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Determination of *N*-Nitrosoproline in Meat Samples

A liquid chromatographic method using a thermal energy analyzer detector for the determination of *N*-nitrosoproline in processed meat samples was studied. The method was shown to have sufficient accuracy and moderate precision in spiked meat samples containing 100 ppb *N*-nitrosoproline. A number of commercial meat samples were also examined and were found to contain very low levels of *N*-nitrosoproline in some samples.

It has long been known that simple nitroso amino acids such as *N*-nitrosoproline (NPRO) can be decarboxylated by heating in dilute alkali to produce the corresponding nitrosamine (Lijinsky et al., 1970). It has also been shown that raw bacon contains only very small quantities of the extremely potent carcinogen, *N*-nitrosopyrrolidine, but when it is fried, considerably higher levels of *N*-nitrosopyrrolidine can be formed from the decarboxylation of NPRO (Pensabene et al., 1974; Kushnir et al., 1975; Warthensen et al., 1976). NPRO has been isolated and identified in bacon at an estimated level of 1 μg/g (Kushnir et al., 1975).

Though the formation of NPRO in nitrite cured meat products is of considerable interest, work in the area has been hampered by a lack of an analytical method that is sufficiently sensitive (ca. 10–100 ng/g) and selective. The gas chromatographic determination of NPRO as a methyl ester derivative (Wolfram et al., 1977) and as a trimethylsilyl derivative (Eisenbrand et al., 1975) have been reported; however, the methods have not been applied to meat samples or other complex systems. A semiquantitative TLC method has also been demonstrated to be useful for commercial bacon containing NPRO (Sen et al., 1977); however, the method requires a lengthy clean-up procedure and has a detection limit of only 100 ng/g.

The analysis of nonvolatile nitrosamines in food products using a thermal energy analyzer (TEA) and high-pressure liquid chromatography has recently been demonstrated (Fine et al., 1976). The TEA detector is useful for analysis of nitrosamines in the 10 μg/mL range (prior to sample concentration), and it is fairly selective for compounds containing the *N*-nitroso group.

The major efforts of the work described in this report were directed toward the development of a high-pressure liquid chromatographic method using the TEA detector for the determination of NPRO in the 10–100 ng/g range in meat samples. In addition to demonstrating the selectivity, precision, and accuracy of the method with spiked beef samples, the method was also used for the analysis of several commercial nitrite preserved meat samples.

MATERIALS AND METHODS

LC and Detector Operating Conditions. The principle of operation of the TEA detector (Fine et al., 1975) and application as a detector in LC analysis (Fine et al., 1976) has been previously reported. The detector unit used in this study was a prototype to Model TEA 502/LC produced by the Thermo Electron Corporation, and it was essentially the same as the units described in the earlier literature. The furnace consisted of a nonglazed ceramic tube ($^1/_8$ in. i.d. × 22 in.); however, it did not contain tungsten oxide powder as described in the earlier report (Fine et al., 1975). The response of the detector varied with pyrolysis temperature and with the condition of the apparatus. The largest response was obtained at temperatures over the range 350–400 °C, but was variable. The flow of oxygen to the ozone generator was adjusted to increase the pressure in the chemiluminescence chamber by 1.0 Torr. Nitrogen was used to purge the solvent cold traps at a flow rate of 50 mL/min (STP), and the cold traps were maintained at 0 °C.

A Waters Associates Model 202 LC equipped with a U6K injector was used. The reverse-phase chromatograms were obtained using a 3.9 × 300 mm μ-Bondapak-C18 column, and 5% v/v glacial acetic acid in water was used as the mobile phase (2.0 mL/min).

Chemicals. *N*-Nitroso-L-proline (mp 104–106 °C, lit. 97.5–98 °C) and *N*-nitrosopipecolic acid (mp 92–93 °C, lit. 91–92 °C) were prepared through the nitrosation of L-proline and racemic pipecolic acid with sodium nitrite (Lijinsky et al., 1970). Vitamin-free casein was used as supplied by Fisher Scientific Co. and the acetonitrile was freshly distilled. All other chemicals and solvents were of standard reagent grade.

General Extraction Procedure. A 20-g meat sample was weighed (20 g of vitamin-free casein for control sample) and transferred to a Waring blender containing 20 mL of water, 0.2 g of sulfamic acid, and 20 mL of 25%

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Table I. Determination of Nitrosoproline (100 ng/g) and Nitrosopipecolic Acid (200 ng/g) in Spiked Casein Samples

sample	% recovery of NPRO	% recovery of NPIC	% recovery NPRO / % recovery NPIC
1	92 ± 27	63 ± 22	1.49 ± 0.41[a]
2	86 ± 8	64 ± 6	1.35 ± 0.19
3	107 ± 29	52 ± 13	2.03 ± 0.17
4	54 ± 5	60 ± 12	0.93 ± 0.23
5	50 ± 14	49 ± 13	1.07 ± 0.38
Av	77 ± 25	58 ± 7	1.37 ± 0.43[b]

[a] Standard deviation of three chromatograms of the same extract. [b] Standard deviation of the value for all extracts.

Table II. Determination of Nitrosoproline in Ground Beef Samples (100 ng/g Nitrosoproline Added to Sample)

sample	% recovery of NPRO	% recovery of NPIC	% NPRO/ % NPIC	NPRO found, ng/g
1	85 ± 15	68 ± 2	1.25 ± 0.19	90 ± 33[a]
2	64 ± 6	34 ± 4	1.90 ± 0.33	144 ± 40
3	71 ± 21	60 ± 14	1.19 ± 0.31	59 ± 16
4	46 ± 14	52 ± 7	0.90 ± 0.16	101 ± 35
5	56 ± 22	47 ± 20	1.23 ± 0.26	119 ± 18
Av	65 ± 15	52 ± 13	1.29 ± 0.31	103 ± 31[b]

[a] Standard deviation of three chromatograms of the same extract. [b] Standard deviation of the value for all extracts.

Na_2WO_4. The samples were blended until uniform (ca. 30 s), then 4.0 μg of the nitrosopipecolic acid (NPIC) internal standard was added (also 2.0 μg of NPRO in the casein control sample), and the sample was blended for an additional 10 s. The mixture was transferred to a beaker with the aid of 80 mL of water, then 20 mL 50% w/v $NaHSO_4$ was added. The mixture was heated in a water bath at 55 °C for 30 min and then filtered through a Buckner funnel lined with Whatman No. 4 filter paper. The filtrate was evaporated under vacuum in a rotary evaporator at 40 °C until dry, 10 mL of benzene was added, and evaporation was continued to remove the last trace of water. The residue was then sonicated with 10 mL of acetonitrile for 20 min, the liquid layer was transferred to a conical tube and was then centrifuged to remove inorganic salts. The clear acetonitrile layer was transferred to a conical vial and evaporated under a steam of nitrogen at 40 °C. Then 400 μL of water was added to the residue in the vial and shaken on a vortex mixer for 2 min. A 50-μL aliquot of the clear solution was used for the chromatographic analysis.

RESULTS AND DISCUSSION

The precision and accuracy of the method were evaluated using samples of casein and ground beef that had been spiked with NPRO and the internal standard NPIC (Tables I and II). The analysis of these samples were conducted over a 6-week period in order that both short-term and long-term experimental variations might be detected.

The percent recoveries for NPRO and NPIC were calculated from a comparison of the LC–TEA peak areas of the final extract and simple aqueous solutions of NPRO and NPIC. The variation of the percent recovery between samples was partially due to errors made in small volume measurements of the final extract and partially due to actual variations in the extent of recovery. It was also observed that the standard deviations of the results of multiple chromatograms of the same extract were fairly large compared to typical LC results. In fact, the variations of the results of the absolute recoveries or the relative recoveries of NPRO and NPIC from multiple chromatograms of the same extract were approximately the same as the standard deviation of the values obtained on different days with different spiked samples. These moderately high standard deviations were primarily due to the poor reproducibility of the TEA detector in the analysis of nitroso compounds of high polarity (Baker and Ma, 1977).

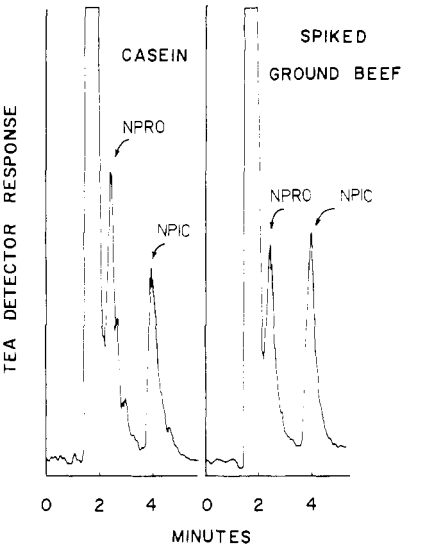

Figure 1. Chromatograms of the extracts of casein and ground beef samples spiked with 100 ng/g and 200 ng/g of NPIC.

The ratio of the relative recovery of NPRO and NPIC was slightly but consistently above 1.0 for both the spiked casein and spiked ground beef model studies. When a simple aqueous model was processed using the extraction scheme, recovery ratios consistently near 1.0 were observed for NPRO and NPIC. Because of the more satisfactory agreement of the casein model with the spiked ground beef study, a casein sample spiked with both NPRO and NPIC was run parallel to all subsequent meat sample determinations as a quantitative control.

The average value of the NPRO determination (103 ng/g) given in Table II was arrived at through a comparison of NPRO and NPIC peak area ratios in the casein control and the peak area ratios of NPRO and the internal standard in the spiked (100 ng of NPRO/g) ground beef samples. From an inspection of the NPRO determinations reported in Table II, it would appear that the method is sufficiently accurate at the 100 ng/g level, but that the precision of the method is slightly poorer. The detection limits of the method were found to vary considerably with the age of the LC column, optimization of TEA detector, and nature of the specific meat sample. In Figures 2 and 3 a fairly new μ-Bondapak-C18 column had been used, and a retention time of 3.4 min was observed for NPRO and it was well resolved from the solvent front. However, as the column aged, the retention time of NPRO and NPIC steadily became shorter (Figure 1), and after a period of 4 months used, the NPRO peak could not be satisfactorily resolved from the solvent front on the low level samples. Though there was considerable variability due to these three factors, the detection limit for NPRO was usually 5 ng/g in the meat samples.

A number of commercial nitrite preserved meat samples (salami, cooked ham, bologna, bacon, corned beef, and hot dogs) were analyzed for their NPRO content. The NPRO content of meat of the samples was at or below the de-

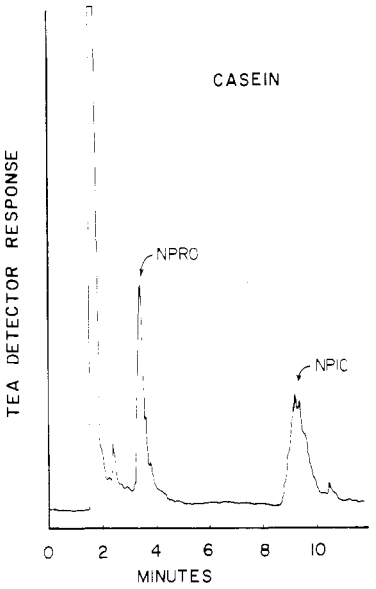

Figure 2. Chromatogram of the extract of a casein sample spiked with 100 ng/g of NPRO and 200 ng/g of NPIC. The same procedures were used as in the previous figure, but the LC column was new.

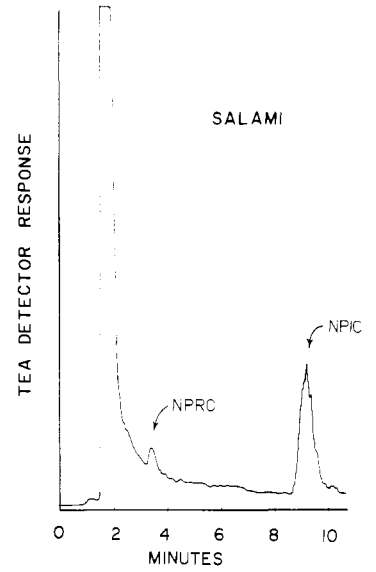

Figure 3. Chromatogram of the extract of a commercial salami sample. 200 ng/g of NPIC was added to the sample as the internal standard. The same column and conditions were used as in the previous figure.

tection limit of the method and was generally two orders of magnitude lower than the earlier estimates (Kushnir et al., 1975). The chromatogram of a sample of salami found to contain 16 ng/g of NPRO is shown in Figure 3 and the corresponding casein control is shown in Figure 2.

In order to determine what the maximum levels of NPRO might approach, a sample of bologna was deliberately nitrosated and then assayed for NPRO content. A 20-g sample was homogenized with 200 mg of $NaNO_2$ at pH 2.0 and allowed to stand at room temperature for 4 h. Then excess nitrite was removed by the addition of sulfamic acid and the NPRO content determined by the standard procedure. The sample was found to contain 30 ± 5 μg/g of NPRO, which was three orders of magnitude larger than in the untreated commercial sample.

For confirmation purposes, the extracts and a NPRO-containing control were irradiated for 3.5 h with ultraviolet radiation (366 nm) and then chromatographed again, and the NPRO and NPIC peaks disappeared. This method which was adapted from that of Doerr and Fiddler (1977) takes advantage of the rapid photolytic decomposition of nitrosamines with this wavelength of ultraviolet light. Ideally, the identity of NPRO in the meat samples should be also confirmed by mass spectrometry; however, it is not likely that the present generation of LC/MS interfaces will be useful at such low levels.

Although the isotope labeling studies of Hwang and Rosen (1976) indicated that preformed NPRO in uncooked meat may be the major precursor of nitrosopyrrolidine in cooked meat, the levels of NPRO found in the present study would be too low to account for the high levels of nitrosopyrrolidine in cooked samples. However, as was found in the commercial bologna sample, the NPRO content can be increased three orders of magnitude in 4 h by the addition of a large excess of nitrite. Because of the very limited number of samples studied, it is still not possible to definitely conclude if the nitrosopyrrolidine found in cooked commercial samples is produced by the decarboxylation of preformed NPRO or if both the formation of NPRO and decarboxylation occur during cooking.

NOTE: Precautions should be exercised in the handling of nitrosamines as they are potential carcinogens.

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