AN ABSTRACT OF THE THESIS OF

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(Name) (Degree)

in FOOD SCIENCE presented on February 20, 1974
(Major) (Date)

Title: THE DETERMINATION OF N-NITROSOPROLINE IN CURED MEATS

Redacted for privacy

Abstract approved: A. F. Anglemier

A method is described for the determination of N-nitrosoproline (NOPRO) in cured meat products. NOPRO was extracted with ethyl acetate from a slurry of cured meat and water after the addition of ammonium sulfamate and acid. This nitrosamino acid was converted to the methyl ester and after further purification, it was quantitated and confirmed by gas liquid chromatography and mass spectroscopy. Recoveries of 10 μg from spiked samples averaged about 70%.

A capillary column was used to confirm the presence of low levels of MeNOPRO in the extracts. An oscilloscope, utilizing horizontal sweep magnification of the mass spectrometer output, was used to detect the elution of MeNOPRO from the column by monitoring the m/e 30 ion. The presence of 5 nanograms per injection of concentrated cured meat extract could be confirmed.

NOPRO was detected in several commercial meat products.

Five of six bacon samples analyzed were found to contain NOPRO at levels ranging from 13-46 ppb. A precooked ham slice and a sample of breakfast beef contained 22 and 62 ppb NOPRO, respectively. NOPRO was not detected in a sample of canned chopped ham nor a wiener sample.

The detection of NOPRO, a non-volatile nitrosamine, in cured meats suggests that the formation of other non-volatile nitrosamines is possible. To date, only volatile nitrosamines have been analyzed and confirmed by mass spectrometry. The ubiquity of proline and the finding of NOPRO in several different cured meat products suggest that NOPRO could be an indicator of nitrosation in foods.

Bacon cured in brines containing 800 and 1600 ppm nitrite contained about 50 and 100 ppm residual nitrite, respectively. Bacons receiving the lower nitrite level contained no NOPRO while the higher nitrite treated bacons contained over 100 ppb NOPRO. Frying of bacon was found to destroy 86 to 100% of NOPRO in the five samples analyzed. NOPRO has been reported to break down during heating to nitrosopyrrolidine (NOPYR). However, the levels of NOPRO determined in raw bacon were not sufficient to produce the amounts of NOPYR reported in fried bacon if the rate of conversion was similar to that previously reported in model studies.

The Determination of N-Nitrosoproline in Cured Meats

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1974

APPROVED:

Professor of Food Science and Technology
in charge of major

Head of Department of Food Science and Technology

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Date thesis is presented February 20, 1974

Typed by Mary Jo Stratton for Francis James Ivey

ACKNOWLEDGEMENTS

I would like to extend my appreciation to the faculty and staff of the Food Science and Technology Department for their cooperation and interest throughout the course of this investigation. Dr. A. F. Anglemier deserves special thanks for his excellent guidance and council. Dr. L. M. Libbey's expertise and assistance in mass spectrometry were essential to this study and greatly appreciated. Dr. R. A. Scanlan is thanked for his advice as well as service on the committee. Dr. R. R. Becker is acknowledged for his advice and cooperation in amino acid analysis in addition to serving on the committee.

Drs. G. H. Arscott and R. W. Thies are recognized for their contributions to the committee. Mr. R. L. Howard is thanked for his aid in amino acid analysis. Mr. Ralph Turner of the O. S. U. meats laboratory is acknowledged for technical assistance in the preparation and smoking of bacon samples processed for this study. Dr. N. E. Pawlowski is thanked for his assistance in NMR and IR interpretation.

Special recognition goes to my wife for her sacrifice and dedication to the completion of this work.

This research was supported by a National Defense Education Act Fellowship (Title IV), and partially by a Food and Drug Administration Grant 5R01-FD-00382.

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INTRODUCTION

The formation of N-nitrosamines in cured meats has become an important problem in food safety. These compounds may have a role in the occurrence of human cancer. N-nitrosamines, which are generally produced by the reaction of nitrite with secondary amines, have been reported in several foods. The carcinogen, dimethylnitrosamine (DMNA) has been reported to occur sporadically in cured meats.

Methods for the detection of N-nitrosamines have become extremely sensitive, with limits as low as ten parts per billion (ppb) for volatile N-nitrosamines. Confirmation of N-nitrosamines by mass spectrometry has been essential in order to avoid reports of hazardous compounds that do not exist. The methods presently available have been limited to volatile nitrosamines which can be analyzed by gas chromatography coupled to mass spectrometry.

N-nitrosopyrrolidine (NOPYR), a known carcinogen in rats, has been reported to consistently occur in fried bacon. The amino acid proline (pro) and its nitrosamine, N-nitrosoproline (NOPRO), have been shown to be precursors of NOPYR under frying conditions.

The purpose of this study was to develop a method to measure

the levels of the non-volatile NOPRO, to determine its presence in cured meats, and to investigate some of the variables affecting the possible formation of NOPRO in bacon.

REVIEW OF LITERATURE

N-nitrosamines, as a class, have been found to be potent carcinogens. As possible products of reactions of amines and nitrite, these carcinogens have become a matter of concern in food safety. Many N-nitrosamines and N-nitrosamides have been found to be carcinogenic to animals (Magee and Barnes, 1956; Druckrey et al., 1967) and these may also be mutagenic and teratogenic as well (Magee and Barnes, 1967). Of the approximately 100 N-nitrosamines tested, about 75% produced lesions in test animals (Wolff and Wasserman, 1972). Experimental results of animal studies suggest that these compounds could be carcinogenic to man (Greenblatt and Lijinsky, 1972a). The relationship between ingestion of DMNA and esophageal cancer has been implicated in man in Africa (McGlashan et al., 1968) and established in rats (Druckrey et al., 1963).

Nitrite in Foods

Salts of nitrite are found in many foods. Nitrite occurs naturally in many plant materials (Phillips, 1968). During storage, spinach has been reported to develop up to 1000 parts per million (ppm) nitrite (Phillips, 1968). Nitrate and in rare cases, nitrite, are present in well water (Alexander, 1973) and nitrite has been detected in saliva (Tannenbaum, 1972).

Nitrite can be added, presently as a legal food preservative to meats and smoked fish as prescribed by food additive regulations CFR 121, 1063, 1064, and 1230 (Code of Federal Regulations). The levels of residual nitrite, however, in cured meat products can be extremely varied. Panalaks et al. (1973) reported an average level of 28 ppm in 197 products tested, with levels ranging from 0 to 252 ppm. Nitrate occurs at higher levels in plants than nitrite. It may be added at higher levels than nitrite to cured meats although it can be reduced by microbial action to nitrite (Phillips, 1968).

Nitrite is added to meat during curing for several purposes. Nitrite acts as a preservative in fixing the red color of cured meats and by inhibiting the outgrowth of Clostridium botulinium, the causitive agent of botulism poisoning (Greenberg, 1972; Hustad et al., 1973). Nitrite is also important in developing the flavor characteristic of cured meat products (Hustad et al., 1973). Recent studies attempted to determine the amount of nitrite required to maintain the safety of temperature-abused cured meats. Canned hams (stored at 21°C) were found to require levels approaching the legal level (200 ppm) of sodium nitrite to maintain safety (Greenberg, 1972). Frankfurters were found to be safe up to 56 days at 21°C with only initial levels of 50 ppm sodium nitrite and longer with 100 ppm sodium nitrite (Hustad et al., 1973). During these studies, nitrate was found to have no significant effect on safety when added in combination with nitrite (Greenberg, 1972; Hustad et al., 1973).

Precursors of Nitrosamines

Many amines that react to form nitrosamines are found in foods. Nitrite reacts with all types of amines, but the formation of nitrosamines generally arises from interaction with secondary amines. Nitrosamides arise from the reaction of secondary amides with nitrite. Nitrosamines have been found to arise from tertiary amines and quaternary ammonium compounds (Fiddler et al., 1972a). Moreover, some primary diamines have been reported to cyclize and nitrosate (Bills et al., 1973) although these are unusual sources of nitrosamines.

Many common amines, which are precursors of N-nitrosamines, have been identified in foods. Trimethylamine and dimethylamine are commonly found in fish products and either amine can react with nitrite to form DMNA (Hein, 1963; Fiddler et al., 1972a). Certain amino acids were noted to form N-nitrosamines when heated with nitrite in dry starch (Ender and Ceh, 1971). These workers found that valine, glycine, and sarcosine produced DMNA while pro formed NOPYR. Citrulline, arginine, methylurea, and methylguanidine can undergo nitrosation to form nitrosamides (Mirvish, 1971a). Fiddler et al. (1972a) found that the quaternary amines such as choline, acetylcholine, neurine and carnitine, could react with nitrite to form trace amounts of nitrosamines.

Spices have become a recognized source of amines. In recent action, the FDA banned the use of commercial spice-nitrite premixes

after finding 2-56 ppm of N-nitrosopiperidine and NOPYR in samples investigated (Federal Register, 1973). Morpholine, a secondary amine used as an anticaking agent in boiler treatment compounds, was also banned from use by food processors after it was found to be a readily nitrosated inadvertent contaminant of smoked cured meats (Federal Register, 1972). Morpholine reacts with nitrite to form the carcinogen, N-nitrosomorpholine.

Several chemicals have been reported to either enhance or reduce the rate of nitrosamine formation from nitrites and secondary amines. Many inorganic ions, notably thiocyanate which occurs in saliva, enhance the rate of nitrosation (Boyland et al., 1971; Fan and Tannenbaum, 1973a). Mirvish et al. (1972) discovered that ascorbic acid could block the reaction of nitrite with amines. When present in high concentrations (twice the molar concentration of nitrite), ascorbate was up to 99% effective in blocking nitrosation reactions. The protective effect of ascorbic acid has been confirmed in mouse feeding studies (Kamm et al., 1973; Greenblatt, 1973), and in the processing of frankfurters (Fiddler et al., 1973a).

Detection Procedures

Concern for the safety of nitrite-containing foods has instigated the development of analytical procedures for detecting and measuring N-nitrosamines. Many procedures have been used to determine the

presence of N-nitrosamines in foods. However, many of the early methods lacked specificity which led to positive reports in negative samples or over-estimation of the levels of nitrosamines present in foods (Wolff and Wasserman, 1972).

Polarography has been used to detect nitrosamines (Lydersen and Nagy, 1967; Devik, 1967) although Heynes and Koch (1970) noted that pyrazines interfere with this method as well as some gas liquid chromatography (glc) methods.

Thin-layer chromatography (tlc) has been used with limited success (Preussman et al., 1964; Sen et al., 1969; Sen and Dalpe, 1972). A color reaction between nitrite released from the N-nitrosamine by heat or ultraviolet irradiation and the Griess reagent was used to detect the N-nitrosamine after development of the chromatogram. Wolff and Wasserman (1972) pointed out that the color reagents could react also with other food components, such as fatty acids and pigments. Colorimetry, utilizing the same color reaction as tlc has been used for detecting non-volatile nitrosamines (Daiber and Preussman, 1964; Fan and Tannenbaum, 1971).

Nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy were used to identify DMNA from a Bantu food by Du Plessis et al. (1969). Since exact levels could not be determined, the authors indicated that the possibility of interfering artifacts did exist.

Glc has been used extensively for determining volatile

nitrosamines (Kroller, 1967; Howard et al., 1970; Fiddler et al., 1971; Telling et al., 1971; Essigman and Issenberg, 1972). Several workers have reported that the use of gas liquid chromatography-mass spectroscopy (glc-ms) in tandem gave superior separation and conformation of identity which other methods lacked (Fiddler et al., 1971; Wolff and Wasserman, 1972).

Although glc-ms techniques are extremely sensitive, such procedures are limited to volatile N-nitrosamines. Several methods have been reported to yield a sensitivity of 10 ppb for DMNA (Fiddler et al., 1971; Fazio et al., 1971; Newell and Sisken, 1972). A multidetection method that measures 14 nitrosamines at levels of 10 ppb has been developed by Fazio, Howard and White (1971).

Most of the methods for glc-ms determinations involve an extraction, followed by steam distillation and then, as additional cleanup, adsorption chromatography, tlc, and/or liquid-liquid extraction. The final step is the separation, detection and confirmation by glc-ms (Fazio, Howard and White, 1971; Telling et al., 1971; Essigman and Issenberg, 1972).

The multidetection method of Fazio, Howard and White (1971) utilized a methanolic potassium hydroxide reflux to liquify the sample, liquid-liquid extraction into methylene chloride (CH_2Cl_2) followed by steam distillation under alkaline conditions. The distillate underwent liquid extraction by acid and then base. The CH_2Cl_2 fraction was then

placed on a silicic acid column so that the proper fraction could be concentrated and later injected on glc. After tentative identification of N-nitrosamines, the sample was then subjected to glc-ms for confirmation.

Oxidation of the N-nitrosamines to the corresponding nitramine, which can then be detected by electron capture has been used as a detection technique (Eisenbrand et al., 1970). This allows the use of the more sensitive electron capture detector, rather than a flame ionization type of detector normally used. The nitramine, however, is very unstable and the use of this method has been limited.

Nitrosamines Reported in Foods

The presence of low levels of N-nitrosamines, particularly DMNA, in foods has been confirmed by using glc-ms. Panalaks et al. (1973) analyzed 197 meat samples and found 57 samples to contain trace amounts (2-12 ppb) of DMNA. Fazio, White and Howard (1971) analyzed 57 meat products but detected only one sample containing DMNA (5 ppb). The presence of DMNA was confirmed in commercial nitrite-treated shad, salmon, and sable (Fazio et al., 1971). Using the multidetection method, Fazio, Howard and White (1971) re-examined these fish samples and found no other nitrosamines to be present. These workers also found no nitrosamines in nitrited fresh-water fish and nitrited salmon roe. Sen (1972) reported five of

59 samples of cured meats contained DMNA at levels ranging from 10-80 ppb. Wasserman et al. (1972) reported DMNA levels of 11, 48, and 84 ppb, respectively, in three of 40 samples of frankfurters. Hustad et al. (1973) investigated franks made with up to 400 ppm sodium nitrite using the multidetection method of Fazio, Howard and White (1971). Whether raw, boiled or fried, the nine samples of franks were found to contain no N-nitrosamines.

NOPYR has been detected in bacon fried in a conventional manner (Crosby et al., 1972; Fazio et al., 1973). This is the only N-nitrosamine that has been detected in cured meat products consistently. NOPYR was not found, however, in raw bacon, fried Canadian bacon, or fried ham (Fazio et al., 1973). In all eight samples of fried bacon tested, these workers found between 10 and 108 ppb NOPYR. These workers also found significant levels of NOPYR in the fat drippings (45-207 ppb). Crosby et al. (1972) reported levels of 16-40 ppb in fried Danish bacon. Sen et al. (1973) also reported measurable levels of NOPYR in eight of 16 fried bacon samples analyzed by tlc (4-25), but confirmed only one sample by ms.

Formation of NOPYR in Bacon

The consistency of formation of NOPYR in bacon during frying has led to investigations into factors affecting its production. Fiddler et al. (1973b) investigated the effects of cooking method, temperature

and ascorbic acid additions on the resultant levels of NOPYR in cooked bacon. Of the cooking methods evaluated, frying yielded the highest levels of NOPYR while microwave cooking produced the least. No NOPYR was formed on cooking at 99°C but the levels produced increased as the temperature increased up to 190°C. The addition of ascorbic acid (1000-2000 ppm) during processing reduced or inhibited the production of NOPYR during frying.

The source of NOPYR in bacon has not been established, although several theories have been proposed. Fazio et al. (1973) suggested that NOPYR was found only in bacon because NOPYR was retained in the fat of the bacon strip and not volatilized as in leaner cuts. These workers stated that NOPYR might come from a decarboxylation of NOPRO or from direct nitrosation of pyrrolidine (Pyr) arising from pro or putrescine. Fiddler et al. (1973b) showed that the rate of decarboxylation with temperature of NOPRO to NOPYR was similar to the rate of NOPYR formation with temperature in bacon. This observation, they stated, substantiated that route of formation of NOPYR. These workers also noted that sufficient free pro existed in pork belly to be the sole source of NOPYR.

Huxel (1973) found that NOPYR could, at frying temperatures (170°C), arise from nitrite and pro, N-acetyl proline, glycyl-proline, prolyl-glycine and collagen. Free pro was found to produce more NOPYR (4 to 10 times more) under the same conditions, than the

dipeptides or pyr. Collagen and nitrite in buffer at pH 6.0 reacted to form NOPYR at temperatures above 120°C (Huxel, 1973), suggesting that during heating, hydrolysis was required before nitrosation occurred. Lijinsky et al. (1972) reported hydrolysis was needed before nitrosation when reacting the N-N-disubstituted amide, dimethylformamide, to form DMNA.

Bills et al. (1973) produced NOPYR, in a model system similar to frying, from NOPRO, pyr, spermidine, pro and putrescine. Their system utilized a boiling flask which contained cooking oil and a small quantity of water. A condensor was used to reflux the water in the container. The compound to be treated was introduced into the flask and then heated in a 170°C oil bath for 20 min. NOPRO gave the highest yield of NOPYR of the compounds tested (2.6% of theoretical). Pyr yielded more NOPYR than did pro (1.0% and 0.4%, respectively).

Proline Nitrosation

The rate of nitrosation of pro was studied by Mirvish (1971b) who found that the reaction rate was dependent on the concentration of pro and the square of nitrite concentration. Pro nitrosated more rapidly than pyr which also suggested that formation of NOPRO followed by decarboxylation was a more likely occurrence than decarboxylation to pyr and then nitrosation to yield NOPYR (Fiddler et al., 1973b).

The nitrosation of pro intragastricularly in rats has been demonstrated by Braunberg and Dailey (1973). Greenblatt and Lijinsky (1972b), however, found that feeding NOPRO or nitrite and pro to Swiss mice for 26 weeks did not increase the incidence of lung adenoma, suggesting that NOPRO is not carcinogenic. Nagasawa et al. (1973) reported the LD_{50} of NOPRO to be 203 mg/kg in Swiss-Webster mice. After feeding NOPRO for eight weeks (274 mg/mouse), these workers observed no gross toxicity manifestations during the subsequent 45 weeks. NOPRO does not inhibit the log phase growth of E. coli, nor inhibit the growth of radicles of mung bean (Phaseolus aureus). These results also indicate a lack of mutagenic activity (Nagasawa et al., 1973).

NOPYR, however, was shown to produce hepatocellular carcinomas in 25 of 25 MRC (Wistar derived) rats fed a total dose of 1340 mg over 67 weeks (Greenblatt and Lijinsky, 1972b). These workers described NOPYR as having a hepatocarcinogenicity comparable to DMNA. The presence, then, of NOPRO in food may be significant only as an indicator of the hazard of further processing which might give rise to the much more potent carcinogen, NOPYR, or more importantly, as a monitor of the level of nitrosation which has occurred involving non-volatile nitrosamines yet to be detected.

METHODS AND MATERIALS

Experimental Precautions

While NOPRO has not been shown to be carcinogenic, its decarboxylated product, NOPYR, has. Therefore, all nitrosamine solutions, spiked samples and concentrates were handled with caution. All work, when practical, with these solutions was done in efficient fume hoods. Rubber gloves were worn and care was taken to avoid skin contact, spills and inhalation. The gas chromatograph used was in a fume hood.

Synthesis of Standards

NOPRO was synthesized by the modified method of Lijinsky et al. (1970). Thirty grams of L-pro (Eastman Kodak Co., Rochester, N. Y.) were dissolved in 20 ml HCl and 100 ml of water, cooled in ice. Twenty-five grams of sodium nitrite (reagent, Mallinckrodt Chemical Works, St. Louis, Mo.) were slowly added. After reacting one hour, NOPRO was extracted three times with equal volumes of ethyl acetate (ETOAC). The ETOAC was removed by rotary evaporation. The resultant crystals were recrystallized from acetone. The NOPRO was stored at $2^{\circ}C$.

The methyl ester of NOPRO (MeNOPRO) was prepared by

dissolving NOPRO in methanol and acidifying to 2% sulfuric acid. After one hour, an equal volume of water was added to the methanol solution and the mixture was extracted with CH_2Cl_2. The CH_2Cl_2 was evaporated by rotary evaporator to give a yellow oil, MeNOPRO. To confirm the identity of this compound, the IR absorption, NMR and mass spectra were determined on a Beckman Model 18A IR spectrophotometer, a Varian HR 100 NMR spectrometer, and a Finnigan Model 1015C mass spectrometer, respectively.

In the determination of NOPRO, N-acetyl proline (ACPRO) (Sigma Chemical Co., St. Louis, Mo.) was added as the internal standard and converted to the methyl ester (MeACPRO). MeACPRO was made by the same method used to make MeNOPRO from NOPRO. ACPRO was dissolved in acidified methanol and extracted after one hour with ETOAC. The resultant MeACPRO was an oil that was shown to be pure by gas chromatography. The mass spectrum was used for confirmation of identity.

NOPRO Extraction

In order to detect low levels of NOPRO, purity of solvents was essential. All reagents used were reagent grade or better and solvents were redistilled before use with the following exceptions: acetone and pentane were technical and practical grades, respectively, and were redistilled prior to use; ethyl ether was an anhydrous,

peroxide-free, analytical grade reagent in one pint cans and was not redistilled. The adsorbents used, alumina and silicic acid, were washed with CH_2Cl_2 and methanol and dried prior to use.

The meat sample to be analyzed was twice ground through a 3/16 inch plate, mixed and a 100 g aliquot was removed. To the 100 g sample in a quart blender jar, 200 ml distilled water and 50 ml of ethyl ether were added. The mixture was blended for five 1-min periods with 15 sec intermissions on an Osterizer. The slurry was centrifuged 10 min at 650 x G. The lipid layer was discarded and the remainder blended 2 min with 25 ml ethyl ether and centrifuged. After this lipid layer was discarded, 10 ml of 5% ammonium sulfamate and 15 ml of 50% sulfuric acid were added and blended for one min. Then 30 g of anhydrous sodium sulfate and 150 ml ETOAC were added and blended for 3 min. After centrifuging for 10 min at 650 x G, the ETOAC layer was removed to a 500 ml round bottom flask and the remainder extracted two more times with 100 ml of ETOAC. The ETOAC layers were combined and reduced to dryness by rotary evaporation.

To the residue from the ETOAC extract, 10 ml methanol and 100 μg ACPRO were added. The methanol solution was acidified by the addition of 0.2 ml concentrated sulfuric acid. One hour after acidification, the methanol solution was placed in a separatory

funnel with 10 ml water. The solution was extracted three times with 25 ml CH_2Cl_2. The CH_2Cl_2 fractions were combined and reduced to about one ml by rotary evaporation. This was placed on 5 g of alumina in a 2 x 20 cm column which was prewashed with 50 ml pentane. The sample was washed with 200 ml pentane. The nitrosamine ester and MeACPRO were removed from the column with 150 ml of 30% acetone in CH_2Cl_2. The acetone-CH_2Cl_2 fraction was reduced by rotary evaporation to about 4 ml and then reduced to about 0.5 ml in a stream of prepurified nitrogen. If further clean-up was required, this sample was then applied to 5 g of silicic acid in a 2 x 20 cm column, washed with 150 ml pentane and the nitrosamine and MeACPRO were eluted with 150 ml of 30% ethyl ether in CH_2Cl_2. This was reduced as above to about 0.5 ml.

Gas Chromatographic Analysis of MeNOPRO

Quantitative determinations were done on a Varian Aerograph Model 1200 equipped with a flame ionization detector. The column was a 10 ft (1/8 in. i.d.) aluminum tube packed with 10% SP222 PS 01-1885 100/120 mesh (a polyester liquid phase; Supelco, Inc., Bellfonte, Pa.). Column temperature was 190°C isothermal and the nitrogen flow rate was 20 ml per min at ambient temperature. Injection port and detector temperatures were 210° and 260°C, respectively.

Peak height (h) times retention time (t_r) was used to quantitatively determine MeNOPRO according to the following formula where f was the detector response factor:

$$\frac{h \times t_r \text{ MeNOPRO}}{h \times t_r \text{ MeACPRO}} = f \frac{\text{mass MeNOPRO}}{\text{mass MeACPRO}}$$

Gas Liquid Chromatography-Mass Spectrometry

A Finnigan Model 1015C glc-ms system which included a Varian Aerograph Model 1400 glc was used to confirm the presence of MeNOPRO in the bacon extracts. A capillary column of 0.03 in. i.d. x 500 ft wall-coated with OV-210 (a silicone liquid phase) was used to separate the nitrosamine. A flow rate of 15 ml/min of helium was used. The oven temperature and injection port temperature were 165° and 210°C respectively. The glc-ms interface was a Gohlke all-glass, jet orifice helium separator. A total ion current monitor provided a chromatogram. The operating conditions were: filament current, 400 μa; electron energy, 70 eV; analyzer pressure 5×10^{-7} torr; and electron multiplier voltage, 1.8 KV. Spectra were scanned from m/e 14 to m/e 175 in 1.0 sec. During analysis, the lower masses of the mass spectrometer output were displayed on a secondary oscilloscope (Type 502A, Textronics, Inc., Beaverton, Ore.). Utilizing a horizontal sweep magnification of 10, the ion range of

m/e 26 to 32 could be isolated. The NO^+ ion has a m/e 30 and by taking spectra when the m/e 30 was maximum, low levels of MeNOPRO could be identified.

Free Proline Determination

Free pro was measured using column chromatography by the method of Moore, Spackman and Stein (1958) after deprotination of the sample by the method of Rajagopalan, Moore and Stein (1966) as modified by Field and Chang (1969). About 10 g of ground and mixed sample were accurately weighed into a blender jar and blended on an Osterizer for 3 min with 100 ml of 1% picric acid. The samples were centrifuged for 10 min at 15,000 x G, the supernatant was filtered, and the residue washed with 20 ml distilled water and filtered. The filtrate was passed over Dowex 1-X8 resin (chloride form, washed with 1N HCl then washed with distilled water until neutral). About 3 cm of 100-200 mesh resin in a 2 x 25 cm column were sufficient to remove the picric acid. The column was washed with 3 x 5 ml of 0.02N HCl. The effluent and wash were collected and dried by rotary evaporation. The amino acids were redissolved in 3 ml of distilled water. One-tenth ml of sample was placed on a 0.9 x 25 cm column of Beckman type 50A resin. The column was developed at 50°C with pH 3.25 citrate-HCl buffer which was 0.2N in sodium with a flow rate of 0.5 ml/min. The buffer was made by

adding 84.0 g citric acid, 33.0 g NaOH (97%) and 42.6 ml concentrated HCl per four liters (Moore, Spackman and Stein, 1958). One ml fractions were collected and reacted with ninhydrin reagent made as described by Moore (1968). Two grams of ninhydrin were dissolved with 0.6 g of hydrindantin in 75 ml of dimethyl sulfoxide. Twenty-five ml of 4N lithium acetate buffer (pH 5.2) were added after it was purged with prepurified nitrogen. The ninhydrin solution was added to the samples in a ratio of 0.5 ml to 1.0 ml and boiled for 15 min. The absorbance of each fraction was measured on a Beckman Model B spectrophotometer at 440 and 570 nm. Free pro was determined from a standard curve.

Determination of Residual Nitrite

Residual sodium nitrite in cured meats was determined by the method of Nicholas and Fox (1973). Five grams of ground or finely chopped sample were weighed into a 50 ml beaker. About 40 ml of nitrite-free 80^{o}C water were added. A glass rod was used to thoroughly disperse the sample. The slurry was transferred to a 500 ml volumetric flask with hot water. Hot water was added to the flask to about 300 ml. The flask was placed on a steam bath for two hours. The flask was filled to the mark after cooling with nitrite-free water. The solution was filtered and a suitable aliquot diluted to 50 ml and reacted with 5 ml of Griess reagent.

Twenty-five min after mixing, the absorbance of the solution was measured at 520 nm on a Beckman Model B spectrophotometer. The amount of nitrite in the diluted sample was determined from a standard curve.

The Griess reagent was made by mixing a solution of 0.5 g sulfonic acid in 150 ml of 15% acetic acid and 0.10 g α-napthyl-ethylenediamine·HCl in 150 ml of 15% acetic acid. The mixture was stored in a brown glass bottle at 2°C.

Amino Acid Analysis of Pork Belly

The amino acid content of pork bellies to be processed into bacon was determined in order to establish background values of the amino acids and to measure the variation between the bellies. Two pork bellies were purchased, labeled A and B, and divided into four sections. One section of each belly was ground and frozen at -35°C for amino acid analysis, while the other sections were cured into bacon as described later. Before analysis, each ground pork belly sample was thawed, mixed, and an aliquot weighed into a drying pan. The samples were dried for 24 hours in a vacuum drying oven at 70°C. The weight was again determined, and the lipid was extracted with ethyl ether on a Goldfisch extraction apparatus. The loss of lipid was determined. The residue from ether extraction was dried and then approximately 3 ml were accurately weighed into an ampule for

acid hydrolysis. The samples were hydrozlyed in 1.0 ml of constant boiling HCl at 110°C for 20 hours. The amino acid hydrolyzates were analyzed by the method of Spackman, Stein and Moore (1958) on a Spinco Model 120B amino acid analyzer.

Commercial Cured Meat Samples

Commercial cured meat samples were purchased in local markets and stored at 2°C until analyzed which was a maximum of three days. The samples purchased were assigned code numbers to avoid designation by brand name. The coded samples are listed in Table 1 with product identification and ingredient information. The brand name and other relevant label information are listed for each sample in the Appendix.

Determination of the Effects of Level of Nitrite in Curing on NOPRO Formation in Bacon

In order to determine the effects of levels of sodium nitrite used during curing of bacon on NOPRO formation, two levels of nitrite were used to cure paired quarters from the same pork belly. Two pork bellies were purchased from Nebergall Meat Packing Co., Albany, Oregon, labeled A and B, and cut into quarters. One quarter of each belly was used for amino acid analysis as described previously. The center quarters of each belly were cured by either of

Table 1. Sample designation and ingredients of commercially cured meats analyzed for NOPRO.

Sample Code Number	Product	Ingredients
1012	Bacon	Cured with: water, salt, sugar, sodium phosphate, sodium ascorbate, sodium nitrite.
1014	Bacon	Cured with: water, salt, sodium tripoly-phosphate, sodium erythorbate, sodium nitrite, and flavorings.
1016	Cured and smoked beef plate	Salt, water, sugar, dextrose, MSG, hydro-lyzed plant protein, sodium nitrite, sodium nitrate, sodium erythorbate and flavorings.
1018	Bacon	Cured with: water, salt, sodium tripoly-phosphate, sodium ascorbate, sodium nitrite, and flavorings.
1020	Ham slice	Cured with: water, salt, sugar, sodium phosphate, sodium ascorbate, sodium nitrite. Fully cooked.
1022	Chopped ham	Ham, ham shank meat, water salt, sugar, sodium tripolyphosphate, sodium ascorbate, sodium nitrite.
1024	Wieners	Beef and pork, water, salt, corn syrup, dextrose, flavoring, sodium ascorbate, sodium nitrite.
1026	Bacon	Cured with: water, salt, sodium tripoly-phosphate, sodium ascorbate, sodium nitrite, and flavorings.
1028	Bacon	Cured with: water, salt, sugar, sodium phosphates, sodium erythorbate, sodium citrate, sodium nitrite.
1030	Bacon	Cured with: water, salt, sugar, sodium phosphates, sodium erythorbate, MSG, sodium nitrite, sodium nitrate.

two treatment levels of nitrite. The normal nitrite treatment contained nitrite at a level used commercially, while the high nitrite treatment contained twice the nitrite level of the normal treatment.

Two gallons of each brine were made with 1.27 kg sodium chloride and 242 g sucrose. For the normal treatment, 7.83 g of sodium nitrite were added, while 15.65 g were added to the high treatment. The pork belly quarters were soaked, submersed for seven days, then smoked for 18 hours to the internal temperature of 61°C. The bacon slabs were then sliced and frozen at -35°C until analyzed. Bacon from belly A was referred to as A Normal from the normal treatment and A High from the high nitrite treatment. B Normal and B High were assigned similarly to the other belly.

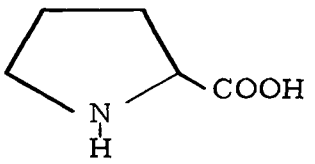
The bacon samples were analyzed for residual nitrite, free pro, and NOPRO.

Determination of the Effects of Frying on NOPRO in Bacon

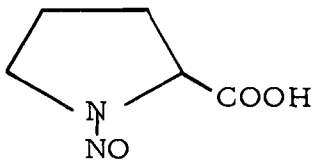
Bacon samples were analyzed for NOPRO before and after frying in order to determine the effects of frying on NOPRO level. Commercial samples 1026, 1028 and 1030 were used as well as one normal nitrite level bacon sample (B Normal) and one high nitrite level bacon sample (B High).

The bacon was fried on a preheated Sunbeam Model RD electric griddle at 185°C for 4 min on each side. The bacon was weighed

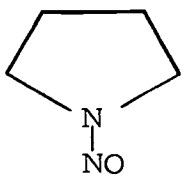
before and after frying. Residual sodium nitrite and NOPRO were determined for each sample before and after frying. Free pro was also determined after frying of the prepared bacon samples, B Normal and B High. Figure 1 shows the chemical structures of compounds discussed in this study.



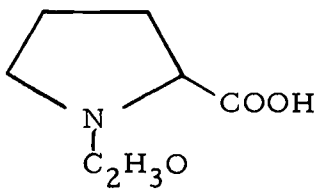
Proline



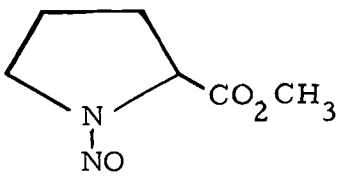
N-Nitrosoproline



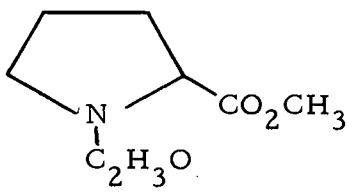
N-Nitrosopyrrolidine



N-Acetylproline



Methyl Nitrosoproline



Methyl Acetylproline

Figure 1. Structures of compounds discussed in this study.

RESULTS AND DISCUSSION

Identification of Standards

To identify NOPRO from cured meats, after methylation, the mass spectra was compared to that of standard MeNOPRO. To insure that the standard MeNOPRO was authentic, the IR and NMR spectra were measured. The reported IR and NMR spectra of NOPRO (Lijinsky et al., 1970) are similar to those of MeNOPRO. The major IR absorption bands of NOPRO were reported to be 1430 and 1730 cm^{-1} (assigned to the N=0 and C=0 stretches, respectively), while the corresponding absorption bands of MeNOPRO were 1435 and 1730 cm^{-1} (Figure 2). The addition of the methyl group accounted for the absorption bands at 1290 and 1205 cm^{-1}.

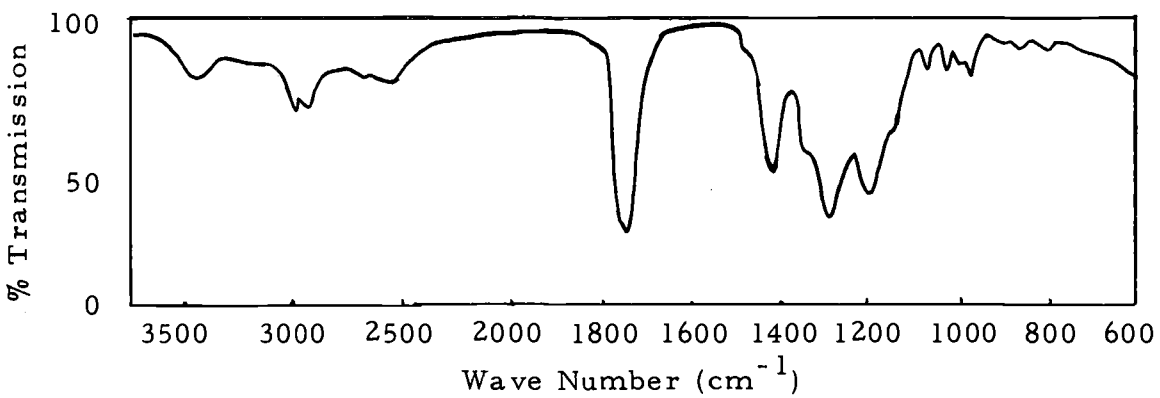


Figure 2. IR spectrum of MeNOPRO.

The NMR spectrum of MeNOPRO in pyridine indicated that MeNOPRO exists in two conformers as does NOPRO. Two doublets of doublets were seen for the methine group on the α-carbon at 4.56 and 5.38τ with a 1:2 ratio. The peaks for the methylene group next to the amine nitrogen were also divided into a 2:1 ratio at 5.68 and about 6.3τ. The hydrogens of the methoxy group gave two very sharp peaks, six cycles apart, in a ratio of about 1:2 on top of the upfield methylene peak centered at 6.34 τ. A multiplet was observed centered at 7.94 τ for the four hydrogens on the 3 and 4 carbons. NOPRO crystallizes in the syn configuration (with the nitroso group nearer to the α-carbon) but equilibrates to an equal amount of syn and anti conformers (Lijinsky et al., 1970). MeNOPRO appeared to prefer the syn conformer about two to one.

The mass spectrum of MeNOPRO is shown in Figure 3. The main characteristic peaks are m/e 99 (base peak), 69, 128 and the parent ion m/e 158. Those reported for NOPRO were m/e 99, 69 and the parent ion m/e 144 (Lijinsky et al., 1970). The ion fragment, m/e 99, was due to the loss of $COOCH_3$ from MeNOPRO and the loss of COOH from NOPRO. The P-30 ion (loss of the NO group) was reported to be absent from the spectrum of NOPRO (Lijinsky et al., 1970) but was observed for MeNOPRO.

The mass spectrum of MeACPRO is given in Figure 4. The characteristic peaks of the spectrum were m/e 70 (base peak), 112

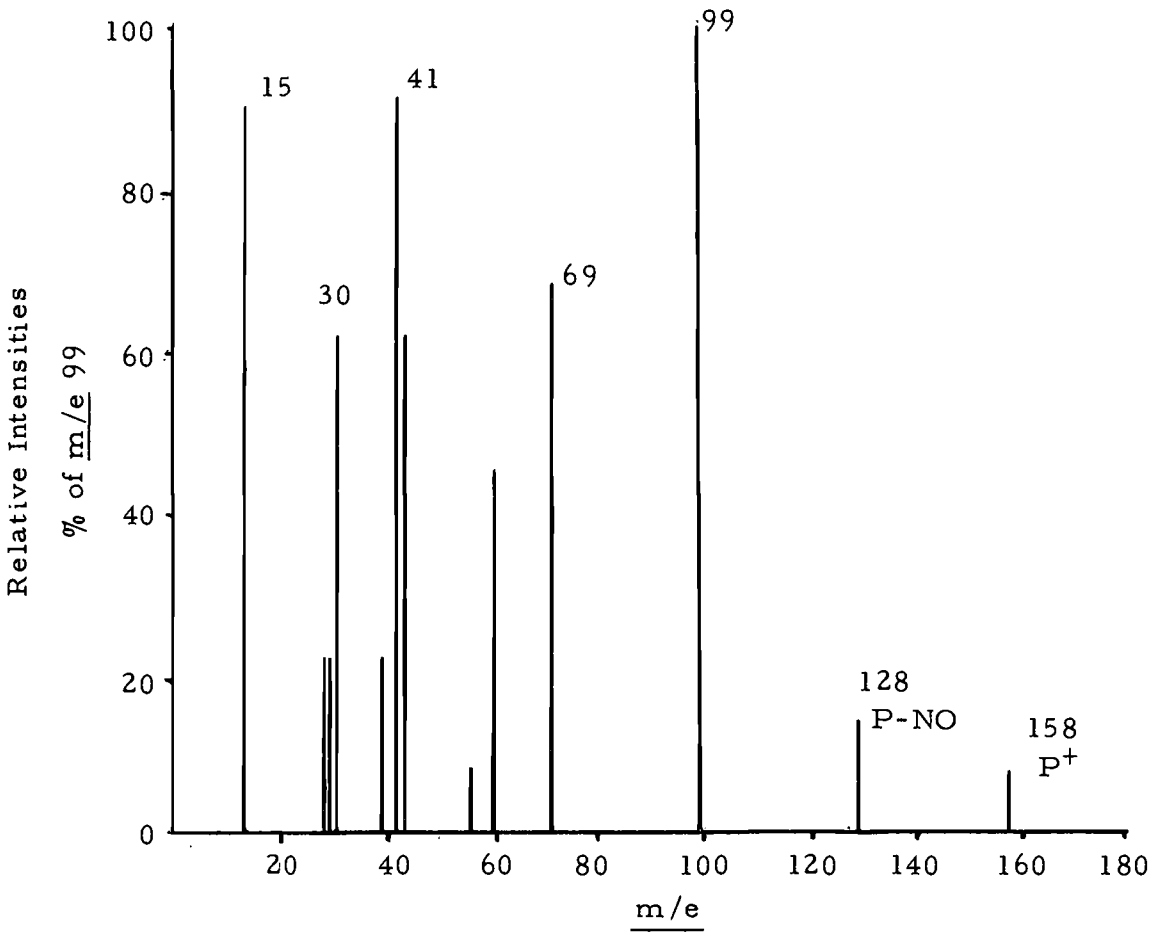


Figure 3. Mass spectrum of MeNOPRO.

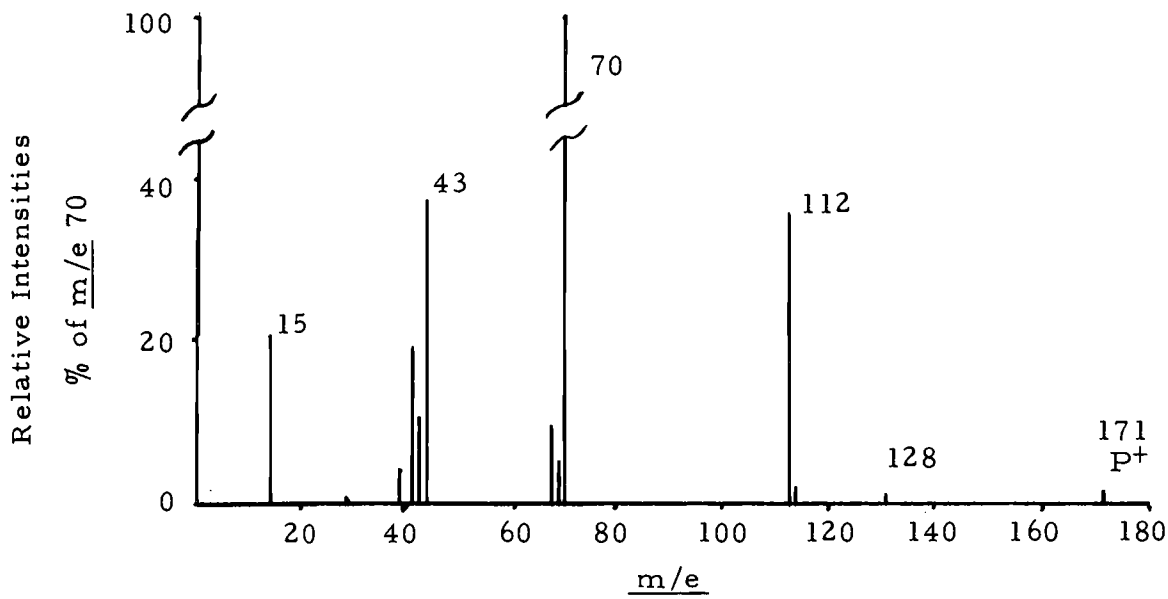


Figure 4. Mass spectrum of MeACPRO.

and the parent ion, m/e 171. The parent ion was consistent with the chemical formula of MeACPRO, $C_8H_{13}NO_3$. The fragmentation was similar to that of MeNOPRO. The loss of $COOCH_3$ and the loss of the acetyl group, $COCH_3$, were noted. The m/e 128 ion, resulting from the loss of the amine-linked group (NO or $OCCH_3$), was present in both spectra.

Extraction of NOPRO

Recoveries of NOPRO from samples of bacon spiked with 10 μg were between 66 and 78%. In one replicate set of four extractions of a bacon sample determined to be void of NOPRO, the average recovery was 72% with a standard deviation of 5.6%. The values for NOPRO from cured meats reported below were not adjusted for the recoveries, however. This was done in order to present levels that were confirmed by ms to be present in the samples.

Brief descriptions of the procedures that were unsuccessful in extracting NOPRO from cured meats are presented in the Appendix.

Extraction of the sample initially with ethyl ether was done to reduce the amount of lipid extracted with the NOPRO thus reducing clean-up requirements. Lijinsky et al. (1970) reported the partitioning of NOPRO between ether and water as 1:9, which would predict a

very low loss of NOPRO in the extraction from 200 ml water with 50 ml ethyl ether. Hamilton and Ortiz (1950) reported no extraction of NOPRO from aqueous solutions above pH 4.0. NOPRO was never detected in the ether extract, although this was checked several times during this study.

In order to extract the nitrosamino acid into an organic solvent, the aqueous phase was acidified to approximately a pH of 1 (Hamilton and Ortiz, 1950). To prevent the acid-catalyzed production of NOPRO from residual nitrite and pro in the samples, an excess of ammonium sulfamate was added just prior to acidification. Ammonium sulfamate, in the presence of acid, reacts very quickly with nitrite to remove it from the system (Fan and Tannenbaum, 1973a). Friedman (1972) reported using 3.5 M urea to remove nitrite from a reaction mixture. However, in preliminary studies, urea was found to be ineffective in removing the residual nitrite completely. Starting with 100 ppm sodium nitrite, ammonium sulfamate removed 100% of the residual nitrite in one min under the extraction conditions employed, while urea removed 91.3% after one min and only 93.2% after 10 min.

Braunberg and Dailey (1973) reported using ethyl ether for extracting NOPRO from the acidified stomach contents of rats fed pro and nitrite. Nagasawa et al. (1973) used ETOAC for the extraction of NOPRO from aqueous acid during synthesis. Preliminary studies indicated that ETOAC was at least 25% more effective in

extracting NOPRO than ethyl ether. This was also reported by Hamilton and Ortiz (1950). Under identical conditions, ET OAC was found to extract seven times as much NOPRO as CH_2Cl_2 which is commonly used to extract volatile nitrosamines.

The formation of the methyl ester of the extracted NOPRO was found to be complete after one hour. There was no increase nor loss of MeNOPRO from the methylation reaction after one hour up to 24 hours.

For quantitation, the detector response factor was found to be equal to 1.0. This was determined by comparing the peak heights of known weights of MeNOPRO and MeACPRO.

To confirm the presence of low levels of MeNOPRO in cured meat extracts, mass spectrometric conditions were optimized by the use of a capillary column. This reduced the background due to column bleed and better chromatographic resolution was obtained than resulted with larger diameter packed columns. The use of a capillary column also gave sharper elution of the nitrosamine ester from the column. Since MeNOPRO was less spread out eluting from the capillary column, a larger amount of sample per unit time went into the mass spectrometer. However, with the sharper elution of sample from the column, the importance of scanning the spectrum at the peak apex was greatly increased. The retention time of the nitrosamine ester on the column was noted to increase about 10 sec through

the course of a day of analysis which, while less than 2% of the total retention time, could result in lowered sensitivity if the retention time was not adjusted. In order to closely monitor the elution of the nitrosamine ester, a secondary oscilloscope using horizontal magnification was used to monitor the output of the spectrometer during analysis. With this oscilloscope, the m/e 30 ion was monitored and elution of NOPRO from the capillary column could be seen and spectra could be taken at the peak maximum. Using these techniques, the presence of about 5 ng per injection of MeNOPRO could be confirmed.

The criteria for positive identification of MeNOPRO in a sample were dependent on the observation of increases of certain critical ions at the retention time of MeNOPRO. A sample was considered negative unless all three ions m/e 99, 30 and 128 were increased simultaneously over the background in the proper ratios. Other ions of importance in the spectra were also measured, but these were considered supporting evidence. It was not necessary for confirmation to have an increase of the parent ion, m/e 158, as this ion comprised only 7% of the base peak m/e 99. However, this was often observed. Figure 5 shows plots of important ions in the fragmentation of MeNOPRO from meat samples as they occurred around the retention time of MeNOPRO. Of the graphs in Figure 5, a and b were confirming evidence while c was considered negative. Similar plots

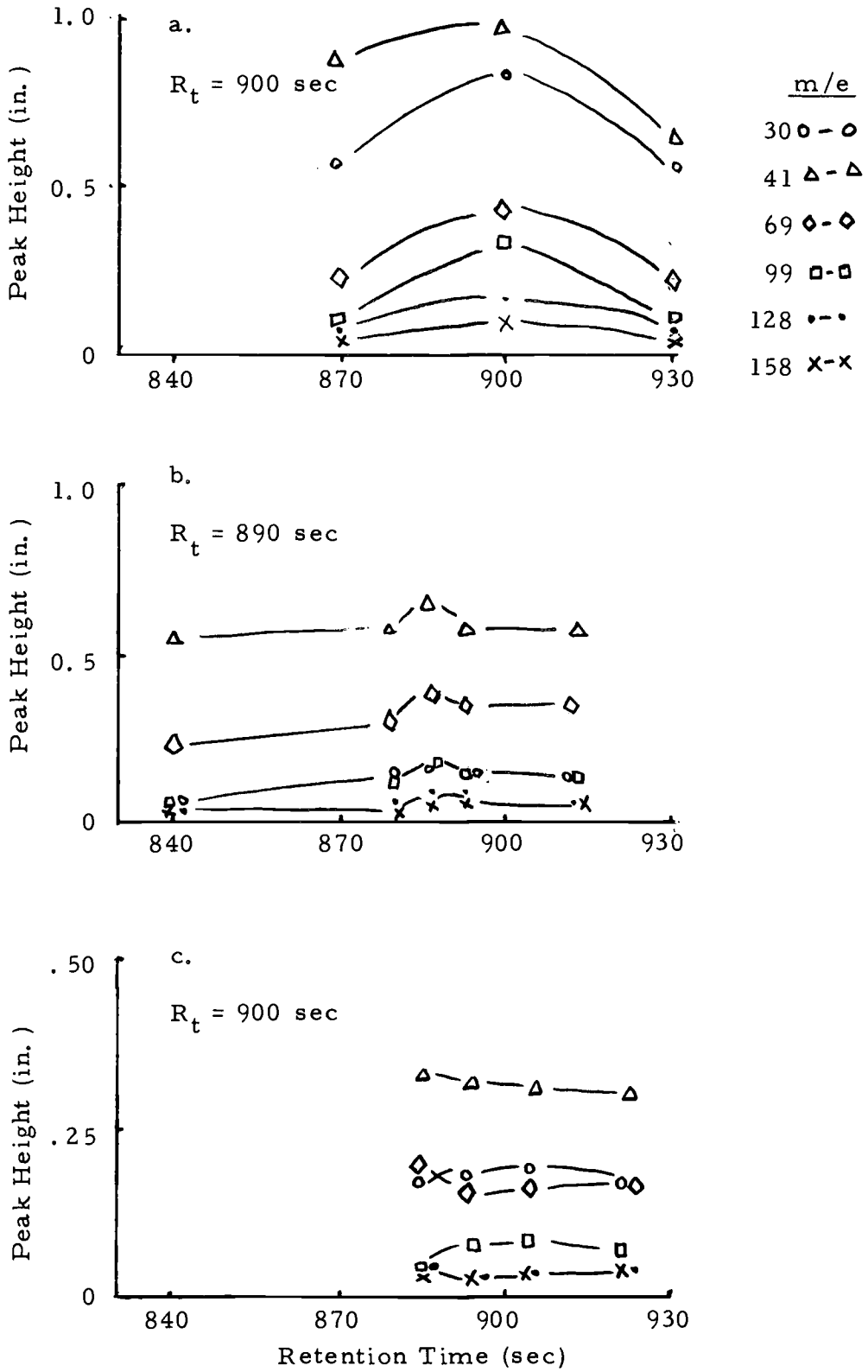


Figure 5. Change of ion intensities with retention time during ms detection of MeNOPRO from bacon samples. Graphs a and b were positive identification of MeNOPRO; c was negative.

have been used to detect DMNA in food extracts (Essigman and Issenberg, 1972).

NOPRO in Commercial Cured Meat Products

Low levels of NOPRO were detected in several cured meat products (Table 2). Seven of the 10 cured meat samples analyzed were confirmed by mass spectrometry to contain from 13 to 62 ppb NOPRO. Of the six bacon samples analyzed, five contained NOPRO. The sample containing the most NOPRO was a baconlike product called breakfast beef, which was made from cured beef plate, the cut analogous to that used for bacon. A precooked ham slice was positive for NOPRO while a canned chopped ham sample was negative. The only wiener sample was also negative. Overall, NOPRO appeared to be frequently formed in detectable amounts in commercially prepared cured meats.

The above data comprise the first report concerning the detection and ms confirmation of a non-volatile N-nitrosamine occurring in cured meats. The finding of low levels of a probable non-carcinogen, such as NOPRO, would be of little importance, except that NOPRO can break down during frying to NOPYR (Fiddler et al., 1973b; Bills et al., (1973). Fazio, Howard and White (1971) stated that while volatile nitrosamines were presently being detected, non-volatile nitrosamines were of equal importance toxicologically. The finding of one non-volatile N-nitrosamine in foods, even a non-carcinogen, greatly

Table 2. Amounts of NOPRO detected in commercial cured meats.

Sample No.	Product	NOPRO[a] (ppb)
1012	bacon	34
1014	bacon	13
1016	breakfast beef	62
1018	bacon	16
1020	ham slice	22
1022	chopped ham	N. D.[b]
1024	wiener	N. D.[b]
1026	bacon	31
1028	bacon	21
1030	bacon	N. D.[b]

[a]The presence or absence of NOPRO was confirmed by ms.

[b]Not detected.

increases the probability of other non-volatile carcinogenic N-nitrosamines being formed.

The Effect of Nitrite Level during Curing on Free Pro and NOPRO in Bacon

Bacon was produced from pork bellies with two levels of nitrite in the cure (800 and 1600 ppm). Two pork bellies were used, and part of each belly was cured in each level. The proximate analysis and amino acid content of each belly are presented in Table 3. The variation between the two bellies was, in general, small. The levels of amino acids in the bellies were within the ranges listed for pork by FAO (1970) except cysteine which was lower than the

Table 3. Proximate and amino acid analysis of pork bellies to be processed into bacon.

	Belly A	Belly B
	(%)	(%)
moisture	37.19	36.36
fat	50.34	51.39
protein	9.08	8.94
	(mg/100 g)	(mg/100 g)
lys	717	796
his	300	313
NH_4	136	134
arg	690	670
asp	862	914
thr	436	402
ser	354	357
glu	1536	1430
pro	536	447
gly	627	474
ala	617	518
cys	54	36
val	473	465
met	227	223
ile	418	456
leu	736	724
tyr	318	331
phe	399	384
hyp	114	64

70-133 mg/100 g listed. Pro, glycine, alanine and glutamic acid were near the higher limits reported by FAO (1970) which may reflect a higher collagen content of pork belly. Belly A had slightly more total pro (536 mg/100 g) than B (447 mg/100 g).

Table 4 lists the residual nitrite, free pro, and NOPRO for each sample tested. The bacons made with the higher level of nitrite had slightly more than twice the residual nitrite of the lower level bacon. Free pro increased approximately 50% during the curing process. During normal aging of porcine muscle, from one to eight days, pro has been observed to increase, going from 6 to 13 mg/100 g (Bowers, 1969). The level of free pro in pork bellies was lower than porcine muscle; however, the content of lean in the bellies was also considerably lower. The standard curves used to determine sodium nitrite and free pro are given in Figures 6 and 7 respectively.

Table 4. Effect of nitrite levels on free pro, residual nitrite and NOPRO in bacon made from the same pork bellies.

Sample	Residual Nitrite (ppm)	Free Pro (mg/100 g)	NOPRO[a] (ppb)
A - fresh	-	2.1	-
A - Normal	50	3.1	N. D.[b]
A - High	119	3.4	116
B - fresh	-	2.6	-
B - Normal	44	3.4	N. D.[b]
B - High	103	3.3	146

[a]The presence or absence of NOPRO was confirmed by ms.
[b]Not detected.

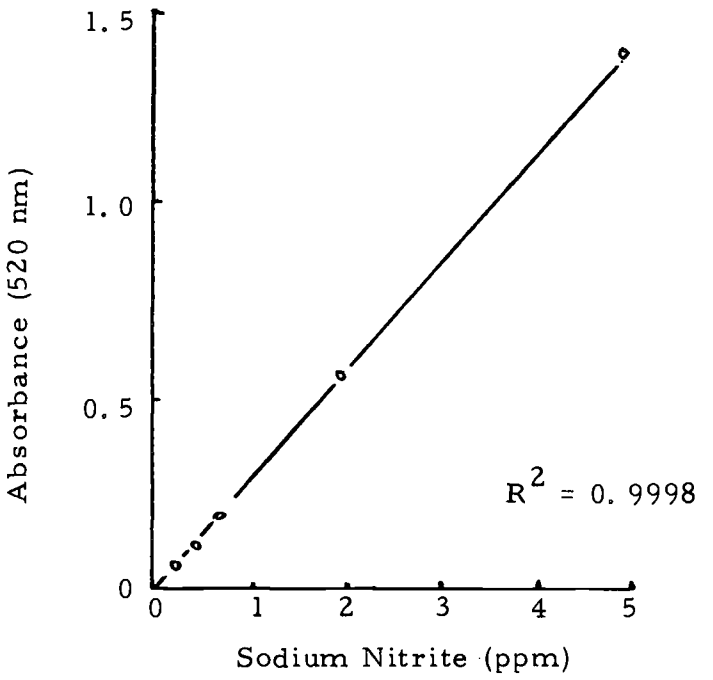


Figure 6. Standard curve for sodium nitrite determinations.

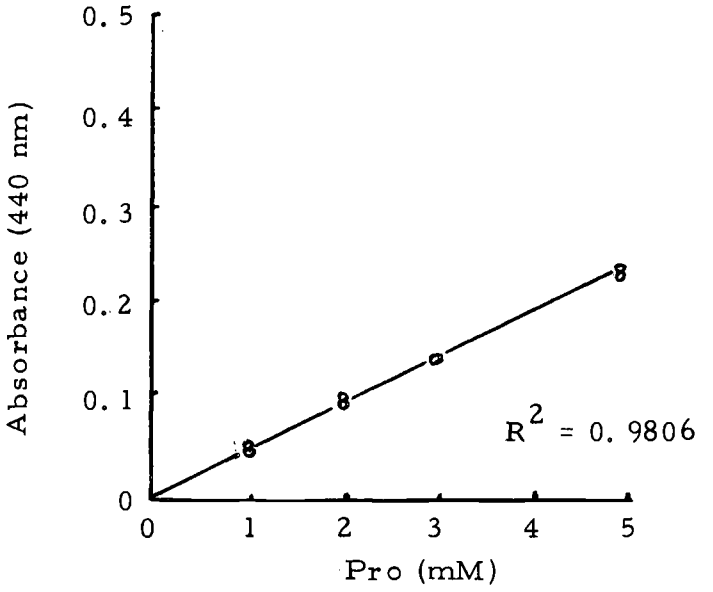


Figure 7. Standard curve for free pro determinations.

NOPRO was detected in only the high nitrite treatment bacons, but the levels found were higher than those detected in commercial bacon (Table 3). The nitrosation rate of many secondary amines, including proline, is third order (Mirvish, 1971b), first order with respect to the amine and second order with respect to nitrite. This would indicate that if the level of nitrite was doubled, the yield of NOPRO should increase by the square of the nitrite concentration. These data suggest an even more pronounced increase of nitrosation as nitrite levels are increased. However, the rate kinetics are effective only in solutions where competition for nitrite does not occur. In a bacon system, it is probable that nitrite is more reactive with other components than with pro. Fan and Tannenbaum (1973b) stated that not all nitrite in food was available for nitrosation reactions. From nitrosation kinetics in a milk system they concluded that complexed forms of nitrite may exist in foods that are not available for nitrosation. However, some of these appeared to be measured as nitrite by the Griess reaction. These workers suggested that the nitrite that can nitrosate amines be called available nitrite. If the same amounts of nitrite were complexed at both the high and low level treatments, doubling the level of nitrite would have the effect of more than doubling the concentration of available nitrite which could react with pro.

Fiddler et al. (1972b) reported that about ten times the legal

level of nitrite was needed to form measurable quantities of DMNA (10 ppb) consistently in frankfurters processed with only the addition of water, salt and nitrite. This was done in order to study the effects of processing parameters on DMNA formation in wieners. The data from Table 4, however, suggest that NOPRO could be consistently formed at levels of nitrite much closer to the legal limits of nitrite addition than that used to produce DMNA. This indicates that NOPRO may be a more valid compound for measuring the level of nitrosation occurring during processing. The use of NOPRO would have the advantages of working with a much less volatile and apparently much less hazardous compound than the known carcinogen, DMNA.

The Effect of Frying on the NOPRO Content of Bacon

Frying has been reported to induce the formation of NOPYR in bacon (Crosby et al., 1972; Fazio et al., 1973). NOPRO has been demonstrated to be a precursor of NOPYR at frying temperatures (Fiddler et al., 1973b; Bills et al., 1973). Fiddler et al. (1973b) hypothesized that NOPRO was formed during frying which was then decarboxylated to NOPYR.

NOPRO appeared to be almost completely degraded during frying. Of the five fried samples analyzed, only one contained a measurable level of NOPRO (Tables 5 and 6). The yield of fried bacon from raw was generally about 25%, and NOPRO, unlike NOPYR, is

Table 5. Effects of frying on nitrite and NOPRO in commercial bacon.

Sample Code Number	Nitrite before frying (ppm)	Nitrite after frying	NOPRO[a] after frying (ppb)	Frying yield (%)
1026	50.4	38.8	16	26.5
1028	82.0	26.6	N. D.[b]	23.4
1030	51.3	39.1	N. D.[b]	28.9

[a]The presence or absence of NOPRO was confirmed by ms.

[b]Not detected

Table 6. The levels of residual nitrite, free pro and NOPRO in bacon prepared from the same belly after frying.

Sample	Residual Nitrite (ppm)	Free pro (mg/100 g)	NOPRO (ppb)
B - Normal	12	7.4	N. D.[a]
B - High	17	9.1	N. D.[a]

[a]NOPRO not detected by glc-ms.

not fat soluble and should be concentrated in the bacon if not destroyed. Thus, sample 1026 which had 16 ppb NOPRO after frying lost approximately 87% of the initial NOPRO detected. Even the high nitrite treatment bacon, B High, was devoid of NOPRO after frying (Table 6).

The stability of NOPRO, under various conditions, has been studied. Fan and Tannenbaum (1972) reported that at 110°C, NOPRO had the highest rate of decomposition at neutral and acid pH's of the nitrosamines tested. These workers also reported that at this temperature, no conversion of NOPRO to NOPYR was observed over a pH range from 2.2 to 12.5. Lijinsky et al. (1970) reported that NOPRO decomposed at 99°C rather than melting. Hamilton and Ortiz (1950) reported a 98% yield of pro from NOPRO after acid hydrolysis. These workers also reported the release of pro from NOPRO during decomposition of NOPRO with uv light.

NOPYR was reported by Fazio et al. (1970) in fried commercial bacon at levels up to 108 ppb with an average of eight samples of 62 ppb. Even higher levels were detected in the fat drippings (45-208 ppb). Other studies have reported slightly less NOPYR in fried bacon. Crosby et al. (1972) reported 15-40 ppb while Sen et al. (1973) reported 4-25 ppb in 8 of 16 samples. The levels of NOPYR in these reports were similar to levels of NOPRO detected in the commercial samples analyzed in this study.

In model systems of frying, the conversion of NOPRO to NOPYR

has been of low efficiency. Fiddler et al. (1973b) reported a maximum yield of 1.8% (uncorrected for 50% recovery) while Bills et al. (1973) reported a 2.6% yield of NOPYR from NOPRO.

From the stability studies and reports of NOPYR yields under frying conditions, it is apparent that NOPRO in raw bacon could not be the only source of NOPYR. That NOPRO formed during processing is a precursor of NOPYR is very probable, but unless NOPRO converts to NOPYR in much higher yields in bacon than in the model systems, it would only account for about 5 to 10% of the total NOPYR reported to be formed during frying.

During frying, the residual nitrite (Table 5) decreased, considering shrinkage, from 80 to 88%. At the temperatures achieved during frying, nitrite would be extremely reactive, and many products of its reaction would be expected. Reaction with free pro during frying could account for a large part of the NOPYR produced. Huxel (1973) observed the production of NOPYR from pro, glycyl-proline, prolyl-glycine as well as collagen at 170°C. Bills et al. (1973) reported 1% yields of NOPYR from spermine and pyrrolidine and 0.4% yield from putrescine under model frying conditions. Thus, there are many amine sources which could contribute to the formation of NOPYR.

Even with these potential sources of NOPYR, Sen et al. (1973) found 8 of 16 bacon samples to contain no NOPYR. Fiddler et al.

(1973b) also reported several negative bacon samples. NOPRO was not found in every bacon sample analyzed in this study (7 of 10 were positive). Further research investigating the possible relationship between NOPRO and NOPYR would be of interest. If NOPYR were found to be roughly related to NOPRO content, this might suggest that the yield of NOPYR from NOPRO was greater than indicated by model systems. More probably, such a relationship would indicate that the level of NOPRO reflects the level of nitrite, the available nitrite as proposed by Fan and Tannenbaum (1973b), which can undergo nitrosation reactions. This available nitrite could then react during frying with free pro and other amines to produce, along with NOPRO decarboxylation, the NOPYR detected. Further research should also be done to determine the maximum level of sodium nitrite that could be added to bacon and have no NOPRO production, inferring that below this level all the added nitrite would be unavailable for nitrosation.

SUMMARY AND CONCLUSIONS

A method was developed to measure the NOPRO content of cured meat products. A sample was first ground and blended with distilled water, then extracted twice with ethyl ether. Ammonium sulfamate was added and the aqueous system acidified to approximately pH 1.0. The NOPRO was extracted with ETOAC, and converted to the methyl ester, MeNOPRO, in acidified methanol. The MeNOPRO was cleaned up on an alumina column and quantitated by glc. The presence of NOPRO in each sample was confirmed by ms. Recoveries of 10 μg in spiked samples averaged above 70% using this method. Ammonium sulfamate was found to be effective in preventing the formation of NOPRO during the acidified portion of the extraction.

In order to confirm the presence of low levels of MeNOPRO in the extracts, a capillary column was used for optimum separation. A secondary oscilloscope utilizing horizontal magnification of the ms output was used to detect the elution of MeNOPRO from the column. Monitoring the m/e 30 ion on the oscilloscope was found to be an extremely effective technique for obtaining the strongest spectra during elution of quantities too small to give individual peaks on the total ionization trace. The presence of 5 ng per injection could be confirmed in this way.

NOPRO was detected in several cured meat products. Seven of

ten samples analyzed contained from 13 to 62 ppb NOPRO. Of these, five of six bacon samples were positive. NOPRO was also found in a breakfast beef sample and a fully cooked ham slice. A chopped ham sample and a wiener sample were negative.

The finding of NOPRO, a non-volatile nitrosamine, suggests that the formation of other non-volatile nitrosamines is also possible. To date, only volatile nitrosamines have been routinely analyzed and confirmed by ms. NOPRO was found to occur frequently in several types of cured meats, while DMNA has been reported to have a low incidence rate in cured meats. This suggests that NOPRO, because its precursor amine is much more common, may be a better indicator of nitrosation in foods.

In bacon made with different levels of nitrite, doubling the level of nitrite was found to increase the level of NOPRO in the bacon. The lower level nitrite treatment bacons (50 ppm residual nitrite) contained no detectable NOPRO while the higher nitrite treatment bacons (100 ppm residual nitrite) contained 116 and 146 ppb.

Frying was found to destroy from 86 to 100% of NOPRO in five bacon samples. However, model studies by Bills et al. (1973) and Fiddler et al. (1973b) reported a maximum of 3% conversion of NOPRO to NOPYR. At this level of conversion, the NOPRO in raw bacon samples would be insufficient to produce the levels of NOPYR reported in fried bacon.

The residual nitrite in bacon was greatly reduced on frying. NOPYR not formed from the NOPRO present in raw bacon would probably be formed by reacting with some of the residual nitrite during frying. This nitrite could react with free pro, or any of the other potential precursors of NOPYR that have been identified.

NOPRO determinations in cured meats may have greatest potential as an index to the level of nitrosation that has occurred in a cured meat during processing or that may occur during further processing or cooking. NOPRO, the nitrosamine of perhaps the most common secondary amine in foods, may reflect the level of available nitrite in a food, or that level of nitrite which could be involved in nitrosation reactions.

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APPENDIX

APPENDIX

Commercial Cured Meat Samples

Table 7 lists the coded meat samples and label information that was not presented in the Methods and Materials section.

Table 7. Additional label information for commercially cured meats analyzed for NOPRO.

Sample Code Number	Label Information
1012	Oscar Mayer Bacon, vacuum seal. Oscar Mayer & Co. Gen. Off. Madison Wisc. EST 537F. Dec. 21. 1 lb.
1014	Armour Star Bacon. Maracure. Armour & Co. Phoenix, Arizona 85077. EST 139. Dec. 24. 1 lb.
1016	Fisher's Home Quality Breakfast Beef. Home Provision Co. Inc. Denver, Colo. 80216. EST 535. Jan 27. 12 oz.
1018	Safeway Bacon. Dist. by Safeway Stores, Inc. Oakland, Calif. 94660. Jan. 07. EST 139. 2 lb.
1020	Oscar Mayer Jubillee Ham, sectioned and formed. Oscar Mayer & Co. Madison, Wisc. EST 537A. Jan. 03. 8 oz.
1022	Dak Chopped Ham. Packed by Dak Meat Packers, Ltd. Gen. Off. DK4000 Roskilde, Denmark. Danmark 210. 1 lb.
1024	Oscar Mayer, all meat wieners, Oscar Mayer & Co. Madison, Wisc. EST 537P. Dec. 06. 1 lb.
1026	Safeway Bacon. Dist. by Safeway Stores, Inc. Head Office, Oakland, Calif. 94660. EST 139. Feb. 12. 2 lb.
1028	Cudahy Bar S Thick Sliced Bacon. Cudahy Foods Inc. (div. Cudahy Company) Gen. Office. Phoenix, Az. 85013. EST 191. Feb. 17. 1 lb.
1030	Oregon Chief Slab Bacon. Portland Provissioner, Inc. Portland, Oregon.

Preliminary Extraction Procedures

Before the method for extraction of NOPRO used in this study was completely developed, several techniques were tried and found to be unsatisfactory.

Since NOPRO has a pH of 3.0 (Lijinsky et al., 1970) and properties similar to free fatty acids, a technique for free fatty acid extraction was tried. The method of Bills et al. (1963) was modified and used to extract NOPRO from cured meats. One hundred grams of ground sample were blended with 100 ml of 0.5 N HCl and 100 ml ETOAC. The lipid layer was removed after centrifugation. The ETOAC portion was dried by rotary evaporation and redissolved in 50 ml of hexane. To the hexane extract, 20 g of Dowex 1-X8 resin (OH form) were added and stirred for 30 min. The fat was washed from the resin by hexane, then ethanol. The resin was then placed in 40 ml of methanol containing 1% HCl. After one hour the methanol was removed to a separatory funnel containing 25 ml distilled water. This was extracted three times with 25 ml CH_2Cl_2. The CH_2Cl_2 extract was reduced to about 4 ml by rotary evaporation and then to about 0.5 ml in a stream of prepurified nitrogen. The internal standard (ACPRO) was added with the methanol. This method resulted in high levels of free fatty acids which could not be completely removed on further purification and subsequently interferred in glc separation.

A modification of the free amino acid extraction procedure of Field and Chang (1969) was investigated. To 200 ml of 1% picric acid 100 g of sample were added. The mixture was blended and centrifuged. The aqueous phase was filtered through Whatman No. 1 paper and passed over a Dowex 2X8 resin (chloride form). Sufficient resin was used to remove a maximum of picric acid (2 x 20 cm column). NOPRO was not retained on the resin. The column was washed three times with 100 ml of ETOAC. The ETOAC fraction was dried by rotary evaporation. NOPRO was quantitated by the procedure described previously. Picric acid was not entirely removed by ion exchange chromatography and after concentration, the residual picric acid was sufficient to interfere with glc. Also, the potential for NOPRO formation existed during this extraction procedure.

Ion exchange chromatography without picric acid was also tried. The cured meat sample was blended with distilled water and centrifuged. The aqueous phase was filtered and then passed over a column of Dowex 2X8 resin (OH form). The column was 2 x 10 cm. The NOPRO was retained on the resin. The column was then washed three times with 25 ml of 0.5 N HCl. The wash was collected and extracted three times with 50 ml of ETOAC. The ETOAC fraction was dried by rotary evaporation. The methylation procedure was that as previously described. With this technique it was difficult to determine if all the NOPRO was retained on the column or when the

ion exchange capacity of the column was exceeded. Also, some free fatty acids were extracted which interferred with glc.