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Effects of Processing on the Amine Content of Pork Bellies

Ann M. Spinelli,* Leon Lakritz, and Aaron E. Wasserman

The concentration of a number of amines was determined in fresh and processed pork bellies. Analyses were conducted for spermine, spermidine, putrescine, cadaverine, histamine, tyramine, tryptamine, and ethanolamine. Fresh, pickled, and fully cured and smoked bellies were obtained from two commercial sources. The amines were recovered from perchloric acid extracts of the lean meat and derivatized with dansyl chloride. The fluorescent derivatives were separated by thin-layer chromatography, extracted, and then quantitated spectrofluorometrically. The concentration per 100 g of tissue ranged from 0.03 mg for cadaverine to 8.1 mg for spermine. There were considerable variations in the levels of the individual amines within different specimens of the same sample. Processing did not significantly alter the levels of the free amines.

N-Nitrosamines are carcinogenic to various species of animals, and pose a potential health hazard to humans (Magee and Barnes, 1956). These compounds are formed through the reaction of nitrite and secondary and/or other amines. Since approximately 70% of the pork produced annually is cured with nitrite salts, and since a nitrosamine, *N*-nitrosopyrrolidine, has been identified in bacon after frying (Pensabene *et al.*, 1974), information about the concentration of amines or their precursors in this product is needed.

A number of amines have been studied in physiological fluids (blood, urine, and semen) and special organs (heart, liver, and nervous tissue) (Frazen and Eysell, 1969; Guggenheim, 1940), but very little is known about amines in skeletal muscle. Putrescine, cadaverine, and tyramine have been reported in spoiled squid and octopus (Takagi *et al.*, 1971), and there are numerous reports on the presence of methyl-, dimethyl-, and trimethylamine in fish (Gruger, 1972). The effect of storage on the concentrations of these amines has also been investigated (Keay and Hardy, 1972).

Although nitrosamines have not been reported to any extent in hams, some of these amines may be nitrosated or act as precursors for other compounds capable of forming nitrosamines. Tabor *et al.* (1958) showed that putrescine is a precursor of spermine and spermidine. Putrescine and cadaverine, on heating, are converted to pyrrolidine and piperidine, respectively (Lijinsky and Epstein, 1970). *N*-Nitrosopyrrolidine and *N*-nitrosopiperidine have been detected in Danish bacon after frying (Crosby *et al.*, 1972) and nitrosopyrrolidine has been reported in bacon by other investigators in levels ranging from 10 to 108 μg/kg (Fazio *et al.*, 1973).

This paper reports on the identification and quantitation of eight amines, spermine, spermidine, putrescine, cadaverine, tryptamine, tyramine, histamine, and ethanolamine, in pork bellies, and the effects of two curing procedures on their concentrations.

EXPERIMENTAL SECTION

Materials. Pork bellies were obtained from two commercial processors who used different techniques for curing their products. Processor A immersed fresh pork bellies (48–72 hr after slaughter) in commercial curing solution for 6–7 days. Since these cures are commercial products, the exact composition varies; usual components include sugar, sodium chloride, sodium nitrite, sodium ascorbate, and sodium tripolyphosphate. Following a 6-hr drying step at 130°F, the bellies were smoked at the same temperature for 18 hr. Processor B utilized the prevalent procedure of stitch pumping the fresh bellies to 8–10% of green weight with a commercial cure, drying for 10–12 hr at 70–90°F, and then smoking for approximately 5.5 hr at 128°F. Several bellies were obtained at three stages of operation at each processor. The first stage sampled consisted of the green bellies prior to pickling. The second set of bellies was taken after pickling but before drying, and the final set consisted of finished bacon.

Each belly was trimmed of visible fat and only the lean tissue (approximately 600 g per belly) was used for analysis. The tissue was ground, mixed, and reground four times to exclude variation in amine content among muscles.

All chemicals and solvents were Ultra Pure, spectral quality, or fluorometric grade where available commercially. Amines for standards and dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride) were purchased from Sigma Chemical Co. Putrescine-*1,4*-^{14}C dihydrochloride (specific activity, 20.92 mCi/mmol) was obtained from New England Nuclear, with a purity greater than 98.5% as determined by thin-layer chromatography

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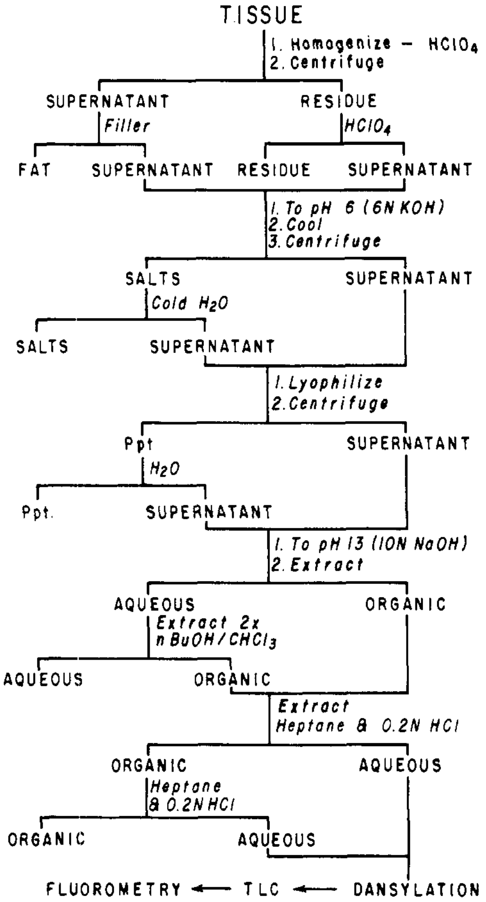

Figure 1. Flow chart of procedure for extraction and concentration of amines.

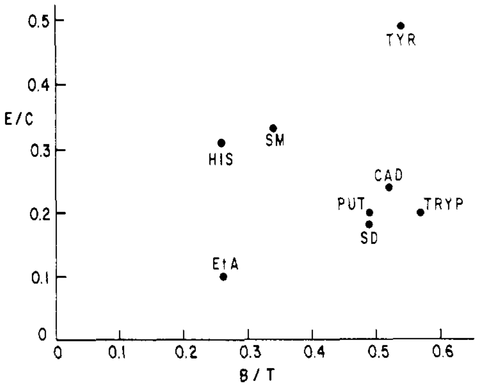

Figure 2. Two-dimensional thin-layer separation of dansyl derivatives of: SM, spermine; SD, spermidine; PUT, putrescine; CAD, cadaverine; TRYP, tryptamine; TYR, tyramine; HIS, histamine; ETA, ethanolamine. Solvent systems: E/C, ethyl acetate/cyclohexane (65/35); B/T, benzene/triethylamine (80/20).

(tlc). No further purification was done. Silica gel plates (PQ5), 500 μ thick, were purchased from Quantum Laboratories.

Methods. The flow chart of the procedure for extraction and concentration of amines is shown in Figure 1. One hundred grams of the ground tissue was homogenized in a blendor with 100 ml of 0.6 N $HClO_4$ and centrifuged. The tissue residue was reextracted twice with 100 ml of perchloric acid, and the supernatants were combined, filtered through glass wool to remove suspended fat, and adjusted to pH 6 with 6 N KOH. The supernatant was cooled to precipitate perchlorate salts which were removed by centrifugation. The precipitate was washed with ice cold deionized water, and washings were added to the supernatant. To facilitate subsequent extractions, the volume of the supernatant was reduced to 50 ml by freeze drying and then centrifuged to remove solids. The precipitate was washed three times with water, and the washes combined with the supernatant. The precipitate was discarded. The supernatant was saturated with NaCl, and the pH adjusted to 13 with 10 N NaOH. Amines were extracted (Fram and Green, 1965) three times with equal parts by volume of a mixture of n-butyl alcohol–chloroform (1:1). The organic phase was simultaneously reextracted with 1.5 vol of heptane and 0.1 vol of 0.2 N HCl. The aqueous phase was removed, and the organic layer reextracted with the HCl. The aqueous layers containing the amine hydrochlorides were combined, and their volume reduced to 25 ml by freeze drying. An internal standard, putrescine-^{14}C, was added to 100 g of meat to establish the quantitative nature of the extraction procedure.

Fluorescent derivatives of the amine hydrochlorides were prepared by adding 1 ml of reagent (40 mg of dansyl chloride dissolved in 10 ml of acetone) to 0.2 ml of the amine-containing sample which had been saturated with $NaHCO_3$. The reaction mixture was wrapped with aluminum foil to shield it from light, and shaken for 12 hr (overnight) on a wrist action shaker. The solution was dried; dansylated amines were extracted three times with ethyl acetate and the volume was reduced under nitrogen to 1.0 ml.

Dansylated amines were chromatographed on silica gel G plates, 500 μ in thickness. The solvent system used in the first direction was ethyl acetate–cyclohexane (65:35), and in the second direction, benzene–triethylamine (80:20) (Figure 2). Three plates were used for each analysis. The extract was spotted on the first and second plates, and a solution containing a mixture of standard dansylated amines was spotted on the second and third plates. The plates were developed together in a tank saturated with the first solvent system, air dried, and then developed in the second system. The spotted plates were developed in the dark, and were handled under subdued light. Plates were viewed under long-wave (365 nm) ultraviolet light, and appropriate areas marked. The silica gel containing the dansyl derivatives was scraped off, extracted with 5 ml of benzene–triethylamine (95:5), and centrifuged (Seiler, 1970). At least three standards for each amine, in different concentrations, were developed concurrently on separate tlc plates. They were processed in the same manner as the samples with one exception in that they were developed only with the benzene-triethylamine solvent.

Samples were quantitated by measuring the fluorescence on an Aminco-Bowman spectrophotofluorometer, equipped with an X-Y recorder. Emission spectra of selected amines (Figure 3) were obtained with the excitation wavelength set at 350 nm for each dansyl derivative, except histamine, where 360 nm was used. Samples were scanned from 250 to 600 nm. Water proved to act as a quenching agent and therefore silica gel plates and solvent systems should be dry. Standard solutions of dansylated amines show a linear response from 20 to 1000 ng.

RESULTS AND DISCUSSION

Preliminary studies on the isolation of amines from a perchloric acid extract of fresh pork bellies revealed a number of fluorescent spots when thin-layer plates containing dansylated material were examined under uv light. Comparison with the spectrophotofluorometric spectra and R_f values on tlc of dansyl derivatives of known amines led to the identification of spermine (SM), spermidine (SD), putrescine (PUT), cadaverine (CAD), tryptamine (TRYP), tyramine (TYR), histamine (HIS), and ethanolamine (ETA). Mass spectrometry aided in the

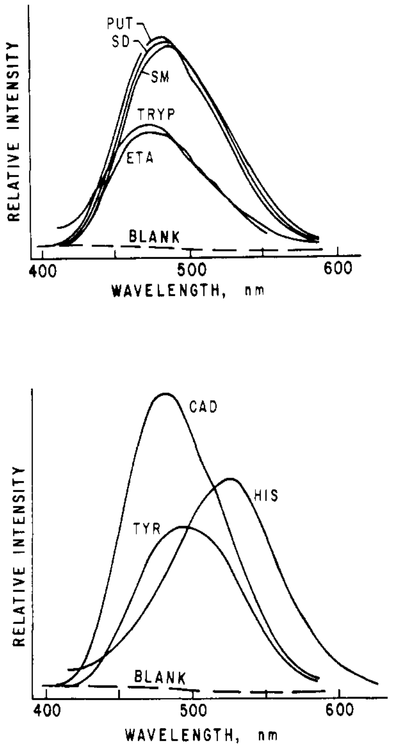

Figure 3. Emission spectra of dansyl derivatives in benzene-triethylamine (95:5): excitation, 350 nm; histamine, 360 nm; SM, spermine; SD, spermidine; PUT, putrescine; CAD, cadaverine; TRYP, tryptamine; TYR, tyramine; HIS, histamine; ETA, ethanolamine.

Table I. Concentration of Free Amines in Fresh and Immersion Cured Bellies from Processor A

	Concn of amines, mg/100 g of tissue							
Sample	SM[a]	SD	PUT	CAD	TYR	TRYP	HIS	ETA
Fresh	0.10	1.45	0.45	1.27	0.14	0.73	0.45	1.01
Pickle	8.10	1.07	0.82	0.33	0.18	2.35	0.48	0.78
	0.29	0.82	0.94	3.89	0.15	0.30	0.26	0.69
Bacon	2.85	0.28	0.23	0.27	0.22	1.34	0.19	2.20
	0.13	0.17	0.30	0.12	0.33	0.35	1.49	0.90

[a] SM, spermine; SD, spermidine; PUT, putrescine; CAD, cadaverine; TYR, tyramine; TRYP, tryptamine; HIS, histamine; ETA, ethanolamine.

Table II. Concentration of Free Amines in Fresh and Stitch Pumped Bellies from Processor B

	Concn of amines, mg/100 g of tissue							
Sample	SM[a]	SD	PUT	CAD	TYR	TRYP	HIS	ETA
Fresh	4.73	0.57	1.09	0.07	0.35	1.10	0.09	0.49
Pickle	2.19	0.36	0.36	0.22	0.10	2.73	0.07	0.14
	4.60	0.70	0.78	0.08	0.45	0.61	0.09	0.54
Bacon	0.03	0.20	2.16	0.03	0.14	0.57	0.15	0.11
	3.67	1.49	3.60	0.33	0.60	0.76	0.57	1.18

[a] SM, spermine; SD, spermidine; PUT, putrescine; CAD, cadaverine; TYR, tyramine; TRYP, tryptamine; HIS, histamine; ETA, ethanolamine.

identification of the following amines: ethanolamine, tryptamine, tyramine, and histamine. Difficulty was encountered with the derivatives of the four polyamines—spermine, spermidine, putrescine, and cadaverine. Although we were not able to successfully reproduce the work, Seiler (1970) has reported mass spectra of the mentioned polyamines. Instrumentation was carried out on the DuPont Model 21-492 spectrometer.

Separation of the eight amines on tlc plates is shown in Figure 2. In addition, there were a number of fluorescent areas on the plates which were not identified. Several radioactive amines were added to the ground pork and carried through the extraction and separation procedures; they showed the final recovery of the labeled amines was only 30% of the original material. Recoveries at various steps of the method were studied with ^{14}C-labeled spermine tetrahydrochloride, ^{14}C-labeled tyramine hydrobromide, and ^{14}C-labeled putrescine dihydrochloride. Large losses of amines were found to occur through entrapment in the ground meat matrix, particularly when 1-kg quantities were used as starting material. Reducing the sample size to 100 g and extracting with perchloric acid 3× improved recovery at this stage. No losses were noted during $HClO_4$ precipitation or freeze-drying steps and 93% of the labeled putrescine was recovered following solvent partitioning in *n*-butyl alcohol-$CHCl_3$. The final overall recovery of labeled amines carried through the modified extraction procedure was 83% of the starting compounds. The modified procedure is presented in the Methods section.

The analyses of free amines in the lean portion of one fresh (green) belly, two bellies after a 7-day immersion in cure solution, and two finished slabs of bacon (processor A) are shown in Table I. Although samples were selected at random and only two items per treatment were examined, it is evident that the concentration of amines is very low in all of the bellies and the finished bacon. The concentration of amine in several instances was somewhat higher in one sample than in the second, *i.e.* SM, CAD, and TRYP in bellies following the cure, or SM, TRYP, HIS, and ETA in the bacon. This was undoubtedly due to variability in the bellies. Inadequacy of the analytical procedure was ruled out by adding putrescine-^{14}C to the ground meat of a fresh belly and to the ground meat of a cured belly. Recovery studies for each sample were done in duplicate and averaged 83 and 80%, respectively. The overall reproducibility of the method was checked with duplicate samples of fresh and pickled bellies. The amine concentrations compared were 95.8 ± 5.5% for fresh bellies, and 81.2 ± 5.5% for pickled.

The presence of relatively constant, low levels (concentrations) of free amines in the bellies and bacon following the immersion cure is of interest. Soaking the bellies in brine solution for extended periods of time lends itself to the development of high microbial contamination. Bacterial concentrations of 10^6 ml have been reported and Cader-Strzelecka and Strzelecki (1967) found more cocci than bacilli in curing brines. The numbers of putrifying pseudomonads are reduced by 5% brine solutions and completely eliminated in 7% brine. Since the bellies in this study were cured in 7.2% brine, the possibility of proteolytic breakdown of proteins and subsequent formation of amines by decarboxylation of amino acids was greatly reduced. Furthermore, it is possible that any amino acids or amines produced could be leached out of the belly and discarded with the brine. Analysis of the rapidly cured stitch pumped bellies revealed free amine concentrations to be of the same order of magnitude as the immersion cured bellies, indicating that little degradation takes place. The results of the bellies from producer B, prepared by stitch pumping and cooked and smoked within 24 hr, are shown in Table II. In spite of the variability between pieces of meat, essentially the same low amine concentrations were found in the stitch pumped as in the immersion cured products.

The variability in amine concentration among pieces of pork belly can be seen by comparing the fresh bellies obtained from the two processors. The amines present are the end products of tissue or bacterial metabolism and may reflect the history (pre- and postslaughter treatment) of the belly. However, since no pattern is evident, *i.e.* no belly or treated product contains a higher concentration of all the amines tested, it appears that the variability is random and differences are not due to either the curing treatment or the cooking–smoking step.

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Changes in Nitrate and Nitrite Content, and Search for Nitrosamines in Storage-Abused Spinach and Beets

Edward G. Heisler,* James Siciliano, Samuel Krulick, Jules Feinberg, and Joseph H. Schwartz

Conditions favoring formation of nitrosamines—simultaneous presence of nitrite and secondary amines at acid pH—can occur during abusive storage of comminuted fresh spinach or beets. Shredded spinach, shredded or ground fresh beets, or beet juice rapidly produced nitrite. One beet sample contained 1000 ppm of nitrite after 2 days at room temperature and still appeared edible. Shredded fresh spinach samples were considered inedible when nitrite levels of 300–500 ppm were reached. Whole fresh and all forms of processed spinach and beets accumulated little nitrite even though containing 1500–2000 ppm of nitrate originally. Selections from these tests, cooked and uncooked, and thawed, frozen, and thawed spinach were analyzed by a glc-mass spectral method for six nitrosamines (methyl-, ethyl-, and methylethylnitrosamine, nitrosopyrrolidine, nitrosomorpholine, and nitrosopiperidine). Results were negative, indicating no detectable formation of these six nitrosamines when fresh or processed spinach or beets are stored even beyond the point of edibility.

The possible occurrence in foods of nitrates and nitrites, and especially of nitrosamines, has recently become a matter of great concern because of the toxic or carcinogenic nature of some of these compounds (Aune, 1972; IFT, 1972; Wolff and Wasserman, 1972). Some vegetables, such as spinach and beets, can accumulate high levels of nitrate, which may be reduced to nitrite during storage after preparation. Earlier concern was with the relation of nitrate and nitrite in the diet to infant methemoglobinemia (Phillips, 1969, 1971; Sinios and Wodsak, 1965; Achtzehn and Hawat, 1970). Numerous instances of this disorder have been related to excessive levels of these ions in well water; in Europe 15 cases of infant methemoglobinemia were traced to feeding with improperly stored home-prepared spinach purees. Processed vegetable products have been shown not to accumulate significant amounts of nitrite during normal storage (Phillips, 1969, 1971; Sinios and Wodsak, 1965).

Since nitrites are known to react with secondary or tertiary amines to form nitrosamines, we felt it prudent to check these vegetables for the presence of the latter compounds. Keybets *et al.* (1970), using methods sensitive to 500 μg/kg of dimethylnitrosamines and 100 μg/kg of diethylnitrosamines, could not detect these nitrosamines in high-nitrite spinach. However, since many nitrosamines are highly potent carcinogens, and since their carcinogenic threshold values in humans are not known, it is important that more sensitive and specific analytical techniques be used.

The purpose of the present study was to examine the development of nitrite in beets and spinach subjected to storage abuse and to analyze high-nitrite samples for several nitrosamines by a method which is both specific and highly sensitive.

EXPERIMENTAL SECTION

Beet and spinach samples, both fresh and processed, were obtained from local retail markets and were subjected to storage at room temperature (25°) and at refrigeration temperature (5°). The lengths of time were from a few days at room temperature to as long as 32 days under refrigeration.

The following types of beet samples were studied: fresh beets, whole and shredded; beet juice; canned beets, sliced, whole, and ground; strained beets (baby food); and borscht. Spinach samples studied were: fresh spinach, whole and shredded; spinach juice; frozen spinach, whole

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