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# DETERMINATION OF N-NITROSOPROLINE AT THE NANOGRAM LEVEL

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## SUMMARY

Two sensitive detection systems are described for the quantitative determination of a nonvolatile nitrosamine, nitrosoproline. One procedure involves denitrosation followed by derivatization of the amino product, proline, with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). The highly fluorescent NBD-proline compound formed is then identified and quantitated by either thin-layer chromatography or high-pressure liquid chromatography(HPLC). In the second system,the volatile methyl ester of the intact nitrosoproline is prepared, then detected by gas–liquid chromatography (GLC), and confirmed by combined gas–liquid chromatography and mass spectrometry (GLC–MS). Both methods permit the quantitative detection of less than 10 ng of nitrosoproline. However, the HPLC fluorescence technique is approximately ten times as sensitive as the GLC method.

## INTRODUCTION

Trace quantities of N-nitrosopyrrolidine (NPyr) have been consistently detected and confirmed in fried bacon[1]. Since this compound is carcinogenic to several species of test animals[2], several studies have been carried out to identify the amino precursors involved in its formation[3–5].

Kinetic data on the nitrosation of pyrrolidine in model systems[6] and the fact that pyrrolidine is not normally found free in animal tissue, suggest that other precursors might be involved in NPyr production. Huxel *et al.*[7] reported that NPyr was formed at elevated temperatures from nitrite and proline. Pensabene *et al.*[5] showed that the decarboxylation of nitrosoproline (NPro) yielded NPyr at temperatures close to those normally recommended for frying bacon. Proline could be a precursor for NPyr since it is a natural component of animal tissue. Bacon contains *ca.* 2% collagen (dry weight basis) and collagen is composed of more than 10% bound proline[7]. Gray and Dugan[4] also suggest that collagen might be the precursor for NPyr in fried bacon. While NPro has been isolated and identified in uncooked bacon[8], at present there is no satisfactory quantitative method for its determination.

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Two different detection systems that utilize three chromatographic techniques for the detection and quantitation of NPro are described herein.

## EXPERIMENTAL

### *Reagents*

Solvents were analytical grade and used without further purification; diethyl ether, however, was redistilled from glass. L-Proline was purchased from Aldrich (Milwaukee, Wisc., U.S.A.)* and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) from Regis (Chicago, Ill., U.S.A.). Nitrosoproline and its methyl ester were prepared and purified as described previously[8,9].

### *Denitrosation*

Aqueous solutions containing $1 \times 10^{-4}$ mmoles of NPro were taken to dryness *in vacuo* on an Evapo-Mix (Buchler) at 50° with continuous shaking. The denitrosation procedure was similar to that described by Eisenbrand and Preussmann[10]. One milliliter of glacial acetic acid containing 15% HBr was added; the test tubes were stoppered and heated for 30 min at 50° in a water-bath. The reaction was terminated by the addition of 1 ml of distilled water to each tube, and the samples were then taken to dryness again as described above.

### *Derivatization*

The denitrosated samples and the standard solutions of L-proline were reacted with NBD-Cl. Each tube received 0.1 ml distilled water and sufficient sodium carbonate to saturate the solution, followed by 0.9 ml of acetonitrile containing 3.0 mg NBD-Cl. The samples were heated in a water-bath at 50° for 30 min, then cooled to room temperature prior to analysis of NBD-proline.

### *Thin-layer chromatography*

For thin-layer chromatography (TLC), the NBD-amino derivatives were applied to 250-$\mu$ silica gel G plates (Analtech, Newark, Del., U.S.A.) that had been activated for 30 min at 100°. The plates were developed in the dark at 4° with a solvent system of hexane–*n*-butanol–ethyl acetate (80:10:10). The plates were air dried and the fluorescent NBD-proline spot ($R_F$ 0.24) was scraped and eluted with *p*-dioxane. The fluorometric analysis was carried out with an Aminco Model 8202 spectrofluorometer at wavelengths of 465 nm for excitation and 520 nm for emission.

### *High-pressure liquid chromatography*

For high-pressure liquid chromatography (HPLC), the NBD-amino derivatives were diluted 1:20 and aliquots were introduced into a Waters Model 6000 liquid chromatograph containing a 25 cm × 2 mm MicroPak LiChrosorb Si 60–10 column (Varian, Palo Alto, Calif., U.S.A.). The HPLC apparatus was coupled to a continuous-flow fluorescence detector. The derivatives were separated with a solvent system of

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

*n*-hexane–ethyl acetate–acetic acid (50:50:0.5) at a flow-rate of 2.0 ml/min. The NBD-proline peak eluted in 2.1 min.

Peak symmetry permitted measurement of peak heights rather than peak areas. A calibration curve was prepared for each experiment using standard proline solutions and a reagent blank. In addition, the relative fluorescence transmission values of NBD-proline samples, from denitrosated NPro, were compared with values of known quantities of NBD-proline prepared from proline standards.

*Esterification*

An aqueous solution of NPro was taken to dryness, and the residue dissolved in 0.5 ml methanol. Two milliliters of an diethyl ether solution containing *ca.* 1% diazomethane were added and the mixture placed in a water-bath at 33° for 10 min. In order to ensure complete esterification, this treatment was repeated. Fresh diazomethane solution was prepared each week from Diazald (Aldrich) and stored in the refrigerator prior to use.

*Gas–liquid chromatography*

A Varian-Aerograph Model 1740-1 gas–liquid chromatograph, equipped with an alkali flame ionization detector described by Howard *et al.*[11], was used to detect nitrosoproline methyl ester. The separation was performed on a 9 ft. × 1/8 in. stainless-steel column packed with 16% Carbowax 20M-TPA on 60–80 mesh Gas-Chrom P. The operating temperatures were: column, 215°; injector, 200°; and detector, 250°. The flow-rates were: helium, 60 ml/min; air, 200 ml/min; hydrogen, 60 ml/min.

The peak heights of esterified NPro samples were compared to those of a standard solution of nitrosoproline methyl ester.

*Combined gas–liquid chromatography and mass spectrometry*

The equipment and conditions used for the combined gas–liquid chromatography and mass spectrometry (GLC–MS) of NPro methyl ester were identical to those described by Kushnir *et al.*[8].

## RESULTS AND DISCUSSION

In an indirect analytical method, developed for the determination of NPro, the N–NO bond was cleaved under anhydrous conditions as described by Eisenbrand and Preussmann[10]. The resulting secondary amino acid, proline, was reacted with NBD-Cl to produce a highly fluorescent derivative using a modification of the procedure employed by Fager *et al.*[12]. Initially NBD-proline was separated on TLC plates, and the spot was scraped off and quantitated. During this study, we investigated the effect of solvents on the elution of NBD-proline from silica gel. The results with $4 \times 10^{-10}$ moles NBD-proline appear in Table I. Generally, the more polar solvents, water and the alcohols, quenched the fluorescent response. Although methylene chloride yielded the best fluorescence, we selected *p*-dioxane because of its lower volatility. The fluorescence of NBD-proline was linear up to 1150 ng, the highest concentration tested, with a minimum detectability of 5 ng of proline. Recovery of proline was 96%

TABLE I

EFFECT OF ELUTION SOLVENT ON THE RELATIVE FLUORESCENCE OF NBD-PROLINE ($4 \times 10^{-10}$ MOLES)

| *Solvent* | *Relative fluorescence % transmittance* |
|---|---|
| Water | 3.0 |
| Ethanol | 19.0 |
| *n*-Butanol | 30.0 |
| Benzene | 45.5 |
| Methyl isobutyl ketone | 51.5 |
| Ethyl acetate | 53.5 |
| *p*-Dioxane | 55.0 |
| Methylene chloride | 66.0 |

from 0.1 μmoles of NPro with the denitrosation and derivatization procedures (Table II). This recovery was similar to that reported by Johnson and Walters[13], who detected the nitrosyl halide cleavage product instead of the secondary amine.

The HPLC procedure was evaluated to determine whether the minimum detection level and analysis time could be reduced compared to the TLC method. Fig. 1 shows typical HPLC chromatograms of (a) standard NBD-proline and (b) the NBD derivative of denitrosated NPro. The NBD-proline peak eluting at *ca.* 2 min is well separated from the void volume with an elution time of 0.75 min. The other peaks in the chromatograms are reaction products of the NBD-Cl, and do not interfere with the quantitation of NBD-proline. Peak height was linearly related to NBD-proline concentration (Fig. 2). The HPLC–fluorescence detection system gave a minimum detection level of 0.0035 nmoles NBD-proline corresponding to 0.5 ng of NPro, thereby making HPLC ten times as sensitive as TLC.

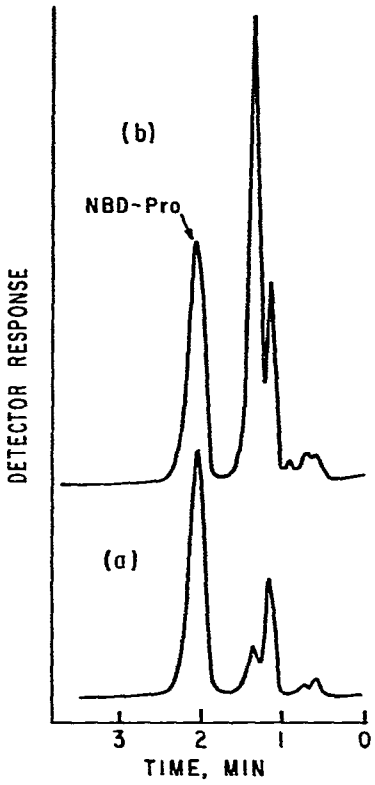
The established procedure of confirming the presence of volatile nitrosamines (NA's) by GLC–MS has proved to be the best method for preventing reports of false positives in food samples[14]. We therefore compared the indirect NBD-proline fluorescence method with another method which would enable the confirmation of the intact nitroso compound by MS. Since NPro is nonvolatile, we prepared and evaluated

TABLE II

CONVERSION OF NITROSOPROLINE TO NBD-PROLINE OR TO THE METHYL ESTER

| *Samples* | *% Conversion* | |
|---|---|---|
| | *NBD-Pro** | *NProMe*** |
| 1 | 86 | 101 |
| 2 | 100 | 88 |
| 3 | 103 | 91 |
| 4 | 100 | 99 |
| 5 | 95 | 101 |
| 6 | 92 | 91 |
| Mean | 96 ± 6.1 S.D. | 95 ± 5.8 S.D. |

* Initial concentration of NPro, 0.1 μmoles.
** Initial concentration of NPro, 0.5 μmoles.



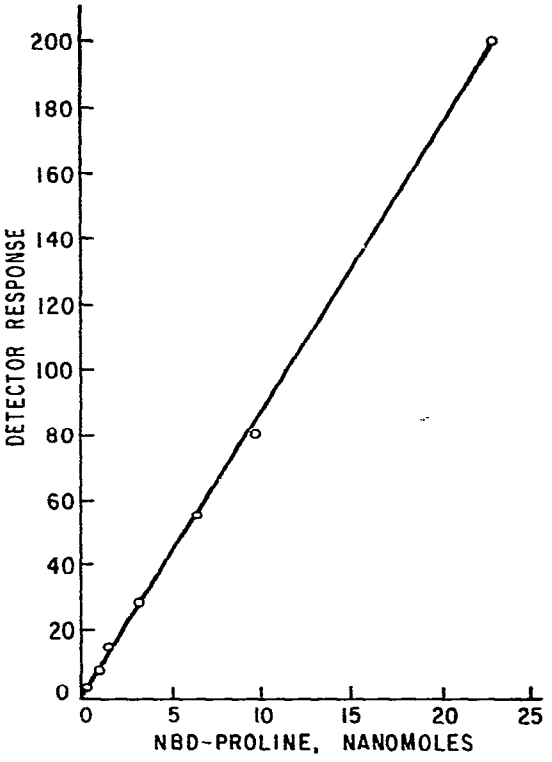




Fig. 1. HPLC of NBD derivatives from: (a) 25 pmoles proline and (b) 25 pmoles nitrosoproline.
Fig. 2. Fluorescence calibration curve of NBD-proline separated by HPLC.

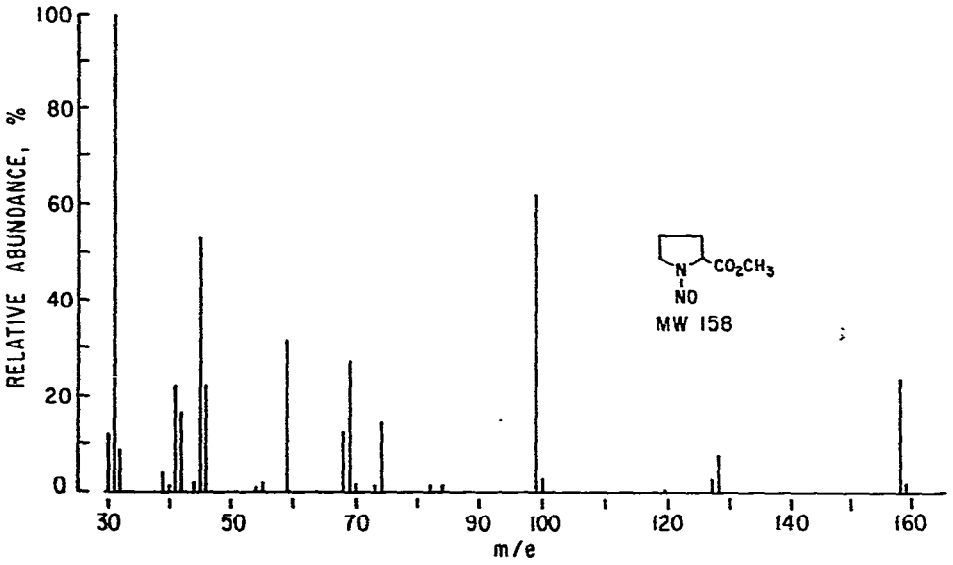



Fig. 3. Low-resolution mass spectrum of nitrosoproline methyl ester.

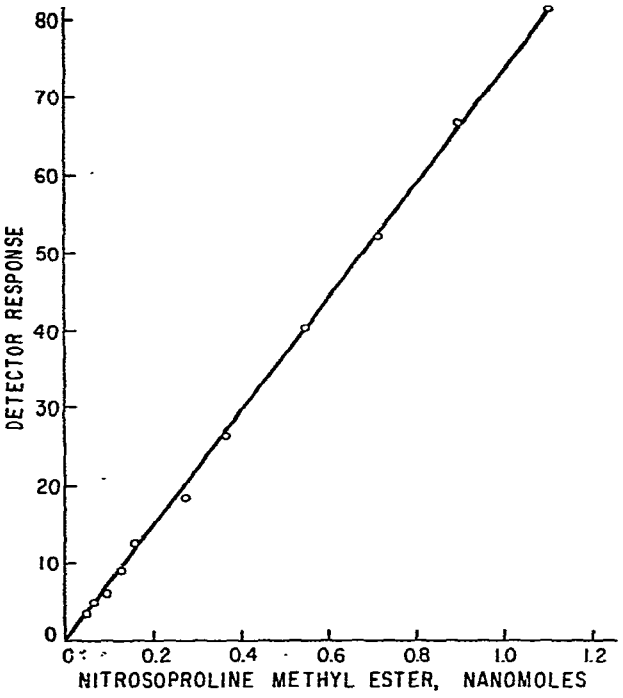



Fig. 4. Calibration curve of nitrosoproline methyl ester determined by GLC using an alkali flame ionization detector.

by GLC–MS the following volatile esters: methyl, ethyl, butyl, isoamyl, hexyl, and benzyl. The methyl ester had the most desirable GC characteristics and mass spectral parent ion response (Fig. 3). Conversion of NPro to its methyl ester was about 95% (Table II). The detection responses of the alkali flame ionization to different concentrations of NPro methyl ester were plotted to give a calibration curve (Fig. 4). The linearity of the peak height responses to NPro methyl ester extended over a twenty-fold concentration range with a minimum detectable quantity of 0.05 nmoles, which corresponds to 7 ng of NPro.

Although the indirect determination of NPro as the NBD derivative by HPLC is approximately ten times as sensitive as the esterification procedure, proline, not NPro, is detected. For food samples, therefore, free proline would have to be removed and generation of proline prior to derivatization would have to be prevented. The fluorescence technique may be most suitable for other nonvolatile NA's for which volatile derivatives are difficult to prepare. While the mass spectral confirmation of volatile derivatives of NA's is desirable, each method offers certain advantages. We are therefore evaluating both analytical methods for the determination of NPro in bacon products.

*Note*

Nitrosamines are potential carcinogens, and should be handled with care.

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