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A GAS CHROMATOGRAPHIC METHOD FOR THE MYCOTOXIN PENICILLIC ACID*

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SUMMARY

Penicillic acid was satisfactorily resolved by gas chromatography either as the trimethylsilyl derivative or in the underivatized form using 3% of Dexsil 300, 3% of OV-17 or 3% of OV-25 liquid phase on Gas-Chrom Q. The limit of quantitation was approximately 25 ng. Extraction and detection of penicillic acid together with another important mycotoxin, patulin, was demonstrated in moldy corn. In addition, a preliminary clean-up procedure using thin-layer chromatography is described for crude extracts, which improves resolution and sensitivity in the gas chromatographic analysis of penicillic acid.

INTRODUCTION

Penicillic acid has been reported to be a mammalian toxicant[1] and carcinogen[2]. It is produced by several fungi that also produce another carcinogen, patulin[2]. In the past, patulin has received more attention because of its higher acute toxicity to animals (LD_{50} = 5 mg/kg). However, both patulin and penicillic acid are carcinogenic to rodents on long-term exposures at 0.2 and 2 mg/kg, respectively. As these toxins may contaminate a variety of agricultural commodities, sensitive analytical methods are needed for monitoring food products.

Several colorimetric and paper and thin-layer chromatographic methods have been published for penicillic acid[3] and patulin[4-6]. However, the sensitivity of colorimetric methods is relatively poor owing to color instability of the derivative produced by reaction with the detecting reagent. At present, the best method available for analysis of penicillic acid is a fluorodensitometric assay with a detection limit of

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1–3 μg (ref. 7). Patulin, on the other hand, has been satisfactorily analyzed as a trimethylsilyl (TMS) ether or acetate derivative using gas–liquid chromatography (GLC) with a detection limit of 50–80 ng (ref. 8). The purpose of this study was to evaluate GLC techniques, with their inherent advantages of resolution and sensitivity, for the quantitative analysis of penicillic acid. In addition, the feasibility of simultaneous detection of pencillic acid and patulin in crude extracts was investigated.

EXPERIMENTAL

Analytical standards

Penicillic acid (γ-keto-β-methoxy-δ-methylene-$\Delta\alpha$-hexenoic acid) was supplied gratis by Dr. THOMAS G. PRIDHAM, Northern Regional Research Laboratory, Peoria, Ill., U.S.A. Patulin [4-hydroxy-4H-furo-(3,2*c*)-pyran-2(6H)-one] was supplied gratis by Dr. ROBLEY LIGHT, Florida State University, Tallahassee, Fla., U.S.A. Purity was confirmed by melting-point determinations and thin-layer chromatography (TLC). Identity of the standards was verified by infrared and mass spectral analyses.

Gas chromatography

A Model 2100 Varian-Aerograph gas chromatograph with a flame-ionization detector, and an accessory Model 480 Varian-Aerograph digital integrator with printout, were used for all analyses. OV-17, OV-25 and Dexsil 300 (3%, w/w, of each) were absorbed on Gas-Chrom Q, 100–120 mesh, and packed into glass U tubes (5 ft. $\times$ 2 mm I.D.). The columns were conditioned for 24 h at 250°. The detector and injector temperatures were 300°; the nitrogen carrier gas flow-rate was 25 ml/min; the hydrogen flow-rate was 35 ml/min; the oxygen flow-rate was 450 ml/min; and the electrometer range was 10^{-11} A/mV.

Method

GLC of penicillic acid was carried out at concentrations of 1–5 μg/μl using tetrahydrofuran, pyridine, absolute ethanol, chloroform or diethyl ether as the solvent. TMS derivatives of both penicillic acid and patulin were prepared with N,O-bis(trimethylsilyl)acetamide (BSA) and trimethylchlorosilane in pyridine (3:1:9, v/v). The reactions were completed within a few minutes and the products were stable in the reaction mixture for two weeks if the derivatives were stored at 5° when not in use.

Moldy corn and rice used to test the applicability of the method to biological samples were extracted with 3% of methanol in chloroform. The crude extracts were silylated at concentrations of 100 μg/μl. If the concentrations of penicillic acid or patulin in the crude extracts were 1% or greater, they were quantified directly. When the concentration was less than 1% of total solid extractives, a preliminary clean-up was effected using preparative 500-μm Silica Gel G TLC plates (Analtech). In this case, 75 mg of crude extract dissolved in chloroform was streaked on to the plate and chromatographed in methanol–chloroform (10:90) in unequilibrated tanks. The silica gel between R_F 0.75 and 0.95 was scraped quantitatively from the plate and the toxins (penicillic acid and patulin) were eluted from the gel with acetone. The acetone was removed by evaporation with air and the dried residue silylated directly. The toxin content of moldy corn was calculated as follows:

$$\frac{\text{Total mg } CHCl_3 \text{ extract} \times \text{g toxin found/75 mg } CHCl_3 \text{ extract}}{\text{Total g corn extracted}} = \frac{\mu\text{g toxin}}{\text{g corn}}$$

Two other TLC solvent systems were used to verify GLC determinations of penicillic acid or patulin in crude extracts. They were diethyl ether–hexane (75:25, v/v) and THF–benzene (20:80, v/v) with corresponding R_F values for penicillic acid of 0.24 and 0.53 and patulin of 0.47 and 0.58, respectively.

RESULTS AND DISCUSSION

Penicillic acid and patulin were resolved as trimethylsilyl derivatives with good peak characteristics on 3% of OV-17, Dexsil 300 and OV-25 by using three different temperature programs (Fig. 1). Limits of detection varied between columns,

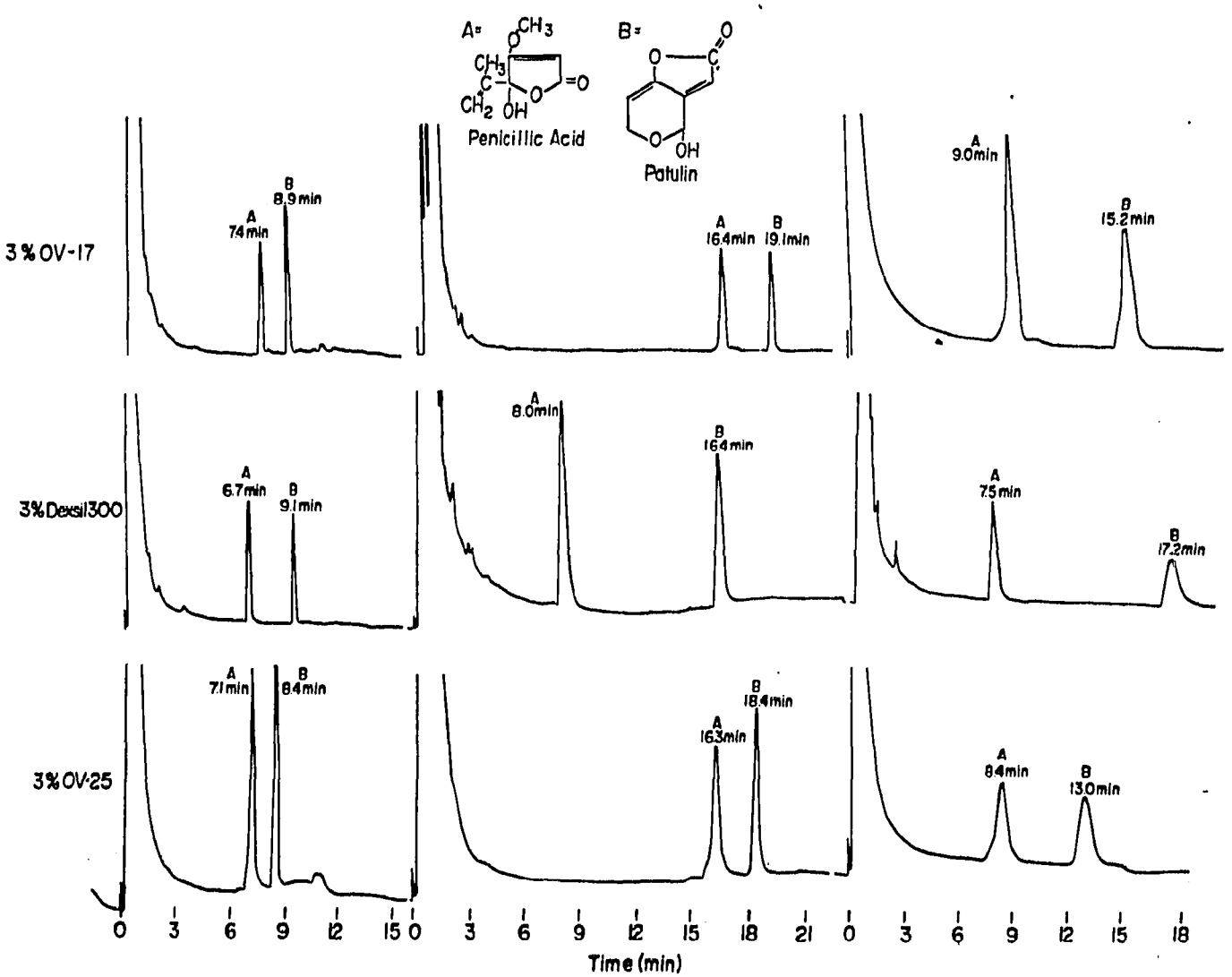

Fig. 1. Gas chromatograms of TMS derivatives of penicillic acid and patulin with retention times and structures of the compounds indicated. The temperature programs from left to right are: (1) 100–250° at 8°/min; (2) 75–250° at 4°/min; and (3) 125°, isothermal.

with the greatest sensitivity being achieved on 3% of Dexsil 300. This was apparently due to the lack of column bleed, which is a consequence and advantage of the high-temperature stability of this phase (480°). The best temperature program for quantitation and analysis was found to be 100–250° at 8°/min. The program from 75° to 250° at 4°/min resulted in considerable variation of retention times and the isothermal trial at 125° gave poorer peak characteristics for quantitation.

A linear relationship between detector response and concentration of TMS-penicillic acid or TMS-patulin was established using 3% of OV-17 (Fig. 2). The minimal quantitative detection limit for each was 0.025 μg.

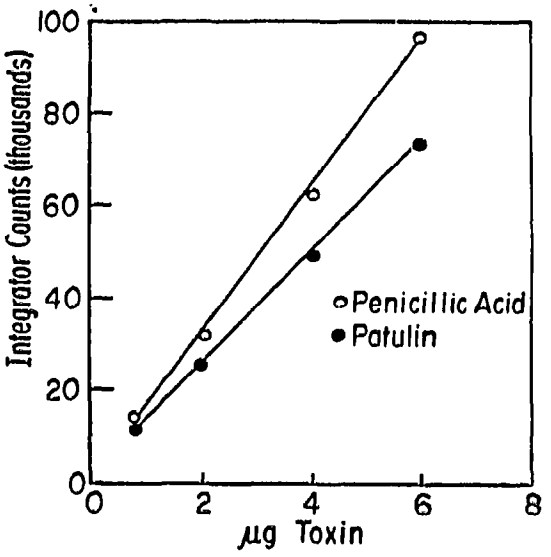

Fig. 2. Quantitative plot showing the linear relationship between the concentration of the TMS derivatives of penicillic acid and patulin with integrator response. Quantitation was carried out on 3% of OV-17 with a temperature program of 100–250° at 8°/min and 64× attenuation with sample sizes of 0.1–0.8 μg.

Underivatized penicillic acid was also analyzed by GLC. When absolute ethanol, chloroform, diethyl ether or tetrahydrofuran was used as the solvent for the toxin, a single peak was obtained at 19.8 min with the temperature program of 75–250° at 4°/min. The detection limit for this peak was about 1 μg. As underivatized penicillic acid can be chromatographed by GC, this technique could be used advantageously as confirmatory evidence of the presence of penicillic acid in experiments with mixtures of toxins. However, the poor sensitivity of the method would limit its usefulness to the analysis of crude mixtures that contain relatively high levels of penicillic acid.

Chloroform–methanol extracts of moldy rice and corn were analyzed for penicillic acid and patulin by GLC. Representative chromatograms using moldy rice extract are presented in Fig. 3. Penicillic acid was resolved satisfactorily using 3% of OV-17 and 3% of Dexsil 300. Patulin was resolved from the other moldy rice components only on 3% of OV-17, indicating the limited usefulness of both the 3% of Dexsil 300 and 3% of OV-25 columns for the analysis of the toxins in whole extracts of rice. Similar results were obtained with a chloroform–methanol extract of moldy corn. The detection limit in the crude extracts was 1 μg of each per 100 μg of extract. However, in combination with TLC preparative analysis, very minute amounts of

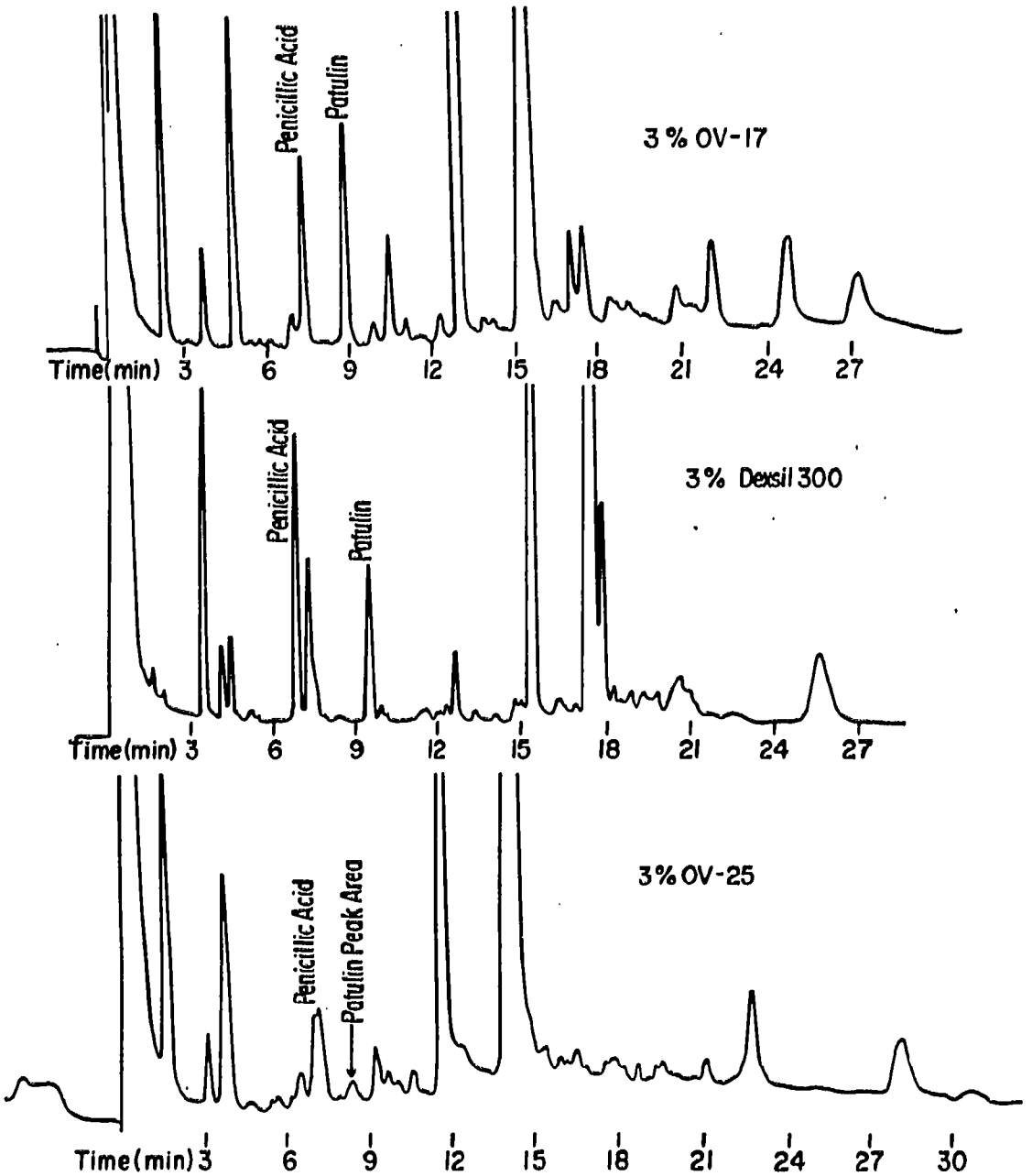

Fig. 3. Gas chromatograms of the TMS derivatives of a moldy rice extract containing penicillic acid and patulin. The temperature program was 100–250° at 8°/min. The sample size was 100 μg and the attenuation was 256×.

penicillic acid can be analyzed in food products by appropriate manipulation of sample size. This is feasible because 100% recovery of penicillic acid can be achieved with the TLC clean-up procedure.

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