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QUANTITATION OF PATULIN PATHWAY METABOLITES USING GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A gas–liquid chromatographic system which allows most of the metabolites of the patulin pathway to be separated and quantitated has been developed. The metabolites are mostly phenols, and after conversion to their trimethylsilyl derivatives they are efficiently separated on a 3.18 mm O.D. stainless-steel column of 10% QF-1 on Gas-Chrom Q using a temperature programme. The detection limits for the three phenolic acids, 6-methylsalicylic acid, *m*-hydroxybenzoic acid and gentisic acid are all markedly lower than those obtained in previous systems. The usefulness of the system has been assessed by quantitating the patulin pathway metabolites present in culture filtrates of *Penicillium urticae* NRRL 2159A.

INTRODUCTION

In an earlier study Murphy *et al.*[1] separated a number of the metabolites of the patulin pathway (*i.e.*, 6-methylsalicylic acid, *m*-cresol, toluquinol, *m*-hydroxybenzyl alcohol, gentisyl alcohol and patulin) using gas–liquid chromatography (GLC) with the following conditions: 7.5% neopentyl glycol succinate (NPGS) on 80–100 mesh Chromosorb in 3 m × 3.175 mm O.D. stainless-steel columns, programmed at 2°/min for 10 min from 120°, with a helium flow-rate of 25 ml/min. The above authors used trimethylchlorosilane (TMCS)–hexamethyldisilazane (HMDS)–pyridine (1.8:3.2:10, v/v/v) as the silylating reagent.

Recently Suzuki *et al.*[2] studied the effectiveness of various silylating reagents in the trimethylsilylation of the mycotoxin, patulin. Using the preferred reagents N,O-bis(trimethylsilyl)acetamide(BSA)–benzene (1:25) or BSA–TMCS–benzene (1:1:25) Fujimoto *et al.*[3] assayed patulin and penicillic acid found in samples of contaminated grain (10% DC-200 plus 15% QF-1 on Gas-Chrom Q in a 2 m × 3 mm I.D. glass column at 175°; 60 ml/min nitrogen; electron capture detection). Since the above authors found BSA to be a more effective silylating reagent (*i.e.*, it gave greater peak heights for equivalent quantities of patulin) than the TMCS–HMDS combination, the BSA reagent was employed in conjunction with the GLC system developed

by Murphy *et al.*[1] in an effort to quantitate all of the patulin pathway metabolites in one assay.

When BSA was used as the silylating agent, and the derivatives were run on the system described by Murphy *et al.*[1] it was found that the column was very inefficient with respect to the quantitation of 6-methylsalicylic acid (6-MSA), *m*-hydroxybenzoic acid (mHOB-acid) and gentisic acid (G-acid). In addition BSA produced a large solvent front, which completely masked the peak of the *m*-cresol derivative. It was therefore necessary to develop a new GLC system, which would give efficient and simultaneous quantitation of as many of the patulin pathway metabolites as possible. Since 6-MSA is the first detectable metabolite which is committed to the pathway[4], the sensitive detection and quantitation of this metabolite was of particular importance. The names and structures of these metabolites as well as the abbreviations used for them in this article, appear in Table I.

MATERIALS AND METHODS

Pure crystalline samples of patulin, and phyllostine and gentisyl alcohol were kindly supplied by Drs. J. W. D. Groot Wassink and J. Sekiguchi, respectively. All other patulin pathway metabolites were obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and Eastman-Kodak (Rochester, N.Y., U.S.A.), with the exception of 6-MSA which was synthesized by the method of Eliel *et al.*[5]. Some of the metabolites were recrystallized before use, and all were checked for purity by sensitive thin-layer chromatography procedures[6].

Peroxide-free diethyl ether (Mallinckrodt, Toronto, Canada) or reagent grade ethyl acetate (Fisher Scientific, Fair Lawn, N.J., U.S.A.) were used to extract metabolites from culture samples. The reagents for trimethylsilylation were either BSA–pyridine (1:1) or TMCS–HMDS–pyridine (1.8:3.2:5); they were of silylation grade and were obtained from Pierce (Rockford, Ill., U.S.A.) except for reagent grade pyridine which was obtained from Fisher and was stored over sodium hydroxide pellets at room temperature in a bottle with a ground glass stopper. After BSA was found to be unsuitable as a silylating agent, TMCS–HMDS–pyridine was used routinely. *p*-Bromophenol (reagent grade; Eastman-Kodak) was used as a convenient internal standard. Hydrogen and nitrogen gasses were L grade, and were obtained from Union Carbide (Toronto, Canada).

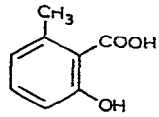
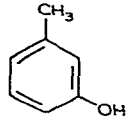
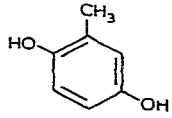
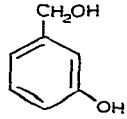
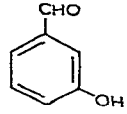
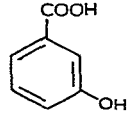
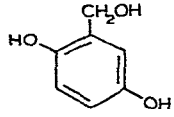
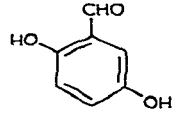
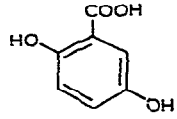
The column packing was prepared from Gas-Chrom Q (80–100 mesh, Applied Science Labs., State College, Pa., U.S.A.) coated with QF-1 (10% w/w; Applied Science Labs.) or NPGS (7.5% or 5% w/w; Chromatographic Specialties, Brockville, Canada) by the slurry and evaporation procedure. The columns were stainless steel (Analabs, North Haven, Conn., U.S.A.) and had dimensions of 1.88 m × 2.16 mm I.D. and 3.18 mm O.D. They were flushed with 300 ml of absolute methanol (reagent grade, Baker) before packing.

Sample preparation and silylation

Submerged cultures of *P. urticae* were sampled during the secondary metabolite production phase. Fermentor cultures (3.5 l) were commonly grown in yeast extract–glucose–buffer medium[7] (yeast extract, 5 g; glucose, 60 g; citrate, 9.8 g; Na_2SO_4, 1.0 g; KH_2PO_4, 13.6 g; 10 ml of trace metal solution[8] per l of distilled water; pH

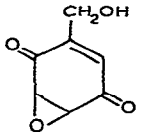
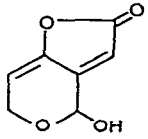
TABLE I

VARIOUS ANALYTICAL PARAMETERS FOR PATULIN PATHWAY METABOLITES

Name	*Metabolite structure*	*Extraction (%)**	*Representative** retention time (min)*		*R for QF-1*** ($\mu g \cdot ml^{-1}cm^{-1}$)*	*Detection limit§ for QF-1 ($\mu g/ml$)*
			QF-1	*NPGS*		
6-Methylsalicylic acid (6-MSA)		96	15.6	19.4	95.4	7
m-Cresol		94	3.9	4.8	47.4	4
Toluquinol		94	10.9	11.4	51.6	4
m-Hydroxybenzyl alcohol (mHOBz-alc)		94	10.9	12.6	51.6	4
m-Hydroxybenzaldehyde (mHOB-ald)		92	11.9	14.8	90.6	6
m-Hydroxybenzoic acid (mHOB-acid)		96	14.7	—	88.1	6
Gentisyl alcohol (G-alc)		78	15.6	16.8	80.1	6
Gentisaldehyde (G-ald)		91	20.5	22.2	85.3	7
Gentisic acid (G-acid)		92	19.2	—	159.4	11

(Continued on p. 20)

TABLE I *(continued)*

Name	*Metabolite structure*	*Extraction (%)* *	*Representative** retention time (min)*		*R for QF-1*** ($\mu g \cdot ml^{-1} cm^{-1}$)*	*Detection limit§ for QF-1 ($\mu g/ml$)*
			QF-1	*NPGS*		
Phyllostine		99	18.4	23	261.6	18
Patulin		93	22.8	32.9	166	12

* Percent of metabolite recovered from 1 ml of culture filtrate after extracting with two 4-ml portions of diethyl ether.

** Exact retention times vary from column to column of the same composition.

*** Representative values of the ratio C_x'/H_x' (defined in eqn. 1) for a particular column in which QF-1 was the liquid phase; when the liquid phase was NPGS, values of R were generally greater.

§ Metabolite concentrations of culture filtrates ($\mu g/ml$) which were less than these values, did not yield an accurate peak height using columns prepared from QF-1; the detection limits for NPGS were generally greater.

adjusted to 6.5 with NaOH). After vacuum filtration through Whatman No. 4 filter paper, a 1-ml aliquot of culture filtrate was acidified to pH 1–2 with 4 *N* HCl and extracted with two 4-ml portions of anhydrous diethyl ether. Although ethyl acetate is usually an excellent solvent for such extractions, citrate was found to be extracted and subsequently silylated to yield an unacceptably large, spurious GLC peak. This did not occur with ether extractions. After drying over anhydrous sodium sulphate the ether was removed by vacuum evaporation at 37° in an Evapo Mix water bath (Buchler). This gave a thin layer of gummy residue which adhered to the walls of the test tube. Typical metabolite recoveries are given in Table I.

Silylation of the above dried culture extracts or, one or more pure metabolites (0.5 mg of each), was accomplished by adding 0.5 ml of pyridine, containing 1 mg/ml of *p*-bromophenol, and either 0.18 ml of TMCS and 0.32 ml of HMDS or 0.5 ml of BSA and allowing the mixture to react for about 20 min at room temperature. Longer reaction times did not increase the peak height. Storage of either of these reaction mixtures for two days at 4°, resulted in a decrease of less than 3% in the amount of all products except the phyllostine derivative which decomposed to two peaks in less than 24 h. The precipitate of ammonium chloride in the reaction mixture could be removed by centrifugation (1,500 *g* for 10 min) if necessary (see below). One microliter of the reaction mixture was injected directly into the gas chromatograph.

In order to eliminate the effect of unknown compounds present in the extract of culture filtrates the residue from a culture filtrate (prepared as described above) was silylated with TMCS–HMDS–pyridine in the normal manner. The reaction mixture was centrifuged, and 1 μl of supernatant was injected into a 10% QF-1 column.

A 0.5-ml aliquot (*i.e.*, half) of this reaction mixture was then diluted two-fold by the addition of silylating reagent (0.18 ml TMCS and 0.32 ml HMDS), and after 20 min was centrifuged and injected as before. Half of this second reaction mixture was again diluted in the same manner to yield an overall four-fold dilution of the original solution. Centrifugation and injection of this third solution was carried out as before. Measurement of the various peaks for each of the three serially diluted samples showed the expected decrease in peak height (*i.e.*, peak heights for the third sample were a quarter of the heights obtained for the first sample, within an acceptable error). This indicated that, using the standard procedure, silylation of the metabolites in crude residues was not inhibited by other residue constituents.

Gas–liquid chromatography

Chromatographic separations were carried out using a Hewlett Packard 5710A gas chromatograph equipped with dual columns prepared as described under Materials and Methods, a flame ionization detector, and a Hewlett Packard 7123 strip-chart recorder. The nitrogen carrier gas (60 p.s.i.) flow-rate was 30 ml/min (QF-1 column) or 27 ml/min (NPGS column), while the flow-rates for hydrogen (15 p.s.i.) and compressed air (27 p.s.i.) were 30 and 240 ml/min, respectively. Both columns were conditioned overnight at 220°, and the optimum temperature programs were found to be 120° for 2 min (QF-1 column) or 4 min (NPGS column), followed by a 4°/min increase in temperature to 200° which was maintained for 4 min (QF-1 column) or 16 min (NPGS column). The injection port and detector were operated at 200° and the instrument was set at range 10 and attenuation 32.

Linearity of silylation and instrument response

The linearity of silylation using TMCS–HMDS–pyridine followed by chromatography of the derivatives on a 7.5% NPGS column was checked in the following manner. A stock solution of 2 mg/ml each of 6-MSA, mHOBz-alc, mHOB-ald, G-ald, patulin and *p*-bromophenol in pyridine (*i.e.*, a representative selection of the compounds to be silylated) was prepared. An aliquot (0.5 ml) of this solution, as well as serial dilutions, all yielding a final volume of 0.5 ml of pyridine, were silylated by the addition of 0.18 ml TMCS and 0.32 ml HMDS, and chromatographed after centrifugation, using 1-μl injections. The sum of all the peak heights obtained for one sample was plotted against the total amount of all of the compounds present in that sample. The linearity of this plot demonstrated that silylation was linear from 0 to 6 mg of total silylated compound and that detector response was linear up to at least 1000 ng per peak (flame ionization detection is commonly linear up to 10,000 ng per peak). Plots of peak height *versus* amount injected for each individual compound were also linear demonstrating that each of the compounds was maximally silylated. This linearity was also true for the 10% QF-1 column.

Determination of metabolite concentration

The concentration (C_x in μg/ml) of a particular metabolite x in the culture filtrate was calculated from the following formula:

$$C_x = H_x \cdot R = H_x \cdot \frac{C_x'}{H_x'} \qquad (1)$$

where H_x and H_x' are the peak heights (cm) for x in a crude and a pure sample of x, respectively. C_x' is the concentration (μg/ml) of x in the standard solution of pure x used to determine H_x'. The ratio R is an indicator of the sensitivity obtained for a particular GLC column (*i.e.*, column aging or repacking requires redetermination of R) under the given operating conditions. The smaller the R value the better the sensitivity.

Small decreases in peak height (H_x) were caused by slightly less than 1 μl of sample being injected into the instrument, due to the presence of the ammonium chloride precipitate in the silylation reaction mixture. This error was eliminated by centrifugation or more commonly by multiplying H_x by the ratio H_i'/H_i, where H_i' and H_i are the peak heights obtained from one microliter of a sample of the internal standard, *p*-bromophenol, which was silylated in the absence of metabolites, then centrifuged, and which was silylated in the presence of metabolites and not centrifuged, respectively. Day-to-day changes in instrument response were eliminated by

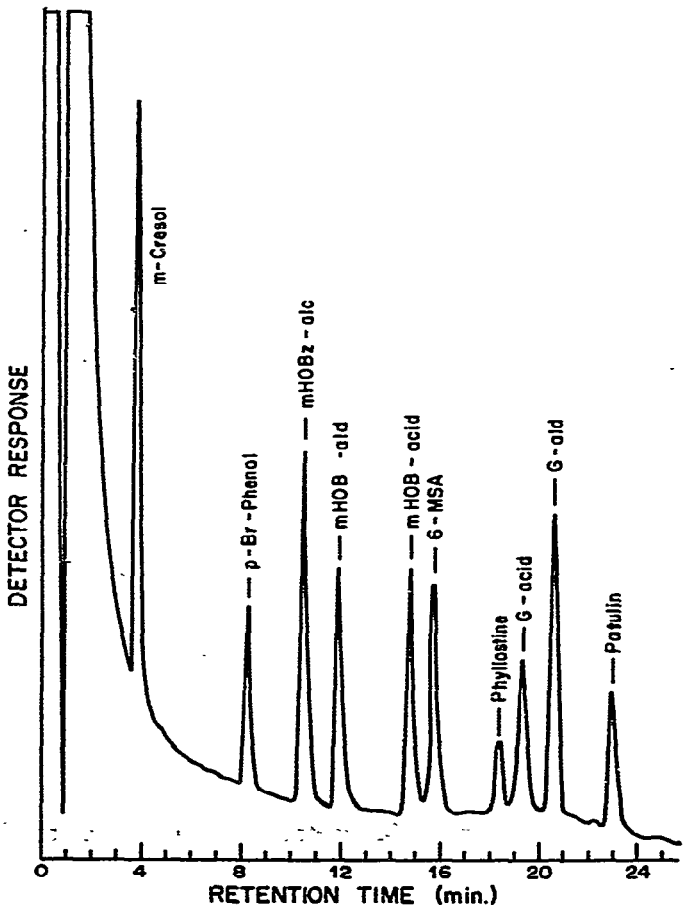


Fig. 1. Gas–liquid chromatogram obtained when a 1-μl sample containing about 575 ng of each pure compound was injected into a 10% QF-1 column.

routinely recalibrating the instrument with a standard solution of silylated 6-MSA. This metabolite was chosen because of its sensitivity to changes in the column used.

RESULTS AND DISCUSSION

Chromatography of pure metabolites

As can be seen from Fig. 1, the 10% QF-1 column gave good resolution of most of the patulin pathway metabolites, reasonably low detection limits (see Table I), and reasonable accuracy (when peak heights were greater than 0.5 cm). Fig. 2 illustrates the less efficient chromatography of metabolites on a 5% NPGS column, which was used in an effort to improve on the efficiency of the 7.5% NPGS column. Detection limits for this column were generally higher, and the peak for 6-MSA is poorly resolved. The peaks for G-acid and mHOB-acid are not visible, although these two acids were present in the sample.

Fig. 3 illustrates the advantages of the 10% QF-1 system over the NPGS

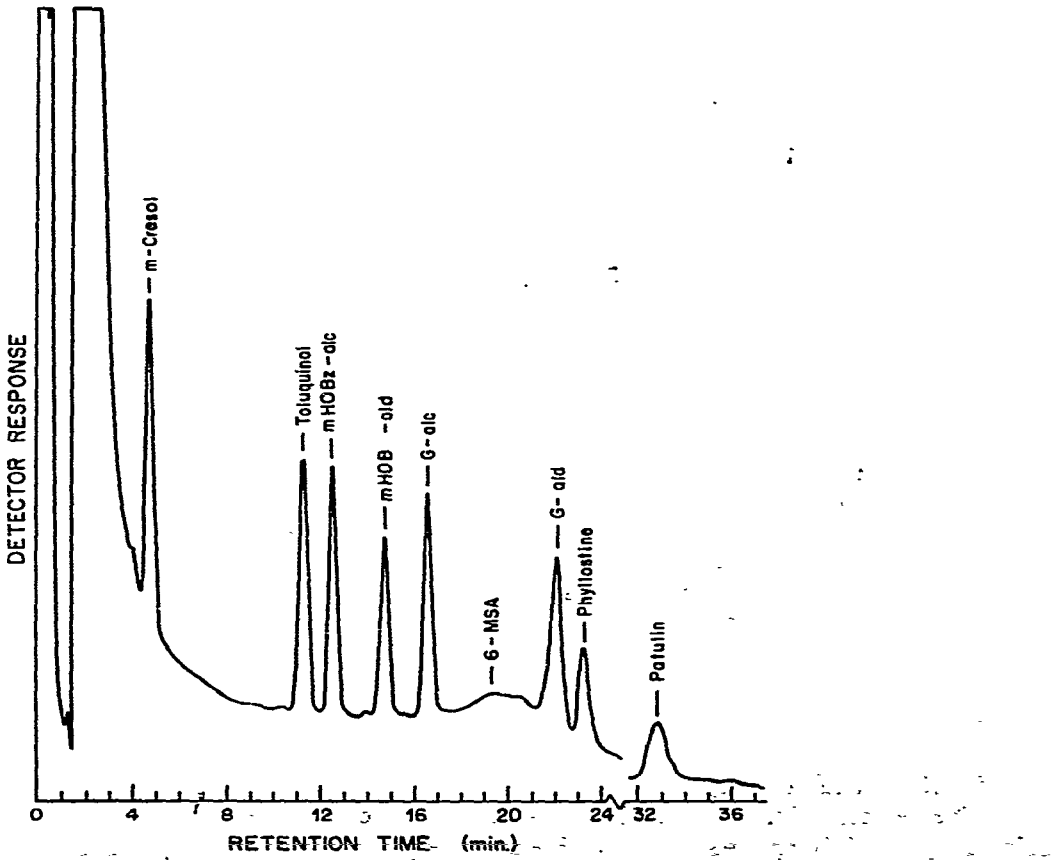


Fig. 2. Gas–liquid chromatogram obtained when a 1-μl sample containing about 575 ng of each pure compound was injected into a 5% NPGS column.

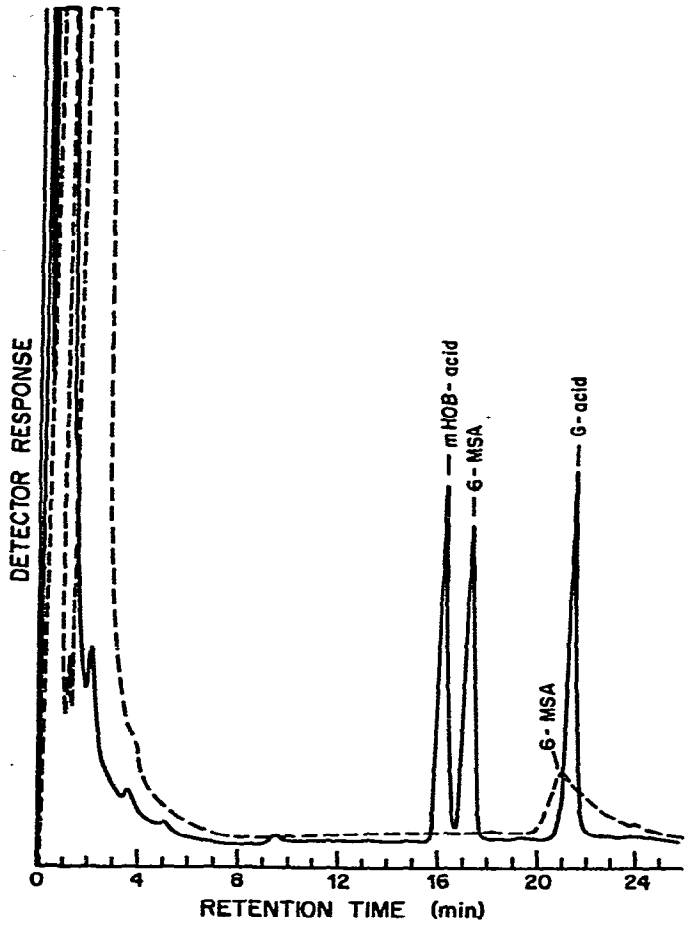


Fig. 3. Gas–liquid chromatograms obtained when a 1-μl sample containing 1000 ng of each of the three pure phenolic acids was injected into (———) a 10% QF-1 column or (– – –) a 5% NPGS column.

system used by Murphy *et al.*[1]. These advantages are principally with respect to the acidic pathway metabolites 6-MSA, mHOB-acid and G-acid. When the NPGS column was used, only the peak representing 6-MSA could be located. The other two acids could not be detected even at the high concentration of 1,000 ng/μl. The reason for this is presumably that in the presence of the base, pyridine, and at the high operating temperatures used, the acids react with the polyester liquid phase, NPGS.

Detection limits and accuracy

The detection limits for a 10% QF-1 column are given in Table I, and were obtained by multiplying the smallest visible peak height (usually 0.07 cm) by the ratio *R* for each of the pure compounds. In the case of *m*-cresol a small spurious peak of constant height was found to have an identical retention time. Its height was therefore

subtracted from the apparent sample peak height before calculating the concentration of *m*-cresol.

Accuracy varied from ±30% at the detection limit (largely error of peak-height measurement) to a limiting value of ±3% at peak heights of greater than 0.7 cm.

Extraction and chromatography of a typical culture filtrate

Fig. 4 shows the chromatogram obtained for a culture filtrate of *P. urticae* cultivated in the medium described above. As can be seen a low and relatively smooth baseline is present, which allows for easy measurement of the various peak heights. The lack of spurious peaks illustrates the selective properties of the column in separating only those compounds which are very closely related in structure, or, as in this sample, are all patulin pathway metabolites except for the internal standard *p*-bromophenol.

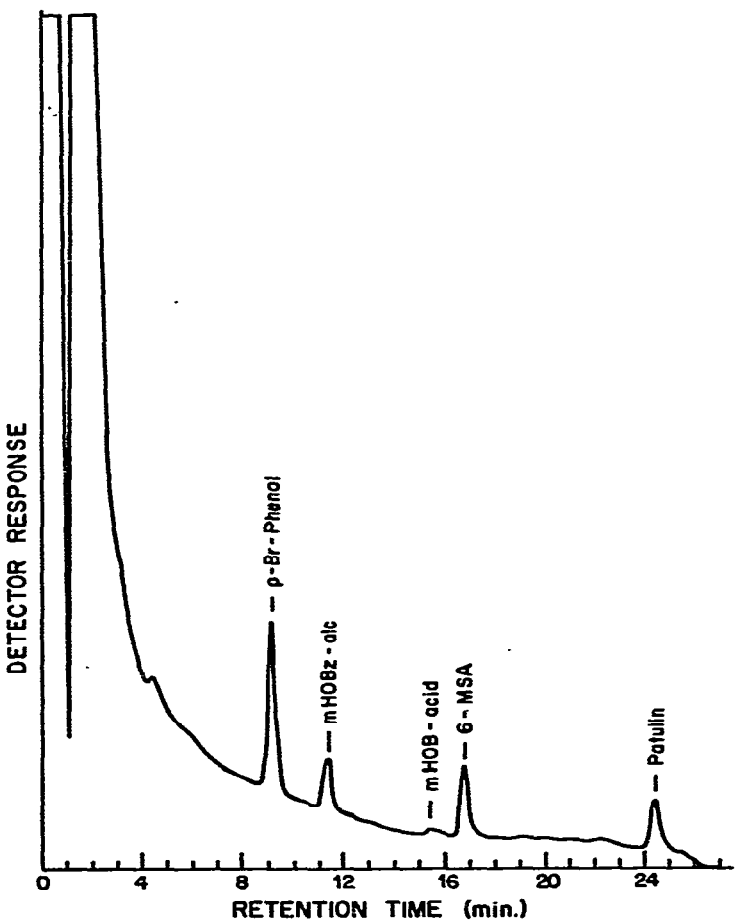


Fig. 4. Gas–liquid chromatogram obtained when a 1-μl sample prepared from 1 ml of a 30-h culture filtrate, as described under Sample preparation, was injected into a 10% QF-1 column. The resultant culture filtrate concentrations of the metabolites shown are: mHOBz-alc, 151 μg/ml; mHOB-acid, 22 μg/ml; 6-MSA, 390 μg/ml; patulin, 589 μg/ml.

Value of the procedure as an analytical tool

Use of the chromatographic procedure developed in this study makes possible the analysis of patulin pathway metabolites present in the culture filtrates of patulin-producing organisms. This means that the relative concentrations of these compounds in various cultures can be quickly and simultaneously assayed.

An earlier and more common assay method which is still in use, is thin-layer chromatography. While this method could be used as a sensitive monitor of a culture's metabolite spectrum (*i.e.*, detection limits are lower than those obtained using GLC[6]), it was somewhat time consuming (optimum color development sometimes required a day or two) and could not yield reliable quantitative data. The present GLC system can yield accurate quantitative data in as little as 40 min.

ACKNOWLEDGEMENT

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