The Eastern Regional Research Center (ERRC), Meat Laboratory initiated studies on nitrosamines (NA's) in cured meat products in 1970. This was shortly after the U.S. Food and Drug Administration (FDA) found dimethyl-nitrosamine (DMNA) in fish products treated with NO₂ and/or NO₃ (1).

Initially, commercial samples of ham were analyzed for DMNA, but none was detected (2). In a survey of frankfurters, 11 to 84 ppb (µg/kg) DMNA was confirmed in 3 of 40 samples analyzed (3). Therefore, two approaches were taken to determine the cause of DMNA formation: to determine chemically whether some biogenic amines had any role in DMNA formation, and to determine the effect of cure ingredients on DMNA formation in processed frankfurters.

In the first study, tertiary amines containing a dimethylamino group were found to produce a greater amount of DMNA than the corresponding quaternary ammonium compounds. These results suggested that tertiary amines, under certain conditions, may pose as great a problem in NA formation as secondary amines (4). Secondly, in processed frankfurters, a concentration of 10 ppb or greater DMNA could not be produced when franks were prepared with the permitted level of ca. 150 ppm (mg/kg) NaNO₂. Ten times this amount was needed to consistently yield 10 ppb DMNA (5) which was the lowest concentration of NA confirmable by mass spectrometry at that time. It was found that added NaNO₃ had little effect on DMNA formation (6), but sodium ascorbate (NaAsc) and sodium erythorbate (NaEry) inhibited its formation (7). At the same time we carried out this work it was also reported that ascorbic acid (AscH) inhibited NA formation from drugs (8). Cure ingredients in a frankfurter model system were also investigated. Generally, AscH, NaAsc, and NaEry were found to inhibit the nitrosation of dimethylamine, while glucono-delta-lactone (GDL) was found to enhance DMNA formation (9).

Preliminary experiments with GDL in frankfurters also showed the same effect. The study on frankfurter cure was not completed because reports by other investigators of the consistent occurrence of nitrosopyrrolidine (NPy) in fried bacon (10,11,12) raised questions of higher priority. Since the consumption of bacon exceeds 1.5 billion pounds annually in the United States, this problem required immediate attention.

The questions that needed to be answered were: how is NPy formed and how do you prevent its formation? Nitrosopyrrolidine may result from the nitrosation of pyrrolidine (Py) derived from putrescine or from the decarboxylation of proline (Pro) or by the formation of nitrosoproline (NPro) and its subsequent decarboxylation. Kinetic data by Mirvish (13) suggests that Pro, a weaker base than Py, would nitrosate more readily. The fact that NPy is not present in uncooked bacon (11,12,14) provides indirect evidence that formation occurs via the NPro pathway. If this is correct, it would theoretically be possible to prevent conversion of NPro to NPy.
Heating NPro in silicone oil in a model system at different temperatures showed that maximum production of NPy occurs at 185°C (365°F), close to the recommended bacon frying temperature, and that little is formed at lower temperatures (14). Therefore, the frying conditions might be important in determining how much NPy is formed in the cooked product.

Two experiments: 1) frying bacon to medium–well done using 4 different temperatures from 99° to 204°C and varying time; and 2) holding the frying time constant at the same temperatures with the degree of bacon crispness variable; showed that NPy formation was primarily temperature dependent. Lower temperatures and longer frying times yielded less NPy (14). Other commonly used cooking methods were also investigated. When bacon was cooked by frying, baking, broiling, using a microwave oven, or an appliance called a "baconer", the least amount of NPy was found in samples cooked by the microwave oven (14).

Next, investigations on bacon storage and processing were conducted to determine their contribution to NPy formation. Vacuum packages of freshly prepared bacon stored at 7°C for up to 10 weeks prior to frying showed a slight decrease in NPy formation (15). Bellies fried and analyzed after cure injection, prior to introduction into the smokehouse, and after the drying step contained ca. 8 ppb NPy. A significant increase in NPy resulted from the remaining heat and smoking treatment (16).

These results suggested that processing time may be an important factor in NPy production since it varies from one manufacturer to another. However, repeated processing of bacon up to 24 hours with sampling at 4-hour intervals showed no clear-cut correlation between NPy concentration and processing time (17).

Investigations were also carried out on "green" uncured bellies. Storage of green bellies under refrigeration conditions up to 2 weeks resulted in no change in either free Pro prior to processing or NPy in the finished bacon (16). However, a significant increase in free Pro was observed in green bellies frozen for 2 weeks and then thawed. A corresponding change in NPy content was observed after processing the bellies into bacon. This finding was clear-cut only when bacon was made without NaAsc or NaEry (18).

Earlier investigations showed higher concentrations of NPy in the cooked-out fat than in fried bacon (11,12). This may result from NPy partitioning into the fat phase due to its fat solubility. When separated bacon adipose and lean tissue were subjected to similar frying conditions, NPy was shown to form only from the adipose tissue (19). Assuming NPro is the precursor for NPy, why doesn't ham also form NPy since uncured ham contains an average of 52 ppm Pro (20) as compared to 17 ppm in green intact bellies (18)? The unique composition of pork belly adipose tissue may explain why ham, Canadian bacon (back bacon), and a beef bacon-like product do not form NPy under similar frying conditions (19). It appears that NPy formation is complex and it is theorized that connective tissue or collagen, known to be composed of
large concentrations of bound glycine, hydroxyproline and proline, is
directly or indirectly responsible. At this time, the role of NPro is
not clear since we have isolated and identified it in uncooked bacon in
concentrations that could yield NPy at the ppb level after frying (21).

Since NPy is formed primarily in the adipose tissue of bacon, the NA
inhibitory activity of NaAsc and NaEry may be limited by their low fat
solubility. Therefore, a number of more fat soluble derivatives of
ascorbic acid, in combination with NaAsc, were evaluated for inhibition
of the nitrosation of Py in a bacon model system. Ascorbyl palmitate
and dipotassium ascorbyl-2-sulfate were particularly effective (22), and
are currently being investigated for their NPy inhibitory activity in
bacon. These studies are being conducted because earlier work in our
laboratory and those sponsored by the American Meat Institute (AMI)
failed to eliminate NPy in bacon prepared with 1000 ppm NaAsc or NaEry.
This approach of interfering with the nitrosation reaction by reducing
the NO\textsuperscript{2} to nitric oxide, a compound that does not normally nitrosate
secondary amines, has to date not been completely successful. The
reductant competes with amines for NO\textsuperscript{2} and is effective if the rate of
reaction is greater. Reducing added NO\textsuperscript{2} is an attractive approach to
decreasing NA's because their formation is generally dependent on the
square of the NO\textsuperscript{2} concentration. However, little information is avail­
able as to how much the NO\textsuperscript{2} can be lowered without having problems with
flavor, color, and more importantly, microbiological preservation, par­
ticularly against \textit{C. botulinum}. Our laboratory has been interested in
two alternatives that would control \textit{C. botulinum} and still permit use of
lower levels of NO\textsuperscript{2}. The first uses the irradiation sterilization of
bacon. This is an extension of our work on ham in collaboration with
the U.S. Army Natick Laboratory (23,24). The other approach, in co­
operation with the AMI, utilizes prefried bacon. Here, \textit{C. botulinum} is
controlled by reduced water activity and high concentration of salt
resulting from the removal of rendered fat and moisture during the brief
prefrying process (25). This work has not been completed.

Several other investigations have been carried out. Recognizing that
gelatin is commonly prepared by the alkaline treatment of collagen,
gelatin could serve as a source of nitrosatable proline that may be
converted to NPy under certain conditions. Therefore, a limited
volatile NA survey was carried out on meat products made with gelatin
and chunks of cured nonskeletal and organ meats. DMNA was confirmed in
8 of 10 samples of souse, head cheese and blood and tongue loaf, but
only a single sample containing NPy. No correlation was found between
residual NO\textsuperscript{2} and the concentration of DMNA (26).

In a study of lecithin and its derivatives, the results suggest that
phospholipid decomposition products may be a source of DMNA (27). The
lecithin content of nonskeletal tissue, including organ tissue, may be
higher than skeletal tissue. In fact, phospholipids have been found in
bovine tongue (28) and the same is probably true of porcine tongue, which
is a cured ingredient of souse.

Some research has been conducted on fermented meat products, specifically
with Lebanon bologna, an all beef product, and with pepperoni in coopera­
tion with our Meat Microbiology group. NA's could possibly be formed in
this product type as a result of its pH, NO\textsuperscript{3} cure and the microorganisms
present. None of the volatile NA's surveyed were found in commercial samples of Lebanon bologna or in those prepared in our pilot plant with natural meat flora or starter culture during the fermentation and mellowing period (29). A similar study of pepperoni prepared in our laboratory also gave negative NA results (30). These studies suggest that volatile NA's were not formed and decomposed during processing and that NA's in fermented meats may pose little or no problem.

In cooperation with the Plant Products Laboratory, samples of fresh and processed spinach and beets originally containing 1500 to 2000 ppm NO\textsubscript{3}, which had been subjected to storage abuse, were analyzed for NO\textsubscript{3} and NA's. No NA's were detected, even in samples stored beyond the point of edibility (31).

Although we have investigated other areas, NPy in fried bacon remains our priority project.

This summary would not be complete without some mention of the analytical procedures employed. The difficulty in the NA analysis has been found to be related to the fat content of the meat product. That is, the complexity and amount of sample clean-up required increased as our analyses progressed from ham to frankfurters, then fried bacon, and finally its cooked-out fat. Sampling, specifically with bacon, is a persistent problem due to its inherent variability. Procedures for the isolation and determination of volatile NA's developed by the U.S. FDA are used for the analysis of meat samples (32,33,34). We have modified them slightly as required. The column chromatographic clean-up step is very critical. The silica gel must have the proper activity in order to obtain a relatively clean sample and good NA recovery so that it can be quantitatively determined and confirmed. The samples are assayed for six volatile NA's which include: dimethylnitrosamine, methylethyl-nitrosamine, diethylnitrosamine, nitrosopiperidine, nitrosopyrrolidine, and nitrosomorpholine. Separation and detection is carried out with a gas chromatograph equipped with a column containing Carbowax 20M-TPA liquid phase and a nitrogen selective alkali flame ionization detection (AFID). In our initial work with ham, a Varian-Aerograph rubidium sulfate pellet detector was used. However, problems were encountered with reproducibility of response toward NA's. A potassium chloride coated Pt-Ir wire coil AFID is currently used. The detector response to NA's is linear over a wide range and 0.1 ng NA is readily detected. Occasionally, sample peaks, which are not NA's, are obtained at the same retention time as NA's. Confirmation of identity of NA's is carried out by gas-liquid chromatography-mass spectrometry (glc-ms). The mass spectrometer is operated in the peak matching mode at a resolution of 1 in 12,000. A glc retention time coincident to an authentic NA and matching the exact mass of the NA parent ion are the criteria for positive confirmation. For example, the parent ion of NPy is m/e 100.06366. Concentrations as low as 3 ppb have been confirmed with this single ion detection technique depending on the amount of interfering sample components present; 5 ppb NPy or higher are confirmed with more consistency.

Some of the same techniques have been applied to the isolation and identification of NPro in uncooked bacon. The volatile methyl ester was
used for gas-liquid chromatographic detection and mass spectral confirmation (21). However, a reliable quantitative procedure for NPro has not been developed. Currently, high-pressure liquid chromatography is being used in an attempt to overcome the isolation problems.

References


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Precaution should be exercised in the handling of nitrosamines since they are potential carcinogens.
SUMMARY OF RECENT DEVELOPMENTS IN NITROSAMINE RESEARCH

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Concern about nitrosamines several years ago led to the appointment of a Department of Agriculture (USDA) Expert Panel on Nitrites and Nitrosamines. This panel was charged with investigating the use of NO₂⁻ and NO₃⁻ and their role in the development of NA's in cured meats and with recommending appropriate changes in Federal Regulations. Recently, three recommendations have been submitted to the Secretary of Agriculture (1). They are: 1) that the use of NO₃⁻ salts be discontinued in all meat products except dry-cured and fermented sausage products, 2) that a standard level of 156 ppm NaN₃ be added to all processed meat products, except bacon and dry-cured products, and 3) the currently permitted 200 ppm residual NO₂⁻ be reduced to 100 ppm in cooked sausage products, 125 ppm in canned cured and pickle cured products and 50 ppm in canned and sterile products. Action on bacon, dry-cured and fermented products is deferred until additional research data becomes available. The general objective is to use ingredients only in the concentrations needed on a product by product basis.

Ham, frankfurters, fermented sausages, bacon, and other cured meats differ from each other in several respects including cure formulation, processing, packaging, storage, and marketing. Therefore, the NO₃⁻ and NO₂⁻ levels for each product class needed for the control of the botulinum hazard, NA's and to a lesser degree flavor, color, and other qualities had to be ascertained. With this in mind cooperative studies have been carried out by the AMI, FDA, and USDA. Results have appeared from investigations of a perishable, canned, comminuted cured meat product (2), weiners or frankfurters (3), and, recently, fermented sausages (4). In no instance was a detectable quantity of 14 volatile NA's found during the preparation and processing of these products. Results of studies on bacon have shown that addition of Asc⁻ or Ery⁻ reduces NPy formation during frying. However, some unexplainable discrepancies and variability in results have been obtained (5). The problem is that bacon produced on a pilot plant scale did not always contain NPy after frying (6), whereas NPy is still present in bacon produced on a commercial scale. Investigations in this area are continuing.

There have been several recent publications directed toward NA formation in bacon. In one, fried samples of bacon prepared with 0 to 200 ppm NaN₂O₃ showed that levels of NPy correlated well with the initial concentrations of NO₂⁻, but not with the residual NO₂⁻ determined prior to frying (7). In a study of volatile amines in pork eye muscle, 200 ppb dimethylamine was found prior to curing and up to 520 ppb in vacuum packaged bacon that had been prepared according to the Wiltshire process. No NA's were found above the detection limit of 1 ppb in any of the samples (8). In another experiment, the same investigators found that NaAsc suppressed DMNA formation in bacon prepared from pork muscle to which dimethylamine had been added. Heat treatment of cans or frying also resulted in lower concentrations of DMNA in the lean tissue as compared to the fried fat (9).
Some recent work has appeared using model systems to determine the precursor(s) of NPy in bacon. The most significant results are the production of NPy from the action of NO$_2^-$ on bovine Achilles tendon collagen (10) and the connective tissue of cured, smoked ham (11).

In the past year, investigators in more countries have become active in NA analysis. The Japanese have been very active in NA research and in an earlier report have detected DMNA in 7 of 23 meat products made with fish and/or whale meat (12). A recent survey of Italian meat products also reported unconfirmed DMNA in concentrations up to 119 ppb in 54 of 250 samples (13). In a Canadian survey of 100 cured meats, DMNA was detected in 29, diethylnitrosamine in 9, and NPy in 17 samples. However, NA's were confirmed by mass spectrometry in only 5 samples (14). In an extensive article by West German workers (15) on cured sausage products, it was generally found that the legally permitted amount of NO$_2^-$ or NO$_3^-$ added resulted in less than 10 ppb NA. However, the authors claim the individual occurrence of higher NA values in products exposed to higher temperatures, and higher NA concentrations were found on addition of AsCH, NaAsc, and GDL. Because of the methods employed, they recommend that additional confirmation such as with glc-ms is required.

After the Canadian Health Protection Branch consistently found NPy and nitrosopiperidine (NPip) in sausage products of one manufacturer, investigations indicated that the cure premix, composed of spices and curing salts, was responsible (16). Buffering the premixes to pH 7.5-8.2 reduced NA formation, but was not reliable in completely eliminating them (17). Similar results were obtained in United States studies. On the basis of this finding, a change in U.S. Federal Regulations was issued banning the use of cure premixes. Now the spices and NO$_2^-$ must be packaged separately (18).

Recently, a new element has entered the NA picture. That is, the question of C-nitrosophenols. In 1971, it was reported that tannins retard NA formation (19). It was thought that simpler phenols might effectively compete with the nitrosation of secondary amines and reduce the potential for NA formation. However, the nitrosophenols produced may be hazardous (20). The report that 4-methylcatechol and chlorogenic acid catalyze NPip formation (21) and the report of the identification of nitrocresols and nitroso- and nitroguaiaicols in smoked bacon (22) has served to confuse the situation. The significance of these findings is not known at this time.

The International Agency for Research in Cancer of the World Health Organization has conducted collaborative studies to obtain more information on the occurrence of NA's in the food supply and to standardize NA analytical procedures. The first study was carried out on aqueous and methylene chloride solutions of diethylnitrosamine (DENA) and NPy utilizing analytical methods currently employed by the analysts in the various countries. This past year a canned luncheon meat containing added DMNA, DENA, dibutylnitrosamine and NPy was analyzed in a similar fashion and the preliminary results have been reported (23).

There are analytical methods available for the determination of volatile NA's in foods. At present there is a trend toward developing methods
for nonvolatile NA's with particular emphasis on nitroso secondary amino acids. High-pressure liquid chromatography has been used to separate standard solutions of NPro, nitrososarcosine and nitroso-2-(ethylamino)-ethanol (24) and nitroso-alkylureas and alkylurethans (25). A major drawback in liquid chromatography is the lack of specific detection systems. For volatile NA's, gas-liquid chromatographic separation and detection is the preferred method. The Alkali Flame Ionization, Coulson or Hall, and Electron Capture detectors are commonly employed. They all involve some specificity to differentiate the NA or its derivative from other sample components, but require additional confirmatory procedures. Currently, a "clean" sample is required for confirmation with multiple ion detection, low resolution mass spectrometry or a "less than clean" sample using high resolution mass spectrometry.

There are two new detection systems that may be applied to NA's. The first, is the Plasma Chromatograph which can be coupled to a gas chromatograph. Utilizing the mobility spectra of standard volatile NA's it is claimed that $10^{-11}$ g of these compounds can be specifically detected (26). The applicability of this detector to NA's is so recent that its effectiveness with food samples has not been determined. The second detector is the Thermal Energy Analyzer which can be used for both GLC and liquid chromatography. It is claimed to be specific for compounds containing the N-NO group and capable of analyzing samples for total NA's in picogram concentrations (27,28). Preliminary results with a few volatile NA's added to fresh beef and herring are encouraging (29).

References


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