Journal of Chromatography, 195 (1980) 416–420
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,833

Note

Gas chromatographic determination of N-nitrosoamino acids

TOHRU ISHIBASHI*

Research Laboratory, Japan Medical Foods Association, 5-3-11 Maesawa, Higashikurume, Tokyo 130-03 (Japan)

TOSHIHARU KAWABATA

Department of Biomedical Research on Foods, National Institute of Health, 2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141 (Japan)

and

HIROYA TANABE

Research Laboratory, Japan Medical Foods Association, 5-3-11 Maesawa, Higashikurume, Tokyo 130-03 (Japan)

(Received March 12th, 1980)

N-Nitrosoamino acids, except for N-nitrososarcosine (NSAR), have been reported to be non-carcinogenic to experimental animals. However, these acids may be converted into carcinogenic N-nitrosoamines by decarboxylation during high-temperature cooking. N-Nitrosoproline (NPRO), for instance, was reported to be converted into carcinogenic N-nitrosopyrrolidine in a model system using conditions simulating the frying of bacon[1].

In the gas chromatographic determination of N-nitrosoamino acids, methods have been reported for the derivatization of the carboxyl groups by methylation[2,3] or silylation[4] to form volatile derivatives.

We have developed a method for the determination of trace amounts of N-nitrosoamino acids, including hydroxylated N-nitrosoamino acids such as N-nitrosohydroxyproline (NHPRO), based on the following principle. The carboxyl groups of NSAR, NPRO, NHPRO and N-nitrosopipecolic acid (NPIC) were esterified with diazomethane, and the nitroso groups further oxidized with peroxytrifluoroacetic acid (PTFA) to give N-nitroamino acid methyl esters. The hydroxyl group of NHPRO methylester was acylated with trifluoroacetic anhydride (TFA).

The derivatized N-nitrosoamino acids can be determined with high sensitivity using a gas chromatograph equipped with an electron capture detector (GC–ECD); in addition, these compounds can be confirmed by gas chromatography–mass spectrometry (GC–MS).

EXPERIMENTAL

Reagents

NSAR, NPRO, NHPRO and NPIC were synthesized by nitrosation of the corresponding amino acids according to the Lijinsky method[5]. Each N-nitrosoamino

0021-9673/80/0000–0000/$02.25

acid was dissolved in ethanol–ethyl acetate (1:4) to a level of 5 μg/ml and this solution was employed as the test solution for derivative formation.

Derivative formation

Esterification. To 0.1 ml of the test solution in a glass-stoppered test-tube was added 1 ml of diazomethane-saturated diethyl ether, the mixture was allowed to stand for 30 min at room temperature, and was then evaporated to dryness under a gentle stream of nitrogen.

Oxidation and acylation. To a test-tube containing an N-nitrosoamino acid methyl ester, 0.2 ml of PTFA reagent[6,7] [0.4 ml of 85–90% hydrogen peroxide and 2.5 ml of TFA were added to a 10-ml volumetric flask containing a few millilitres of dichloromethane; after gently shaking this mixture, CH_2Cl_2 was added to make the volume exactly 10 ml] and 1 ml of CH_2Cl_2 were added.

After brief shaking, the tube was heated under reflux for 2 h at 40°C. Following the oxidation reaction, the reaction mixture was evaporated to dryness under a gentle stream of nitrogen and 0.2 ml of TFA was added to the tube, which was then allowed to stand for 30 min at room temperature. The reaction mixture was then evaporated to dryness under a gentle stream of nitrogen. The resulting derivative was dissolved in 1 ml of CH_2Cl_2 and employed for GC–ECD analysis.

Operating conditions for GC–ECD and GC–MS

A Shimadzu GC-4BF gas chromatograph equipped with an ^{63}Ni electron-capture detector was used for the GC analysis of the derivatives of N-nitrosoamino acids; a glass column (2 m × 3 mm I.D.) packed with 3% DEGS–0.5% H_3PO_4 on Chromosorb W (60–80 mesh) pre-treated with HMDS was employed. The carrier gas was nitrogen at a flow-rate of 40 ml/min. The temperature of both the column and the detector was 170°C. A Shimadzu-LKB 9000 gas chromatograph–mass spectrometer was used for GC–MS analysis of the derivatives of test N-nitrosoamino acids. The glass column (2 m × 3 mm I.D.) was packed with the same materials employed for GC–ECD analysis. The carrier gas was helium at a flow-rate of 30 ml/min. The temperatures of the injection port and column oven were 240 and 180°C, respectively, and those of the separator and ion source were 250 and 260°C, respectively. The electron energy was 20–70 eV, the accelerating voltage was 3.0 kV and the trap current was 60 μA.

RESULTS AND DISCUSSION

There are two different ways of carrying out the derivatization of N-nitrosoamino acids to form N-nitroamino acid methyl esters. Firstly, after esterifying the carboxyl group of an N-nitrosoamino acid with diazomethane, the resulting N-nitrosoamino acid methyl ester can be oxidized with the PTFA reagent to form the N-nitroamino acid methyl ester. In the second method, the nitroso group of the N-nitrosoamino acid is oxidized with the PTFA reagent to give the N-nitroamino acid, followed by esterification with diazomethane to yield the N-nitroamino acid methyl ester. In the present study, we chose the former method. In a previous paper[3], we reported the rates of esterifications of N-nitrosoamino acids by diazomethane to be more than 95% for four test N-nitrosoamino acids.

The rates of oxidation of N-nitrosoamino acid methyl esters to form N-nitroamino acid methyl esters were determined in the following way. A 5-mg amount of each N-nitrosoamino acid was esterified with diazomethane and the resulting methyl ester was oxidized with PTFA reagent; then the unreacted N-nitrosoamino acid methyl ester was determined using a gas chromatograph equipped with an alkali flame-ionization detector. It was found that no appreciable amounts of the N-nitrosoamino acid methyl esters could be detected, indicating that the conversion of an N-nitrosoamino acid methyl ester to the corresponding N-nitroamino acid methyl ester may proceed almost to completion. It has been reported that the methyl ester of NHPRO exhibits a fairly low sensitivity to GC analysis, and this might be caused by non-specific adsorption of this compound, which has an OH group, on the packing materials[2,4,8]. We found that N-nitrohydroxyproline methyl ester showed a similar low sensitivity to GC analysis, probably for the same reason as the NHPRO methyl ester; the gas chromatogram of this compound is illustrated in Fig. 1. When the OH group of N-nitrohydroxyproline methyl ester was acylated with TFA reagent, the TFA derivative exhibited a fairly high sensitivity to GC analysis, as can be seen in Fig. 2.

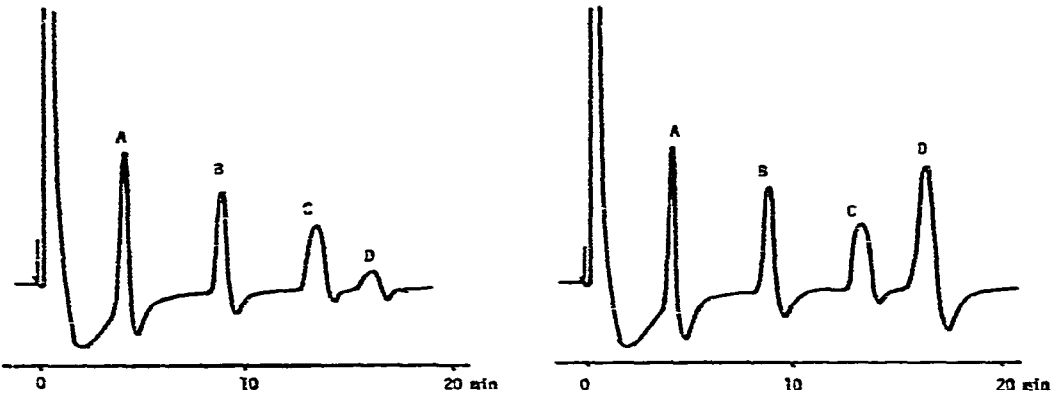

Fig. 1. Gas chromatogram of the esterified and oxidized derivatives of N-nitrosoamino acids before acylation with TFA. Sample size: 500 pg of each N-nitrosoamino acid. Peaks: A = NSAR; B = NPIC; C = NPRO; D = NHPRO.

Fig. 2. Gas chromatogram of the esterified and oxidized derivatives of N-nitrosoamino acids after acylation with TFA. Sample size and peaks as in Fig. 1.

Mass spectra of the four N-nitroamino acid methyl esters are shown in Fig. 3. Although no parent ions of the four derivatives could be detected, some abundant and characteristic fragments were observed. That is, fragments showing the loss of *m/e* 59 from the respective N-nitroamino acid methyl ester were *m/e* 89 for N-nitrosarcosine methyl ester, *m/e* 115 for N-nitroproline methyl ester, *m/e* 227 for N-nitrohydroxyproline methyl ester and *m/e* 129 for N-nitropipecolic acid methyl ester.

The detection limits of these N-nitroamino acid methyl esters by GC–ECD analysis were as low as 200 pg and, as shown in Fig. 4, linear relationships were observed in the range from 200 to 800 pg.

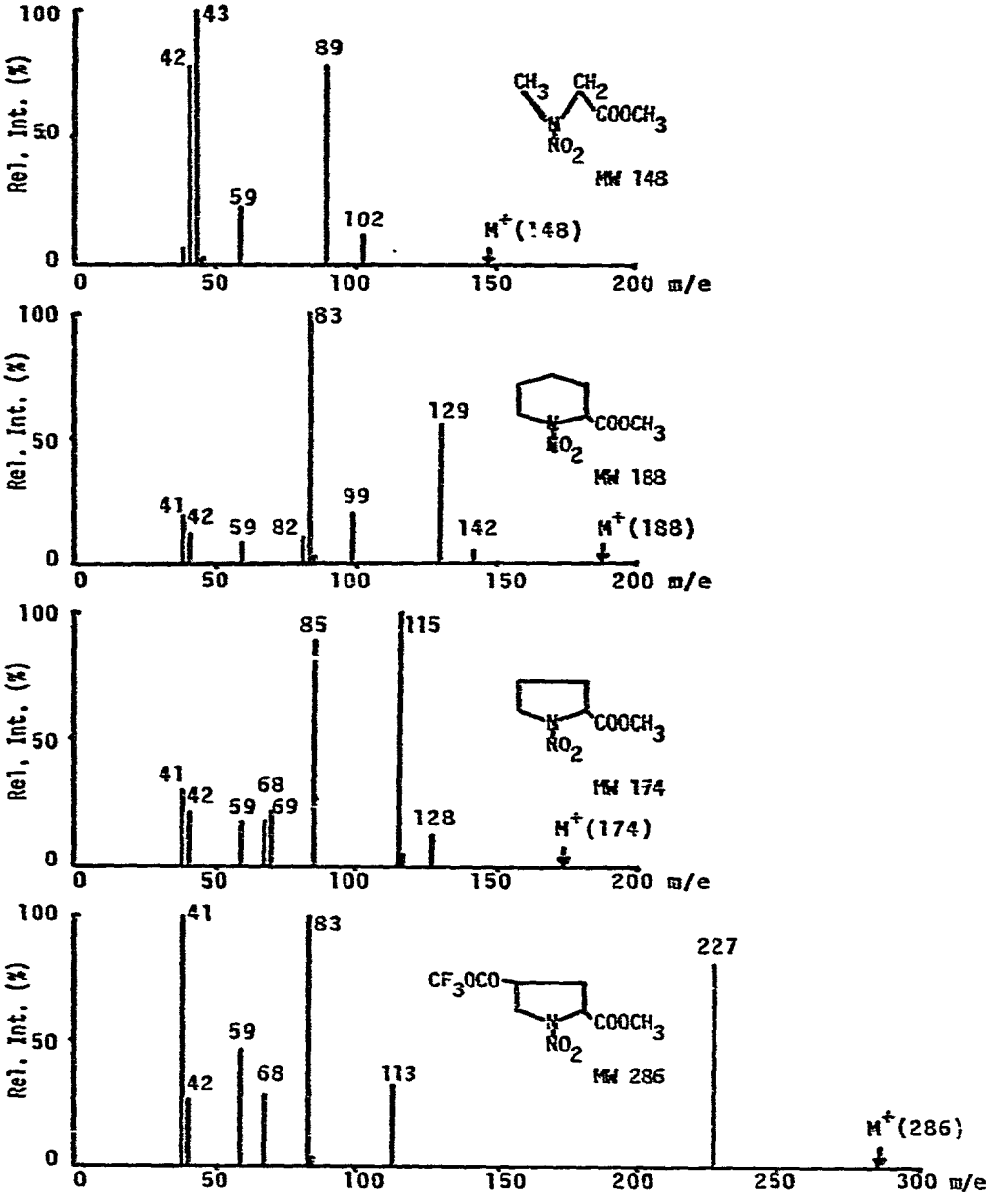

Fig. 3. Mass spectra of the esterified and oxidized derivatives of N-nitrosoamino acids after acylation with TFA.

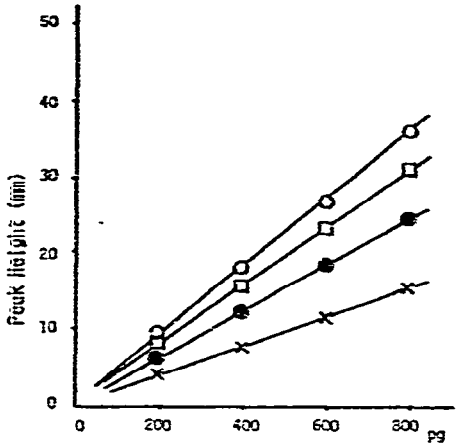

Fig. 4. Calibration graphs for the esterified and oxidized derivatives of N-nitrosoamino acids after acylation with TFA. ○, NSAR; □, NHPRO; ●, NPIC; ×, NPRO.

REFERENCES

1 M. Nakamura, N. Baba, T. Nakaoka, Y. Wada, T. Ishibashi and T. Kawabata, *J. Food Sci.*, 41 (1976) 874.
2 N. P. Sen, B. A. Donaldson, S. Seaman, J. R. Iyengar and W. F. Miles, in E. A. Walker *et al.* (Editors), *Environmental Aspects of N-Nitroso Compounds*, IARC Scientific Publications No. 19, IARC, Lyon, 1978, p. 374.
3 T. Ishibashi, M. Matsui and T. Kawabata, *Bunseki Kagaku (Jap. Anal.)*, 24 (1975) 107.
4 G. Eisenbrand, C. Janzowski and R. Preussmann, *J. Chromatogr.*, 115 (1975) 602.
5 W. Lijinsky, L. Keefer and J. Loo, *Tetrahedron*, 26 (1970) 5139.
6 N. P. Sen, *J. Chromatogr.*, 51 (1970) 301.
7 J. Althorpe, D. A. Goddard, D. J. Sissons and G. M. Telling, *J. Chromatogr.*, 53 (1970) 371.
8 H. Ohshima, M. Matsui and T. Kawabata, *J. Chromatogr.*, 169 (1979) 279.