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GAS CHROMATOGRAPHIC DETERMINATION OF THIABENDAZOLE IN FRUITS AS ITS METHYL DERIVATIVE

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SUMMARY

Quantitative determination of thiabendazole, as its methyl derivative, has been achieved by gas–liquid chromatography with flame ionization detection and a column of 10% of DC-200 on Gas-Chrom Q at 240°. Determination was possible with 10 to 200 ng per μl of the methylated reaction mixture. For determining thiabendazole in fruits, clean-up of the crude extracts by liquid–liquid partition allows satisfactory elimination of interference and permits determination in concentrations down to 0.1 ppm. The recovery of thiabendazole added to fruits at the 0.5-ppm level ranges from 90.3 to 94.9%. The methyl derivative was identified as N-methylthiabendazole by its elementary composition, by its melting-point, and by ultraviolet, infrared, mass and nuclear magnetic resonance spectroscopy.

INTRODUCTION

Thiabendazole [2-(4-thiazolyl)benzimidazole] is an anthelmintic[1] and also a post-harvest fungicide on citrus fruits and bananas, and in several countries the official residue-tolerance limit has been established. The various methods used for determining thiabendazole include colour reaction and absorption measurement, ultraviolet (UV) and infrared (IR) spectrophotometry, fluorimetry and gas chromatography. Hey[2] and other investigators[3–7] briefly studied the determination of thiabendazole by gas–liquid chromatography (GLC) and found it difficult to obtain good accuracy and sensitivity; they recommended use of another method. However, we have found that the methyl derivative is more sensitive than thiabendazole in GLC and can be quantitatively prepared by a reaction with dimethylformamide dimethylacetal[8] (DMF-DMA) in acetonitrile medium[9].

Thiabendazole is soluble in organic solvents, especially ethyl acetate, and also in dilute acids because of salt formation with the secondary amine group, and therefore ethyl acetate and dilute hydrochloric acid were used as extractants[3,5,7,10,11]. The purified extract from the fruits was directly analyzed by GLC after methylation; recovery of thiabendazole added to fruits was satisfactory. The GLC method is simple and sensitive and offers a practical means of determining thiabendazole used for preserving fruits.

MATERIALS AND METHODS

Reagents and apparatus

The thiabendazole (obtained from Merck, Rahway, N.J., U.S.A.) was of a special high grade and was re-crystallized three times from ethanol before use; its m.p. was 301–302°, slightly lower than the value reported in the Merck Index[12]. The standard solution was prepared by dissolving thiabendazole in acetone to give a concentration of 10 μg/ml. The DMF-DMA used in the methylation stage was obtained from Tokyo Kasei Kogyo (Tokyo, Japan), and the internal standard solution for GLC was prepared by dissolving 100 μg of 4,4'-dinitrobiphenyl in 1 ml of acetonitrile. The column-packing materials for GLC, *viz.*, Gas-Chrom Q, DC-200, SE-30, QF-1, OV-1, OV-17, OV-101 and DEGS, were of high purity and were obtained from Nishio (Tokyo, Japan). All other reagents and solvents were of high purity and were obtained from Wako (Osaka, Japan).

For identification of the methyl derivative of thiabendazole, several types of equipment were used. For m.p. measurement, an electrothermal capillary apparatus was employed. The UV and IR spectra were obtained with a Hitachi EPS-032 and a Jasco spectrophotometer (model IR-G), respectively. The mass spectra were recorded on a Shimadzu LKB-9000 mass spectrometer, and the nuclear magnetic resonance (NMR) spectra were measured at 60 Hz with a Varian T-60 spectrometer and tetramethylsilane as internal standard.

Preparation of the methyl derivative

A suitably diluted thiabendazole or purified extract dissolved in acetone was placed in a Pyrex test-tube (10 cm × 9 mm I.D.), and the solvent was removed by evaporation with a stream of dry air. To the dried residue were added 0.5 ml of internal-standard solution and 50 μl of DMF-DMA, and the mixture, in a stoppered test-tube, was heated at 120° for 40 min in an oil-bath, then cooled; 2 μl of the final solution were injected into the gas chromatograph.

Gas–liquid chromatography

A Shimadzu GC-5AIFF gas chromatograph with a flame ionization detector was used for all analyses. The column consisted of a glass tube (1.5 m × 3 mm I.D.) packed with 10% of DC-200 on Gas-Chrom Q (80–100 mesh) and was conditioned at 240°; the detector and injector temperatures were 260°. The flow-rates of nitrogen carrier gas, hydrogen and air were 40, 40 and 800 ml/min, respectively, and the electrometer range was 10^3 MΩ × 0.16 V.

Calibration graph

A series of working-standard thiabendazole solutions was prepared by diluting the stock solution with acetone; aliquots were taken into test-tubes, and the solvent was removed by evaporation. After methylation by addition of DMF-DMA and the internal-standard solution to the residue, a 2-μl aliquot of the reaction mixture (550 μl) was injected into the GLC column. The concentration range of the thiabendazole standard was 10–200 ng/μl. As shown in Fig. 1, the retention time of the methyl derivative relative to that of 4,4'-dinitrobiphenyl was 0.52. The peak height ratios of the methyl derivative to 4,4'-dinitrobiphenyl were plotted against the amount of thiabendazole analyzed; a typical standard graph is shown in Fig. 2.

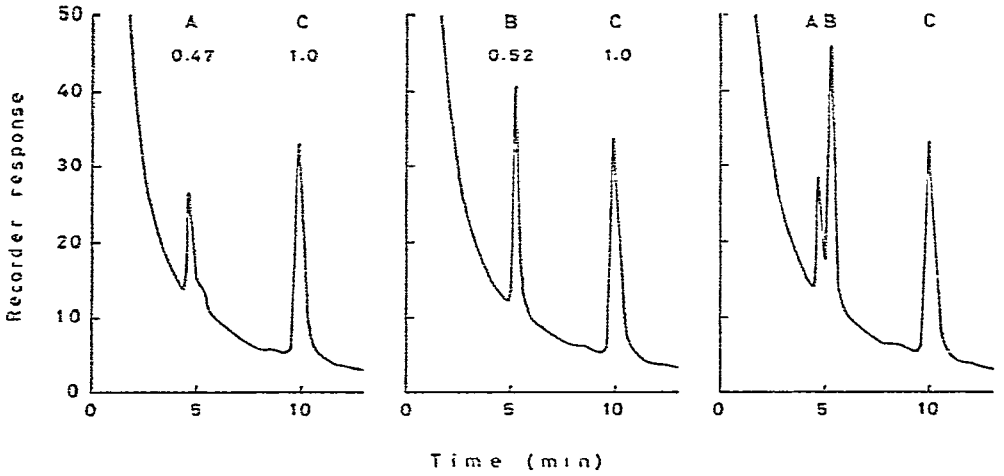

Fig. 1. Gas chromatograms of thiabendazole (A) and the methyl derivative (B), with retention times relative to that of the internal standard (C). The methyl derivative was obtained in crystalline form by methylation of thiabendazole. A 2-μl portion of a mixture of the internal-standard solution and thiabendazole (and/or the methyl derivative) was directly injected into the gas chromatograph.

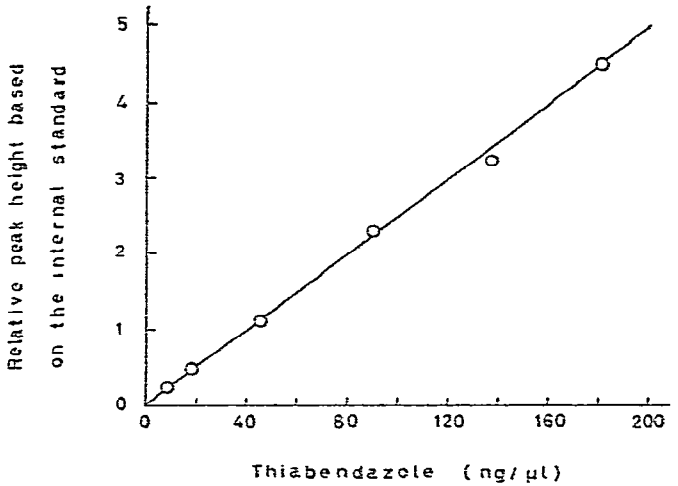

Fig. 2. Calibration graph for thiabendazole. Methylation was at 120° for 40 min; the sample size for GLC was 2 μl; the column temperature was 240° and the nitrogen flow-rate was 40 ml per min. The abscissa shows the thiabendazole content of the reaction mixture, and the ordinate the detector response measured as peak height relative to the internal standard (4,4'-dinitrobiphenyl; 90.91 ng per μl of reaction mixture).

Preparation of fruit extracts and determination

The accurately weighed sample (generally about 50 g) of chopped fruits was placed in the 500-ml stainless-steel container of a homogeniser, 150 ml of ethyl acetate and 50 ml of buffer solution (0.4 *M* in sodium acetate and 3.4 *M* in sodium chloride) were added, and the mixture was homogenised at high speed for 5 min. The

contents of the vessel, with rinsings, were transferred to a 300-ml centrifuge tube and centrifuged at 1400 *g* for 5 min, and the supernatant ethyl acetate phase was transferred to a 500-ml separating-funnel; to the residual plant material were added another 70 ml of ethyl acetate, and the operations were repeated. The combined ethyl acetate solutions were then washed with 50 ml of 1 *M* sodium hydroxide, followed by 50 ml of water. Thiabendazole was extracted from the washed ethyl acetate phase with two 50-ml portions of 0.1 *M* hydrochloric acid, the acid solution was made alkaline with 20 ml of 1 *M* sodium hydroxide, and the alkaline solution was re-extracted with two 30-ml portions of ethyl acetate. This ethyl acetate extract was washed with water and dried with anhydrous sodium sulphate, then the solvent was evaporated at 50° under reduced pressure. The residue was dissolved with two 2-ml portions of acetone, and the mixture was methylated and analyzed by GLC as described above.

RESULTS AND DISCUSSION

Standard assay

As shown in Fig. 2, the calibration graph was rectilinear for 10 to 200 ng of thiabendazole per μl of the reaction mixture. The average deviations of 4 determinations were 0.5% for 20 or 50 ng of thiabendazole and 1.0% for 100 ng, and the reproducibility was considered satisfactory.

Condition for methylation

Solvent system. To 25 μg of thiabendazole were added 50 μl of DMF-DMA (itself a solvent for thiabendazole), and methylation was achieved by heating at 120° for 40 min. When 0.5 ml of some other solvent such as acetonitrile or pyridine were also added, the relative yield of methyl derivative was higher, although it was lower if chloroform was used. The relative yields obtained from the gas chromatograms are shown in Table I. It is assumed that the reaction is influenced by polarization of the

TABLE I

SOLVENT DEPENDENCE OF PRODUCTION OF THE METHYL DERIVATIVE OF THIABENDAZOLE

The methylation and GLC conditions were as for Fig. 2. Each reaction mixture contained thiabendazole (25 μg) and DMF-DMA (50 μl).

*Solvent**	*Relative peak height (%)*
Acetonitrile	100
Pyridine	99.0
Nitrobenzene	82.5
Ethyl acetate	67.0
Methanol	51.4
Acetone	46.8
Benzene	36.7
Chloroform	5.5
—**	36.7

* 4,4'-Dinitrobiphenyl (50 μg) was dissolved in 0.5 μl of each solvent.

** After the methylation with DMF-DMA, 0.5 ml of internal-standard solution was added just before GLC.

solvent molecules, and, in order to obtain a high yield of the methyl derivative, acetonitrile or pyridine could be added, although the reaction mixture subsequently darkened, especially with pyridine. We chose acetonitrile because of its good solvent properties for thiabendazole and 4,4'-dinitrobiphenyl.

Combination of reagents. If we assume that 1 mol of DMF-DMA reacts with 1 mole of thiabendazole, then 12.5 μg of DMF-DMA is required for 25 μg of thiabendazole. The relative yields (%) of the methyl derivative for various amounts of DMF-DMA added to a mixture of 25 μg of thiabendazole and 0.5 ml of internal-standard solution were 24.8 for 10 μl of DMF-DMA, 100 for 50 μl, 98.2 for 100 μl, 93.6 for 500 μl and 56.0 for 1 ml at 120° for 40 min. To some extent, therefore, addition of DMF-DMA in excess gave a good result, and, in practice, 50 μl of DMF-DMA were used. As already mentioned, an addition of solvent promotes methylation. Under conditions in which various amounts of the internal standard solution were added to the mixture of 25 μg of thiabendazole and 50 μl of DMF-DMA, the relative yields (%) of the methyl derivative were 89.0 for 0.1 ml of internal standard solution, 100 for 0.5 or 1.0 ml, and 77.1 for 2.5 ml at 120° for 40 min. Thus, the use of 0.5 ml of internal standard solution was adopted.

Temperature. The production of the methyl derivative at several temperatures was studied by mixing 25 μg of thiabendazole, 50 μl of DMF-DMA and 0.5 ml of the internal standard solution. The relative yields (%) obtained after 40 min were 38 at 30°, 39 at 50°, 92 at 100°, 100 at 120° or 150°, and 57 at 200°; therefore, 120° was adopted.

Reaction time. The time course of production of the methyl derivative at 120° is shown in Fig. 3. The experimental results can be expressed approximately by the equation

$$x = a\frac{k_1}{k_1 - k_2}\left(e^{-k_2 t} - e^{-k_1 t}\right) \qquad (1)$$

where x is the amount of the methyl derivative at time t, a is the initial amount corresponding to thiabendazole and k_1 and k_2 are the rate constants. From the observed values, k_1 and k_2 were 6.35 and 0.032 h^{-1}, respectively. The maximum value of x ($x_{max.}$) was 0.974 when a was 1, and t at $x_{max.}$ ($t_{max.}$) was 50.2 min. After $t_{max.}$, the amount of methyl derivative gradually decreased, suggesting the formation of another compound and the necessity of checking at around $t_{max.}$. The value of x at 40 min was calculated as being 99.6% of $x_{max.}$, and, since the difference between $x_{max.}$ and x at 40 min was not significant, the amount of methyl derivative was checked at 40 min after addition of the reagents to thiabendazole.

Gas chromatographic sensitivity

Columns containing DC-200 (10%, w/w), SE-30 (5%, w/w), QF-1 (8%, w/w), OV-1 (5%, w/w), OV-101 (5%, w/w), OV-17 (5%, w/w) or DEGS (5%, w/w), on Gas-Chrom Q, were tested. Except for OV-17 and DEGS, the columns showed the peak for the methyl derivative; particularly good peak characteristics and sensitivity were achieved with DC-200 under the conditions described above.

A high temperature and a short column were preferable for the GLC of the methyl derivative. At 240°, a 1.5-m column containing DC-200 on Gas-Chrom Q

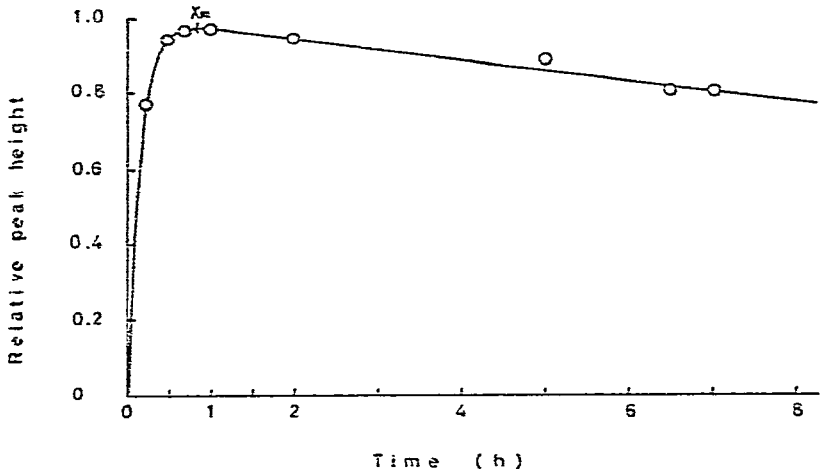

Fig. 3. Time course of production of methyl derivative. To 25 μg of thiabendazole were added 50 μl of DMF-DMA and 0.5 ml of the internal standard at 120°, and the product was analyzed by GLC. The curve was calculated from eqn. 1.

gave a good gas chromatogram, the retention times of thiabendazole and the methyl derivative relative to that of the internal standard were 0.47 and 0.52, respectively. The ratio of the peak height for the same molar concentration of both thiabendazole and the methyl derivative was 1:2.3, but the peak characteristics of the derivative were better than those of thiabendazole (see Fig. 1).

After methylation, the reaction mixture should be injected into the gas chromatograph as soon as possible; under refrigeration, the sample was stable for at least 4 h, but, after 24 h, the content of methyl derivative decreased to 89%.

Extraction and clean-up procedures

Influence of evaporation of the solvent. During the extraction procedure, or before methylation, ethyl acetate or acetone that contains thiabendazole is evaporated off. In order to examine possible loss in these procedures, 25 μg of thiabendazole were dissolved in 60 ml of the solvent, and the solution was analysed during or after evaporation of the solvent; no loss was observed. After the solvent had been removed and thiabendazole remained as the residue, no loss occurred during heating for at least 10 min at 50° under reduced pressure.

Influence of interference. Thiabendazole is basic and can therefore be extracted from organic solvents by acidic aqueous solutions and from basic aqueous solutions by organic solvents. The simple and rapid extraction and clean-up procedures based on this principle permit the determination of thiabendazole in fruits by GLC without effect from interfering substances. To investigate the effect of an essential oil on the determination, 25-μg portions of thiabendazole were added to 0.1 to 30 mg of limonene, and each mixture was analyzed without clean-up procedures. The relative recorder response on the gas chromatogram was in proportion to the amount of limonene and, at the same time, the accuracy decreased. As shown in Table II, the presence of over 20 mg of limonene made accurate determination impossible. How-

TABLE II

INFLUENCE OF LIMONENE ON RECOVERY OF THIABENDAZOLE

Each amount of limonene was added to a mixture of 25 μg of thiabendazole. 50 μl of DMF-DMA and 0.5 ml of internal-standard solution. Methylation and GLC conditions were as for Fig. 2.

Limonene added (mg)	*Recovery (%)*
0.0	100
0.1	99.8
0.5	99.1
1.0	98.3
3.0	98.3
5.0	98.3
10.0	93.4
20.0	—*
30.0	—*

* Quantitative determination impossible.

ever, even if much limonene was present (for example, 25 μg of thiabendazole and 100 mg of limonene), the clean-up procedure described removed most of it. As shown in Fig. 4, methylated extracts obtained from fruits gave gas chromatograms with good peak characteristics. Besides the peak for the methyl derivative, there was another peak (generally a double peak), of which the retention time relative to that of the internal standard was 0.28 to 0.31. However, this peak did not interfere with quantitation of the methyl derivative.

Other possible interfering substances are such preservatives as biphenyl or *o*-phenylphenol added to fruits. These preservatives were eliminated during the clean-up procedure, and an addition of 100 μg of biphenyl or *o*-phenylphenol to 25 μg of thiabendazole did not affect the determination.

Application and recoveries

Thiabendazole added to orange, lemon, grapefruit and banana was determined separately in the peel and in the pulp and calculated, as necessary, for the whole fruit. From an average sample (five to ten fruits) the peel was carefully removed, and the peel or pulp from each fruit was chopped and blended into a slurry. To 50 g of slurry was added thiabendazole, and the samples so obtained were analyzed by our GLC procedure. The recoveries of 0.5 ppm of thiabendazole added to various samples are shown in Table III. These recoveries ranged from 90.3 to 94.9%, and thiabendazole could be determined at the level of 0.1 ppm, the detection limit being 0.05 ppm. When thiabendazole was determined in grapefruit peel, the peak was sometimes insufficiently clear for accurate determination; however, the chromatogram for the sample obtained from the whole fruit was satisfactory.

Identification of the methyl derivative of thiabendazole

For quantitative analyses, to a small amount of thiabendazole were added DMF-DMA in excess and solvent. To obtain the methyl derivative, to 0.50 g of thiabendazole and 0.296 g of DMF-DMA (molar ratio 1:1) were added 5 ml of acetonitrile, and the mixture was heated under reflux at 120° for 40 min; when reaction

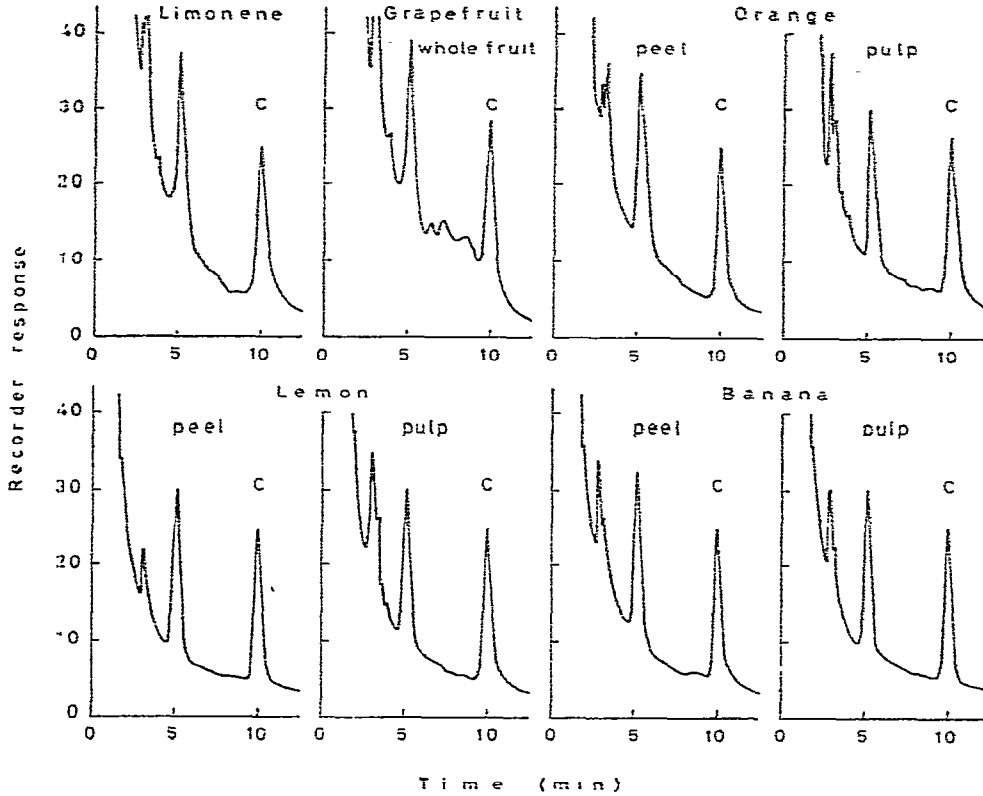

Fig. 4. Gas chromatograms of methylated extracts of orange, lemon, grapefruit and banana; 25 μg of thiabendazole were added to 50 g of fruit before extraction. With limonene, 25 μg of thiabendazole were added to 100 mg of limonene, and the mixture was treated by the same clean-up procedures as used with fruits. Methylation and GLC conditions were as for Fig. 2. C = 4,4'-dinitrobiphenyl (90.91 ng/μl).

TABLE III

PERCENTAGE RECOVERIES OF THIABENDAZOLE ADDED TO VARIOUS SAMPLES AT THE 0.5-ppm LEVEL

Each result is the average of four determinations.

Sample	*Peel*	*Pulp*	*Whole fruit*
Orange	91.3	93.4	90.4
Lemon	90.7	92.1	91.7
Grapefruit	90.3	92.9	90.9, 92.5*
Banana	93.5	94.9	94.2

* Thiabendazole added at 1-ppm level.

was complete, the mixture was cooled and the crystals were collected on filter paper and dissolved in benzene–*n*-hexane (1:1, v/v). The purified product was colourless needles (m.p. 150°); Brown *et al.*[1] reported that the m.p. of N-methylthiabendazole was 140°. A solution of the product in chloroform was stable, even at low or high pH.

The elementary composition of the product was 61.35% of C; 4.21% of H; 19.53% of N; 14.91% of S; for N-methylthiabendazole, $C_{11}H_9N_3S$, the theoretical values are C 61.28%; H 4.08%; N 19.20%; and S 15.44%.

Thin-layer chromatography and gas chromatogram. Thin-layer plates (20 × 20 cm) were coated with a 0.3-mm layer of silica gel HF_{254} (Merck) and activated at 110° for 2 h before use. Portions (1 μl) of acetone solutions containing 25 μg of thiabendazole or the methyl derivative were spotted on a plate, and development was carried out with benzene–methanol (38:3, v/v) in an equilibrated tank; the R_F values were 0.32 for thiabendazole and 0.43 for the methyl derivative. The methyl derivative gave a single peak on the gas chromatogram, with a retention time of 0.52 relative to 4,4'-dinitrobiphenyl; the relative retention time for thiabendazole was 0.47. The peak characteristics were as shown in Fig. 1.

Ultraviolet absorption spectrum. As shown in Fig. 5, each methanol solution of thiabendazole or the methyl derivative has a different characteristic UV spectrum. That of thiabendazole has a maximum at 302 nm, two shoulders (one slight at 294 nm and the other distinct at 310 to 314 nm) and other maxima at 243 and 235 nm. The spectrum of the methyl derivative has maxima at 298 and 236 nm and between them plateaux at 244 and 270 nm; it has no shoulder at 310 to 315 nm. The molar absorptivity of thiabendazole and the methyl derivative were 24,924 at 302 nm and 19,350 at 298 nm, respectively.

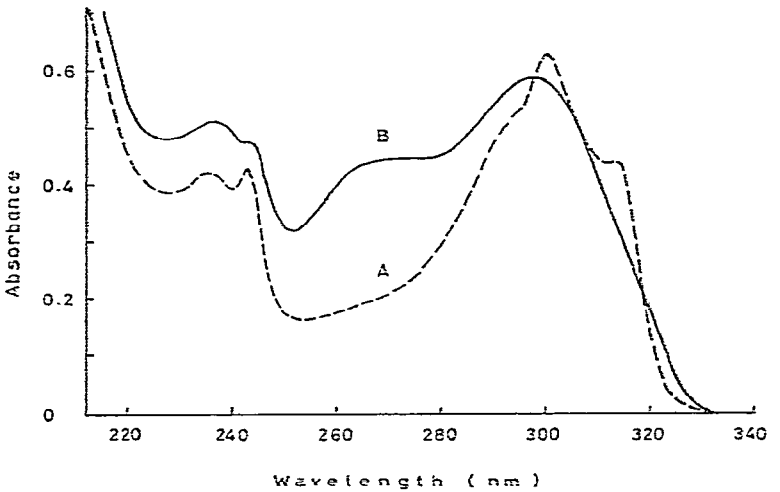

Fig. 5. UV spectra of thiabendazole (A) and the methyl derivative (B), at concentrations of 5 ppm in methanol. Spectra were recorded in 10-mm cells.

Infrared spectrum. The IR spectra (potassium bromide technique) of thiabendazole and the methyl derivative are shown in Fig. 6. The intense absorption at 740 cm^{-1} in both spectra shows that no substitution occurred in the benzene ring during methylation. With the spectrum of the methyl derivative, the absorption at 1580 cm^{-1} in the thiabendazole spectrum has disappeared, and a weak absorption at 2940 cm^{-1} is observed. This may be ascribed to alkylation in the N-position of thiabendazole.

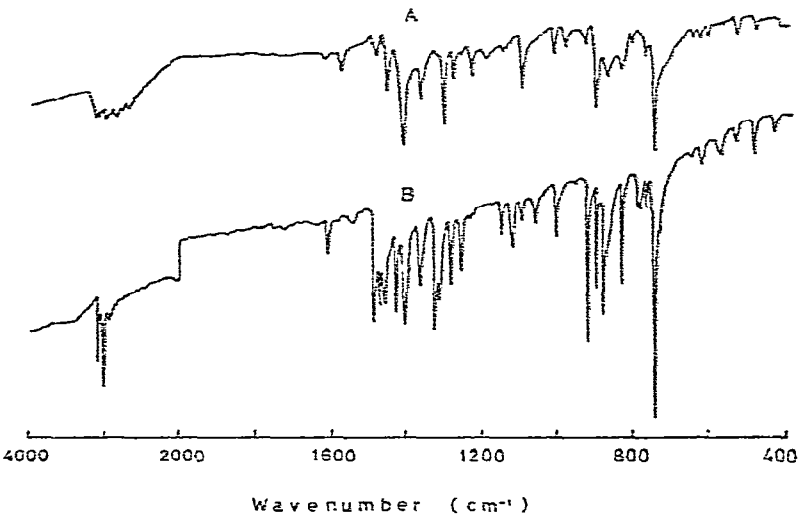

Fig. 6. IR spectra of thiabendazole (A) and the methyl derivative (B) in potassium bromide.

Mass spectrum. The mass spectrum of thiabendazole exhibited ion peaks at *m/e* 201 (M^+), 174 (M^+ — HCN), 129 (—SCH), 117 (—C) and 90 (—HCN). The fragmentation pattern of the methyl derivative is as shown in Fig. 7, *viz.*, *m/e* 215 (M^+), 188 (M^+ — HCN), 155 (—SH), 143 (—C), 131 (—C), 104 (—HCN) and 90 ($-CH_2$). The parent peak (*m/e* 201) for thiabendazole and that at *m/e* 215 for the methyl derivative correspond to the molecular weight of each compound. The shift of the peaks from *m/e* 201 to 117 for thiabendazole and that from 215 to 131 for the

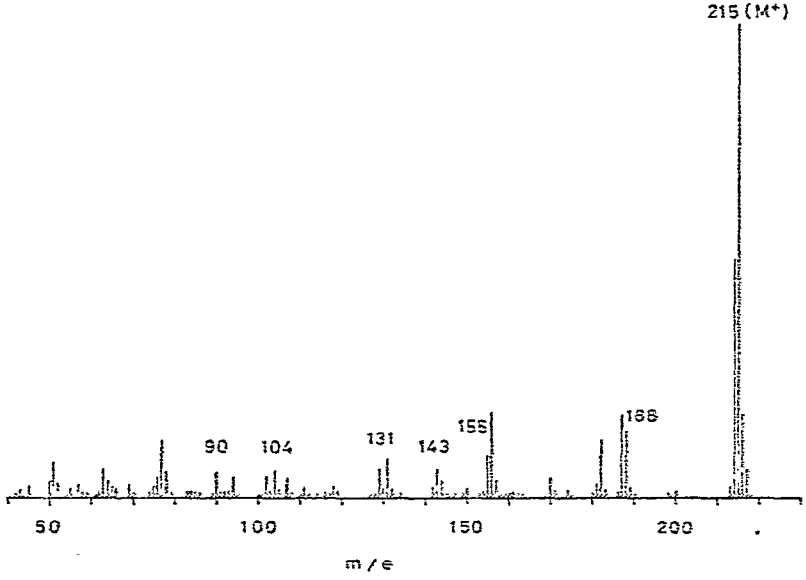

Fig. 7. Mass spectrum of the methyl derivative of thiabendazole: sample temperature, 290°.

methyl derivative could be attributed to initial degradation of thiazole ring, and the subsequent shift from 131 to 90 for the methyl derivative could be ascribed to N-demethylation.

Nuclear magnetic resonance spectrum. In the NMR spectrum of thiabendazole dissolved in dimethyl sulphoxide, signals appear at $\delta = 8.48$ and 9.31 ppm (doublet; 2H; $J = 2$ Hz) and at $\delta = 7.10$–7.82 ppm (multiplet; 4H); this is indicative of a heterocyclic compound. As shown in Fig. 8, the spectrum of the methyl derivative dissolved in the same solvent additionally shows a significant singlet at $\delta = 4.22$ ppm (3H), which suggests a signal of due to the N-methyl group. The difference in the chemical shifts makes it possible to distinguish both thiabendazole and the methyl derivative.

From this series of experiments, it was concluded that the methyl derivative was N-methylthiabendazole.

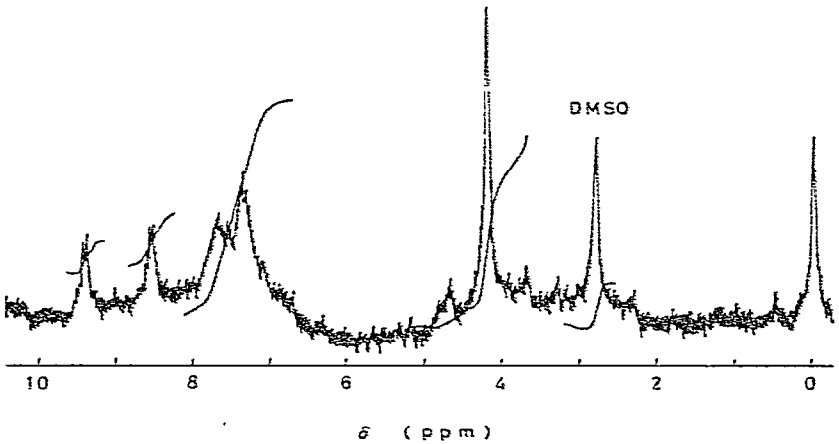

Fig. 8. NMR spectrum of the methyl derivative of thiabendazole in dimethyl sulphoxide (DMSO) at 60 Hz; the internal standard is tetramethylsilane.

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