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GAS CHROMATOGRAPHIC DETERMINATION OF NITRITE IN FOODS AS TRIMETHYLSILYL DERIVATIVE OF 1*H*-BENZOTRIAZOLE

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SUMMARY

1,2-Diaminobenzene reacts with nitrite in acidic solution to form 1*H*-benzotriazole, which can be extracted into ethyl acetate. After evaporation of the ethyl acetate, 1*H*-benzotriazole is determined as its trimethylsilyl derivative by gas–liquid chromatography on a column of 15% SE-30 on Chromosorb G HP at 200 °C with flame-ionization detection. The nitrite concentration is calculated from the peak height. Amounts of 0.5–10 μg of nitrite-nitrogen can be determined. For the determination of nitrite in foods, clean-up of the crude extracts by ion-exchange column chromatography allows the satisfactory elimination of interferents and permits concentrations down to 0.41 ppm to be determined. The recovery of nitrite added to foods at the 4.1 ppm level ranges from 94.6 to 98.7% and at the 8.2 ppm level it ranges from 95.2 to 98.8%. The trimethylsilyl derivative of 1*H*-benzotriazole was identified as 1-trimethylsilylbenzotriazole by combined gas chromatographic–mass spectrometric examination and nuclear magnetic resonance spectrometry.

INTRODUCTION

Sodium nitrite is widespread in nature and is also used as a food preservative. In recent years, there has been concern over the potential health danger from nitrite additives in foods because of the possibility that nitrite may react with secondary amines present in the body and form carcinogenic nitrosamines[1]. In several countries official tolerance limits have been established, and it is important that sensitive and accurate methods be available for the determination of nitrite. Such methods should also be simple and rapid and capable of determining nitrite in various types of real samples.

There are numerous methods for determining nitrite, including colour reactions and absorption measurement, UV and IR spectrophotometry, fluorimetry, polarography and gas chromatography. Many colorimetric methods[2–6] have been reported and in more recent methods sulphanilic acid is diazotized and coupled with 1-naphthylamine or N-(1-naphthyl)ethylenediamine to form a coloured azo dye[7,8]. All of these colorimetric methods are limited by the fact that occasionally turbid and

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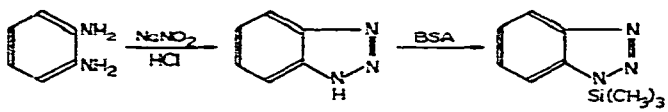
slightly coloured food extracts can affect the colour of the azo dye and, consequently, the accuracy of the nitrite determination.

Recently, the determination of nitrite by gas–liquid chromatography (GLC) has been described[9–11]. Wu and Peter[11] applied an electron-capture detector (ECD) to nitrobenzene after nitration of nitrite and benzene, and found a detection limit of about 0.04 ppm of nitrite; however, this method was not suitable for routine use because of the complex procedure and the vigorous reaction conditions required. Akiba *et al.*[9] studied the determination of nitrite as 1*H*-benzotriazole after reaction with 1,2-diaminobenzene[12] in acidic solution by GLC with a flame-ionization detector (FID) and found it difficult to obtain good accuracy and sensitivity; they recommended the use of another method.

However, we have found that the trimethylsilyl (TMS) derivative of 1*H*-benzotriazole is over 40 times more sensitive than 1*H*-benzotriazole in GLC; it can be prepared quantitatively by reaction with N,O-bis(trimethylsilyl)acetamide (BSA) in ethyl acetate. This reaction scheme is as follows:



The TMS derivative was detected quantitatively with a detection limit of 0.5 ng for nitrite-nitrogen (NO_2-N). Nitrite in foods was extracted with an alkaline solution and purified by ion-exchange column chromatography[10,13] [Dowex 1-X4 (Cl^-)]. This GLC method is simple and sensitive and offers a practical means of determining nitrite in various foods. The recovery of nitrite added to foods was satisfactory.

EXPERIMENTAL

Reagents and apparatus

All water used for preparing solutions was triply distilled and deionized. Sodium nitrite was dried at 100 °C under vacuum immediately before use. A stock nitrite solution was prepared by dissolving 0.493 g of sodium nitrite in distilled water and diluting to 1000 ml to give a concentration of 10 μg/ml of NO_2-N. 1,2-Diaminobenzene solution (0.1 %, w/v) was prepared by dissolving 0.1 g of the reagent (special high grade material, recrystallized three times from benzene before use) in 100 ml distilled water.

The ion-exchange resin Dowex 1-X4 (Cl^-) used in the clean-up stage was obtained from Muromachi Kagaku Kogyo (Tokyo, Japan). The silylating reagents used were BSA–trimethylchlorosilane (TMCS), BSA, TMCS–hexamethyldisilazane (HMDS), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N-trimethylsilylimidazole (SIM), all obtained from Tokyo Kasei Kogyo (Tokyo, Japan). The internal standard solution for GLC was prepared by dissolving 50 μg of fluorene in 1 ml of ethyl acetate.

The column packing materials for GLC, *viz.*, Chromosorb G HP, SE-30, DC-200, OV-101, OV-17 and Triton X-305 were obtained from Nishio (Tokyo, Japan). All other reagents and solvents were of high purity and were obtained from Wako (Tokyo, Japan). For identification of the trimethylsilyl derivative of 1*H*-benzotriazole,

a Shimadzu LKB-9000 combined gas chromatograph–mass spectrometer was used; for GC, a glass tube (1.5 m × 3 mm I.D.) packed with 15% of SE-30 on Chromosorb G HP (80–100 mesh) was fitted. The flow-rate of helium was 30 ml/min, and the column temperature was 200 °C. For mass spectrometry (MS), the separator temperature was 260 °C and that of the ion source was 290 °C. The trap current was 60 μA. The electron energy was 70 eV and the accelerating potential was 3.5 keV. Nuclear magnetic resonance (NMR) spectra were measured at 60 Hz with a Varian EM-60 spectrometer.

Preparation of TMS derivative of 1H-benzotriazole

A suitably diluted solution of nitrite or the effluent from the ion-exchange column was placed in a 100-ml beaker and adjusted to pH 1.0–1.5, then 1 ml of 1,2-diaminobenzene solution was added. After reaction at 80 °C with occasional shaking for 10 min in a water-bath and cooling to room temperature, the solution was re-adjusted to pH 2.0–2.5 and transferred into a 100-ml separating funnel, then 5 g of sodium chloride and 10 ml of ethyl acetate were added. The mixture was shaken vigorously for 5 min and the ethyl acetate layer separated and dried with 2 g of anhydrous sodium sulphate. The ethyl acetate extract was placed in a 50-ml round-bottomed flask with a ground-glass stopper, and the solvent was removed by evaporation under reduced pressure at room temperature. To the dried residue was added 1 ml of internal standard solution and 50 μl of BSA, and the reaction was allowed to proceed at room temperature for 10 min (although it was usually complete after 5 min). A 3-μl volume of the final solution was injected into the gas chromatograph.

Gas–liquid chromatography

A Shimadzu GC-5AIFF gas chromatograph with an FID was used for all analyses. The column consisted of a glass tube (1.5 m × 3 mm I.D.) packed with 15% of SE-30 on Chromosorb G HP (80–100 mesh) and was conditioned at 200 °C; the detector and injector temperature were 290 °C and the flow-rates of nitrogen carrier gas, hydrogen and air were 40, 40 and 800 ml/min, respectively.

Calibration graph

A series of working-standard nitrite solutions were prepared by diluting the stock solution with water. Aliquots were placed in a beaker to give amounts of 0.5, 1.0, 3.0, 5.0, 7.5 and 10.0 μg of NO_2-N. According to the procedure described above, 10 ml of ethyl acetate extract were obtained in each instance, and then removed by evaporation. After trimethylsilylation by addition of BSA and the internal standard solution to the residue, a 3-μl aliquot of the mixture (1050 μl) was injected into the GLC column. As shown in Fig. 1, the retention time of the TMS derivative relative to that of fluorene was 0.63. The peak-height ratio of the TMS derivative to fluorene was plotted against the amount of NO_2-N analysed; a typical standard graph is shown in Fig. 2.

Extraction and clean-up procedure

To 10 g of finely ground sample in a 100-ml flask with a ground-glass stopper were added 40 ml of hot (70–80 °C) water (pH 9.0); after occasional shaking in a water-bath at 80 °C for 40 min and cooling to room temperature, the extracted solution was

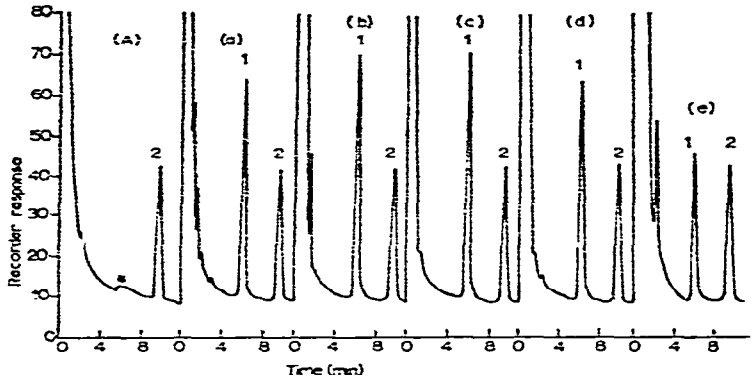


Fig. 1. Gas chromatograms of 1*H*-benzotriazole (A) and the TMS derivative (a–e). The silylating agents added to 42.5 μg of 1*H*-benzotriazole were (a) HMDS–TMCS, (b) BSA, (c) BSA–TMCS, (d) BSTFA and (e) SIM. The reagents were dissolved in 1 ml of ethyl acetate, and the sample size was 3 μl. Peaks: 1, TMS derivative of 1*H*-benzotriazole; 2, fluorene; *, 1*H*-benzotriazole.

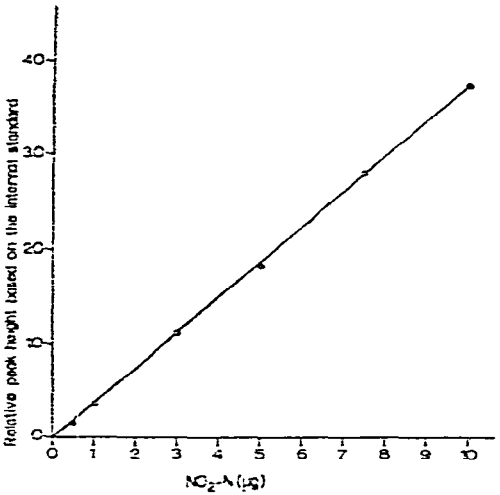


Fig. 2. Calibration graph for nitrite-nitrogen. Silylation was carried out at room temperature for 10 min. The sample size for GLC was 3 μl; the column temperature was 200 °C and the nitrogen flow-rate was 40 ml/min. The abscissa shows the nitrite-nitrogen content of the reaction mixture, and the ordinate the detector response measured as the peak height relative to the internal standard (fluorene; 50 ng per μl of reaction mixture).

filtered and diluted accurately to 100 ml with water. A 40-ml volume of the filtrate was decanted and passed through an ion-exchange column (30 × 1.0 cm I.D.) containing Dowex 1-X4 (which was regenerated with 1 *N* sodium hydroxide solution and 1 *N* hydrochloric acid before use). The column was then eluted successively with 200 ml of water, 50 ml of 0.1% sodium chloride solution and 25% sodium chloride solution at a rate of 1 ml/min. The elution with 25% sodium chloride solution was continued until the effluent volume reached 25 ml. After adjusting the pH to 1.0–1.5, the eluate was reacted with 1,2-diaminobenzene, extracted with ethyl acetate and then evaporated as described above.

The dry residue was silylated and analysed by GLC as described above. The contents of nitrite in foods were determined from the peak heights relative to that of the internal standard on the gas chromatograms, and comparison with calibration graphs.

RESULTS AND DISCUSSION

Standard assay

For the GLC assay using the described procedure, there was a linear relationship between peak height and amount of NO_2-N. As shown in Fig. 2, the calibration graph was linear from 0.5 to 10 μg of NO_2-N, and the average relative standard deviations of four determinations were 0.4% for 1.0 μg, 0.5% for 5 μg and 1.0% for 10 μg of NO_2-N; the reproducibility was considered to be satisfactory.

Production of 1H-benzotriazole

The influence of pH on the reaction of 1,2-diaminobenzene with nitrite to form 1*H*-benzotriazole was studied by mixing 5.0 μg of NO_2-N and 1 ml of 1,2-diaminobenzene solution. The relative yields obtained after 15 min were 86.0% at pH 0.5, 100% at pH 1.0, 99.2% at pH 1.5, 98.6% at pH 2.0, 88.3% at pH 2.5, 83.3% at pH 3.0 and 46.5% at pH 3.5; therefore, pH 1.0 was adopted as optimal.

The course of the reaction at different temperatures is shown in Fig. 3. A constant peak height was obtained after 10 min at room temperature and 3 min at 80 °C. After 10 min at 80 °C, the amount of 1*H*-benzotriazole present slowly decreased, and therefore the reaction was further studied at high temperatures. The relative yields obtained after 20 min were 93.6% for 100 °C and 63.2% at 150 °C. The use of a long reaction time at high temperatures was not desirable, and therefore 80 °C was adopted.

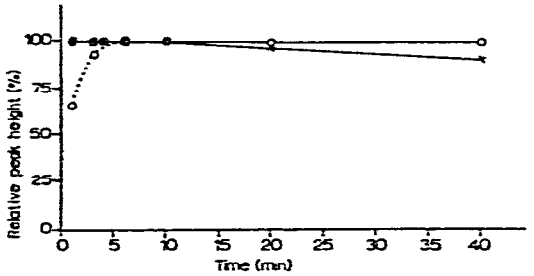


Fig. 3. Time course of production of the 1*H*-benzotriazole. To 5 μg of NO_2-N was added 1 ml of 0.1% 1,2-diaminobenzene at various temperatures, followed by silylation according to the described procedure and analysis by GLC. O—O, Room temperature; O—O, 50 °C; ×—×, 80 °C.

If we assume that 1 mol of 1,2-diaminobenzene reacts with 1 mol of nitrous acid, then 39 μg of 1,2-diaminobenzene is required for 16.8 μg of nitrous acid (5 μg of NO_2-N). The relative yields of 1*H*-benzotriazole for various amounts of 1,2-diaminobenzene added to 16.8 μg of nitrous acid in a total of 26 ml of solution were 96.5% for 50 μg of 1,2-diaminobenzene, 99.4% for 100 μg, 99.8% for 200 μg, 100% for 300 μg, 98.9% for 500 μg and 99.7% for 1000 μg at 80 °C with a reaction time

of 10 min. To some extent, therefore, addition of 1*H*-benzotriazole in excess gave a good result, and in practice 1 ml of a 0.1% reagent solution was used.

Extraction

1*H*-Benzotriazole can be extracted into an organic solvent over the pH range 2–7. When the pH of the aqueous phase is higher than the pK_a (the acid dissociation constant of monoprotonated 1,2-diaminobenzene), the excess of reagent would be extracted into an organic solvent together with the 1*H*-benzotriazole. Consequently, 1*H*-benzotriazole should be extracted at a pH lower than the pK_a. When the pH of the aqueous phase was 4.0 or above, it was impossible to analyse 1*H*-benzotriazole. Therefore, the optimum pH range of the extraction adopted was 2.0–2.5. The use of various solvents as extractants was examined. When a polar solvent such as ethyl, *n*-propyl or *n*-butyl acetate was used, the extraction yield of 1*H*-benzotriazole was high, but it was lower if a non-polar solvent such as *n*-hexane was used. Thus, ethyl acetate, the most volatile of the selected polar solvents, was adopted.

Influence of evaporation of the solvent on the recovery of 1H-benzotriazole

Prior to trimethylsilylation of 1*H*-benzotriazole it was necessary to evaporate the ethyl acetate, with the risk of loss of volatile 1*H*-benzotriazole. A 10-ml volume of ethyl acetate which contained 42.5 μg of 1*H*-benzotriazole (corresponding to 5 μg NO_2-N) was evaporated under reduced pressure at room temperature. No loss of 1*H*-benzotriazole during the evaporation was observed. However, after the ethyl acetate had been removed and 1*H*-benzotriazole remained as the residue, the decrease in the amount of 1*H*-benzotriazole was significant, as shown in Table I. When the evaporation was performed at up to 40 °C, a significant decrease in 1*H*-benzotriazole was observed. The recoveries obtained after 15 min were 73.4% at 50 °C, 23.5% at 80 °C and 20.9% at 100 °C. Therefore, the sample should be trimethylsilylated within 1 min after removal of the ethyl acetate at room temperature.

TABLE I

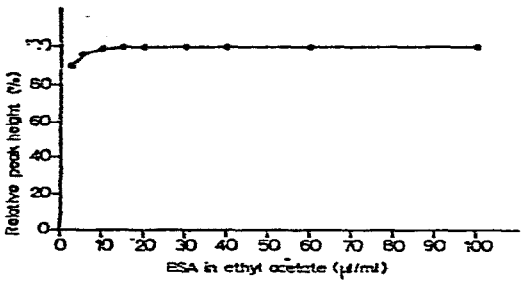
DECREASE OF 1*H*-BENZOTRIAZOLE RESIDUE AFTER EVAPORATION OF THE SOLVENT

1*H*-Benzotriazole content before evaporation was 42.5 μg (NO_2-N: 5.0 μg). The tests were carried out at room temperature.

Time after completion of evaporation (min)	*1H-Benzotriazole recovery (%)*	*Time after completion of evaporation (min)*	*1H-Benzotriazole recovery (%)*
0	100	5	100
1	100	10	97.5
2	100	15	92.7
3	100	30	90.7

Trimethylsilylation of 1H-benzotriazole

The chromatograms of the TMS derivative of 1*H*-benzotriazole are shown in Fig. 1. The retention time of the TMS derivative was 6.0 min. The optimal amount of reagent and the optimal reaction time were investigated by using BSA, and the



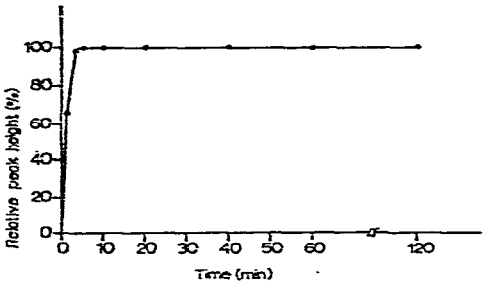


Fig. 4. Effect of amount of BSA on production of the TMS derivative of 1*H*-benzotriazole. To 42.5 μg of 1*H*-benzotriazole (corresponding to 5.0 μg NO_2-N) was added BSA in 1 ml of ethyl acetate at room temperature, and the product was analysed by GLC after 15 min.

Fig. 5. Time course of TMS derivative production after addition of BSA to 1*H*-benzotriazole. To 42.5 μg of 1*H*-benzotriazole (corresponding to 5.0 μg NO_2-N) was added BSA in ethyl acetate (50 μl/ml) at room temperature, and the product was analysed by GLC.

results are shown in Figs. 4 and 5. For 42.5 μg of 1*H*-benzotriazole (corresponding to 5.0 μg NO_2-N), at least 72.5 μg of BSA in 1 ml of ethyl acetate were required. The reaction proceeded fairly rapidly and when BSA solution in ethyl acetate (50 μl/ml) was added to the solid residue of 1*H*-benzotriazole, the yield of the TMS derivative reached 100% within 10 min.

On comparing the reactivities of various trimethylsilylating reagents towards 1*H*-benzotriazole, it was observed that the reaction of BSTFA was slower that those with reagents BSA–TMCS, BSA and TMCS–HMDS and that the reaction with SIM was not complete even after 24 h. BSA–TMCS, BSA and TMCS–HMDS gave good chromatograms and these reagents are suitable for the silylation of 1*H*-benzotriazole. *n*-Hexane, ethyl acetate, cyclohexane, 4-methylpentan-2-one, dimethyl sulphoxide, tetrahydrofuran and acetonitrile were tried as reaction solvents. The most suitable were ethyl acetate and *n*-hexane and the least suitable pyridine and methanol, as shown in Table II. We chose ethyl acetate because of its good solvent properties for 1*H*-benzotriazole and fluorene.

Gas chromatographic sensitivity

Columns containing SE-30 (15%, w/w), DC-200 (15%, w/w), OV-17 (15%,

TABLE II

SOLVENT DEPENDENCE OF PRODUCTION OF THE TMS DERIVATIVE OF 1*H*-BENZOTRIAZOLE

Silylation and GLC conditions as in Fig. 2. Each reaction mixture contained 42.5 μg of 1*H*-benzotriazole (corresponding to 5 μg NO_2-N) and 50 μl of BSA.

*Solvent**	*Relative peak height (%)*	*Solvent**	*Relative peak height (%)*
Ethyl acetate	100	Dimethyl sulphoxide	21.8
n-Hexane	100	Dimethylformamide	16.4
Acetonitrile	100	Pyridine	7.3
Tetrahydrofuran	81.8	Methanol	3.6
Acetone	45.5		

* Fluorene (50 μg) was dissolved in 1 ml of each solvent.

w/w), OV-101 (15%, w/w) and Triton X-305 (15%, w/w), on Chromosorb G HP, were tested. Except with Triton X-305, the columns showed the peak for the TMS derivative of 1*H*-benzotriazole; particularly good peak characteristics and sensitivity were achieved with SE-30 under the conditions described above. A high temperature and a short column were preferable for the GLC of the TMS derivative of 1*H*-benzotriazole. At 200 °C, a 1.5-m column containing SE-30 on Chromosorb G HP gave a good gas chromatogram, the retention times of 1*H*-benzotriazole and the TMS derivative relative to that of the internal standard were 0.60 and 0.64, respectively. The ratio of the peak height for the same molar concentration of 1*H*-benzotriazole and the TMS derivative was 1:40, and therefore the peak characteristics of the TMS derivative were better than those of the parent 1*H*-benzotriazole (see Fig. 1). After trimethylsilylation, the reaction mixture should be injected into the gas chromatograph as soon as possible; at room temperature, the sample was stable for at least 24 h, but the content of the TMS derivative decreased to 94.8% in this period.

Interferences

Sodium nitrite can be extracted from foods with an alkaline solution and subsequently separated from the alkaline solution by ion-exchange chromatography. The simple and rapid extraction and clean-up procedures based on this principle permit the determination of nitrite in foods by GLC without effects from interfering substances. To investigate the effects of preservatives such as sorbic acid, benzoic acid, dehydroacetic acid, butylhydroxyanisole and butylhydroxytoluene on the determination, 42.5-μg portions of 1*H*-benzotriazole (corresponding to 5 μg NO_2-N) were added to 0.5–10.0 mg of various preservatives, and each mixture was analysed by direct silylation without clean-up procedure. As shown in Table III, when more than 5 mg of most preservatives were present (for example, 5 μg of NO_2-N and 10 mg of preservatives), the clean-up procedure described removed most of the

TABLE III

INFLUENCE OF FOOD ADDITIVES ON RECOVERY OF NITRITE-NITROGEN

Each amount of food additive was added to a mixture of 42.5 μg of 1*H*-benzotriazole (corresponding to 5 μg NO_2-N), 50 μl of BSA and 1 ml of internal standard solution. Silylation and GLC conditions as in Fig. 2.

Additive	*Amount added (mg)*			
	0.5	*1.0*	*5.0*	*10.0*
Sorbic acid	100	100	100	61.3 (98.2)**
Benzoic acid	100	100	100	50.2 (99.1)
Dehydroacetic acid	100	100	95.0	25.0 (98.5)
Butylhydroxyanisole	—* (100)	—* (100)	—* (98.7)	— (77.9)
Butylhydroxytoluene	93.0 (100)	60.0 (100)	40.0 (100)	— (82.3)

* Quantitative determination impossible.

** Values in parentheses are recoveries after clean-up.

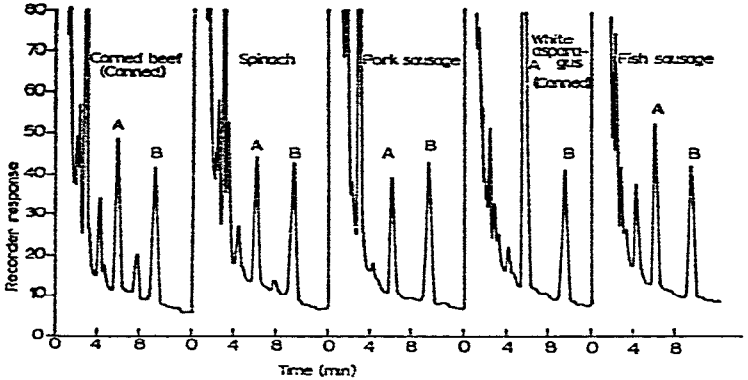


Fig. 6. Gas chromatograms of silylated extracts of various foods. Sample size, 3 μl. Peaks: A, 1-trimethylsilylbenzotriazole; B, fluorene.

amount present. As shown in Fig. 6, the silylated extracts obtained from foods gave gas chromatograms with good peak characteristics.

Application and recoveries

Nitrite added to 10-g samples of pork sausage, corned beef (canned), fish sausage, spinach and white asparagus (canned), chopped and then ground in a porcelain pestle and mortar, was determined by the proposed method. The recoveries of 4.1 and 8.2 ppm of nitrite, given in Table IV, ranged from 94.6 to 98.7% for 4.1 ppm and 95.2 to 98.8% for 8.2 ppm. The detection limit was 0.31 ppm.

TABLE IV

PERCENTAGE RECOVERIES OF NITRITE ADDED TO VARIOUS FOODS AT THE 4.1 AND 8.2 ppm LEVELS

Each result is the average of four determinations.

Sample	*Amount of nitrite-nitrogen added (μg)*	
	12.5	*25.0*
Fish sausage	98.7	98.4
Corned beef (canned)	97.5	98.1
Pork sausage	94.6	95.2
White asparagus (canned)	98.4	98.8
Spinach	96.7	97.3

Identification of the TMS derivative of 1H-benzotriazole

GC–MS. The mass spectrum of the product from the reaction of 1,2-diaminobenzene and nitrous acid was identical with the standard spectrum of 1*H*-benzotriazole, with ion peaks at *m/e* 119 (M^+), 91 ($M^+—N_2$) and 76 (—NH). The mass spectrum corresponding to the peak obtained by silylation and GLC separation of the 1*H*-benzotriazole are shown in Fig. 7, *viz.*, *m/e* 191 (M^+), 176 ($M^+—CH_3$), 118 (—Si (CH_3)), 90 ($—N_2$) and 75 (—NH). The parent peak (*m/e* 119) for 1*H*-benzotriazole and at *m/e* 191 for the TMS derivative correspond to the molecular weight of each compound. The shift of the peaks from *m/e* 191 to 118 for the TMS derivative could be ascribed to 1-de-trimethylsilylation and the subsequent shift from *m/e* 118

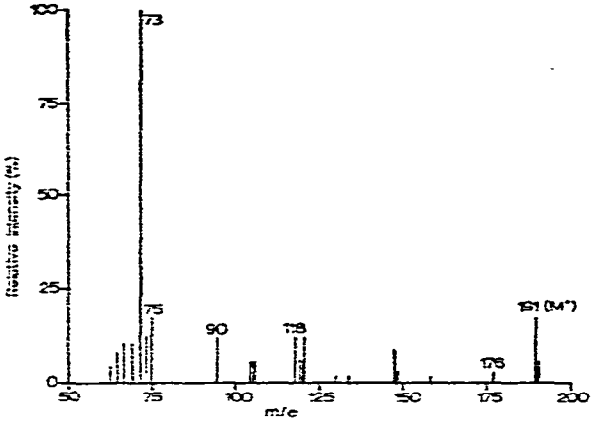


Fig. 7. Mass spectrum of 1-trimethylsilylbenzotriazole.

to 75 for the 1*H*-benzotriazole ion could be attributed to degradation of the triazole ring.

NMR spectrometry. Portions of 30 mg of 1*H*-benzotriazole dissolved in acetone were silylated with 100 μl of BSA. In the NMR spectrum of 1*H*-benzotriazole dissolved in acetone, signals appear at $\delta = 7.35$–7.93 ppm (multiplet; 4H) which is indicative of an aromatic compound, and at $\delta = 14.56$ ppm (wide singlet peak; 1H), which is indicative of the NH group. As shown in Fig. 8, in the NMR spectrum of the TMS derivative dissolved in acetone the singlet peak (H) at $\delta = 14.56$ ppm has disappeared, which suggests the loss of the NH group in the triazole ring. The difference in the chemical shifts makes it possible to distinguish 1*H*-benzotriazole and the TMS derivative.

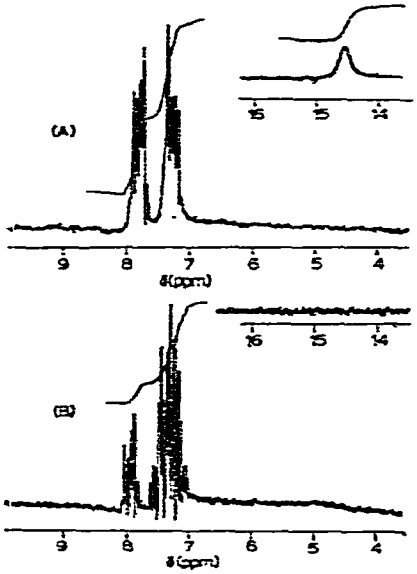


Fig. 8. NMR spectra of 1*H*-benzotriazole (A) and 1-trimethylsilylbenzotriazole (B) in acetone at 60 Hz.

From this series of experiments, it was concluded that the TMS derivative was 1-trimethylsilylbenzotriazole.

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