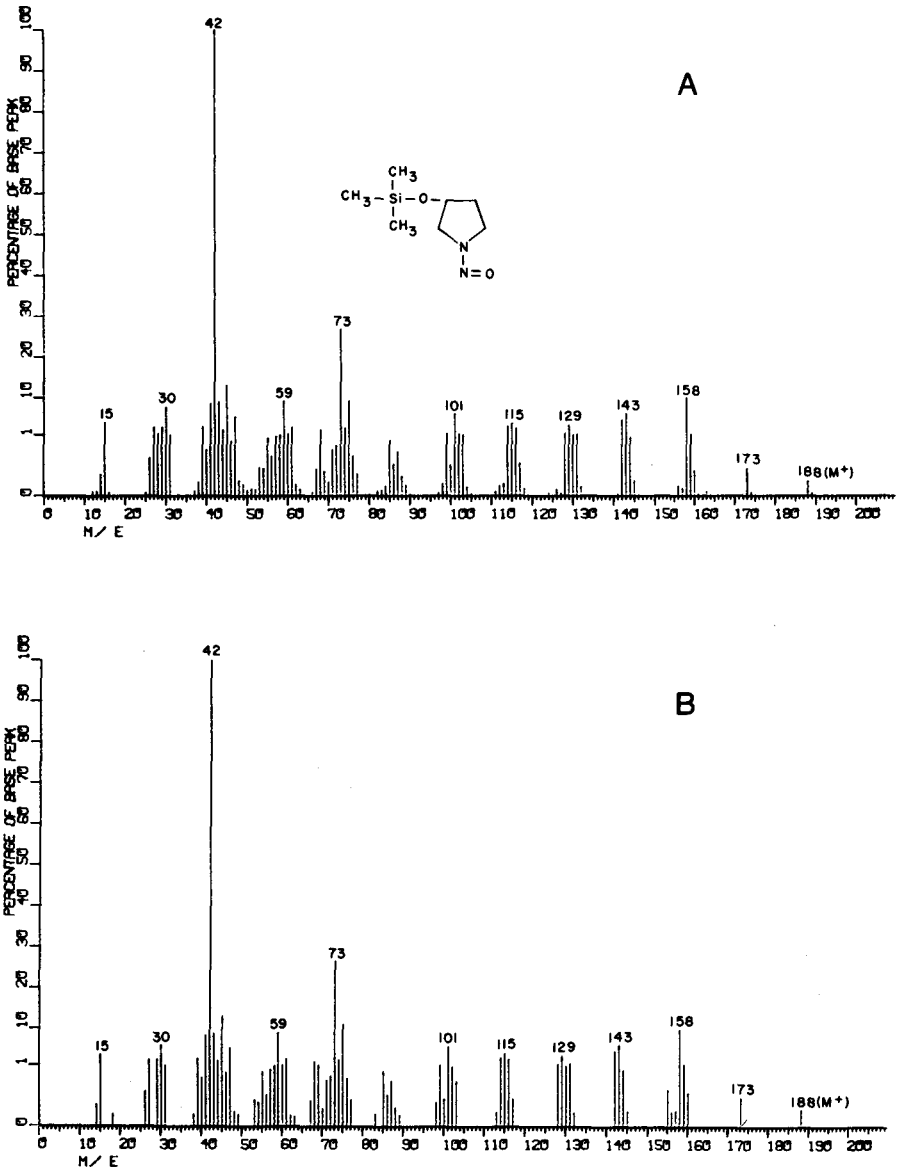

Figure 1. Mass spectra of trimethylsilyl derivative of 3-hydroxy-N-nitrosopyrrolidine: (A) authentic compound synthesized in our laboratory; (B) derivative of compound isolated from fried bacon.

To check that HNPYR was not formed in the analytical procedure by the decarboxylation of HNPRO, 200 ml of the solvent mixture was spiked with 200 µg of HNPRO. As with the model systems above, the spiked solvent was carried through the analytical procedure and checked for the absence of HNPRY by GC/TEA.

RESULTS

Unequivocal identification of HNPYR as the TMS derivative is based on: positive GC/TEA response at the correct retention time, peak enhancement by adding the authentic derivative, and GC/MS in which the fragmentation patterns of TMS-HNPYR from fried bacon and fried-out fat and the authentic compound are practically identical (Fig 1).

Table 1 gives a summary of the recovery data for six replicate analyses for both fried bacon and fried-out fat.

TABLE 1

Recovery of 3-Hydroxy-N-nitrosopyrrolidine

	n	$\bar{x}$	s	95% confidence limit
Fried Bacon	6	61.6	5.4	67.26 - 55.94
Fried-out Fat	6	62.7	3.75	66.67 - 58.77

Thus recoveries of approximately 60% can be expected for the analytical procedure reported here.

Results from the artifact study indicated that no, or less than 0.07 ppb of HNPYR was generated during the analysis of 100 g of fried bacon. Similarly, decarboxylation checks indicated that no or less than 0.005% decarboxylation had occurred during the analytical procedure (0.07 ppb and 0.005% decarboxylation were the lower detection limits in the two experiments).

Quantitative data for the levels of HNPYR in several commercial fried bacons is reported in Table 2.

TABLE 2

3-Hydroxy-N-nitrosopyrrolidine Levels[a,b] in Commercial Fried Bacon and Fried-out Fat

Sample	Fried Bacon	Fried-out Fat
1	0.4	0.3
2	2.2	2.2
3	2.0	1.2
4	2.6	2.3
5	3.9	1.9

a Amounts in ppb, relative to the weights of fried bacon and fried-out fat

b Corrected for recovery

DISCUSSION AND CONCLUSIONS

Although NPYR is a well known carcinogen, it is not known whether HNPYR is also carcinogenic. It is prudent to consider all N-nitrosamines as carcinogens and handle them with adequate precautions. Similarly it is not known if the trace levels of HNPYR reported here are of significance to human health. The number of samples analyzed in this study was limited, yet our data indicate that ppb levels of HNPYR appear fairly consistently in fried bacon.

Although we were successful in using the TMS derivative for the identification of HNPYR in fried bacon, the molecular ion in the mass spectrum is of low abundance (Fig 1). We suspect that the O-methyl ether derivative of HNPYR (SEN et al 1976) may be preferable, especially in GC/MS specific ion monitoring (SIM) of the molecular ion. High resolution MS SIM has been suggested (SEN et al 1976) (STEPHANY et al 1976) as the only reliable, unequivocal technique for the analysis of nitrosamines, but we feel that this is true only when adequate resolution (10,000 or above) is used and when a unique choice can be made among possible structures written for an empirical formula.

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