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Note

Occurrence and determination of N-nitroso-3-hydroxypyrrolidine in cured meat products

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Analytical methods for the determination of volatile nitrosamines in food are well established[1,2]. However, less volatile or non-volatile N-nitroso compounds have not been investigated so far, because suitable analytical methods were not available.

Recent studies have shown that the poorly volatile N-nitroso-3-hydroxypyrrolidine (NHPYR) is formed by heat-induced decarboxylation of N-nitrosohydroxyproline in a model system as well as in foods by the normal frying procedure[3]. It can be assumed that N-nitrosohydroxyproline is formed in foods from hydroxyproline during processing with nitrate or nitrite. Preliminary analytical studies showed that NHPYR is present in several fried cured-meat products marketed in Germany[3].

In this paper we describe an analytical method which has been developed for the isolation and determination of NHPYR from foods, and its application to a limited range of cured meat products.

The clean-up procedure consists of methanol–water extraction of the food followed by several liquid–liquid partitions. NHPYR is determined by gas chromatography (GC) of the concentrate before and after trifluoroacetylation; a thermal energy analyzer (TEA)[4] is used as the GC detector. The identity is confirmed by GC–high-resolution mass spectrometry. N-Nitroso-4-hydroxypiperidine (NHPIP) is used as an internal standard.

MATERIALS AND METHODS

NHPYR and NHPIP were synthesized from the corresponding amines by a modification of a method described by Krüger and Bertram[5]. The purity of the compounds was established by thin-layer and gas chromatography and by UV and mass spectrometry. The solvents were of analytical grade or were purified by distillation. Basic alumina and SE-cellulose were obtained from Serva, Heidelberg, G.F.R. N-Methylbis(trifluoroacetamide) (MBTFA, Macherey, Nagel & Co., Düren, G.F.R.) was used for trifluoroacetylation. Food samples were obtained from local stores, and 50-g amounts were cooked for 6 min in a preheated open pan with 5 ml of vegetable oil at 180–200°. After cooling, the sample was analyzed for NHPYR.

Extraction and clean up

The minced sample, fortified with 0.2 μg of NHPIP (4 ppb), was homogenized with 20 g of Celite 545 in 150 ml of methanol–water (9:1). The homogenate was filtered and the extraction of the sample was repeated twice. The extracts were combined and concentrated in a rotary evaporator to a volume of 25 ml. The concentrate was saturated with sodium chloride and extracted first with 100 ml of *n*-pentane and then with two 100-ml portions of *n*-pentane–dichloromethane (1:1); the organic layers were discarded.

The aqueous phase was adjusted to pH 9 with sodium hydroxide and extracted three times with 120 ml of ethyl formate. The organic phases were combined and concentrated to a small volume in a rotary evaporator (water-bath temperature, 20°). The concentrate was finally adjusted to 0.2 ml in a graduated vial under a stream of nitrogen.

Some samples needed further purification by column chromatography. In these cases the concentrate was placed on a column (6 mm I.D.) of basic alumina (1 cm) covered with 1 cm of strongly acidic SE-cellulose. The column was eluted with a mixture of acetone–dichloromethane (1:1). The first 20 ml of the eluate were concentrated to 0.2 ml. Aliquots of 4 μl were injected into the gas chromatograph.

GC determination with the TEA detector

Separations were carried out on a glass column (1.2 m × 2.0 mm I.D.) packed with 10% Carbowax 20M–terephthalic acid on Gas-Chrom Q (80–100 mesh). The column was connected to the TEA-detector oven by a heated glass capillary (1 mm I.D.). A three-way valve was inserted between the pyrolytic oven and the cold trap to allow venting of solvent to the atmosphere. Carrier gas (helium) flow-rate, 45 ml/min. Injection-port temperature, 200°. Column-oven temperature, 10 sec isothermal at 150° then programmed at 10°/min to 190°.

When the TEA-detector indicated the presence of NHPYR, MBTFA (100 μl) was added to 100 μl of the sample and the mixture was heated in a sealed vial at 60° for 2 h. After cooling, aliquots of 4 μl were injected into the gas chromatograph. Separations were carried out on a glass column (3 m × 2.0 mm I.D.) packed with 3% QF-1 on Gas-Chrom Q (100–120 mesh). Carrier gas (helium) flow-rate, 45 ml/min. Injection-port temperature, 200°. Column-oven temperature, 10 sec isothermal at 120° then programmed at 4°/min to 140°.

GC–MS

A Varian MAT 311 mass spectrometer, coupled to a Pye Unicam 106 gas chromatograph, was used. The molecular ion of the NHPYR-TFA derivative was monitored by the peak-matching technique at a resolution of 10,000, using an appropriate fragment of perfluorokerosene as reference. A glass column (1.2 m × 2.0 mm I.D.) packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh) was used for the separations. Carrier gas (helium) flow-rate, 14 ml/min. Injection-port temperature, 180°. Oven temperature, 150°. Separator: slit type; temperature, 250°.

RESULTS AND DISCUSSION

Methanol–water (9:1) was found to be the most suitable solvent system in

model experiments for the extraction of NHPYR from the sample matrix. After removal of the organic solvent the aqueous concentrate is extracted with non-polar solvent systems to remove lipophilic contaminants. By subsequent partitioning at pH 9, NHPYR is transferred into ethyl formate. The alkaline pH ensures that N-nitrosohydroxyproline, which could possibly generate NHPYR artifacts by heat-induced decarboxylation during GC, is retained in the aqueous phase.

NHPIP was selected as internal standard because its physicochemical and chemical properties were deemed to be similar to those of NHPYR. In order to verify the suitability of NHPIP, comparative recovery experiments were carried out with both compounds at concentrations of 4 ppb. Uncured bacon, which had been tested before for the absence of NHPYR and NHPIP, was used in these studies. The mean recoveries were 58% for NHPYR (9 tests, $s = 8$) and 45% for NHPIP. (9 tests, $s = 6$). Additional column chromatographic purification diminished the recoveries for both compounds by *ca.* 10%. These results demonstrate the suitability of NHPIP as the internal standard, assuming that this compound does not occur naturally in foods. Examination of 21 samples of fried cured-meat products gave no indication of the presence of NHPIP. The recoveries of the internal standard in the present study (33 samples) were in the same range as in the recovery experiments with uncured bacon. This confirms the assumption that NHPIP was not initially present in the samples.

Samples which had indicated the presence of NHPYR by direct GC–TEA were trifluoroacetylated and reexamined. A positive response of the TEA detector for NHPYR and its trifluoroacetyl (TFA) derivative under different chromatographic conditions corroborated the identity of the nitrosamine. The TEA results could be confirmed by GC–high-resolution mass spectrometry of the TFA derivative when

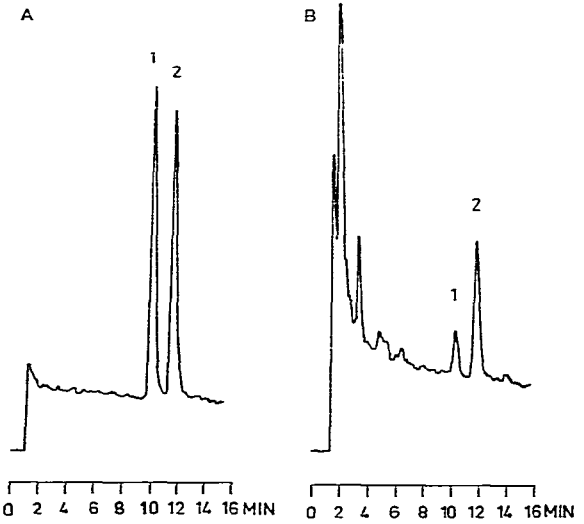

Fig. 1. Gas chromatogram of TFA derivatives of N-nitroso-3-hydroxypyrrolidine (1) and N-nitroso-4-hydroxypiperidine (2) analyzed by the TEA detector. (A) 4 μl (2 ng) of each nitrosamine were injected; (B) 50-g sample containing 1 ppb of N-nitroso-3-hydroxypyrrolidine. N-Nitroso-4-hydroxypiperidine was added as internal standard. 4 μl were injected.

concentrations $\geqslant$ 5 ppb were present (samples 7–9, Table I). A typical TEA gas chromatogram of a food extract after trifluoroacetylation is shown in Fig. 1. NHPYR-TFA is eluted prior to NHPIP-TFA. Under the described conditions, an effective baseline separation of the two compounds could be achieved. The peaks are narrow, and the detection limit is well below 0.2 ng (signal-to-noise ratio, 4:1).

In the present study, 33 fried samples (10 of bacon, 10 of boiled ham and 13 of bologna sausage) were analyzed for NHPYR with the described method. Nine samples contained detectable amounts of the nitrosamine in concentrations ranging from 1 to 7 ppb (Table I). The results confirm those of our previous study[3]. They show that NHPYR occurs in different types of fried cured-meat products at concentrations below or near 10 ppb. Similar concentrations have been found by Sen *et al.*[6] in fried bacon by a different analytical method[7].

TABLE I

AMOUNTS OF N-NITROSO-3-HYDROXYPYRROLIDINE FOUND IN 9 OUT OF 33 FRIED CURED-MEAT PRODUCTS

No.	*Sample*	*Amount of NHPYR (ppb)*
1	Bacon	1
2	Bacon	1
3	Bacon	1
4	Boiled ham	1
5	Bologna sausage	1
6	Bologna sausage	2
7	Bologna sausage	7*
8	Bologna sausage	6*
9	Bologna sausage	7*

* Confirmed by high-resolution mass spectrometry.

No data on the carcinogenicity of NHPYR have been available until now. This compound is presently being tested in our laboratory by chronic oral application in rats. It has been found to be a mutagen in *Salmonella typhimurium*[8]; it might also be carcinogenic.

GC analysis of the food extracts also showed some unidentified peaks, which, because of the high specifity of the TEA for N-nitroso structures, are believed to be N-nitroso compounds. Attempts at their identification are currently being carried out.

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