Journal of Chromatography, 463 (1989) 201–206
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 021

Note

Separation and assay of N-nitroso compounds by high-performance liquid chromatography with chemiluminescence detection

C. PINCHE, J. P. BILLARD, A. M. FRASEY, H. BARGNOUX, J. PETIT and J. A. BERGER
Laboratoire de Chimie analytique et Bromatologie de la Faculté de Pharmacie et Institut de la Viande des Universités, BP 38, 63001 Clermont-Ferrand Cédex (France)
and
B. DANG VU and J. YONGER
Laboratoire de Chimie analytique et Bromatologie, Faculté de Pharmacie, 4, Avenue de l'Observatoire, 75006 Paris (France)
(First received April 28th, 1988; revised manuscript received September 26th, 1988)

Assay of nitrite and total N-nitroso compounds can be carried out by chemiluminescence detection after chemical denitrosation and recovery of the liberated nitric oxide [1–4]. In addition, nitrite can be differentiated from N-nitroso compounds by carrying out an initial selective reduction of nitrite, which is thereby eliminated, enabling the N-nitroso compounds alone to be assayed[5–7]. We developed a method for separately assaying nitrite and total N-nitroso compounds based on these reactions that can be applied to various types of sample, liquid and solid, including foodstuffs and, in particular, pork products[8,9].

Concomitantly, we set out to improve further this method by its combination with high-performance liquid chromatography (HPLC). Such analysis by HPLC with denitrosation and chemiluminescence detection allows separate assay of individual N-nitroso compounds. The method was developed using mixtures of reference N-nitrosamines, according to the modified technique of Rühl and Reusch[10]. Evaluation of the method was satisfactory.

MATERIALS AND METHODS

General principle

The N-nitroso compounds were first separated by HPLC and then chemically denitrosated. The nitric oxide evolved was assayed in a chemiluminescence analyser. The study was performed on a mixture of four reference N-nitrosamines.

Apparatus (Fig. 1)

The apparatus consisted of an HPLC instrument, a chemical denitrosation reactor and attachments and a chemiluminescence detector + integrator + recorder.

Two chromatographs were used: a Waters instrument equipped with a Type 6000 A pump suitable for micro-flow-rates, and a Rheodyne Type 7125 injection loop (v = 20 μl); a Hewlett-Packard HP 1081 B equipped with a variable volume automat-

0021-9673/89/$03.50

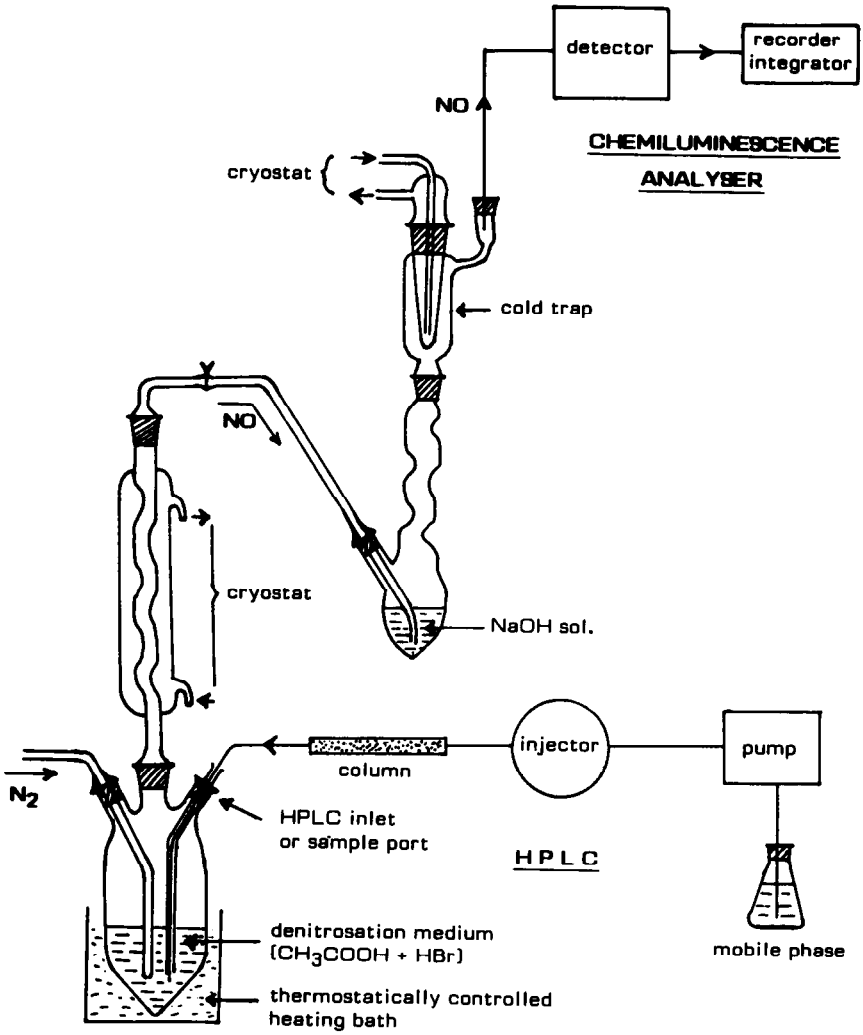


Fig. 1. Apparatus.

ic injector (79841 A). The column (250 mm × 2.1 mm) was packed with Spherisorb 5-μm silica. The column outlet was connected to the denitrosation reactor via a PTFE capillary inserted through a glass tube.

The eluate was led directly into the reaction medium of 15 ml of 48% hydrogen bromide solution in 100 ml acetic acid, kept at a constant temperature of 80°C in a three-necked flask immersed in a thermostatically regulated heating bath. Denitrosation was immediate, and the nitric oxide liberated was swept out of the flask by a stream of nitrogen through three traps designed to remove water and reactants. These were, successively, a condenser, a bubbler containing 33% (w/v) aqueous sodium hydroxide and a cold trap. The condenser and the cold trap were fed with ethanol–water (2:3) at −4°C from a cryostat.

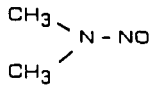
A classical atmospheric nitrogen oxide detector was used "Topaze" (COSMA). The nitric oxide, carried by the nitrogen stream, was mixed with ozone and then drawn by a pump through calibrated capillary tubes fitted to flow regulators. The gas flow was then led to a reaction chamber kept under vacuum by a second pump. The oxidation of nitric oxide by ozone generates excited nitrogen dioxide which releases photons. These are captured by a photomultiplier.

$$NO + O_3 \rightarrow O_2 + NO_2^* \rightarrow NO_2 + h\nu$$

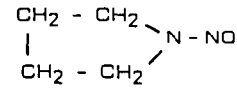
The signal was amplified and converted either into vpm (volumes per million) of NO after calibration with a reference gas mixture (79 vpm NO) or recorded on an integrator (HP 3393 A).

Reagents

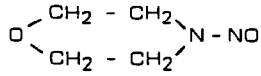
Glacial acetic acid and 48% hydrogen bromide were of RPE ACS quality (Carlo-Erba). Hexane and isopropanol were of HPLC quality (Rathburn). Reference N-nitrosamines were obtained from Sigma; they were: N-nitrosodiphenylamine (NDPhA) powder in sealed ampoules (Isopac); N-nitrosomorpholine (NMor) powder in sealed ampoules (Isopac); N-nitrosodimethylamine (NDMA) liquid in sealed ampoules (Isopac); N-nitrosopyrrolidine (NPyr) liquid in sealed ampoules (Isopac).



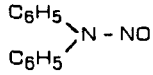
NDMA $[C_2H_6N_2O]$ $m = 74.8$



NPyr $[C_4H_8N_2O]$ $m = 100.11$



NMor $[C_4H_8N_2O_2]$ $m = 116.11$



NDPhA $[C_{12}H_{10}N_2O]$ $m = 198.2$

HPLC procedure

Stock solutions of N-nitrosamines were made up in the mobile phase: NDPhA, 3.48; NMor, 2.14; NDMA, 2.42; NPyr, 1.82 μmol/ml. The mobile phase was hexane–isopropanol (96.5:3.5), ultrasonically degassed. The flow-rate was 400 μl/min, and the volume injected was 20 μl.

RESULTS

Evaluation of the method

Specificity. Only substances that liberate nitric oxide, *i.e.*, nitrites and nitroso compounds under the operating conditions can be detected. Hence the method is absolutely specific to the N-nitrosamines studied.

Resolution. Under the HPLC conditions used, satisfactory separation of the four reference N-nitrosamines was achieved in 18 min (Fig. 2). The denitrosation reaction was rapid and did not affect the chromatographic resolution.

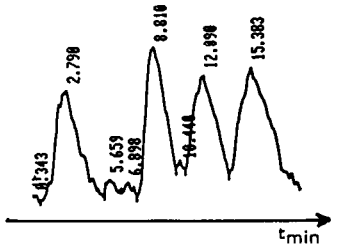


Fig. 2. HPLC chromatogram of a mixture of four N-nitrosamines (2 nmol injected). Mobile phase: hexane–isopropanol (96.5:3.5). Flow-rate: 400 μl/min. Retention times: NDPhA, 2.79; NMor, 8.81; NDMA, 12.09; NPyr, 15.38 min.

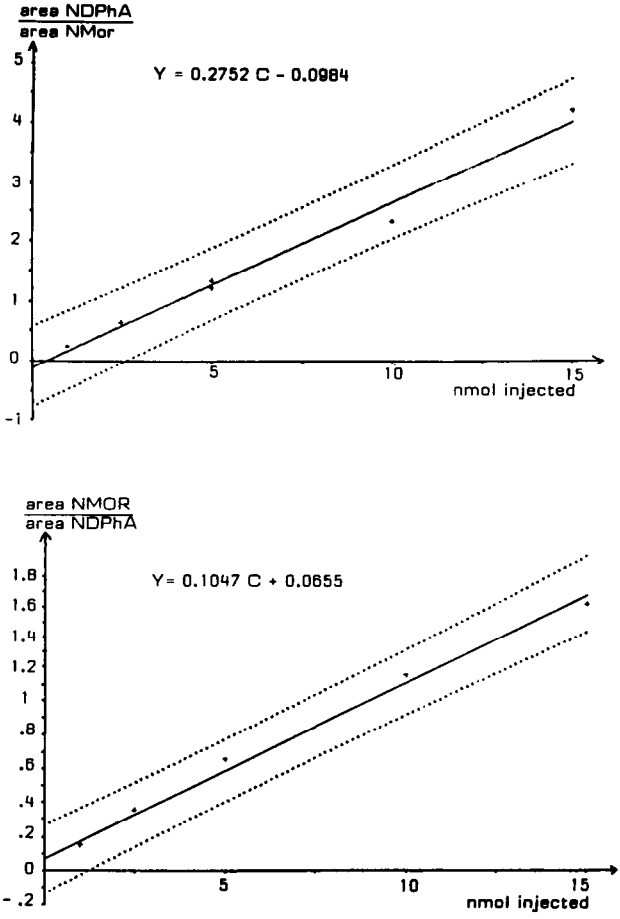


Fig. 3.

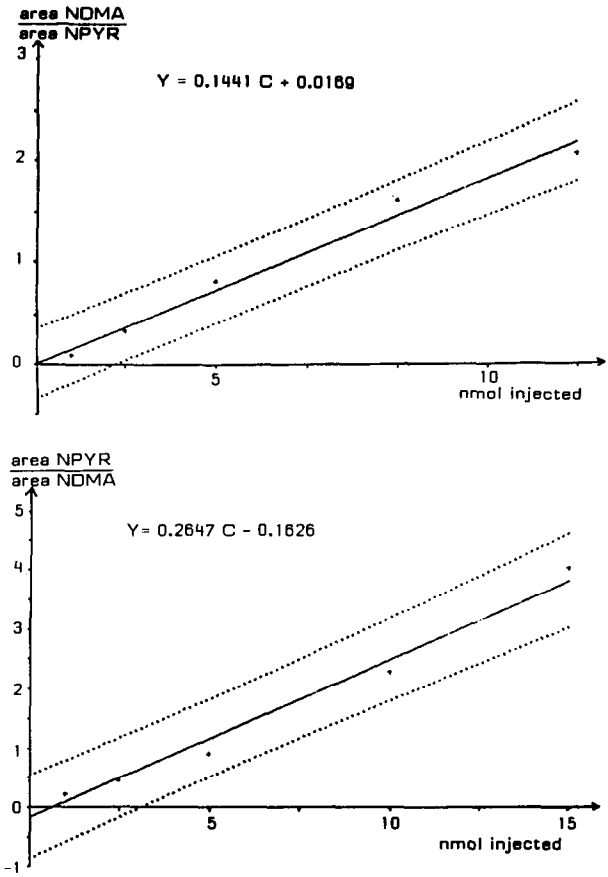


Fig. 3. Calibration graphs for the four N-nitrosamines used.

Linearity. Calibration curves for each N-nitrosamine were obtained over the range 0–15 nmol injected (0–0.75 μmol/ml), using one of the others as the internal standard (5 nmol). The detector response was linear over this range (Fig. 3). The correlation coefficients were: NDPhA, 0.992; NMor, 0.996; NDMA, 0.993; NPyr, 0.992.

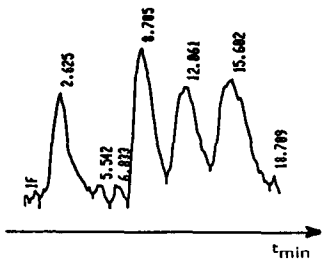


Fig. 4. Chromatogram of a pork product spiked with nitrosamines.

Precision. The repeatability and reproducibility were assessed using various dilutions of the stock solutions of N-nitrosamines in the HPLC mobile phase. The results are given in Table I.

Limits of detection. The detection threshold calculated from the mean value of the background noise ($n = 10$) plus three standard deviations ($\bar{M} + 3$) was of the order of 1 nmol injected: NDPhA, 0.6; NMor, 0.8; NDMA, 1.0; NPyr, 1.2 nmol.

Applications

This method was applied to the differential assay of N-nitrosamines in foodstuffs and especially in pork products. According to Klein and Debry[11], the extraction is performed with dichloromethane (2 × 5 ml) from a suspension of 1 g mixed sample previously homogenized with an Ultra Turrax in 3 ml distilled water. The pooled extracts are dried on anhydrous sodium sulphate and concentrated to 1 ml at 50°C in an evaporator Type Kuderna Danish before HPLC with chemiluminescence detection.

Fig. 4 shows a typical chromatogram of a dry cured ham spiked sample with 100 nmol/g of each N-nitrosamine studied. Their mean recovery is about 80%.

CONCLUSIONS

HPLC with denitrosation and chemiluminescence detection is applicable to the separation and individual assay of N-nitrosamines in solution. The method is specific to nitroso compounds, since it is based on their ability to release nitric oxide by chemical denitrosation. Evaluation of the resolution, linearity and reproducibility is satisfactory.

Therefore, this method can be applied to the differential assay of nitrosamines in pork products. Moreover, the application of this procedure to various kinds of samples including liquid and solid foodstuffs from animal or vegetal origin can be considered without major modifications.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Agriculture (Research contract No. 85/29 "06.12.85"). We also thank Mr. J. Queuille, glassblower at the Faculty of Pharmacy, Clermont-Ferrand, who made the glassware.

REFERENCES

1 T. Y. Fan, D. Fine and A. Lafleur, in P. N. Cheremisinoff and H. J. Perlis (Editors), *Analytical Measurements and Instrumentation for Process and Pollution Control*. Ann. Arbor Sci. Publ., Ann Arbor, MI, 1981, p. 273.
2 G. S. Drescher and C. W. Frank, *Anal. Chem.*, 50 (1978) 2118.
3 R. C. Massey, J. M. Baily, P. E. Key, D. J. McWeeny and M. E. Knowles, *Food Add. Contam.*, 1 (1984) 237.
4 N. P. Sen and S. J. Kubacki, *Food Add. Contam.*, 4 (1987) 357.
5 R. D. Cox, *Anal. Chem.*, 52 (1980) 332.
6 R. C. Doerr, J. B. Fox, L. Lakritz and W. Fiddler, *Anal. Chem.*, 53 (1981) 381.
7 C. L. Walters, P. N. Gillatt, R. C. Palmer and P. L. R. Smith, *Food Add. Contam.*, 4 (1987) 133.
8 B. Dang Vu, J. L. Paul, O. G. Ekindjian, J. Yonger, M. Gaudric and J. Guerre, *Clin. Chem.*, 29 (1983) 1860.
9 C. Pinche, J. P. Billard, A. M. Frasey, B. Dang Vu, J. Yonger, J. P. Poma, M. Saudan, H. Bargnoux, J. Petit and J. A. Berger, *Food Add. Contam.*, submitted for publication.
10 C. Rühl and J. Reusch, *J. Chromatogr.*, 328 (1985) 362.
11 D. Klein and G. Debry, *Ann. Nutr. Aliment.*, 34 (1980) 779.