

N-Nitrosothiazolidine-4-carboxylic acid (NTHZC) was recently identified in a variety of smoked fish and cured meat products. While the toxicological properties of NTHZC are currently unknown, it has been speculated that *N*-nitrosothiazolidine (NTHZ) in fried bacon may be caused in part by decarboxylation of NTHZC. A limited survey of uncooked cured meat products failed to demonstrate any correlation between levels of NTHZC and NTHZ. However, NTHZC levels were significantly higher, ranging from 8 to 1400 ppb, than the corresponding NTHZ levels, which ranged from <2 to 18 ppb. Decarboxylation of NTHZC to NTHZ in a model system occurs at 110°C, which is significantly higher than the average bacon processing temperature of 54°C, but lower than the normal frying temperature of 177°C recommended for bacon. Results from both model system and frying experiments with NTHZC and its precursors suggest that NTHZC decarboxylation to NTHZ is not the principal pathway to NTHZ formation in uncooked bacon.

N-Nitrosothiazolidine-4-carboxylic acid (NTHZC) was recently identified in some smoked fish and cured meat products (1, 2). It has been hypothesized that this nitrosamine can form by the reaction of cysteine in foods with formaldehyde in smoke to yield thiazolidine-4-carboxylic acid (THZC), which can then be nitrosated either by nitrite in the product or by nitrogen oxides generated during the smoking process. The presence of NTHZC in foods is possible because THZC has been shown to nitrosate 250–500 times more rapidly than proline (3), which is thought to be one of the precursors for *N*-nitrosopyrrolidine found in fried bacon. In addition, NTHZC detected in the urine of normal people is thought to have a better potential as an indicator of *in vivo* nitrosamine formation than nitrosoproline (3). Part of the total amount of NTHZC found in the urine, however, may be contributed by foods containing preformed nitrosamines.

Although the toxicological properties of NTHZC are unknown, it may undergo decarboxylation to form *N*-nitrosothiazolidine (NTHZ), which has been reported to form a mutagenic compound when prepared from cysteamine and formaldehyde (4). In addition, Helgason et al. (1) hypothesized a relationship between NTHZ ingestion and congenital diabetes found in Iceland.

Recently, Sen et al. (2) reported a good correlation between NTHZC levels in raw bacon and NTHZ levels in fried bacon. However, they found no correlation between NTHZC and NTHZ in raw bacon. Previously, we reported (5) that NTHZ levels were higher in the raw bacon than in the fried product, which indirectly suggests that NTHZC was not responsible for NTHZ in fried bacon, contrary to the report by Sen et al. (2). To resolve this discrepancy in results, we developed a method for determining NTHZC that was applicable to a wide variety of smoked cured meat products, and then conducted a limited survey to determine levels of NTHZC in uncooked food products in addition to bacon. We also conducted experiments to determine whether NTHZC had the potential to be a precursor of NTHZ in raw or fried bacon. The results are reported herein.

METHOD

Note: *N*-Nitrosoamines are potential carcinogens. Exercise care in handling these materials.

Reagents

- Methanol, ethyl acetate, and dichloromethane.*—Burdick and Jackson Distilled-in-Glass solvents.
- Sulfamic acid.*—1% in 1N sulfuric acid.
- Diazomethane.*—Prepared from Aldrich *N*-methyl-*N*-nitroso-*p*-toluene-sulfonamide as directed.
- N-Nitrosopipercolic acid (NPIC) internal standard solution.*—0.20 µg NPIC/mL in methanol.
- Methyl esters of N-nitrosothiazolidine-4-carboxylic acid (NTHZC) and NPIC.*—GC working standard, each 0.20 µg/mL in dichloromethane.
- Cured meat products.*—Random samples purchased from local retail outlets, ground, and thoroughly mixed before analysis.
- Other reagents.*—Purchased from local suppliers and used without further purification. NTHZ, NTHZC, and NPIC were synthesized from their corresponding amines and sodium nitrite, and purified by either fractional vacuum distillation (NTHZ) or by recrystallization (NTHZC, NPIC), according to general procedure published previously (6).

Apparatus

Usual laboratory equipment and the following items:

- Homogenizer.*—Virtis Co., Inc., Model 45 with 250 mL flask.
- Rotary evaporator and Evapo-Mix.*—Buchler Instrument Co.
- Refrigerated centrifuge.*—Sorvall Model RC-5B.
- Gas chromatograph-thermal energy analyzer (GC-TEA).*—Varian Aerograph gas chromatograph Model 1700, or equivalent, interfaced with thermal energy analyzer Model 502. Operating conditions: 1.8 m × 2 mm glass column packed with 5% Silar 10CP on 100–120 mesh Supelcoport; helium carrier gas, 35 mL/min; column temperature programmed from 150 to 250°C at 4°/min; injector port, 200°C; TEA furnace, 450°C; TEA vacuum, 0.5 mm; liquid nitrogen–ethanol cold trap.
- Gas chromatograph-mass spectrometer (GC-MS).*—Hewlett-Packard Model 5992B low-resolution quadrupole mass spectrometer. Operating conditions: 10 m × 0.20 mm glass capillary column coated with methyl silicone (fused silica); helium flow rate through column, 0.6 mL/min; column temperature maintained at 20°C for 2 min, then programmed at 10°/min to 250°C; injector port, 150°C.

If ions with *m/z* 30, 45, 59, 87, 146, and 176 were present before and absent after UV photolysis (365 nm), using same procedure described previously (7), the presence of NTHZC as its methyl ester was considered confirmed.

Procedure

- Sample analysis, NTHZC.*—A flow diagram of this method is shown in Figure 1. Accurately weigh 20.0 ± 0.1 g of ground meat sample into a 250 mL Virtis flask. Add 1.0 mL NPIC internal standard solution (equivalent to 10 ppb) to

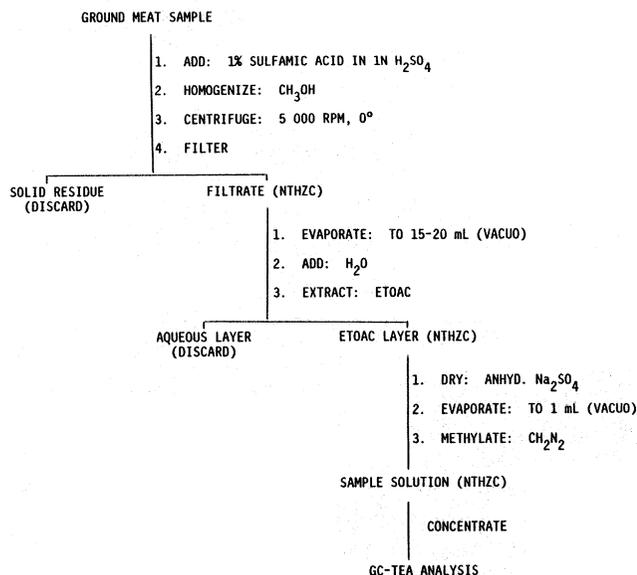


Figure 1. Schematic of procedure for determination of *N*-nitrosothiazolidine-4-carboxylic acid in cured meat products.

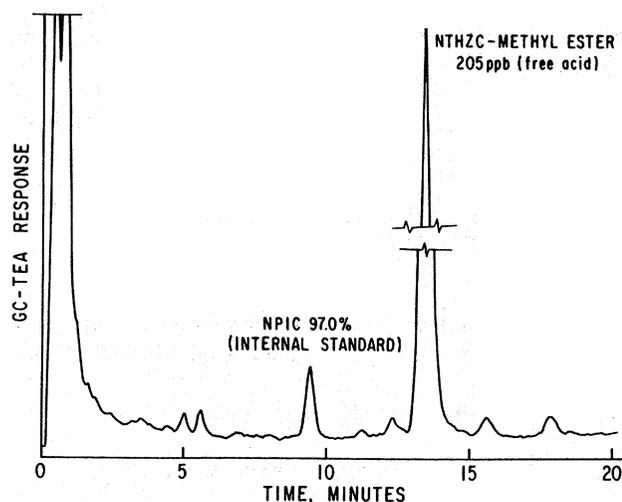


Figure 2. GC-TEA chromatogram of *N*-nitrosothiazolidine-4-carboxylic acid in uncooked bacon.

sample with 1.0 mL transfer pipet, then add 100 mL methanol and 10 mL sulfamic acid solution to flask to destroy residual nitrite. Homogenize sample 3 min at medium setting of 40. Quantitatively transfer sample, using methanol, to 150 mL glass bottle and centrifuge 25 min at 5000 rpm at 0°C. Filter samples through glass wool into 250 mL round-bottom flask, add 2 aluminum boiling stones, and then reduce solvent to 15–20 mL on vacuum rotary evaporator (water bath at 40°C). Quantitatively transfer sample with 50 mL water to 125 mL

separatory funnel. Extract sample 2 times with 50 mL ethyl acetate. Combine extracts and slowly pass them through 35 g anhydrous sodium sulfate, held in 60 mL coarse fritted-glass funnel. Collect effluent in 250 mL round-bottom flask, and reduce ethyl acetate on vacuum rotary evaporator to 1 mL. Quantitatively transfer sample, using 1–2 mL methanol, to 16 × 145 mm test tube. Reduce volume to ca 0.5 mL on Evapo-mix, and then add 3 mL ether solution containing diazomethane while test tube is heated and shaken at 38°C for 20 min. Quantitatively transfer solution to 4 mL concentrator tube by using dichloromethane, and concentrate solution to 1 mL in 70°C water bath.

(b) *NTHZ analysis and determination.*—Carry out procedure and necessary calculations for determining NTHZ in cured meat products as described previously (8).

(c) *Model system reaction.*—Heat for 6 min in closed glass pressure flask a mixture consisting of 4×10^{-4} mole cysteine, formaldehyde, and sodium nitrite in 10 mL of pH 5.6 phthalate buffer. Extract contents of cooled flask twice with 25 mL DCM. Wash combined DCM extracts once with 10 mL 5N NaOH, then dry and concentrate to 1.0 mL as before.

(d) *Bacon system.*—Add precursor (NTHZC, THZC, THZ) to ground raw bacon containing 36 ppm residual NaNO₂ and 870 ppb NTHZC before frying at 177°C for 6 min. Analyze fried product for NTHZ as described previously (8).

(e) *NTHZC determination.*—Inject 8.0 μL GC-TEA working standard at lowest attenuation that yields TEA response at least one-third full scale on recorder; measure peak heights. Repeat standard injection to assure reproducibility of retention time and response. Inject 8.0 μL sample solution and measure peak heights. Calculate NTHZC, from its methyl ester, in ppb. Typical chromatogram from cured meat extract is shown in Figure 2. Minimum level of reliable measurement, using this method, was 5 ppb NTHZC.

(f) *Mass spectral confirmation.*—Mass spectral confirmation was carried out on those samples containing 100 ppb or more NTHZC. All other samples containing lower values for NTHZC were considered “apparent” NTHZC.

(g) *Sodium nitrite analysis.*—Residual sodium nitrite content was determined on 10 g raw, comminuted sample by modified Griess-Saltzman procedure (9).

(h) *Statistical analyses.*—Statistical analyses were carried out according to methods of Snedecor and Cochran (10).

Results and Discussion

To determine the within-laboratory repeatability of the NTHZC method, 8 samples containing NTHZC at levels ranging from 5 to 500 ppb were analyzed in duplicate. An analysis of variance showed that the repeatability coefficient of variation was 5.22% (4.29% in samples corrected for recovery of the internal standard). The repeatability coefficient of variation of the NPIC internal standard recovery was 5.95%.

Sample type	NTHZC, ppb			NTHZ, ppb			NaNO ₂ , ppm	
	No. pos./total	Range	Av.	No. pos./total	Range	Av.	Range	Av.
Cure-pumped bacon	10/10	4–1400	370	10/10	2.0–13.1	5.4	5–75	36
Dry-cured bacon	2/2	45, 281	163	2/2	3.6, 6.2	4.9	51, 58	55
Dry-cured ham	4/4	8–103	61	3/4	3.2–17.6	8.9	2–101	50
Lebanon bologna	5/5	8–276	127	4/5	3.0–8.3	5.5	1–2	1
Poultry franks	2/2	69, 173	121	2/2	3.1, 8.3	5.7	34, 45	40
Pepperoni	2/2	39, 79	59	2/2	4.3, 5.8	5.1	2, 2	2
Hot dogs	3/3	12–83	58	2/3	7.2, 9.8	8.5	2–15	8
Beef, pork strips	2/2	19, 28	24	2/2	7.5, 11.8	9.7	4, 6	5
Other cured products	4/4	8–18	12	4/4	1.7–2.2	1.9	2–10	6

Table 2. Effect of added precursor on nitrosothiazolidine formation in fried bacon

Precursor	Added, ppm	NTHZ, ppb
Control	none	8.5
Nitrosothiazolidine-4-carboxylic acid	0.1	11.1
	1	12.2
	10	133.0
	100	1031.0
Thiazolidine-4-carboxylic acid	100	19.0
	1000	46.5
	1000	1548.0

Table 3. Effect of frying on nitrosothiazolidine formation in bacon

Sample	Raw bacon			Fried bacon	
	NaNO ₂ , ppm	NTHZC, ppb	NTHZ, ppb	NTHZC, ppb	NTHZ, ppb
1	51	1022	6.9	645	8.0
2	35	798	5.7	403	4.2
3	35	418	ND	271	2.3
4	32	120	4.9	138	2.3
5	47	173	3.8	163	2.8
6	2	223	2.7	169	1.5

The results of our limited survey of smoked cured meat products for both NTHZC and NTHZ are shown in Table 1. All of the samples analyzed contained NTHZC ranging from 8 to 1400 ppb. These values were significantly higher than the NTHZ concentrations in the same samples, which ranged from <2 to 18 ppb in 32 of 34 samples analyzed. Analysis of the data indicated no correlation between residual sodium nitrite in any of the products surveyed, and either NTHZC or NTHZ. It is also interesting to note that the one bacon sample that had the NTHZC value of 1400 ppb had a residual nitrite level of 75 ppm, but other bacon samples that had comparable levels of residual nitrite contained significantly lower levels of NTHZC, which again suggested that no nitrite-NTHZC correlation was evident. Lebanon bologna, which is subjected to the longest smoking period of any of the products surveyed, showed a NTHZC average equivalent to the lightly smoked poultry franks. This is consistent with our previous finding, where we hypothesized that the low NTHZ values observed may be due to the acidic nature of this type of fermented product. Despite the higher levels of NTHZC in the surveyed samples, no correlation between NTHZC and NTHZ was found, which is in agreement with Sen et al. (2), who also found no correlation between NTHZC and NTHZ in the uncooked bacon only. Our results suggest that the NTHZC-amino precursor was either present in higher concentrations or was more readily nitrosatable than the NTHZ precursor in the uncooked meat products.

Although NTHZC does not appear to be the primary precursor for NTHZ in uncooked bacon, its parent compound, thioproline (THZC), could decarboxylate to form THZ, which could then nitrosate to form NTHZ. However, we found by thermogravimetric analysis that THZC does not decarboxylate appreciably until 200°C, which is significantly higher than the average internal temperature (54°C) used in processing bacon. These results support the previous findings and suggest that THZC does not contribute to NTHZ formation unless the product contains some unknown factor(s) that might facilitate decarboxylation.

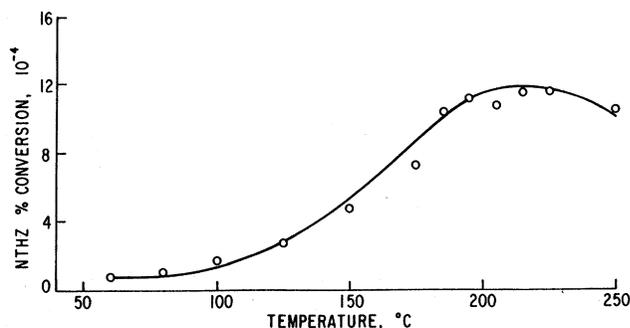
Mandagere et al. (11) reported that NTHZC decarboxylates to NTHZ at 110°C, which is lower than the bacon frying temperature of 177°C recommended by many bacon producers. However, since our previous work did not show an

increase in NTHZ levels when uncooked bacon was fried (5), we investigated, in a model system, whether adding high levels of NTHZC to the uncooked bacon could significantly increase the levels of NTHZ found in the product after it was fried at 177°C. Table 2 shows the NTHZ content after frying when NTHZC, THZC, and THZ were added to ground bacon containing 36 ppm residual nitrite, 870 ppb NTHZC, and 8.5 ppb NTHZ before frying. When 1 ppm NTHZC was added to the bacon, the NTHZ value increased only 3.7 ppb (8.5 to 12.2 ppb); whereas, at the 10 ppm fortification level, NTHZ increased markedly (8.5 to 133.0 ppb). These bacon model system results indicate that the product may need a very high level of NTHZC to make a significant contribution to NTHZ.

As predicted from the decarboxylation temperature (200°C), THZC does not appear to be a major contributor to NTHZ formation, since 100 ppm added to the uncooked bacon only increased the NTHZ level by 10.5 ppb (8.5 to 19.0 ppb). When 100 ppm THZ was added to the bacon, the NTHZ level increased by 134.5 ppb (8.5 to 143.0 ppb), which shows that the bacon used in these experiments was capable of nitrosating the exogenous amines or amine precursors to form NTHZ.

The previous model system results suggest that NTHZC may not be the primary precursor of NTHZ in fried bacon unless present in large amounts or if higher cooking temperatures were employed. To confirm this hypothesis, selected samples of uncooked bacon containing NTHZC levels >100 ppb were fried; NTHZ and NTHZC values were determined both before and after frying. The results in Table 3 show that NTHZC levels decreased in 5 of 6 samples after frying, but NTHZ increased slightly in only 2 of 6 samples; even here, the increase was not considered significant. The difference between our results and those reported by Sen et al. (2) may be due to the higher concentrations of NTHZC or NaNO₂ in the bacon that they analyzed. In our case, the samples contained less than 50 ppm residual nitrite, the concentration normally found in bacon at the retail level.

Up to now, we have shown that NTHZC and THZC are not likely precursors for NTHZ in either raw or typically fried bacon. However, we considered it necessary to investigate the remaining pathway to NTHZ formation, that is, via cysteine. In a pH 5.6 aqueous model system, we reacted, at various temperatures, cysteine, formaldehyde, and sodium nitrite to determine if and how much NTHZ would form. The system was closed during heating to prevent loss of any volatile components generated. The results presented in Figure 3 show that the optimum temperature for conversion of the reactants to NTHZ occurred at 215°C, which is higher than either the decarboxylation temperature for NTHZC to NTHZ (110°C) or for THZC to THZ (200°C), but below the reported temperature threshold of 230°C for cysteine decarboxylation (12). Even though formation of NTHZ from cys-

**Figure 3. N-Nitrosothiazolidine formation from cysteine-formaldehyde-nitrite in a model system.**

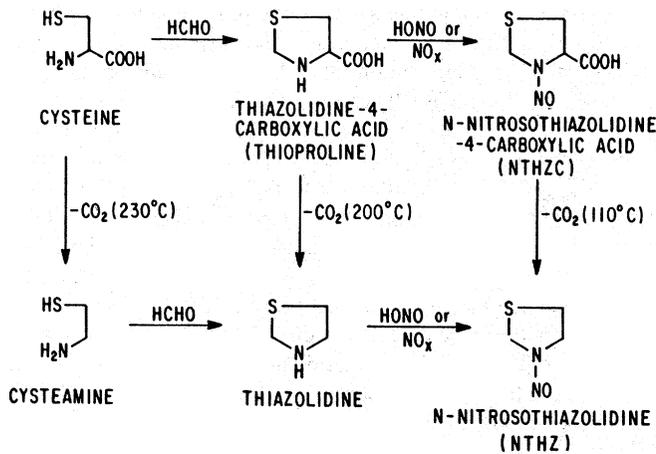


Figure 4. Possible pathways of *N*-nitrosothiazolidine formation.

teine in a simple model system is minor at 215°C ($12 \times 10^{-4}\%$), at normal frying temperatures (177°C), the rate of decarboxylation might be sufficient to form NTHZ, but the rate of decarboxylation at typical bacon processing temperatures (54°C) is far too low to account for formation by this pathway.

The possible pathways of NTHZ formation are presented in Figure 4, with the decarboxylation temperatures indicated in parentheses. We have shown that there is a high probability that NTHZ in raw bacon does not occur by decarboxylation of cysteine, THZC, or NTHZC. As we hypothesized previously (13), NTHZ appears to form via the cysteamine-thiazolidine pathway. However, at present there has been no published data on either cysteamine or thiazolidine content in meat products. Therefore, we are currently evaluating precooked cured bacon for these compounds to confirm that this indeed is the pathway to NTHZ. We have also shown in a bacon model system that NTHZC must be present in large amounts to contribute to NTHZ formation when bacon is

fried. However, our results indicated that while NTHZC was present in amounts considerably higher than normally obtained for NTHZ, the concentrations were sufficiently low as not to significantly contribute to NTHZ formation during normal frying.

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