

FOOD COMPOSITION

Determination by Liquid Chromatography with Electrochemical Detection of Cysteamine and Cysteine, Possible Precursors of *N*-Nitrosothiazolidine

A method is described that is selective, sensitive, rapid, and accurate for the quantitative measurement in meat products of both cysteamine and cysteine, potential precursors for *N*-nitrosothiazolidine (NTHZ) and *N*-nitrosothiazolidine-4-carboxylic acid (NTHZC), respectively. In general, a ground meat sample is homogenized with acetonitrile-formate buffer in the presence of dithiothreitol, and then is centrifuged, filtered, and re-centrifuged in a disposable microfilter. The thiols are quantitated by liquid chromatography using an amperometric detector equipped with a gold/mercury electrode operated in the oxidative mode. Cysteamine was found in 6 of 20 samples of raw pork belly in concentrations ranging from 150 to 450 ppb, and cysteine was found in all samples in concentrations ranging from 2.4 to 36.5 ppm. Analysis for the thiols and their corresponding nitrosamines—NTHZ and NTHZC—of bacon before and after processing showed no correlation between cysteamine and cysteine levels before processing nor with nitrosamine levels after processing. Liquid chromatography with electrochemical detection was found to be an extremely selective technique to measure the 2 free sulfhydryl compounds in a complex food substrate.

The occurrence of *N*-nitrosothiazolidine (NTHZ) in smoked, cured meat products, particularly bacon, prior to home cooking has led to an investigation for the precursors of and mechanism for NTHZ formation. Unlike *N*-nitrosopyrrolidine, whose precursors are present in the adipose tissue of bacon (1), the precursors for NTHZ were found in the lean tissue (2). Several possible pathways have been proposed to account for the formation of NTHZ in bacon. In a model system study (3), it was found that cysteamine in meat can react with formaldehyde from smoke to form thiazolidine, which in turn reacts with nitrite to form NTHZ. It has also been hypothesized (4, 5) that cysteine reacts similarly to form *N*-nitrosothiazolidine-4-carboxylic acid (NTHZC), which can then thermally decarboxylate to form NTHZ. Although no correlation has been found between NTHZ and NTHZC in raw bacon (4), Sen et al. (6) reported a good correlation between NTHZC before and NTHZ after frying bacon. In elucidating the mechanism of NTHZ formation, it is important to ascertain whether cysteamine and cysteine are present in sufficient quantities to favor a particular pathway. Given this information, it will be easier to develop potential inhibitors or treatments to reduce the content of these nitrosamines in foods. This work takes on added importance since NTHZC has been found in human urine, and it also is claimed to be an indicator of *in vivo* nitrosamine formation (7).

At present, the only information available on the cysteamine content of edible meats comes from the analysis of kidney, liver, heart, and brain tissue from pork and beef (8, 9); for cysteine, the values are usually reported as the disulfide, cystine. This is due, in part, to the lack of an accurate quantitative method for these compounds in food products

because cysteamine and cyteine are highly reactive and readily oxidized. However, Lunte (10) recently reported an effective method for the detection of cysteine and cystine in urine by liquid chromatography with electrochemical detection (LCEC). Using a gold/mercury electrode, we have developed a selective, rapid, and accurate method for determining low concentrations of cysteamine and cysteine in meat products. A description of the method and the results from the analysis of pork bellies for the 2 thiols in question are reported herein.

METHOD

Reagents

(a) *Acetonitrile*.—Distilled-in-glass solvent (American Burdick and Jackson Labs, Muskegon, MI 49442).

(b) *Dithiothreitol*.—(Aldrich Chemical Co., Milwaukee, WI 53201.) Prepare fresh weekly at a concentration of 5 mg/mL in acetonitrile-formate buffer.

(c) *Cysteamine and cysteine*.—(Aldrich Chemical Co.) 1.0 ng/ μ L each in acetonitrile-formate buffer solution as standard in liquid chromatography with electrochemical detection.

(d) *Other reagents*.—Purchased from local suppliers and used without further purification.

(e) *Formate buffer*.—0.2M formic acid, 0.1 M potassium hydroxide, 0.0002M EDTA in 2 L water (pH 3.5).

(f) *Mobile phase*.—Acetonitrile-formate buffer solution (50 + 50 v/v).

(g) *Meat products*.—Fresh pork bellies were obtained from a local supplier within 2 h of slaughter and frozen at -18°C until analyzed. Thawed bellies were ground and thoroughly mixed prior to analysis. Bacon was prepared as described previously (3).

Apparatus

Usual laboratory equipment and the following items:

(a) *Homogenizer*.—VirTis Model 45 (VirTis Co., Inc., Gardiner, NY 12525) with 100 mL flask and U-shaped "Turbo-Shear" blades (No. 16-107).

(b) *Refrigerated centrifuge*.—Sorvall Model RC-5B (DuPont Co., Wilmington, DE 19898).

(c) *Microfilter*.—Centrex disposable microfilter unit (No. DF 101-1), 0.2 μm nylon (purchased from Schleicher and Schuell, Inc., Keene, NH 03431).

(d) *Liquid chromatograph-electrochemical detector*.—Altex Model 100A pumping system (Altex, Berkeley, CA 94710) with Rheodyne Model 7125 injector (Rheodyne, Cotati, CA 94928) and a 25 cm \times 4.6 mm id 10 μm Partisil PXS 10/25 SCX ion exchange column (purchased from Whatman Ltd., Clifton, NJ 07014) interfaced to a Bioanalytical Systems LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN 47906) equipped with a Au/Hg electrode operated in the oxidative mode (+0.15 V) vs Ag/AgCl (10). Mobile phase flow rate, 1.1 mL/min. Under these operating

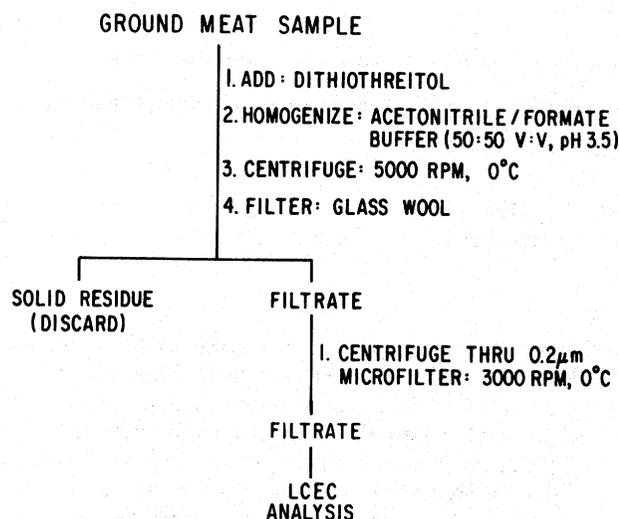


Figure 1. Flow diagram of cysteamine-cysteine LCEC Method.

conditions, the Au/Hg electrode is usually operational with good sensitivity and repeatability for ca 2 weeks before it is necessary to recoat the electrode.

Procedure

Note: *N*-Nitrosamines are potential carcinogens. Exercise care in handling these compounds.

(a) *Cysteamine-cysteine analysis.*—A flow diagram of this method is shown in Figure 1. Accurately weigh 10.0 ± 0.1 g ground pork belly into 100 mL VirTis flask. Add exactly 40 mL acetonitrile-formate buffer solution and 0.5 mL dithiothreitol solution. Homogenize sample 2 min at medium setting. Quantitatively transfer sample, using 9.5 mL-acetonitrile-formate buffer (total volume of solution is 50 mL), to 150 mL glass centrifuge bottle and centrifuge 30 min at 5000 rpm at 0–5°C. Filter sample through glass wool into 125 mL Erlenmeyer flask and transfer a 3 mL aliquot of filtered solution to the 2-stage microfilter unit. Centrifuge unit 20 min at 3000 rpm at 0–5°C. Remove lower section of

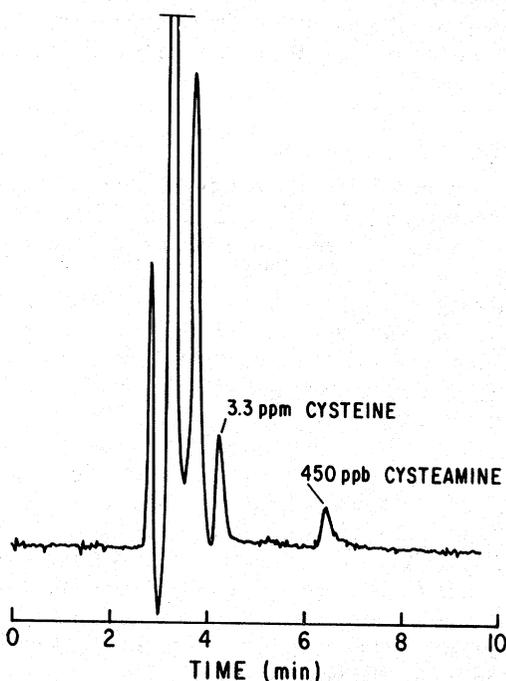


Figure 2. LCEC chromatogram of unprocessed pork belly.

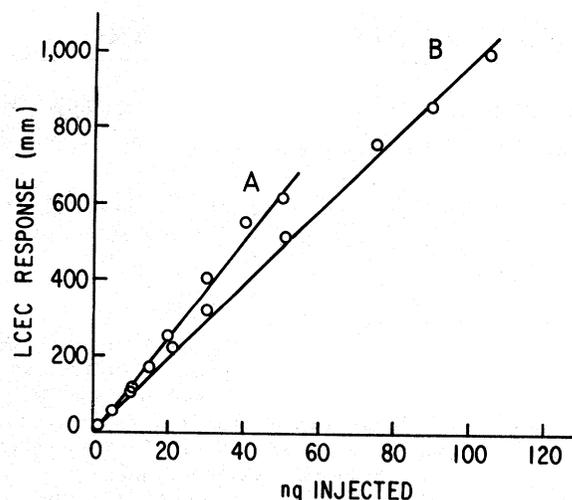


Figure 3. LCEC response for A, cysteamine, and B, cysteine.

microfilter, which contains the sample, for quantitation by LCEC.

(b) *Cysteamine-cysteine determination.*—A sample chromatogram of unprocessed pork belly is shown in Figure 2. Inject $15.0 \mu\text{L}$ cysteamine-cysteine standard ($1 \text{ ng}/\mu\text{L}$) into LCEC at lowest attenuation that yields a peak suitable for quantitation. Repeat standard injection to ensure reproducibility of retention time and response. Inject $15.0 \mu\text{L}$ sample solution and measure peak heights. Calculate cysteamine-cysteine based on 10.0 g sample in 50 mL solution. The minimum detectable level for cysteamine was 100 ppb and for cysteine, 200 ppb.

(c) *N-Nitrosothiazolidine (NTHZ) and N-nitrosothiazolidine-4-carboxylic acid (NTHZC) analysis and determination.*—Perform procedure and requisite calculations for determining NTHZ and NTHZC in cured bacon as described previously (4, 11).

(d) *Statistical analysis.*—Perform according to the methods of Snedecor and Cochran (12).

Results and Discussion

The isolation and quantitation of cysteamine and cysteine from fresh or processed meat samples was performed using an extraction procedure designed to prevent the further oxidation of the free thiols. This was accomplished by adding the antioxidant dithiothreitol (DTT). Cleland (13) reported that in a model system at pH 7 or above, DTT is capable both of maintaining monothiols in the reduced state and of reducing disulfides quantitatively. We found, however, that although DTT at neutral or alkaline pHs can reduce cysteamine (the disulfide of cysteamine), the rate of oxidation of cysteamine back to cysteamine or another disulfide was so rapid under conditions present in a meat sample that cysteamine could not be accurately quantitated. Dupré and Aureli (14) also found that the oxidation of thiols to disulfides was faster in the alkaline range, especially in the presence of catalytic amounts of copper or other cations under mild oxidizing conditions. We found that by extracting cysteamine and cysteine in an acidic medium in the presence of DTT, both thiols remained intact and the symmetric disulfides—cysteamine and cysteine—if present, were quantitatively reduced to their corresponding thiols. Caldwell (15) also successfully reduced disulfides to thiols at pH 3.2 in the presence of DTT by heating the reaction to 100°C for 5 min. We found, however, that the reaction is so facile that the heating step

Table 1. Cysteamine and cysteine in raw pork belly

Sample No.	Cysteamine, ppb	Cysteine, ppm
1	198	17.9
2-5	ND ^a	10.5, 13.6, 7.8, 20.0
6	150	14.5
7	450	3.3
8	292	26.1
9	218	21.6
10-12	tr ^b	23.4, 16.1, 12.1
13, 14	ND	6.1, 7.6
15	170	36.5
16-18	ND	10.3, 10.3, 8.3
19, 20 (ham)	ND	14.5, 2.4

^a ND = none detected.

^b tr = trace (i.e., <100 ppb).

was unnecessary. In addition, extraction of the meat samples with the LCEC mobile phase solvent system both increased the recovery of the thiols and eliminated erratic detector response. Recovery of cysteamine and cysteine during the procedure was verified by adding known amounts of these compounds to the meat sample prior to analysis. Recovery of cysteamine fortified at the 200 ppb level was $82 \pm 6.0\%$; recovery of the more prevalent cysteine spiked at the 2.5 ppm level was $89 \pm 2.1\%$. The LCEC response for both cysteamine and cysteine was linear (Figure 3) over the range of 1 to 50 ng injected for cysteamine (equivalent to 100 ppb to 16 ppm) and from 1 to 110 ng injected for cysteine (equivalent to 200 ppb to 32 ppm).

Some typical concentrations of cysteamine and cysteine in unprocessed pork belly are shown in Table 1. Cysteamine concentrations ranged from none detected to 450 ppb; cysteine, from 2.4 to 36.5 ppm. To determine if there was a correlation between these 2 compounds and their corresponding nitrosamines, cysteamine and cysteine were measured before and after processing; NTHZ and NTHZC were measured after processing. Results are shown in Table 2. No correlation was found between cysteamine levels before and NTHZ levels after processing, nor between cysteine and NTHZC in the 9 pork belly samples. It is interesting to note that NTHZ was present in a few samples that contain no detectable cysteamine in the preprocessed pork belly. Because the NTHZ amine precursors were previously found in the lean tissue (2), and to eliminate the dilution effect of the adipose tissue, lean tissue was physically separated from the pork belly before processing. Again, no apparent correlation was found between the thiols and their corresponding nitrosamines.

It now appears that the mechanism of NTHZ formation is more complex than previously thought. In model system studies, it was shown that NTHZ forms from thiazolidine 3 times faster than from cysteamine; hence, the formation of thiazolidine from cysteamine and formaldehyde may be the limiting factor (3). Formaldehyde might also be limited in either concentration or exposure to the amine precursor, which may be present in the interior of the meat rather than just on the surface. The high concentrations of cysteine present in the samples analyzed suggest that it may contribute to NTHZ formation indirectly, even though no correlation was found between cysteine and NTHZ. Although the thermal conditions during processing are not sufficiently high for NTHZC or cysteine decarboxylation, as indicated in model systems (4), other components in meat may favor this pathway. Certainly, enzymatic decarboxylation would not be a consideration because meat enzymes are deactivated by the

Table 2. Cysteamine, cysteine, and nitrosamines in smoke-house processed bacon

Sample No.	Before processing		After processing			
	Cysteamine, ppb	Cysteine, ppm	Cysteamine, ppb	Cysteine, ppm	NTHZ, ^a ppb	NTHZC, ^a ppb
1	tr ^b	12.1	ND ^c	3.9	ND	350
2	ND	7.6	ND	5.3	10.8	510
3	ND	23.4	ND	17.4	ND	ND
4	ND	16.1	ND	13.9	ND	ND
5	292	26.1	ND	11.1	ND	ND
6	218	21.6	ND	14.4	ND	ND
7	ND	10.3	ND	2.1	9.8	453
8	ND	8.3	ND	2.3	12.7	368
9	ND	10.3	ND	8.0	4.3	576
10 ^d	189	30.3	ND	4.7	1.5	498
11	114	8.0	ND	0.8	7.1	586
12	ND	12.6	ND	2.1	7.7	547
13	337	16.3	ND	19.2	5.9	1055
14	137	8.6	ND	1.2	7.3	486
15	159	9.9	ND	1.4	7.5	542

^a NTHZ = *N*-nitrosothiazolidine; NTHZC = *N*-nitrosothiazolidine-4-carboxylic acid.

^b tr = trace (i.e., <100 ppb).

^c ND = none detected.

^d Sample 10-15 are lean tissue.

heat treatment. The low levels of cysteamine compared to those of cysteine also suggest that cysteamine may be much more reactive than cysteine, thereby forming reaction products that might be much less efficient in forming NTHZ than cysteamine itself, but might be present in sufficiently high concentrations to contribute to the total NTHZ content of bacon.

In conclusion, we have shown that liquid chromatography with electrochemical detection is a sensitive technique for measuring cysteamine and cysteine in extracts from a complex food substrate. The low redox potential of the gold/mercury electrode resulted in a high degree of selectivity. This method should be applicable to these and other thiol compounds in a wide variety of food products and biological samples.

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