Journal of Chromatography, 151 (1978) 215–221

CHROM. 10,541

DETECTION AND DETERMINATION OF N-NITROSAMINO ACIDS BY THIN-LAYER CHROMATOGRAPHY USING FLUORESCAMINE*

J. CHRISTOPHER YOUNG

Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario K1A 0C6 (Canada)

(First received May 27th, 1977; revised manuscript received August 4th, 1977)

SUMMARY

A novel procedure is described for the detection and determination of N-nitrosamino acids (NAAs) on activated silica gel thin-layer chromatographic plates. N-Nitrososarcosine, N-nitrosoproline, and N-nitroso-4-hydroxyproline could be detected as fluorophors at the 200-pmole level (20–30 ng) after being irradiated with ultraviolet light and sprayed with fluorescamine reagent. Spectrophotometric determination of the relative fluorescence of 0.4–40 nmoles of NAAs gave rise to similar calibration curves when plotted on a log–log scale. An application of this method to the detection of NAAs in uncooked bacon is described.

INTRODUCTION

N-Nitrosamino acids (NAAs) are considered to be potential precursors to the carcinogenic N-nitrosamines (NAs)[1–11]. N-Nitrosoproline (N-Pro) has been converted to N-nitrosopyrrolidine (N-Pyr) on heating in model food systems[4–6,8,9] and by microbial action[12], and N-nitroso-4-hydroxyproline (N-HO-Pro) has been converted to N-nitroso-3-hydroxypyrrolidine (N-HO-Pyr)[11]. N-Pro has been detected in raw but not in cooked bacon, whereas N-Pyr is found in cooked but not in raw bacon[13], and N-HO-Pyr has been detected in cooked bacon[14]. Production of NAs from various amino acids and sodium nitrite has been demonstrated[7,15].

Volatile NAs are generally determined directly by gas chromatography (GC) combined with high-resolution mass spectrometry (MS). Methodology for analysis of NAs has been recently reviewed[16,17]. NAAs have been detected or determined by thin-layer chromatography (TLC) on paper, cellulose powder or silica gel[18–31]; liquid chromatography (LC)[18,32,33]; polarography[34–39]; thermal energy analysis[32,40,41]; by conversion to their corresponding alkyl[6,9,42–46] or tetramethylsilyl[47] esters followed by GC or GC–MS; cleavage in solution by ultraviolet (UV) irradiation[48,49], hydrobromic acid in glacial acetic acid[50], or thionyl chloride[51] to give nitrite, which is then complexed and determined colorimetrically, or to give the free amino acid, which is converted to a fluorescent derivative[46] and then quantitated by TLC or LC; and

* Contribution Number 980.

cleavage by hydrobromic acid to give nitrosylbromide, which is quantitated by chemiluminescence[52].

Detection of NAAs on TLC plates has been accomplished with a wide variety of reagents including: bromocresol green[19], bromophenol blue[20], diazotized *o*-dianisidine[20], 4-dimethylaminobenzaldehyde[20,21], 4-dimethylaminocinnamaldehyde[20], Dragendorff reagent[20,22], ferric chloride[20], fluorescamine[23], Griess reagent (UV irradiation and 1-naphthylamine–sulfanilic acid)[24–26], isatin[21], iodine[19,27,28], NEDSA reagent (UV irradiation and N-1-naphthylenediamine–sulfanilic acid)[29], ninhydrin[20–22,26,30], 4-nitrobenzenediazonium fluoborate[20], phosphomolybdic acid[20], Preussmann reagent (UV irradiation and palladium chloride–diphenylamine)[24,29], and sulfuric acid and heat[28]. The majority of these reagents have been used only for diagnostic purposes; detection limits of 2, 20, and 500 μg have been reported for NEDSA[29], iodine and sulfuric acid[28], and Preussmann reagents[29], respectively.

In earlier studies it was observed that most NAs[53] and the NAA N-nitroso-N-(phosphonomethyl) glycine[23] could be detected and determined by TLC at the picomole level with fluorescamine. This paper reports an extension of the use of fluorescamine to the detection and determination of other NAAs on TLC plates and its application to some samples of bacon.

EXPERIMENTAL

Materials

L-Proline, 4-hydroxy-L-proline, and sarcosine were purchased from Aldrich (Milwaukee, Wis., U.S.A.). The corresponding NAAs were synthesized by the method of Hansen *et al.*[27] and gave infrared (IR) and nuclear magnetic resonance (NMR) spectra consistent with those reported by Lijinsky *et al.*[54]. Methyl esters were prepared by treatment of the parent NAA with ethereal diazomethane. Standard solutions of NAAs in methylene chloride were freshly prepared before each determination and stored in a refrigerator. Solutions of 0.1 mg/ml fluorescamine (Fisher Scientific, Pittsburgh, Pa., U.S.A.) in acetone were prepared and stored at room temperature in a stoppered flask. Eastman Chromagram sheets (No. 13179; 0.1 mm silica gel) without fluorescent indicator (Fisher) were stored in a dry atmosphere and activated by heating at 105° for 1 h prior to use. Acidic aluminum oxide (W200 acid Woelm from ICN Pharmaceuticals, Cleveland, Ohio, U.S.A.) (10 g) was washed first with 20% hydrochloric acid (100 ml) and then sufficient water until the eluate was neutral, heated at 400° for 4 h, cooled, mixed with water (0.3 ml), placed in a stoppered flask, and kept overnight in a dessicator prior to use. Bacon was purchased from local grocery stores. All other reagents and solvents were of reagent grade and used as received from commercial sources.

Spectral determinations

IR spectra were determined in chloroform or nujol mull on a Beckman IR-20A spectrometer. NMR spectra were determined in pyridine-d_5 on a Varian T-60 spectrometer. Mass spectra were determined on a Finnigan Model 9500 gas chromatograph (1.8 m × 6.5 mm O.D. glass column containing 3% SE-30 ultraphase on high-performance Chromosorb W, 80–100 mesh) coupled to a Model 3100D quadropole mass spectrometer and a Model 6000 computer controlled data aquisition system.

Thin-layer chromatography

Standard solutions of NAAs were spotted, developed in 95% ethanol–benzene–water (4:1:1)[25], dried for 15 min in a vacuum desiccator, and irradiated with UV light using the apparatus previously described[53]. The plates were then sprayed sequentially with fluorescamine solution and triethanolamine (10% in methylene chloride) and viewed under long-wave UV light. Fluorescence intensities were determined[53] and the relative values normalized by assigning to 1.0 nmole of each NAA a value of 10. Detection limits were determined by spotting decreasing volumes of standard solutions of each NAA, developing, irradiating, spraying with fluorescamine reagent, and then viewing under UV light. Mixtures of methanol–chloroform (4:1) and acetonitrile–chloroform–95% ethanol–acetic acid (100:100:97:3) were also used as developers.

Gas chromatography

The methyl esters of NAAs were examined on a Pye Model 104 gas chromatograph (1.5 m × 6.5 mm O.D. glass column containing 5% Carbowax 20M-TPA on high-performance Chromosorb W, 80–100 mesh) fitted with both a flame ionization detector and an alkali flame ionization detector.

Column chromatography

Mixtures to be analyzed were dissolved in methylene chloride (25 ml) and passed through a column of acidic aluminum oxide (2 g). The column was then eluted successively with ethyl acetate, acetone–methanol (3:1), and water (25 ml each), and 20% aqueous acetic acid (50 ml). The final fraction was evaporated to dryness under reduced pressure, dissolved in acetonitrile, filtered if necessary, and analyzed by TLC using methanol–chloroform (4:1) as developer.

Extraction and analysis of bacon

The clean-up procedure is based on one developed by Sen *et al.*[14]. A 50-g sample of ground bacon was extracted in a blender for 10 min with acetonitrile (125 ml), allowed to settle for several minutes and the supernatant filtered through glass wool. The residue was treated in a similar manner and the combined filtrates washed with heptane (2 × 250 ml) and evaporated to dryness under reduced pressure. A methylene chloride solution (25 ml) of this residue was further purified by column chromatography and then analyzed by TLC. Samples spiked by addition of NAAs to the ground bacon were also analyzed.

To confirm the identity of NAAs, samples were spotted, developed in methanol–chloroform (4:1), developed in the other dimension with acetonitrile–chloroform–95% ethanol–acetic acid (100:100:97:3), irradiated, sprayed with fluorescamine reagent and viewed under UV light. In addition, samples were spotted, developed in methanol–chloroform (4:1), irradiated, developed in the other dimension first with a 0.01% solution of fluorescamine in hexane–acetone (4:1)[55] and then with methanol–chloroform (4:1), sprayed with triethanolamine solution and viewed under UV light. Alternatively, after development, the adsorbent at the appropriate R_F was scraped off, collected using the apparatus of Clemett[56], and eluted with 300 μl of ethereal diazomethane. The eluate was concentrated under a stream of dry nitrogen and then examined by GC or GC–MS.

Safety precautions

Many NAs and some NAAs are known to be potent carcinogens. Thus, safety precautions to prevent skin contact and inhalation must be exercised at all times.

RESULTS AND DISCUSSION

Alkyl and some heterocyclic NAs can be cleaved by UV light on activated silica gel TLC plates to give products that yield fluorophors after being sprayed with fluorescamine reagent[53]. Under similar conditions, NAAs also yield fluorophors. This technique is very sensitive and can detect as little as 200 pmoles. Detection limits and R_F values for the three NAAs studied are given in Table I. In this context, detection limit is the minimum amount of an NAA that ultimately gives a detectable visual fluorescence. The NAA N-nitroso-N-(phosphonomethyl) glycine can be detected at the 50-pmole level[23].

TABLE I

THIN-LAYER CHROMATOGRAPHIC R_F VALUES AND VISUAL FLUORESCENCE DETECTION LIMITS OF N-NITROSAMINO ACIDS

N-Nitrosamino acids are determined on activated silica gel by spotting, developing, irradiating with UV light, spraying with fluorescamine reagent, and viewing under long-wave UV light. Amino acids are detected by spraying with ninhydrin reagent and heating. Solvent systems: A, 95% ethanol–benzene–water (4:1:1); B, methanol–chloroform (4:1); C, acetonitrile–chloroform–95% ethanol–acetic acid (100:100:97:3).

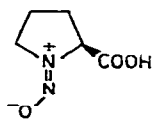
Compound	R_F			*Detection limit**	
	A	*B*	*C*	*Nanograms*	*Picomoles*
N-Nitrososarcosine	0.64, 0.70	0.71	0.75	20	180
Sarcosine	0.23	0.10**	0.02		
N-Nitroso-L-proline	0.63, 0.69	0.72	0.21	30	200
L-Proline	0.33	0.12**	0.03		
N-Nitroso-4-hydroxy-L-proline	0.60, 0.66	0.67	0.13	33	210
4-Hydroxy-L-proline	0.28	0.09**	0.01		

* Determined in solvent system A.

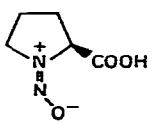
** Streak.

Fig. 1 shows that the calibration curves for the NAAs are not significantly different.

There is a 23-kcal/mole barrier to rotation[57] about the nitrogen–nitrogen bond in the N–N=O system due to contribution from the zwitterionic form. Since NAAs are unsymmetrical, (*E*)- and (*Z*)-conformers (*e.g.* I and II, respectively, for L-proline) are possible and can be resolved by LC[18,33] or TLC[18,19,26,31,58]. In this study it was



(I)



(II)

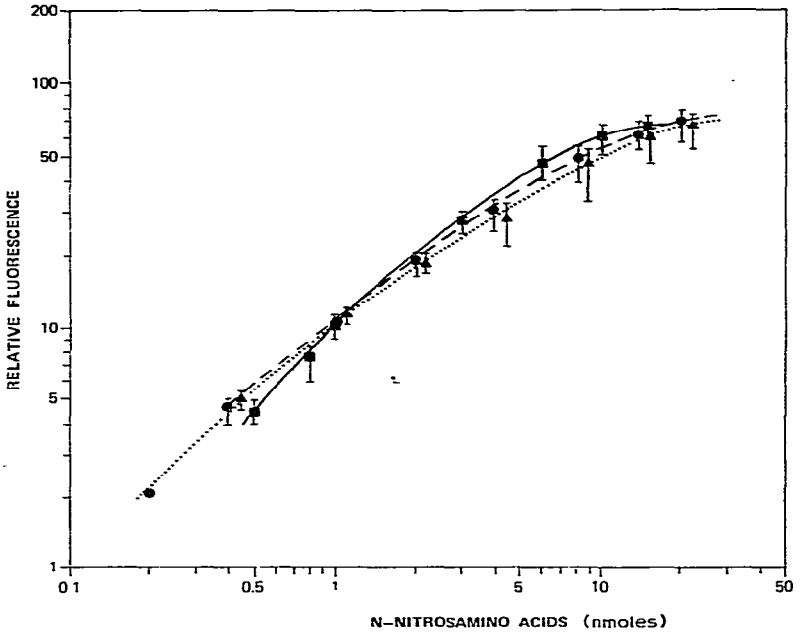


Fig. 1. Calibration curves for UV-irradiated and fluorescamine-treated N-nitrososarcosine (--▲--), N-nitroso-L-proline (—■—), and N-nitroso-4-hydroxy-L-proline (· · ·●· · ·) on silica gel TLC plates. Fluorescence values normalized so that 1.0 nmole gave a relative fluorescence of 10. Error bars are standard deviations calculated from measurements made in triplicate.

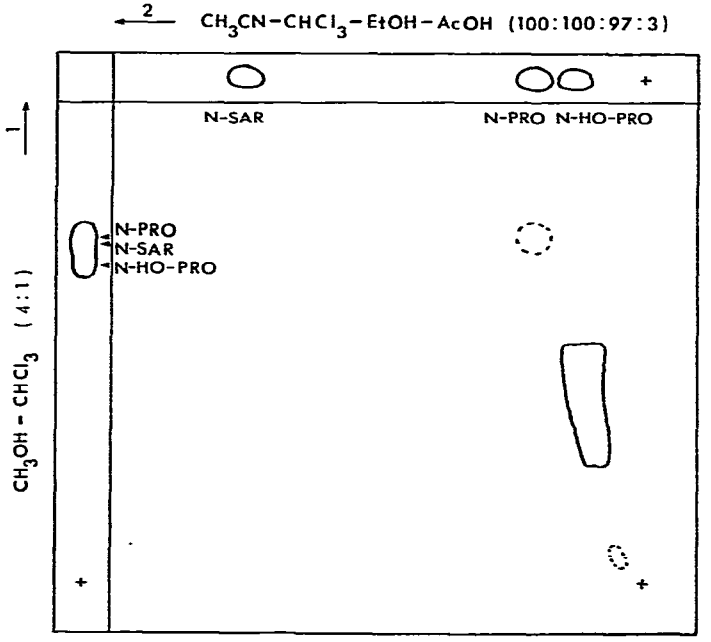


Fig. 2. Two-dimensional thin-layer chromatogram of purified uncooked bacon extract. Silica gel plate was developed, irradiated with UV light, sprayed with fluorescamine reagent, and viewed under long-wave UV light. Mixture of N-nitrososarcosine (N-SAR), N-nitrosoproline (N-PRO), and N-nitrosohydroxyproline (N-HO-PRO) standards spotted alongside.

observed that the conformers could be separated with the ethanol–benzene–water (4:1:1) developer and their ratios determined directly from the TLC plate, thus complementing LC[18,19] or NMR[54] methods of ratio determination. Agreement with the NMR method was good.

Recovery of NAAs in bacon samples spiked at the 1-ppm level from columns of aluminum oxide was variable, ranging from 25–80% (median, 30%) and depended not only upon the brand of adsorbent but also upon the individual lot. The highest recoveries were obtained from W200 acid Woelm (aminotropic) that had been acid washed, heated to 400°, and made to 3% water. This brand was used for the analysis.

Two samples each of two brands of uncooked bacon were examined for the presence of NAAs. In only one sample of one brand was N-Pro detected at the ppm level. The presence of N-Pro was confirmed by two-dimensional TLC (Fig. 2), by comparison of R_F values of the fluorescamine derivative[55] of the UV photolysis product, and by GC and GC–MS of the methyl ester. N-Pro has been reported at the 1-ppm level in some uncooked bacon samples[5] and not observed in others[9]. N-Nitrososarcosine and N-HO-Pro were not detected in any samples at this level.

ACKNOWLEDGEMENTS

The author wishes to thank Mr. J. Lapointe for technical assistance and Mr. D. Dobson of the Analytical Chemistry Services for obtaining mass spectra.

REFERENCES

1 T. Fazio, R. H. White, L. R. Dusold and J. W. Howard, *J. Ass. Offic. Anal. Chem.*, 56 (1973) 919.
2 N. P. Sen, B. Donaldson, J. R. Iyengar and T. Panalaks, *Nature (London)*, 241 (1973) 473.
3 W. Fiddler, J. W. Pensebene, J. C. Fagan, E. J. Thorne, E. G. Piotrowski and A. E. Wasserman, *J. Food Sci.*, 39 (1974) 1070.
4 J. W. Pensebene, W. Fiddler, R. A. Gates, J. C. Fagan and A. E. Wasserman, *J. Food Sci.*, 39 (1974) 314.
5 I. Kushnir, J. I. Feinberg, J. W. Pensebene, E. G. Piotrowski and W. Fiddler, *J. Food Sci.*, 40 (1975) 427.
6 D. D. Bills, K. I. Hildrum, R. A. Scanlan and L. M. Libbey, *J. Agr. Food Chem.*, 21 (1973) 876.
7 F Ender and L. Ceh, *Z. Lebensm.-Unters.-Forsch.*, 145 (1971) 133.
8 N. P. Sen, B. Donaldson, S. Seaman, J. R. Iyengar and W. F. Miles, *J. Agr. Food Chem.*, 24 (1976) 397.
9 M. Nakamura, N. Baba, T. Nakaoka, Y. Wada, T. Ishibashi and T. Kawabata, *J. Food Sci.*, 41 (1976) 874.
10 L. S. Hwang and J. D. Rosen, *J. Agr. Food Chem.*, 24 (1976) 1152.
11 J. S. Lee, D. D. Bills, R. A. Scanlan and L. M. Libby, *J. Agr. Food Chem.*, 25 (1977) 422.
12 T. Kawabata and S. Miyakoshi, in E. A. Walker, P. Bogovski and L. Griciute (Editors), *Environmental N-Nitroso Compounds Analysis and Formation*, IARC Scientific Publications No. 14, Lyon, 1976, p. 261.
13 J. I. Gray, *J. Milk Food Technol.*, 39 (1976) 686.
14 N. P. Sen, D. E. Coffin, S. Seaman, B. Donaldson and W. F. Miles, in B. J. Tinbergen and B. Krol (Editors), *Second Symposium on Nitrite in Meat Products, Zeist, Sept. 1976*, Centre for Agricultural Publishing and Documentation, Wageningen (The Netherlands), 1977, p. 000.
15 J. J. Warthesen, D. D. Bills, R. A. Scanlan and L. M. Libbey, *J. Agr. Food Chem.*, 24 (1976) 892.
16 W. Fiddler, *Toxicol. Appl. Pharmacol.*, 31 (1975) 352.
17 J. K. Foreman and K. Goodhead, *J. Sci. Food Agr.*, 26 (1975) 1771.
18 W. T. Iwaoka, T. Hansen, S.-T. Hsieh and M. C. Archer, *J. Chromatogr.*, 103 (1975) 349.
19 B. Liberek, J. Augustyniak, J. Ciarkowski, K. Plucińska and K. Stachowiak, *J. Chromatogr.*, 95 (1974) 223.

20 L. Reio, *J. Chromatogr.*, 88 (1974) 119.
21 F. Marcucci and E. Mussini, *J. Chromatogr.*, 18 (1965) 431.
22 L. Reio, *J. Chromatogr.*, 68 (1972) 183.
23 J. C. Young, S. U. Khan and P. B. Marriage, *J. Agr. Food Chem.*, 24 (1977) 918.
24 R. Preussmann G. Neurath, G. Wulf-Lorentzen, D. Daiber and H. Hengy, *Z. Anal. Chem.*, 202 (1964) 187.
25 H. T. Nagasawa, P. S. Fraser and D. L. Yuzon, *J. Med. Chem.*, 16 (1973) 583.
26 M. Yamamoto, Y. Takashi and A. Tanimura, *Shokuhin Eiseigaku Zasshi*, 15 (1974) 461; *C.A.*, 82 (1975) 110518.
27 T. Hansen, W. T. Iwaoka and M. C Archer, *J. Label. Compounds*, 10 (1974) 689.
28 G. S. Rao and E. A. Bejnarowicz, *J. Chromatogr.*, 123 (1976) 486.
29 C. L. Walters, E. M. Johnson, N. Ray and G. Woolford, in P. Bogovski, R. Preusmann and E. A. Walker (Editors), *N-Nitroso Compounds Analysis and Formation*, IARC Scientific Publication No. 3, Lyon, 1972, p. 79.
30 D. Myhill and D. S. Jackson, *Anal. Biochem.*, 6 (1963) 193.
31 B. Liberek, J. Ciarkowski, K. Steporowska, K. Stachowiak and E. Jereczek, *Rocz. Chem.*, 46 (1972) 1895.
32 D. H. Fine, F. Huffman, D. Rounbehler and N. M. Belcher, in E. A. Walker, P. Bogovski and L. Griciute (Editors), *Environmental N-Nitroso Compounds Analysis and Formation*, IARC Scientific Publications No. 14, Lyon, 1976, p. 43.
33 W. Iwaoka and S. R. Tannenbaum, *J. Chromatogr.*, 124 (1976) 105
34 K. Hasebe and J. Osteryoung, *Amer. Chem. Soc. Annu. Meet. Abstr.*, 172 (1976) AGFD 115.
35 C. L. Waters, E. M. Johnson and N. Ray, *Analyst (London)*. 95 (1970) 485.
36 J. Velisek, J. Davidek and S. Klein, *Z. Lebensm.-Unters.-Forsch.*, 155 (1974) 203.
37 S. K. Chang and G. W. Harrington, *Anal. Chem.*, 47 (1975) 1857.
38 K. Hasebe and J. Osteryoung, *Anal. Chem.*, 47 (1975) 2412.
39 J. O. Bronstad and H. O. Friestad, *Analyst (London)*, 101 (1976) 820.
40 D. H. Fine, F. Rufeh, D. Lieb and D. P. Rounbehler, *Anal. Chem.*, 47 (1975) 1188.
41 D. H. Fine and D. P. Rounbehler, *J. Chromatogr.*, 109 (1975) 271.
42 F. J. Ivey, *Ph.D. Thesis*, Oregon State University, Corvallis, 1974; *Diss. Abstr.*, 35B (1974) 879.
43 J. J. Warthesen, R. A. Scanlan, D. D. Bills and L. M. Libbey, *J. Agr. Food Chem.*, 23 (1975) 898.
44 T. Ishibashi, M. Matsui and T. Kawabata, *Bunseki Kagaku (Jap. Anal.)*, 24 (1975) 107.
45 T. Kawabata, in P. Bogovski and E. A. Walker (Editors), *N-Nitroso Compounds in the Environment*, IARC Scientific Publications No. 9, Lyon, 1975, p. 154.
46 J. H. Wolfram, J. I. Feinberg, R. C. Doerr and W. Fiddler, *J. Chromatogr.*, 132 (1977) 37.
47 G. Eisenbrand, C. Janzowski and R. Preussmann, *J. Chromatogr.*, 115 (1975) 602.
48 J. Sander, *Hoppe Seyler's Z. Physiol. Chem.*, 348 (1967) 852.
49 T Y. Fan and S. R. Tannenbaum, *J. Agr. Food Chem.*, 19 (1971) 1268
50 E. M. Johnson and C. L. Walters, *Anal. Lett.*, 4 (1971) 383.
51 T. G. Lunt, D. G. Fueggle and C. L. Walters, *Anal. Lett.*, 6 (1973) 369.
52 M. J. Downes, M. W. Edwards, T. S. Elsey and C. L. Waters, *Analyst (London)*, 101 (1976) 742.
53 J. C. Young, *J. Chromatogr.*, 124 (1976) 17.
54 W. Lijinsky, L. Keefer and J. Loo, *Tetrahedron*, 26 (1970) 5137.
55 H Nakamura and J. J. Pisano, *J. Chromatogr.*, 121 (1976) 33.
56 C. J. Clemett, *Anal. Chem.*, 43 (1971) 490.
57 J. D. Cooney, S. K. Brownstein and J. W. ApSimon, *Can. J. Chem.*, 52 (1974) 3028.
58 A. Mannschreck, H. Munsch and A Mattheus, *Angew. Chem., Int. Ed. Engl.*, 5 (1966) 728.