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Note

Trace analysis of 3-hydroxy-1-nitrosopyrrolidine, a non-volatile N-nitrosamine, by combined gas chromatographic–mass spectrometric method

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During the past few years considerable progress has been made in the field of trace analysis of volatile N-nitrosamines[1–3], which are potent carcinogens[4,5]. Similar progress has, however, been lacking for non-volatile nitrosamines mainly because of the lack of adequate clean-up techniques suitable for trace analysis of complex mixtures. In all of the published methods for determining volatile nitrosamines in foods some sort of distillation step constitutes an essential part of the clean-up process. Such clean-up techniques by distillation are, however, not applicable to non-volatile nitrosamines. Since the ultimate purpose is to use these techniques for trace analysis of foods or other environmental materials, it would be a great advantage to convert a non-volatile nitrosamine to a stable volatile derivative that is amenable to purification by distillation.

Theoretically, many non-volatile nitrosamines can be present in the environment, but the relative importance of these compounds with regard to their biological or toxicological significance is unknown at the moment. Traces of nitrosopyrrolidine (a volatile nitrosamine) have been consistently detected in cooked bacon[6–8], and occasionally in other cured meat products[9,10]. Nitrosopyrrolidine is believed to be formed by the interaction of added nitrite and proline or pyrrolidine[6,11]; the latter two compounds are naturally occurring in pork bellies[12,13]. Since the corresponding hydroxy compounds, namely, 3-hydroxyproline and 3-hydroxypyrrolidine, are also known to be present in pork[13], there is a good likelihood that traces of 3-hydroxy-1-nitrosopyrrolidine (HNPY) may be produced during cooking of bacon or other cured meat products. However, owing to the lack of suitable analytical methods it has not been possible to determine whether this is true. In this communication, we wish to describe a highly specific gas chromatographic–mass spectrometric (GC–MS) technique whereby HNPY is determined at nanogram quantities after conversion to a stable volatile derivative, namely, 3-methoxy-1-nitrosopyrrolidine (MEONPY).

MATERIALS AND METHODS

Methyl iodide was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.) and sodium hydride (50% mineral oil dispersion) was purchased from J. T. Baker (Phillipsburgh, N.J., U.S.A.) Small amounts of sodium hydride were taken out, washed with anhydrous *n*-hexane and stored in a tightly closed screw-cap vial. All solvents were glass-distilled.

Preparation of HNPY

HNPY was prepared by nitrosation of 3-pyrrolidinol (Aldrich, Milwaukee, Wisc., U.S.A.) by the method of Krueger and Bertram[14] with minor modifications. About 0.435 g (5 mmole) of 3-pyrrolidinol was dissolved in 10 ml of water and the pH of the mixture was adjusted to 3 with gradual addition of 10 *N* or 1 *N* hydrochloric acid. Sodium nitrite, 1.035 g (15 mmole), dissolved in 5 ml of water was gradually added to the mixture with continuous stirring over a period of 30 min, and the nitrosation reaction was allowed to proceed at room temperature for another 2 h. The reaction mixture was kept overnight in a refrigerator and then made alkaline (pH *ca.* 10) by slow addition of 3 *N* potassium hydroxide solution. The solution was saturated with sodium chloride and extracted with three 50-ml portions of ethyl acetate. The unreacted pyrrolidinol was removed by extracting the combined ethyl acetate layer with 20 ml of 1 *N* hydrochloric acid saturated with sodium chloride. The ethyl acetate layer was washed with 20 ml of 20% potassium carbonate solution, and then dried over anhydrous calcium sulphate (Drierite). The dried ethyl acetate extract was filtered, and concentrated in a flash evaporator to near dryness.

The residue was dissolved in 10 ml of dichloromethane and 1–5-μl aliquots were examined by thin-layer chromatography (TLC) using dichloromethane–diethyl ether–*n*-hexane–methanol (100:70:50:22) as the developing solvent and NEDSA* spray as the visualization reagent as described previously[14,15]. After exposing the plate to ultraviolet light a strong purple spot at R_F 0.36 (due to HNPY) and two minor spots at higher R_F were observed. These fast moving impurities were removed by passing the dichloromethane extract through a silica gel column (2 × 5 cm bed volume) and washing the column successively with 100 ml of dichloromethane and 200 ml of dichloromethane containing 25% ethyl acetate. The washings were discarded. Finally, HNPY was eluted from the column with 200 ml of ethyl acetate and the eluate concentrated to dryness in a flash evaporator. The yield was about 100 mg.

The purity of HNPY was checked by TLC (as described above), MS and nuclear magnetic resonance spectroscopy. High-resolution (10,000) MS analysis confirmed the correct molecular formula, $C_4H_8N_2O_2$. The sample also produced a clean low-resolution mass spectrum (direct probe sample) which contained the necessary fragment ions, namely, the molecular ion peak (M^+), NO^+, and $(M-NO)^+$ peak (Fig. 1).

Preparation of O-methyl ether derivative

An aliquot (1, 10 or 100 μg) of HNPY, dissolved in anhydrous ethyl acetate was taken in a glass-stoppered test tube and the solvent was evaporated with a gentle stream of nitrogen. About 0.5 ml of anhydrous ethyl acetate (dried over Drierite) 0.5 ml of methyl iodide and 15–20 mg of powdered sodium hydride were added and the sample was mixed vigorously using a Vortex mixer. The contents were mixed again after 5 min and then allowed to stand at room temperature for 3 h or an overnight period. After the reaction was over, about 1 ml of water was carefully added dropwise to the mixture with gentle shaking. The product (MEONPY) was extracted with three 2-ml portions of diethyl ether by vigorous mixing in a Vortex mixer and each time the ether layer was carefully withdrawn with a pasteur pipette and dried by passing through a small column of anhydrous sodium sulphate. The combined ether

* NEDSA = N-1-napthylethylenediamine + sulphanilic acid.

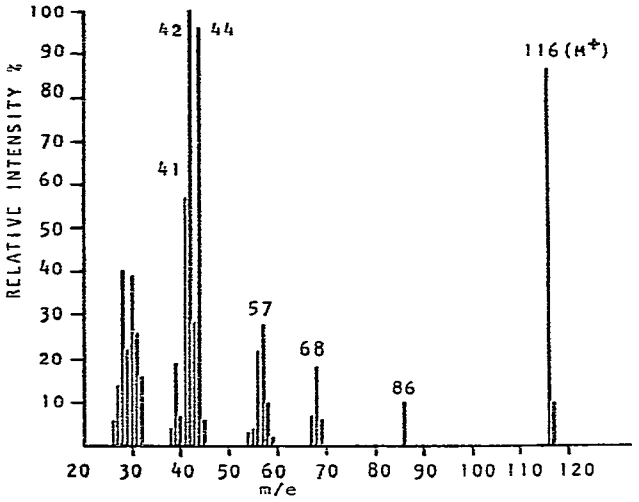

Fig. 1. Mass spectrum of HNPY (probe sample).

extracts were taken in a graduated test tube and concentrated to 0.5–1 ml at about 30° in a stream of nitrogen. Aliquots (1–5 μl) were used for GC–MS analysis.

Vacuum distillation of MEONPY

When the derivative was isolated by distillation, the ether extraction steps, as described above, were omitted and the sample was transferred directly into a 250-ml distillation flask. About 100 ml of 1 *N* potassium hydroxide were added to the sample and the mixture was distilled under vacuum using an all-glass vertical-type (Buchler Instruments) rotary evaporator (water-bath 50–60°). During the distillation, the collection flask was kept immersed in a mixture of dry ice and methanol, and ice-cold water was circulated through the condenser. The distillation was continued until only 1–5 ml of residue was left in the distillation flask.

The frozen distillate was allowed to thaw at room temperature, the solution was then made alkaline by adding solid potassium carbonate (20%, w/v), and the mixture extracted with two 100-ml portions of dichloromethane. The condenser was carefully rinsed with dichloromethane and the washings were combined with the above dichloromethane extracts. The combined organic extract was dried over anhydrous sodium sulphate, filtered, and concentrated in a flash evaporator to 2–5 ml (care should be taken not to evaporate to complete dryness). The solution was quantitatively transferred into a graduated glass-stoppered test tube and concentrated to 0.5–1 ml at 30° in a stream of nitrogen.

GC–MS analysis

A Varian MAT (Model 311A) high-resolution mass spectrometer with an electron impact ionization source and coupled (via an all-glass Watson–Biemann separator) to a Varian Aerograph Model 1400 gas chromatograph was used for the analysis. The mass spectrometer was operated in the specific ion monitoring mode for NO^+ (m/e = 29.9980) or the molecular ion peak (m/e = 130.0742) at a resolution of 5,000. Operating conditions: source temperature, 250°; emission current, 3 mA;

electron voltage, 70 eV; and accelerating voltage, 3 kV. The temperature of the interface was kept at 210–220° or at 270° while monitoring for the molecular ion peak or NO^+, respectively.

RESULTS AND DISCUSSION

The methylation technique used in this study is very similar to that used by Lawrence and Iverson[16] for alkylation of certain pesticides. Similar techniques have been successfully used in the past for alkylation of amines[17], amides[18] and alcoholic[19] compounds. The main difference was that ethyl acetate was used in this study as the reaction medium instead of benzene, toluene or dimethylsulphoxide as used by the above-mentioned investigators. The reaction proceeded smoothly at room temperature and gave an yield of 90% or more after overnight standing. Both low-resolution and high-resolution mass spectra confirmed the structure of the derivative.

Fig. 2 gives typical examples of tracings obtained during GC–MS monitoring for the two specific ions as mentioned above under Materials and methods. It can be seen that both mode of operations are quite sensitive; the minimum detection limit will be about 1 ng. It should be pointed out that MEONPY can also be analyzed by a conventional GC technique using a Coulson electrolytic conductivity detector (pyrolytic mode) under conditions similar to that used for the detection of volatile nitrosamines[20]. The GC–MS technique is, however, more specific, and, hence, it will be more useful for the analysis of environmental samples.

Besides the procedure described above, two other methylation techniques were

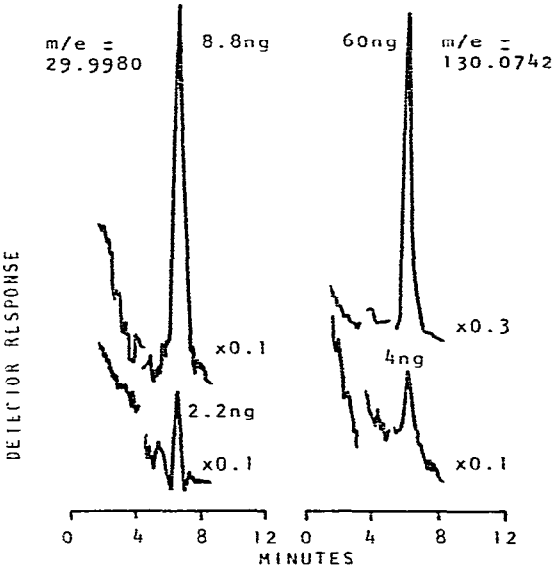

Fig. 2. Chromatograms showing specific in monitoring by GC–high-resolution MS for NO^+ (left) and M^+ (right) of the methyl ether derivative of HNPY. Amounts shown correspond to HNPY. GC conditions: 6 ft. × 1/8 in. stainless-steel column packed with 10% Carbowax 20M on Chromosorb W (80–100 mesh, hexamethyldisilazane treated). Column temperature 180°, helium flow-rate 30 ml/min. For MS conditions (note: interface temperatures), see text.

examined. Methylation by diazomethane, even with boron trifluoride etherate as a catalyst[21], gave only 30% yield. The other technique involved gradual addition of dimethylsulphate[21] to an alkaline solution (1 *N* sodium hydroxide) of HNPY over a period of 3 h which gave an yield of about 80%. This latter technique might be useful in methylating HNPY in samples containing excessive amounts of moisture or other interfering compounds.

High-resolution GC–MS techniques are considered to be the only reliable technique available today for unequivocal confirmation of the presence of nitrosamines. To our knowledge, this appears to be the first successful method reported for the analysis of HNPY. The methylated ether derivative is easy to prepare, and at the same time the product is stable and volatile enough to undergo the vacuum distillation clean-up process as described above. It is hoped that the technique will be useful for trace analysis of HNPY and possibly other hydroxynitrosamines.

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