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DETERMINATION OF PENICILLIC ACID AND PATULIN BY GAS–LIQUID CHROMATOGRAPHY WITH AN ELECTRON-CAPTURE DETECTOR

YOSHINORI FUJIMOTO, TOSHIMASA SUZUKI and YŌJI HOSHINO
Institute of Public Health, Kamiōkubo, Urawa (Japan)
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SUMMARY

Quantitative and concurrent determinations of penicillic acid and patulin have been achieved after separation of these compounds, as their trimethylsilyl derivatives, by gas–liquid chromatography with electron-capture detection and a column of 10% of DC-200 plus 15% of QF-1 on Gas-Chrom Q; the minimum determinable amounts of penicillic acid and patulin were 0.05 and 0.1 ng, respectively. The procedure devised for determining these compounds in grain involves extraction with solvents, followed by further clean-up by thin-layer chromatography; the detection limit of this procedure is about 0.02 ppm, and recovery at the level of 0.05 ppm is about 90%. The significant separation of penicillic acid and patulin produced in moldy rice is demonstrated on the gas chromatograms, and this makes possible a micro-assay for these toxic compounds in contaminated grain.

INTRODUCTION

A sensitive analytical method for determining penicillic acid and patulin is needed for monitoring these toxins in food products, and several colorimetric and paper and thin-layer chromatographic methods have been devised. Recently, a precise and sensitive gas chromatographic method has been described[1–5]. Penicillic acid and patulin have been assayed as trimethylsilyl (TMS) ether derivatives[1–3,5] or as acetate derivatives[1,2] by gas–liquid chromatography (GLC) with a flame ionization detector (FID). The TMS derivatives were quantitatively detected with a limit of 100 ng for patulin[1], 30 ng for penicillic acid and 50 ng for patulin[2] or 25 ng (ref. 3) or 10 ng (ref. 5) for both compounds.

Pohland *et al.*[1] made a brief study with an electron-capture detector (ECD) and the chloroacetate derivative of patulin, and found a lower detection limit of about 12 ng of patulin; however, they did not regard this improvement in sensitivity as sufficient to warrant further work.

In order to evaluate currently available GLC techniques, an investigation of the behaviour of the TMS derivatives of penicillic acid and patulin was carried out with ECD. By varying the experimental parameters, we have improved the sensitivity and precision of the method, and have been able to determine both penicillic acid and patulin in fungally contaminated grain.

MATERIALS AND METHODS

Reagents and solvents

Penicillic acid and patulin were of a special high grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan). The stock solution was prepared by dissolving 1 mg of penicillic acid or patulin in 10 ml of benzene–acetonitrile (49:1) and was diluted with benzene for use.

The silylating reagents used were N,O-bis(trimethylsilyl)acetamide (BSA)–benzene (1:25) and BSA–trimethylchlorosilane–benzene (1:1:25, v/v); they were of high grade and were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). In preliminary experiments[6], it had been established that the reactivity of these two reagents was almost the same, and in the present work the former was generally used.

The internal standard solution for GLC was prepared by dissolving 1.5 μg of β-BHC in 1 ml of benzene. The column-packing materials for GLC, *viz.*, Gas-Chrom Q, OV-17, SE-30, QF-1 and DC-200, were of high purity and were obtained from the Nishio Industrial Co. (Tokyo, Japan). The other reagents and solvents were of high purity and were obtained from Wako Pure Chemical Industries.

Preparation of TMS derivatives

The solution or purified extract containing penicillic acid or patulin was placed in a 10-ml test-tube with a ground-glass stopper, and the solvent was removed by evaporation under reduced pressure. To the dried residue was added 0.5 ml of the TMS reagent, and the reaction was allowed to proceed at room temperature for 10–15 min (although it was usually complete in 10 min). The products were stable in the reaction mixture for at least 2 weeks if stored at 5° (ref. 6). The sample solution for GLC was prepared by adding 0.5 ml of internal standard solution to 0.5 ml of the mixture of TMS derivatives, and 1 μl of the final solution was injected into the gas chromatograph.

Gas–liquid chromatography

A Shimadzu GC-4BM gas chromatograph with an electron-capture detector (^{63}Ni; 10 mCi) was used, with a glass column (2.0 m $\times$ 3 mm I.D.) packed with a 1:1 (v/v) mixture of DC-200 (10%, w/w) and QF-1 (15%, w/w) each absorbed on Gas-Chrom Q (80–100 mesh). The column was conditioned at 175°, and the detector and injector temperatures were 190°. The nitrogen carrier gas flow-rate was 60 ml per min, and the electrometer range was 10^2 MΩ $\times$ 0.16 V.

Standard curves

A series of working standards was prepared by diluting the stock solution with benzene, and aliquots were placed in test-tubes and the solvent was removed. After silylation by addition of 0.5 ml of the TMS reagent, 0.5 ml of the internal standard solution was added, and a 1.0 μl of the mixture was injected into the GLC column. The concentration range of the penicillic acid standard was 0.2 to 1.2 ng per μl and that of patulin was 0.1 to 2.5 ng per μl. The retention times of the TMS derivative of penicillic acid and that of patulin relative to β-BHC were 0.36 and 0.78, respectively. The peak-height ratios of penicillic acid and patulin to β-BHC were plotted against the amounts of the toxins injected; typical standard curves are shown in Fig. 1.

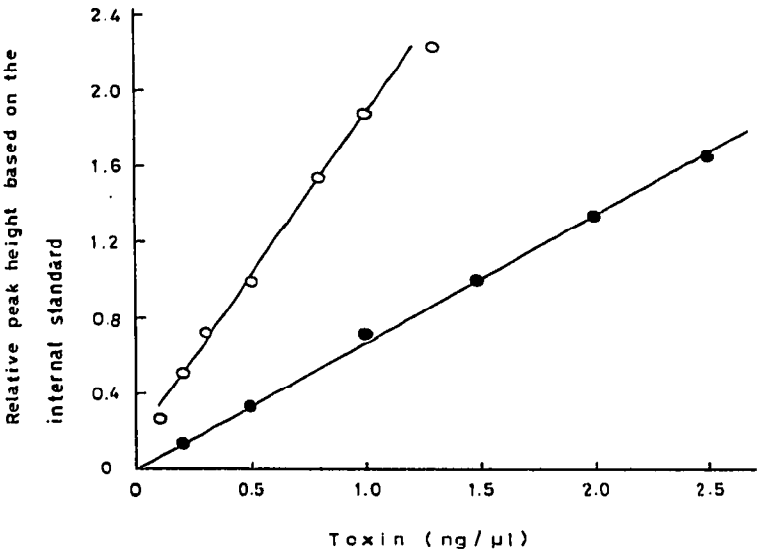

Fig. 1. Calibration curves for penicillic acid and patulin; the detector response was measured as the relative peak height based on the internal standard (β-BHC; 1.5 ng per μl in benzene). ○, Penicillic acid; ●, patulin. The GLC conditions were as for (c) in Fig. 2.

Extraction procedures

Method 1. To 20 g of finely ground sample in a 500-ml flask with a ground-glass stopper were added 100 ml of methanol–5% aq. sodium chloride (1:1, v/v) and 75 ml of *n*-hexane; after vigorous shaking for 30 min, the methanol–sodium chloride layer was filtered, and 50 ml of the filtrate were washed with 30 ml of *n*-hexane. From the washed solution, methanol was removed by evaporation at below 50°, and residual solution was cooled and extracted with two 25-ml portions of ethyl acetate. The ethyl acetate extract was dried with anhydrous sodium sulphate, then evaporated at below 50°, and the residue was dissolved in 0.5 ml of benzene–methanol (97:3, v/v) for clean-up by TLC.

Method 2 (for samples that contain only patulin). To 20 g of finely ground sample in a 500-ml flask with a ground-glass stopper was added 200 ml of ethyl acetate, and the flask was vigorously shaken for 30 min; the extraction was repeated with another 200 ml of ethyl acetate. The combined extracts were evaporated to dryness at below 50°, and the residue was dissolved in 50 ml of acetonitrile (saturated with *n*-hexane) and washed with 25 ml of *n*-hexane, the *n*-hexane washings being extracted with 15 ml of acetonitrile saturated with *n*-hexane. The combined acetonitrile extracts were re-washed with 25 ml of *n*-hexane and then dried with anhydrous sodium sulphate; the solvent was evaporated to dryness under reduced pressure, and the residue was dissolved in 0.5 ml of benzene–methanol (97:3, v/v) for clean-up by TLC.

Clean-up by TLC

This stage was introduced in order to eliminate interferences with the subsequent GLC. Thin-layer plates (20 × 20 cm) were coated with a 0.3-mm layer of Mallinckrodt AR-7GF silica gel or Kieselgel G (Merck) and activated at 100° for

2 h before use. With a 100-μl micro-syringe, half of the 0.5 ml of benzene–methanol extract was applied as a streak about 9 cm long and 3 cm from the lower edge of the plate; about 3 cm apart on the same line, 5 μl of the stock solutions of penicillic acid and patulin were spotted for reference. The plate was then developed with benzene–methanol–acetic acid (18:1:1, v/v) in an equilibrated tank, and after the solvent front had travelled about 10 cm, the plate was removed and dried at room temperature. That part of the layer containing the standards was lightly sprayed with 1% aqueous ammonia and then with 4% aqueous phenylhydrazine hydrochloride and dried under a gentle stream of warm air from a hand-drier. Patulin gave a yellow spot and penicillic acid a yellow fluorescence in 360-nm radiation. The R_F values of both compounds were identical (about 0.25). A band of adsorbent about 2.5 cm wide was scraped from the unsprayed area of the plate at the same R_F as that of the standards, and the penicillic acid and patulin were eluted from the silica gel with 10 ml of benzene–methanol (97:3, v/v). The eluate was transfered via a sintered-glass filter to a 10-ml flask with a ground-glass stopper, and was evaporated to dryness.

Determination

The material obtained as a dry solid after clean-up by TLC was directly silylated and analyzed by GLC as described above. The contents of penicillic acid and patulin in unknown samples were determined from the peak heights relative to the internal standard on the gas chromatograms, comparing them with those of the standard curves.

RESULTS AND DISCUSSION

Standard assay

For the GLC assay using the procedure described, there was a linear relationship between detector response and amount of the TMS derivative of penicillic acid or patulin. As shown in Fig. 1, calibration graphs were linear for 0.2 to 1.2 ng of penicillic acid and 0.1 to 2.5 ng of patulin. The minimum determinable amounts of penicillic acid and patulin were 0.05 and 0.1 ng, respectively, and the average deviations in values were 3.0% for 0.2 ng of penicillic acid and 2.7% for 0.5 ng of patulin.

Gas chromatographic sensitivity

The gas chromatograms of the TMS derivatives of penicillic acid and patulin were highly dependent on the column packings, carrier gas flow-rate and temperature. Columns containing OV-17 (5%, w/w), SE-30 (5%, w/w), QF-1 (8%, w/w), DC-200 (10%, w/w), and DC-200 (10%, w/w) plus QF-1 (15%, w/w) (1:1, v/v), each adsorbed on Gas-Chrom Q, were tested. As shown in Fig. 2, the good peak characteristics and sensitivity were achieved on DC-200, or DC-200 plus QF-1, with a nitrogen flow-rate of 60 ml per min and a column temperature of 175°.

In Figs. 3 and 4 are shown curves of peak height *vs.* retention time obtained at various nitrogen flow-rates and temperatures on a column of DC-200 plus QF-1. The retention times of the derivatives of penicillic acid and patulin for β-BHC were 0.36 and 0.78, respectively (relative to β-BHC), and these values were independent of nitrogen flow-rate (40 to 90 ml per min) and column temperature (160 to 205°). The highest peaks occurred with a nitrogen flow-rate of 60 ml per min, and the higher the

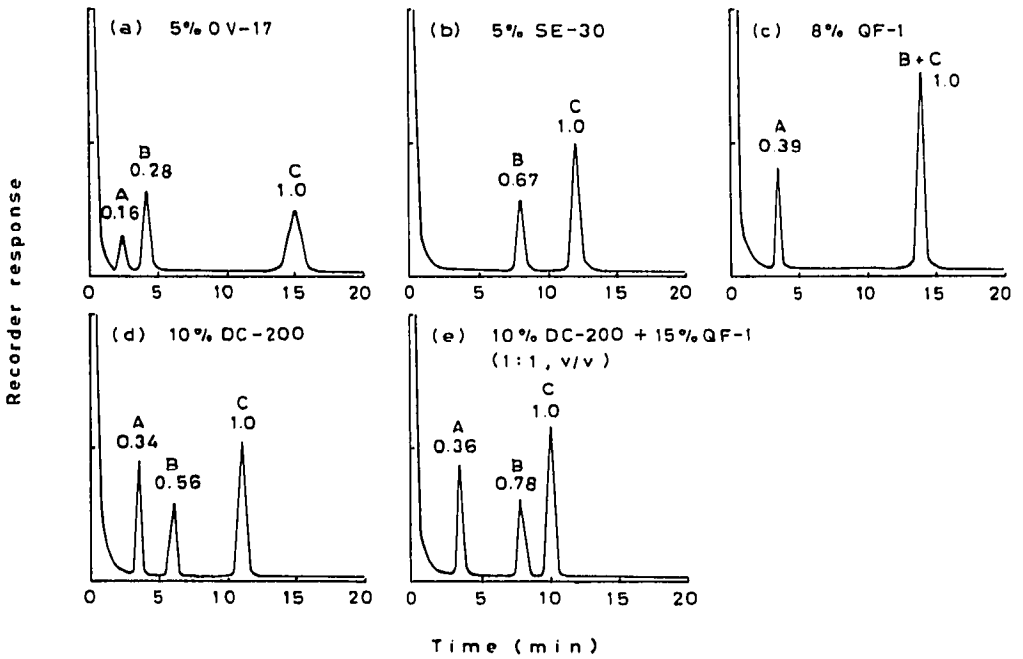

Fig. 2. Gas chromatograms of the TMS derivatives of penicillic acid (A, 0.30 ng) and patulin (B, 0.75 ng), with relative retention times relative to that of the internal standard (C, β-BHC, 1.5 ng). The sample size was 1 μl, and the nitrogen flow-rate was 60 ml per min. The temperatures of the column and the detector and injector were: (a) 165° and 180°; (b) 150° and 165°; (c) 160° and 175°; (d) 175° and 190°; and (e) 175° and 190°.

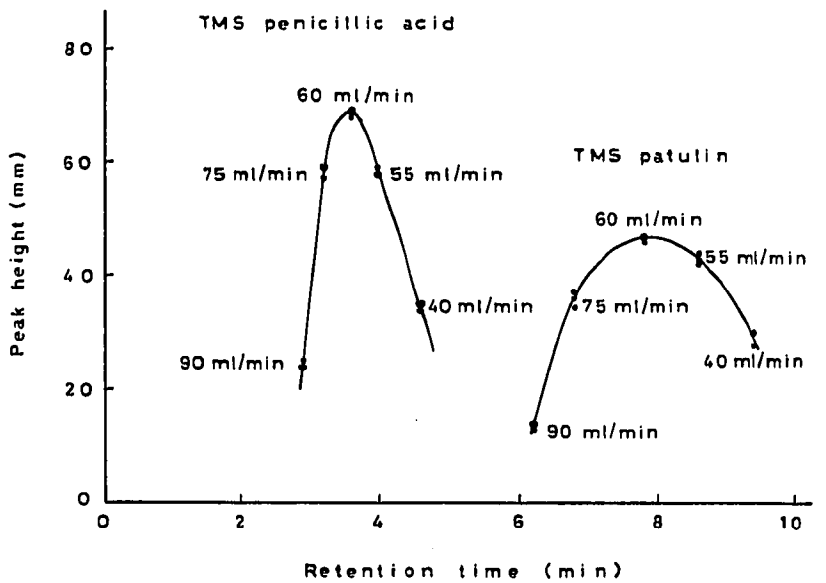

Fig. 3. Curves of peak height *vs.* retention time on the gas chromatograms of the TMS derivatives of penicillic acid and patulin at the nitrogen flow-rates indicated. The GLC conditions were as for (c) in Fig. 2.

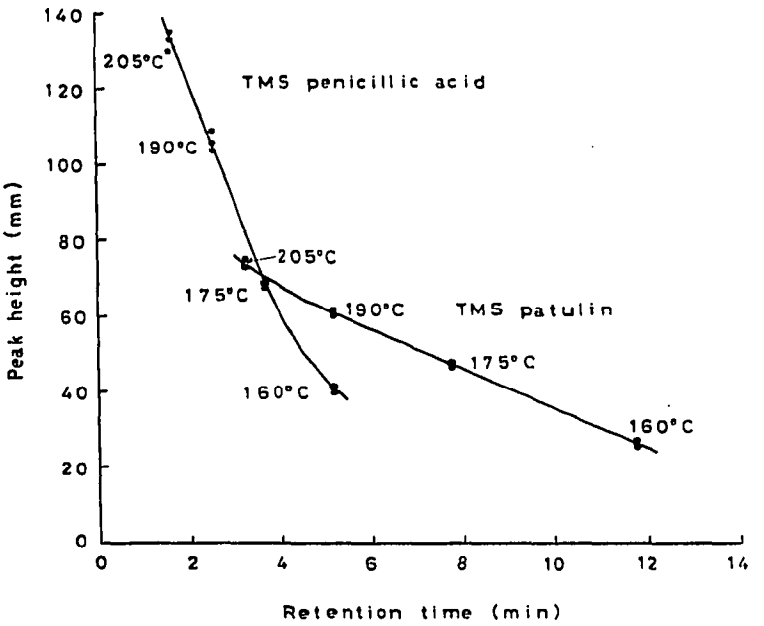

Fig. 4. Curves of peak height *vs.* retention time on the gas chromatograms of the TMS derivatives of penicillic acid and patulin at the column temperatures indicated. The GLC conditions were as for (c) in Fig. 2, except that both the detector and injector were at 220°.

column temperature, the better was the sensitivity. It is noteworthy that there was a linear relationship between peak height and retention time for column temperatures between 160 and 205°. At higher temperatures, the peak characteristics were somewhat unstable, especially for penicillic acid, so that for the concurrent detection of penicillic acid and patulin, GLC was carried out at 175° with a nitrogen flow-rate of 60 ml per min.

Extraction and clean-up procedures, and recovery

Because GLC with ECD was very sensitive, the extraction and clean-up procedures proposed by others were not always adequate. Several workers[2,3] reported that, if the concentrations of penicillic acid and patulin in crude extracts were high, they could be determined directly by GLC with FID; however, there were many difficulties associated with analyses by GLC with ECD, because of interfering background material. On the gas chromatograms of the silylated crude extracts after the extraction by method 1 or 2, the peaks of the derivatives of penicillic acid and patulin were subject to interference from unknown contaminants; removal of such interference was achieved by the TLC clean-up procedure described, and the silylated extracts gave the gas chromatograms with good peak characteristics (see Figs. 5 and 6).

The recoveries of both penicillic acid and patulin added at the level of 0.05 ppm to various samples are shown in Table I. In the concurrent determinations in which the extraction procedure of method 1 was used, the recoveries of penicillic acid were greater than 87.6%, whereas those of patulin were generally less than 80%. Therefore, extraction method 1 was not always suitable for the determination of

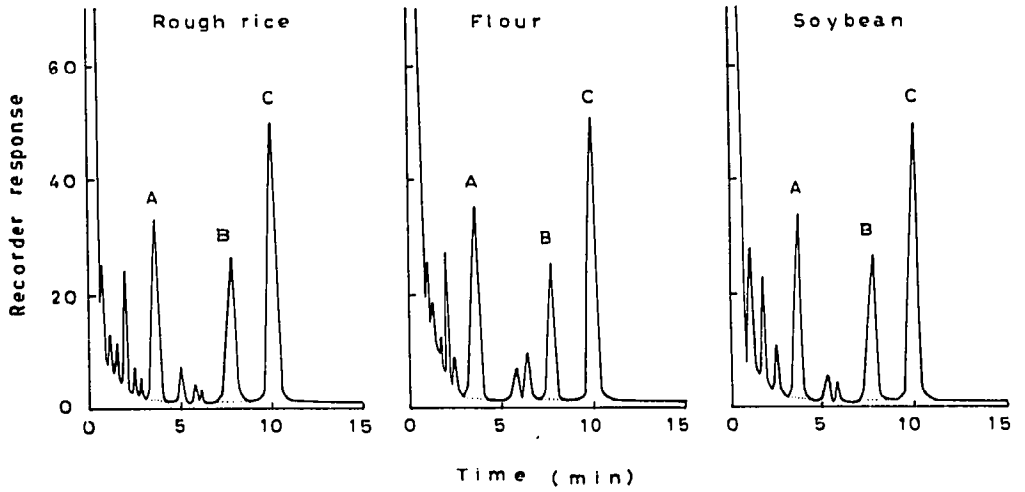

Fig. 5. Gas chromatograms of the silylated extracts of rough rice, flour and soybean, to which penicillic acid and patulin were added at the level of 0.05 ppm. The sample size was 1 μl. A, TMS derivative of penicillic acid; B, TMS derivative of patulin; C, β-BHC (1.5 ng/μl). The dotted lines show the shape of chromatograms for samples to which penicillic acid or patulin was not added.

patulin. When extraction method 2 was applied to patulin, recoveries ranged from 98.0 to 85.0%.

By use of the procedures described, analyses were carried out with a detection limit of 0.02 ppm. Although an extraction should lead to high recovery, the low detec-

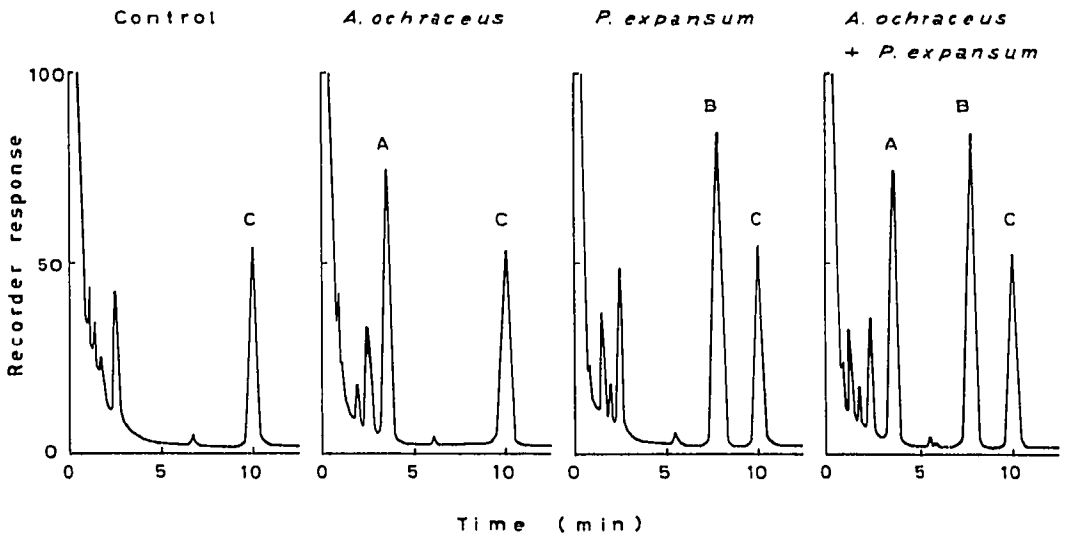

Fig. 6. Gas chromatograms of the silylated extracts of moldy rough rice. The mold was incubated at 25° for 10 days, and the sample size was 1 μl. A, TMS derivative of penicillic acid; B, TMS derivative of patulin; C, β-BHC (1.5 ng/μl).

TABLE I

PERCENTAGE RECOVERIES OF PENICILLIC ACID AND PATULIN ADDED TO VARIOUS SAMPLES AT THE LEVEL OF 0.05 ppm

Each result is the average of five determinations.

Sample	*Extraction and clean-up by*		
	*Method 1 and TLC**		*Method 2 and TLC***
	Penicillic acid	*Patulin*	*Patulin*
Diluted stock solution	99.0	86.0	98.0
Rough rice	88.8	78.3	91.9
Flour	87.6	74.4	83.4
Soybean	91.6	79.8	85.0

* Concurrent determination of penicillic acid and patulin.
** Patulin only.

tion limit and the good reproducibility permitted the use of the proposed procedures for the concurrent detection and determination of micro amounts of penicillic acid and patulin.

Application

The assay procedure proposed here has been used for determining penicillic acid and patulin produced in mouldy rice. *Aspergillus ochraceus* NHL-5132 and *Penicillium expansum* NHL-6152 were individually grown in sterilized rough rice at 25° for 10 days, and equal volumes of the moldy rices in each flask were combined. The samples were extracted according to method 1 and cleaned-up by the TLC technique described. The gas chromatograms of TMS derivatives in moldy rice extracts are shown in Fig. 6. The column packings were DC-200 plus QF-1 on Gas-Chrom Q, and the retention times of the distinct peaks (relative to β-BHC) were 0.36 and 0.78. Moreover, when a column containing 10% of DC-200 on Gas-Chrom Q was used, the relative retention times of two significant peaks on the chromatograms were 0.34 and 0.56. Thus, the products in moldy rice were identified, from their retention times, as penicillic acid and patulin.

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