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A GAS–LIQUID CHROMATOGRAPHIC EXAMINATION OF STILBENE DERIVATIVES

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SUMMARY

Trimethylsilyl ether derivatives of twenty hydroxystilbenes were separated by gas–liquid chromatography on Apiezon-L, OV-1, OV-17, and SE-54. Relative retention times were highly dependent upon the degree of hydroxylation and methoxylation, the positions of these groups and on *cis–trans* isomerism. OV-1 was the best liquid phase for separating compounds with different numbers of trimethylsilyl ether groups while OV-17 was best for separating on the basis of different numbers of methoxyl groups. The method holds promise for analysis of stilbene derivatives in plant extracts.

INTRODUCTION

Stilbenes are an important group of natural products found in wood, bark and leaves of a variety of forest trees[1]. These compounds are noted for their fungi-toxic properties[2,3], their inhibition of wood delignification by acid sulphite pulping methods[4] and discoloration of wood[5]. Pinosylvin and pinosylvin monomethyl ether were found in the heartwood but not sapwood of all pine species investigated[6,7], and pinosylvin dimethyl ether was present in the bark of *P. banksiana*[8]. 3,5,4′-Trihydroxy-3′-methoxystilbene 3-β-D-glucoside[9] and glucosides of 3,5,3′,4′-tetrahydroxystilbene[10] were isolated from spruce bark. HATHWAY[11] found 3,5,4′-trihydroxystilbene and its 3-β-D-glucoside in the heartwoods of a group of *Eucalyptus* species but not in *E. wandoo* sapwood[12]. Both 3,5,2′,4′-tetrahydroxystilbene[13] and 3,5,3′,4′,5′-pentahydroxystilbene[14] were found in wood. The glucosides of 3,5,3′,4′-tetrahydroxy-, of 3,5,3′-trihydroxy-4′-methoxy- and of 3,5,4′-trihydroxystilbene were isolated from the leaves of *E. sideroxylon*[15–17]. The 4,4′-dihydroxy-3,3′-dimethoxystilbene was formed during bisulphite pulping of *P. radiata* and caused a pink coloration of the pulp[18].

HILLIS AND ISHIKURA[19] recently surveyed the paper and thin-layer chromatographic behaviour and spectral properties of stilbenes. Gas–liquid chromatography of trimethylsilyl ether (TMS) derivatives of plant polyphenols showed GLC to be a very useful and sensitive adjunct to PC and TLC in the analysis of flavonoids[20] and lignans[21]. Analysis of stilbenes by GLC would appear to hold similar promise but

little work has been reported to date. SATO *et al.*[22] studied GLC of TMS-pinosylvin and TMS-pinosylvin monomethyl ether on DC-430 liquid phase but did not separate these two compounds[22]. However, they did separate the stilbenes from the flavonoids, TMS-pinocembrin and TMS-pinobanksin as well as the lower molecular weight phenols usually present in plant extracts. SATO AND VON RUDLOFF[23] were able to separate TMS-pinosylvin from TMS-pinosylvin monomethyl ether using a 15% SE-52 liquid phase. SINSHEIMER AND SMITH[24] used GLC for the quantitative analysis of 2-hydroxy-, 4-hydroxy-, 4,4'-dihydroxy- and 4-hydroxy-3-methoxystilbenes as TMS derivatives on SE-52. This paper reports a survey of the separations obtained with twenty stilbenes on four different liquid phases.

EXPERIMENTAL

Gas chromatography

All analyses were carried out on a Beckman GC-4 gas chromatograph with a flame ionisation detector using glass columns (2 m × 3 mm I.D.). The nitrogen carrier gas flow rate was 50 ml/min while hydrogen and air flow rates were 30 and 275 ml/min, respectively. All liquid phases were coated on acid-washed, silylated, 80–100 mesh Chromosorb W* using the filtration method. The liquid phases studied were: (1) 2% Apiezon-L* (hydrocarbon), (2) 1% OV-1* (methyl-silicone gum), (3) 2% OV-17* (methyl-phenyl-silicone gum), and (4) 2% SE-54* (methyl-phenyl-vinyl-silicone gum). Light loading of liquid phase was required for analysis of stilbene glucosides. Oven temperatures varied depending on the group of stilbenes to be separated and on the liquid phase but the detector and injection port temperatures were controlled to at least 25° above oven temperature.

Silylation of stilbenes

The silylating reagent contained 1 ml of N,O-bis(trimethylsilyl) acetamide (BSA)**, 2 ml of hexamethyldisilazane (HMDS)** and 1 ml of trimethylchlorosilane (TMCS)** in 10 ml of dry pyridine. Mixtures of pure stilbenes (0.2–0.5 mg each) were dissolved in 1 ml of the silylating reagent in 1-ml glass vials with silicone gum septum stoppers. Aglucones were reacted for 10 min at room temperature while glucosides were heated to 105° for 5 min and injected within 10–15 min after heating. As a further precaution against moisture, vials were stored in a desiccator over P_2O_5.

Preparation of cis-stilbenes

cis-Stilbenes were prepared by irradiating ethanol solutions of the pure *trans*-isomers with UV light at a wave length of 254 nm. The isomerisation was followed by changes in the UV spectra. After the samples had been irradiated for 24 h, they were divided, a portion silylated and analysed, and a larger amount was applied to Whatman No. 3 paper. The paper chromatograms were developed with 6% acetic acid and the band containing the *cis*-isomer was extracted with diethyl ether. The

* 80–100 mesh Chromosorb W was obtained from Varian Pty. Ltd., Australia, and acid washed and silylated in our laboratory. Apiezon-L was from Assoc. Elect. Ind. Ltd., Great Britain. OV-1, OV-17 and SE-54 were all obtained from Varian Pty. Ltd., Australia.

** BSA was obtained in 1-ml glass ampoules from Pierce Chem. Co., U.S.A. HMDS and TMCS were obtained from Varian Pty. Ltd., Australia.

ether solution was washed twice with distilled water and dried over sodium sulphate before the evaporated extract was dried under high vacuum at room temperature. A sample of this material was silylated and analysed by GLC.

Preparation of wood extracts

Chips from the heartwood of a *Pinus radiata* tree grown near Penola, South Australia, were freeze dried, ground to pass a 40-mesh screen on a Wiley mill and the ground wood soaked in 40–60° petrol for two days. The wood was air dried and then soaked in ethanol–acetone (50 : 50) at room temperature for three days. The filtrate was evaporated under high vacuum and analysed by TLC and after silylation by GLC.

RESULTS AND DISCUSSION

Silylation conditions

The TMS-ether derivatives of stilbene aglucones had sharp individual peaks after a reaction period of 10 min at room temperature when silylated with either HMDS–TMCS in pyridine (2 : 1 : 10) or BSA–HMDS–TMCS in pyridine (1 : 2 : 1 : 10). Additional peaks were sometimes observed after 2–3 h of storage of sample vials over P_2O_5 in a desiccator.

Silylation of the glucosides was more difficult than of the aglucones. Attempts to use HMDS and TMCS at increasing concentrations in pyridine continued to give low yields. Addition of BSA and 5 min of heating at about 100° were necessary to increase yields significantly. More care in the exclusion of moisture from the TMS-stilbene glucosides appeared to be necessary than with the corresponding aglucones, since additional peaks were observed to develop more rapidly. Injection of the solution within 10–15 min after heating gave best results. Solutions were injected directly into the gas chromatograph without prior separation of the ammonium chloride precipitate and no difficulties arising from this procedure were evident. Attempts to use dimethylformamide instead of pyridine gave lower yields and additional peaks.

Relative retention times of TMS-stilbene derivatives

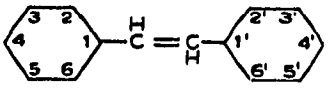
The relative retention times of the TMS-stilbene aglucones were determined with respect to TMS-4,4′-dihydroxystilbene on four different liquid phases and are summarised in Table I. 4,4′-Dihydroxystilbene was chosen as an internal standard because it does not occur in nature, was readily obtainable, and had a central retention time. The relative retention times for the glucoside derivatives were calculated with respect to TMS-3-methoxy-4′-O-β-D-glucosylstilbene. This compound has not been found in plant extracts.

Isomers

On the liquid phase OV-1, the relative retention times of the *cis*-stilbenes were approximately 0.38 those of the corresponding *trans*-isomers. There was only a small change in the relative retention times of these geometrical isomers with increasing degree of hydroxylation (Table II). The *cis*-isomers are frequently observed in paper chromatograms of plant extracts, *e.g.* ref. 11. BATTERSBY AND GREENOCK[25] among

TABLE I

RELATIVE RETENTION TIMES OF STILBENE AND TMS-STILBENE DERIVATIVES



Stilbene	*Liquid phase and oven temperature*			
	Apiezon-L 215°	*OV-1* 180°	*OV-17* 195°	*SE-54* 215°
Aglucones[a]				
cis-Stilbene	—	—	—	0.033
trans-Stilbene	—	—	—	0.062
4-Hydroxy-	0.34	0.22	0.27	0.30
Pinosylvin monomethyl ether (3-hydroxy-5-methoxy-)	0.51	0.44	0.62	0.36
4-Hydroxy-3-methoxy-	0.51	0.43	0.57	—
Pterostilbene (4'-hydroxy-3,5-dimethoxy-)	1.62	1.43	2.36	0.94
4-Hydroxy-4',3-dimethoxy-	1.25	1.19	1.87	—
Pinosylvin (3,5-dihydroxy-)	0.53	0.55	0.58	0.40
4,4'-Dihydroxy-	1.00	1.00	1.00	1.00
4,4'-Dihydroxy-3,3'-dimethoxy-	2.58	2.96	4.26	1.89
Resveratrol (3,5,4'-trihydroxy-)	1.84	2.35	2.13	1.30
3,5,3',4'-Tetrahydroxy-	2.97	4.58	3.58	2.38
3,5,2',4'-Tetrahydroxy-	2.39	4.05	3.58	—
	Apiezon-L 275°	*OV-1* 225°	*OV-17* 240°	*SE-54* 250°
Glucosides[b]				
3-Methoxy-4-O-β-D-glucosyl-	1.00	1.00	1.00	1.00
Pinosylvin monomethyl ether glucoside (5-methoxy-3-O-β-D-glucosyl-)	—	0.92	—	0.92
4',3-Dimethoxy-4-O-β-D-glucosyl-	2.00	1.10	2.58	—
Piceid (5,4'-dihydroxy-3-O-β-D-glucosyl-)	1.79	2.62	1.77	2.11
Rhapontin (5,3'-dihydroxy-4'-methoxy 3-O-β-D-glucosyl-)	2.19	3.30	2.50	2.62
Astringin (5,3',4'-trihydroxy-3-O-β-D-glucosyl-)	2.93	3.83	2.34	2.87

[a] Aglucone relative retention time with respect to 4,4'-dihydroxystilbene.
[b] Glucoside relative retention time with respect to 3-methoxy-4-O-β-D-glucosylstilbene.

many others have observed the facile photo-chemical isomerisation of *trans*- to *cis*-stilbenes. Consequently, care must be taken in this respect when preparing samples and interpreting chromatograms.

There was often a large difference in retention time for various structural isomers (Table III). Compounds that had TMS-ether or methoxyl groups on both rings of the molecule showed longer retention times than compounds with the same number of the same groups on one ring. TMS-4,4'-dihydroxystilbene had a much longer retention time than TMS-3,5-dihydroxystilbene on all liquid phases. On Apiezon-L, TMS-3,5,3',4'-tetrahydroxystilbene had a significantly longer retention time than TMS-3,5,2',4'-tetrahydroxystilbene. There was little separation of TMS-3-hydroxy-5-methoxy- from TMS-4-hydroxy-3-methoxystilbene on all liquid phases.

TABLE II

RELATIVE RETENTION TIMES OF STEREOISOMERS OF STILBENES

Stilbene	*Relative retention time using liquid phase OV-1*
cis-Stilbene	0.36
trans-Stilbene	1.00
cis-4-Hydroxy-[a]	0.38
trans-4-Hydroxy-[a]	1.00
cis-4,4'-Dihydroxy-[a]	0.40
trans-4,4'-Dihydroxy-[a]	1.00

[a] TMS derivatives.

OV-17 gave the best separation of this pair with a separation factor of 1.09. These compounds are more easily separated by TLC[19] but since 4-hydroxy-3-methoxystilbene has not been isolated from plants, the GLC method is suitable for phytochemical studies. There was a large separation of TMS-4'-hydroxy-3,5-dimethoxystilbene from TMS-4-hydroxy-4',3-dimethoxystilbene on all liquid phases. Despite the large increase in molecular size and weight of the only pair of isomeric glucosides studied, the separation of the pair was similar to that obtained with the aglucone pair (Table III).

Effect of the number of TMS-ether groups

Although there were exceptions, an increase of one TMS-ether group resulted in about twice the relative retention time (Fig. 1). The methyl-silicone gum OV-1 liquid phase gave the largest separation of compounds with different numbers of TMS-ether groups. Another methyl-silicone gum (SE-30) also gave the largest separation of flavonoids with different numbers of TMS-ether groups[21]. OV-17 and Apiezon-L gave somewhat less separation, while SE-54 gave the least separation

TABLE III

RELATIVE RETENTION TIMES OF TMS DERIVATIVES OF STRUCTURAL ISOMERS OF STILBENES

Stilbene isomers	*Liquid phase*		
	Apiezon-L	*OV-1*	*OV-17*
3,5-Dihydroxy-	0.53	0.55	0.58
4,4'-Dihydroxy-	1.00	1.00	1.00
3-Hydroxy-5-methoxy-	1.00	1.02	1.09
4-Hydroxy-3-methoxy-	1.00	1.00	1.00
4'-Hydroxy-3,5-dimethoxy-	1.30	1.20	1.26
4-Hydroxy-4',3-dimethoxy-	1.00	1.00	1.00
3,5,3',4'-Tetrahydroxy-	1.24	1.13	1.00
3,5,2',4'-Tetrahydroxy-	1.00	1.00	1.00
3-Methoxy-4-O-β-D-glucosyl-	—	1.10	—
5-Methoxy-3-O-β-D-glucosyl-	—	1.00	—

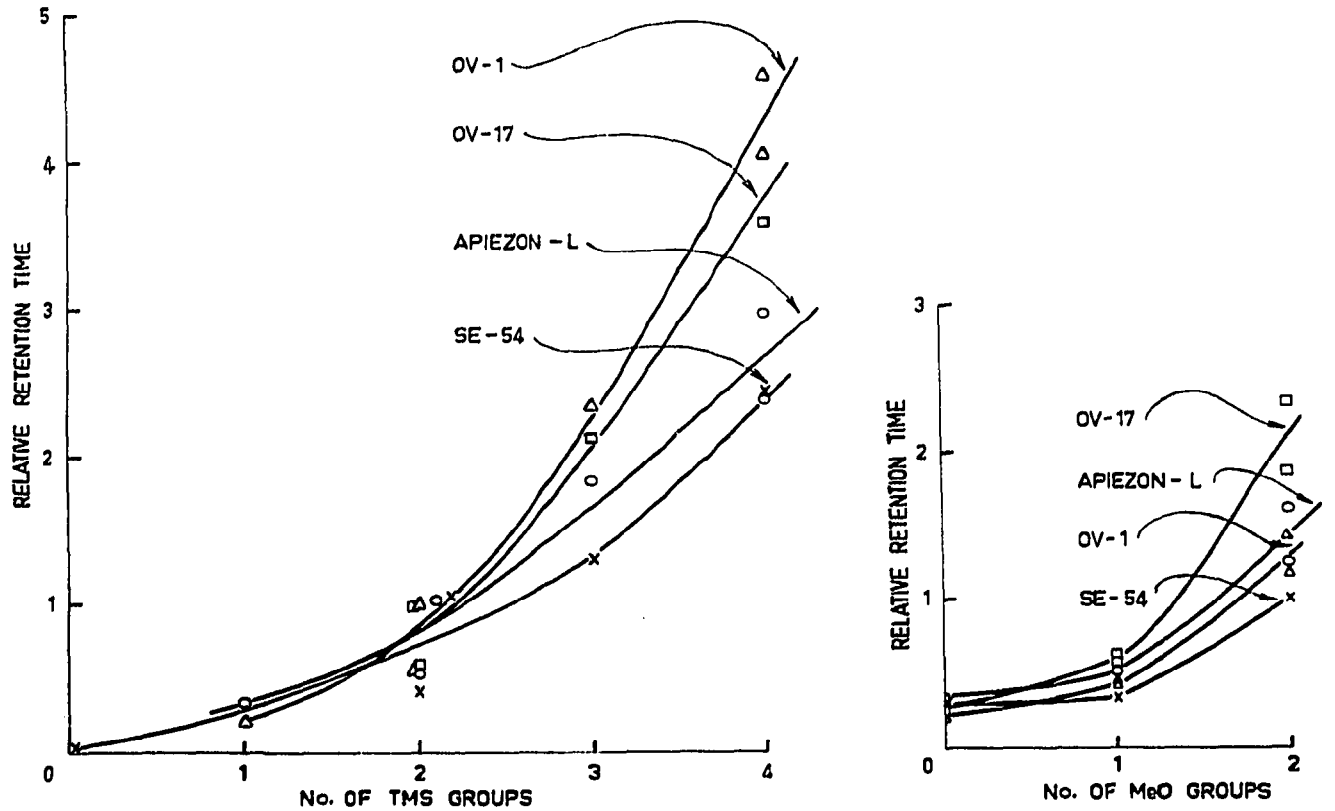


Fig. 1. Influence of the number of TMS-ether groups on the relative retention times of stilbene derivatives chromatographed on four liquid phases. □, OV-17; △, OV-1; ○, Apiezon-L; ×, SE-54.

Fig. 2. Influence of the number of methoxyl groups on the relative retention times of stilbene derivatives chromatographed on four liquid phases. □, OV-17; △, OV-1; ○, Apiezon-L; ×, SE-54.

studied. Addition of the first two TMS-ether groups increased retention times less than addition of the third and fourth.

TMS derivatives of the stilbenes on OV-1 showed some peak tailing, as did TMS-flavonoids on SE-30 (ref. 21). However, the previous work with flavonoids indicated that peak symmetry could be improved by temperature programming with a temperature increase of about 1°/min[21]. The other liquid phases did not show a significant amount of tailing of compounds with large numbers of TMS-ether groups.

Effect of number of methoxyl groups

The increase in retention time resulting from an increase in the number of methoxyl groups was greater than or equal to that caused by increasing an equal number of TMS-ether groups (Fig. 2). Increasing numbers of methoxyl groups caused an exponential increase in retention time on all liquid phases. OV-17 gave the best separation of compounds with different numbers of methoxyl groups. There was little difference between Apiezon-L and OV-1 in this respect, while the SE-54 again gave the least separation based on the number of methoxyl groups. The first

based on the number of TMS-ether groups. The increase in relative retention time with increasing number of TMS-ether groups was exponential on all liquid phases. The second methoxyl increased retention times significantly more, to a minimum of 2.5 times for SE-54 and 3.5 times for OV-17.

Glucosides

The TMS-stilbene glucosides usually gave sharp individual peaks on Apiezon-L, OV-17, and SE-54 liquid phases. Peak tailing was small but significant on the OV-1 liquid phase and this occurred also with the aglucones that had a large number of TMS-ether groups. It was apparent that OV-1 gave the best separation of compounds with different numbers of TMS-ether groups and that OV-17 gave the best separation based on the number of methoxyl groups. Increasing the number of methoxyl groups from one to two increased the retention time to 2.5 times on the OV-17 liquid phase. The retention time of TMS-5,4'-dihydroxy-3-O-β-D-glucosylstilbene increased to 1.5 times its original value with an additional TMS-ether group at the 3' position when chromatographed on the OV-1 liquid phase. The OV-1 liquid phase was not effective in the separation of TMS-4',3-dimethoxy-4-O-β-D-glucosylstilbene from TMS-3-methoxy-4-O-β-D-glucosyl- or TMS-5-methoxy-3-O-β-D-glucosylstilbenes. However, the former two stilbene glucosides are not natural products. TMS-3-methoxy-

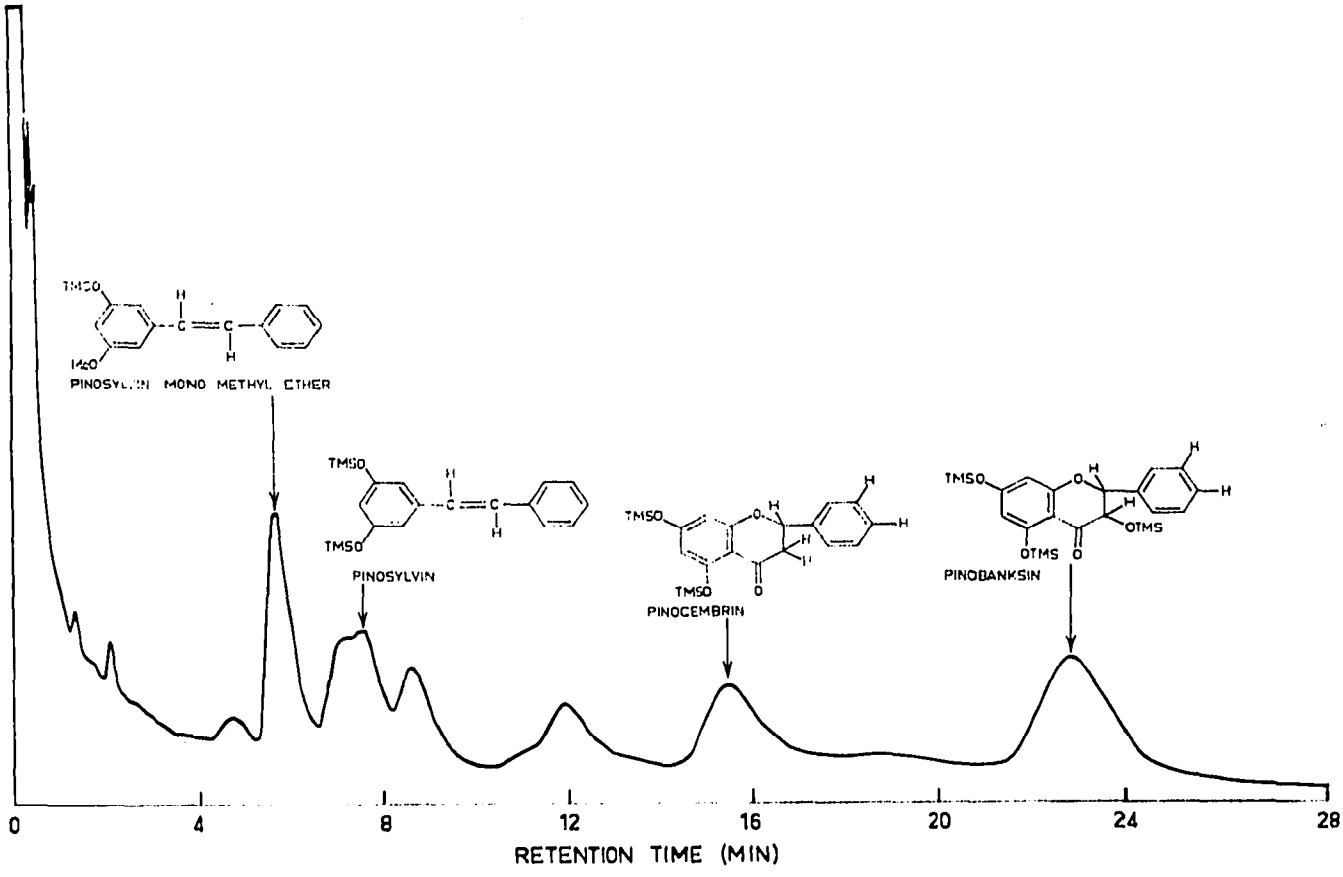


Fig. 3. Gas–liquid chromatogram of the silylated products of the ethanol–acetone soluble components of *Pinus radiata* heartwood. Chromatographic conditions: oven temperature, 150°; injection port and detector temperatures 200°; carrier gas flow 50 ml/min; column, 2 m × 3 mm I.D. glass packed with 2% OV-1 on 80–100 mesh DMCS-treated Chromosorb W.

methoxyl on a monohydroxystilbene increased the relative retention time to a minimum of 1.2 times on SE-54 and to a maximum of 2 times for OV-1 and OV-17. 4-O-β-D-glucosylstilbene was a useful internal standard; it was close to but adequately separated from TMS-5-methoxy-3-O-β-D-glucosylstilbene.

Application to pine heartwood

Compounds previously found in *Pinus radiata* heartwood include pinosylvin, pinosylvin monomethyl ether, pinocembrin and pinobanksin[6,7]. Both TLC and GLC of the wood sample we have studied indicated significant quantities of additional compounds. Unfortunately one unknown compound has a relative retention time very similar to that of TMS-pinosylvin on an OV-1 liquid phase (Fig. 3). A better separation was obtained by lowering the oven temperature to 140°.

CONCLUSIONS

Both stilbene glucosides and their aglucones were successfully silylated and separated by GLC. The relative retention times obtained for various stilbene derivatives were highly dependent upon the degree of hydroxylation and methoxylation. OV-1 was the best liquid phase for separating on the basis of number of hydroxyls while OV-17 was best for separating compounds with differing numbers of methoxyls. Relative retention times were also highly dependent upon the geometry of the compound both with respect to *cis*- and *trans*-isomers and the position of substituent groups. While analyses of natural extracts may be complicated by peaks from unidentified compounds, this problem can probably be overcome. GLC of silylated plant extracts appears to hold considerable promise as an analytical tool.

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