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GAS CHROMATOGRAPHY OF FLAVONOIDS

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SUMMARY

The gas chromatography of some trimethylsilyl ethers of flavonoids and glycosides was carried out using the OV series of silicone polymers as liquid phases, and useful correlations between the KOVÁTS retention indices and the substituents in flavonoids in some crude drugs by temperature-programmed gas chromatography were devised. It was attempted to determine rutin quantitatively in a crude drug by chromatography.

INTRODUCTION

Recently, some reports on the detection of flavonoids by gas chromatography have been published. NARASIMHACHARI AND VON RUDLOFF[1,2] showed that methyl ethers of flavonoids could be chromatographed on an SE-30 silicone polymer column. FURUYA[3] reported the gas chromatography of flavonoids as trimethylsilyl (TMS) derivatives using SE-30 as a stationary liquid. KEITH AND POWERS[4] described the detection of anthocyanidins and other flavonoids as TMS ethers on SE-30 and SE-52 as liquid phases. In 1970, the gas chromatography of TMS derivatives of stilbenes and a few flavonoids was carried out on OV-1, OV-17 and SE-54 by HEMINGWAY *et al.*[5].

This paper describes gas chromatographic studies on some flavonoids and their glycosides which were converted into the respective TMS ethers with bistrimethylsilylacetamide and trimethylchlorosilane in anhydrous pyridine, using OV-1, OV-17, OV-25, OV-210 and OV-225 as liquid phases.

EXPERIMENTAL

Materials

Kaempferid was obtained from the rhizome of *Alpinia officinarum*, baicalin, baicalein and wogonin from the root of *Scutellaria baicalensis*, and alpinon, izalpinin and 3-acetylalpinon from the seed of *Alpinia japonica*, by methanol extraction. Rutin

hesperidin, naringin, quercitrin and quercetin were commercially available. Hesperetin and naringenin were obtained by hydrolysis of hesperidin and naringin, respectively, and cholesteryl *n*-alkanoates were prepared by the method of MAHADEVAN AND LUNDBERG[6].

Gas chromatography

All analyses were carried out on a Shimadzu 5A gas chromatograph with a flame ionization detector and glass columns (1.0, 1.5 or 2.0 m length, 3.0 mm I.D.). All liquid phases were coated on Gas-Chrom Q by the filtration method. The liquid phases and column lengths studied were: 0.5 % OV-1, 1.5 m; 0.5 % OV-17, 1.5 m; 0.5 % OV-25, 2.0 m; 2.0 % OV-210, 1.5 m; and 0.5 % OV-225, 2.0 m. Light loading of the liquid phases was required for the analysis of flavonoid glycosides. The oven temperature was 210° for flavonoids, 260° on OV-1 and OV-25 columns, and 270° on the OV-17 column for glycosides. The flow-rate of nitrogen carrier gas was 40 ml/min. About 1 mg of flavonoids or glycosides was dissolved in 0.2 ml of pyridine, and to this solution were added 0.2 ml of BSA and 0.1 ml of TMCS. After heating for 2 h at 60°, an aliquot of the reaction mixture was directly injected on to the column, which was operated at a suitable temperature as described above. The results are given in Tables I and III.

Temperature-programmed gas chromatography

The components in the crude drugs were detected by temperature-programmed gas chromatography. One of the extracts was obtained from the bud of *Sophora japonica* by extraction with methanol, and another was obtained from the seed of *Alpinia japonica* with ether. About 50 mg of an extract were dissolved in 3 ml of dried pyridine and trimethylsilylated as described above, and then an aliquot of this solution was injected on to a 0.5 % OV-17 column (1.0 m × 3 mm I.D.). The separations were carried out by temperature programming from 150 to 270° at 5°/min for the extract of *Alpinia japonica* and from 170 to 270° at 5°/min for that of *Sophora japonica*. The chromatograms are shown in Fig. 3. The assignments of the peaks were confirmed by adding authentic samples and measuring the increase in the areas of the corresponding peaks.

Calibration graph

Rutin, the standard sample for quantitative work, obtained from the National Institute of Hygienic Sciences, Tokyo, was purified by passing through a Sephadex LH-20 column[7], and recrystallised from distilled water and then methanol so as to remove a small amount of quercetin. It was shown by elemental analysis that this sample contains three molecules of water of crystallisation.

In each of several glass tubes accurate amounts of rutin (10–20 mg) and cholesteryl benzoate in the weight ratio from 0.5 to 2.0 were placed. The materials were dissolved in 1 ml of anhydrous pyridine and to these solutions were added 1 ml of BSA and 0.3 ml of TMCS. After the mixture had been kept at 60° for 2 h, 1–2 μl of this solution was applied on to a 0.5 % OV-17 column (1.0 m × 3 mm I.D.) operated at 260°. A chromatogram obtained is shown in Fig. 5. The peak areas were measured with a Shimadzu ITG-2A digital integrator (sensitivity 10^2 mΩ, threshold level 500 μV, peak detector 10 μV/min). Each value of the area is the average of the results of five duplicate analyses. The weight ratio of rutin to cholesteryl benzoate was plotted

against the ratio of the areas of the peaks corresponding to samples. The calibration graph was obtained from these values by the regression method, as shown in Fig. 6.

Quantitative determination of rutin in the bud of Sophora japonica

UV spectrometry. According to the procedure in the literature[8], the sample was prepared by extracting the crude drug with methanol in a Soxhlet apparatus for 2 h, followed by a further extraction for 4 h with more methanol. The solvent was evaporated off, water was added to the residue and the mixture was washed twice with ether so as to remove quercetin. Methanol was added to this aqueous solution and to an aliquot of this mixture was added a solution of aluminium sulphate and potassium acetate. The solution obtained was analysed by UV spectrometry at 418 nm in comparison with the standard solution of rutin.

Gas chromatography. The extract was obtained by heating 100 mg of crude drug with 10 ml of methanol under reflux for 2 h, and then with a further 10 ml of methanol for 4 h. The solvent was evaporated off and the residue was dried in a desiccator. 20 mg of cholesteryl benzoate were added to the dried extract, and the mixture was dissolved in 2 ml of anhydrous pyridine and treated with 2 ml of BSA and 0.6 ml of TMCS at 60° for 2 h. The quantitative analysis was carried out as in the previously described method in order to obtain the calibration graph. The average rutin contents in Table IV were obtained from determinations on five samples, and each value for a sample is the average of five duplicate determinations.

Gas chromatogrphy (direct silylation method). To 100 mg of the dried pulverised crude drug were added 20 mg of cholesteryl benzoate, 3 ml of anhydrous pyridine and 2 ml of BSA, and the mixture was kept for 6 h at 120° with occasional shaking. The gas chromatography was carried out as above with the supernatant fraction of the reaction mixture.

TABLE I

RETENTION INDICES OF FLAVONOIDS AT 210°

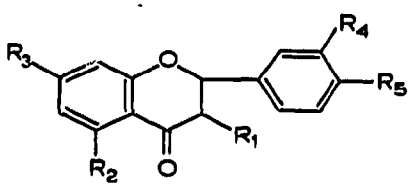
Flavonoid	*OV-1*	*OV-17*	*OV-25*	*OV-210*	*OV-225*
Kaempferid	2737	2917	3037	3139	3225
Quercetin	3236	3335	3425	3642	3653
Hesperetin	2922	3218	3361	3475	3610
Naringenin	2843	3095	3241	3361	3416
Wogonin	2753	3045	3233	3424	3557
Baicalein	2810	3072	3216	3383	3478
Izalpinin	2692	2969	3124	3143	3331
Alpinon	2533	2843	3004	2886	3146
3-Acetylalpinon	2588	3044	3256	3225	3498
2′,3′-Dimethoxyflavone	2515	2910	3016	3223	3434
	Retention times of n-alkanes (internal standards) (min)				
C-24	1.6				
C-26	3.05				
C-28	5.8	3.5	2.45	2.1	
C-30	10.95	7.0	4.65	3.5	
C-32	20.75	13.85	8.75	5.8	4.2
C-34	39.3	26.1	16.75	9.45	7.35
C-36			30.6	15.45	12.9
C-42				67.4	69.5

RESULTS AND DISCUSSION

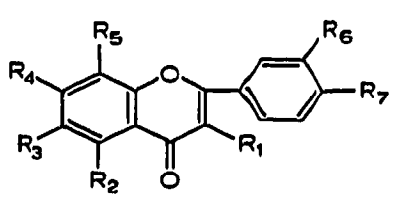
Detection of flavonoids

With columns containing 0.5 % of OV-1, OV-17, OV-25 and OV-225 and 2 % of OV-210 on Gas-Chrom Q (ref. 9), satisfactory gas chromatograms of flavonoids were obtained at 210°, but on the 0.5 % OV-210 column at 190° the flavonoids gave a slightly broad peak. The KOVÁTS retention indices (RI)[10] of the TMS ethers of flavonoids are given in Table I.

Comparison of the abilities of liquid phases to separate these flavonoids indicates that all except OV-225, a cyanoethyl phenylsilicone polymer, are unable to separate some of the flavonoids. On OV-1, a non-polar methylsilicone polymer, the kaempferid (VI) and wogonin (VIII) pair and the baicalein (IV) and naringenin (III) pair could not be separated from each other. On OV-17 and OV-25, weakly polar methylphenylsilicone polymers, baicalein, wogonin and naringenin appeared as a single peak. On OV-210, a weakly polar trifluoropropylsilicone polymer, izalpinin (V) and kaempfrid; and baicalein and naringenin, were not well separated.



R_1,R_2=OH R_3=OCH_3 R_4,R_5=H	Alpinon	(I)
R_2,R_3,R_4=OH R_5=OCH_3 R_1=H	Hesperetin	(II)
R_2,R_3,R_5=OH R_1,R_4=H	Naringenin	(III)



R_2,R_3,R_4=OH R_1,R_5,R_6,R_7=H	Baicalein	(IV)
R_1,R_2=OH R_4=OCH_3 R_3,R_5,R_6,R_7=H	Izalpinin	(V)
R_1,R_2,R_4=OH R_7=OCH_3 R_3,R_5,R_6=H	Kaempferid	(VI)
R_1,R_2,R_4,R_6,R_7=OH R_3,R_5=H	Quercetin	(VII)
R_2,R_4=OH R_5=OCH_3 R_1,R_3,R_6,R_7=H	Wogonin	(VIII)

Baicalein and its methyl derivative wogonin, which are constituents of the root of *Scutellaria baicalensis*, were well separated from each other on OV-1, but not on OV-17 and OV-25. The retention time of wogonin was shorter than that of baicalein on OV-1 and OV-17 ,but on the more polar OV-25, OV-210 and OV-225 baicalein was eluted faster than wogonin. Almost all of the flavonoid peaks were symmetrical, but baicalein on OV-225 appeared as a tailing peak.

It was observed that the RI value increases when the number of hydroxyl substituents in the flavonoid increases, and also when there is a double-bond at the C-2 position. These observations agree with the conclusion proposed by FURUYA[3].

The variations of RI on five liquid phases are shown in Fig. 1. It can be seen

that the RI values of these flavonoids increase according to the polarity of the liquid phase, and a remarkable increase is observed for baicalein and wogonin. On the OV-210 column, an interesting behaviour of the flavonoids was observed. In order to illustrate these points, the differences in the retention indices between OV-210 and OV-1 (ΔI^{210-1}), between OV-225 and OV-210 ($\Delta I^{225-210}$), and between OV-225 and OV-25 (ΔI^{225-25}), and the ratios of these differences in retention indices, $\Delta I^{225-210}/\Delta I^{225-25}$, were calculated and compared (Table II). These comparisons show some structural dependences of these values. It can be seen that the ΔI^{210-1} values of flavones that do not have a hydroxyl group at the C-3 position, such as baicalein and wogonin, are greater than those of flavonols, such as izalpinin, kaempferid and quercetin (VII). A similar relationship is observed for flavanones and flavanonols, such as hesperetin (II), naringenin and alpinone (I).

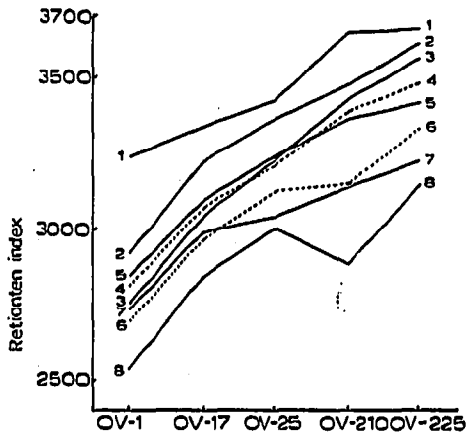


Fig. 1. Relationship between KovÁts retention indices of flavonoid TMS ethers and different stationary liquids: (1) quercetin; (2) hesperetin; (3) wogonin; (4) baicalein; (5) naringenin; (6) izalpinin; (7) kaempferid; (8) alpinon.

TABLE II

RELATIONSHIPS BETWEEN RETENTION INDICES AND SUBSTITUENTS

Compound	ΔI^{210-1} [a]	$\Delta I^{225-210}/\Delta I^{225-25}$ [a]
Alpinon	353 (3-OH)	1.831 ($-OCH_3$)
Hesperetin	553	0.542 ($-OCH_3$)
Naringenin	518	0.314
Baicalein	573	0.363
Wogonin	671	0.411 ($-OCH_3$)
Izalpinin	451 (3-OH)	0.908 ($-OCH_3$)
Kaempferid	402 (3-OH)	0.457 ($-OCH_3$)
Quercetin	406 (3-OH)	0.048
2',3'-Dimethoxyflavone	708	0.505 ($-OCH_3$)

[a] ΔI^{210-1} = (RI on OV-210) — (RI on OV-1).
$\Delta I^{225-210}$ = (RI on OV-225) — (RI on OV-210).
ΔI^{225-25} = (RI on OV-225) — (RI on OV-25).

The existence of a methoxyl group in molecules of flavonoids, especially at the C-7 position, gives higher values of the ratio $\Delta I^{225-215}/\Delta I^{225-25}$ than those of flavonoids that do not contain the methoxyl group.

Detection of flavonoid glycosides

The gas chromatography of flavonoid glycosides has been attempted previously by KEITH AND POWERS[4], and rutin, hesperidin and quercitrin were chromatographed on SE-30 and SE-52 columns. It was found that, in general, glycosides could be detected at a lower temperature than their aglycones. However, in our studies, a higher column temperature was required for the gas chromatography of glycosides (such as rutin) than for that of their aglycones (such as quercetin). Of the liquid phases used, OV-1, OV-17 and OV-25 gave a fairly symmetrical peak, but OV-210 and OV-225 were unsuitable for the chromatography of glycosides because the peaks obtained on these columns were broad and tailing. On OV-1, rutin gave a comparatively symmetrical peak, while hesperidin and naringin appeared as slightly tailing peaks. Also on the OV-25 column, glycosides with monosaccharides, such as quercitrin, gave fairly symmetrical peaks, but glycosides with disaccharides, such as rutin, naringin and hesperidin, gave slightly tailing peaks. It seems that the OV-17 column is the most suitable for the detection of flavonoid glycosides.

Under the conditions used for the gas chromatography of glycosides, *n*-paraffins were eluted so fast that they were unsuitable as internal standards for calculating relative retention values. It was found that cholesteryl *n*-alkanoates[6] (from caproate to palmitate) have suitable retention times for use as standard compounds. The relationships between the number of carbon atoms in cholesteryl *n*-alkanoates (CNCA values) and RI values on OV-1, OV-17 and OV-25 liquid phases are illustrated in Fig. 2. As the polarity of the liquid phase increases, higher RI values are obtained for each CNCA value.

The corresponding carbon numbers of cholesteryl *n*-alkanoates of flavonoid glycosides are given in Table III. CNCA values of the flavonoids decrease when the polarity of the liquid phase increases, and they have almost the same values on OV-17 and OV-25.

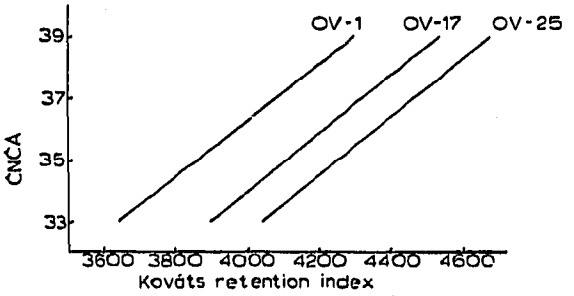


Fig. 2. Relationships between RI and CNCA values for OV-1, OV-17 and OV-25 stationary phases.

Temperature-programmed gas chromatography of some plant extracts

Temperature-programmed chromatograms of the methanol extract obtained from the bud of *Sophora japonica* (A) and the ether extract of the seed of *Alpinia japonica* (B) are shown in Fig. 3.

TABLE III

CARBON NUMBERS OF CHOLESTERYL *n*-ALKANOATES (CNCA VALUES) OF FLAVONOID GLYCOSIDES

Flavonoid glycosides		*OV-1*	*OV-17*	*OV-25*
Rutin		40.18	37.46	37.38
Quercitrin		35.48	33.46	33.30
Naringin		40.42	38.47	38.44
Hesperidin		42.14	40.94	41.13
Baicalin		35.12	34.73	35.07
	Retention times of cholesteryl n-alkanates (min) (CNCA)			
Caproate	(33)		3.3	5.0
Caprylate	(35)	6.5	5.4	7.95
Caprate	(37)	10.7	8.8	12.7
Laurate	(39)	17.3	14.35	20.3
Myristate	(41)	27.6	23.1	32.45
Palmitate	(43)	44.9		51.9

The extracts were treated with trimethylsilylating reagent in pyridine and chromatographed on 0.5 % OV-17 by temperature programming from 150 to 270° at 5°/min, and each peak obtained was confirmed by adding the authentic samples. These chromatograms show that our gas chromatographic method is very useful for the detection and determination of flavonoids in natural products.

Semi-quantitative analysis of rutin

Concerning the quantitative determination of flavonoids by gas chromatography in 1970 ANDERSEN AND VAUGHN[11] reported on quercetin in plant leaves by means of electron capture detection, and also rutin was converted into quercetin and then determined. In 1971, COLLIER AND MALLOWS[12] determined the flavanols in tea by temperature-programmed gas chromatography. However, no reports have appeared on quantitative studies on flavonoid glycosides using gas chromatography.

In this paper, a method for determining rutin (quercetin-3-rutinoside) in the pulverised bud of *Sophora japonica* by gas chromatography of its trimethylsilyl derivative is described.

The various reagents, such as bistrimethylsilylacetamide (BSA), bistrimethylsilyltrifluoroacetamide (BSTFA), trimethylsilylimidazole (TSIM), trimethylsilyldimethylamine (TMSDMA) and hexamethyldisilazane (HMDS) were used for the trimethylsilylation of amino acids, steroids and sugars, and frequently trimethylchlorosilane (TMCS) was incorporated as a catalyst. For the silylation of flavonoids, HMDS alone[13] and mixtures of HMDS with TMCS[3,4,14], BSA with TMCS[12], and BSA with HMDS and TMCS[5] were used.

Before the quantitative analysis of rutin, a comparison of the reactivity of these reagents was carried out in order to find the optimum silylation conditions. The rate of derivatization of rutin by these mixed reagents is shown in Fig. 4. A combination of HMDS–TMCS, used in the early studies on flavonoids, was unsuitable for flavonoid glycosides because the rate of silylation with this reagent was very low. With BSA–

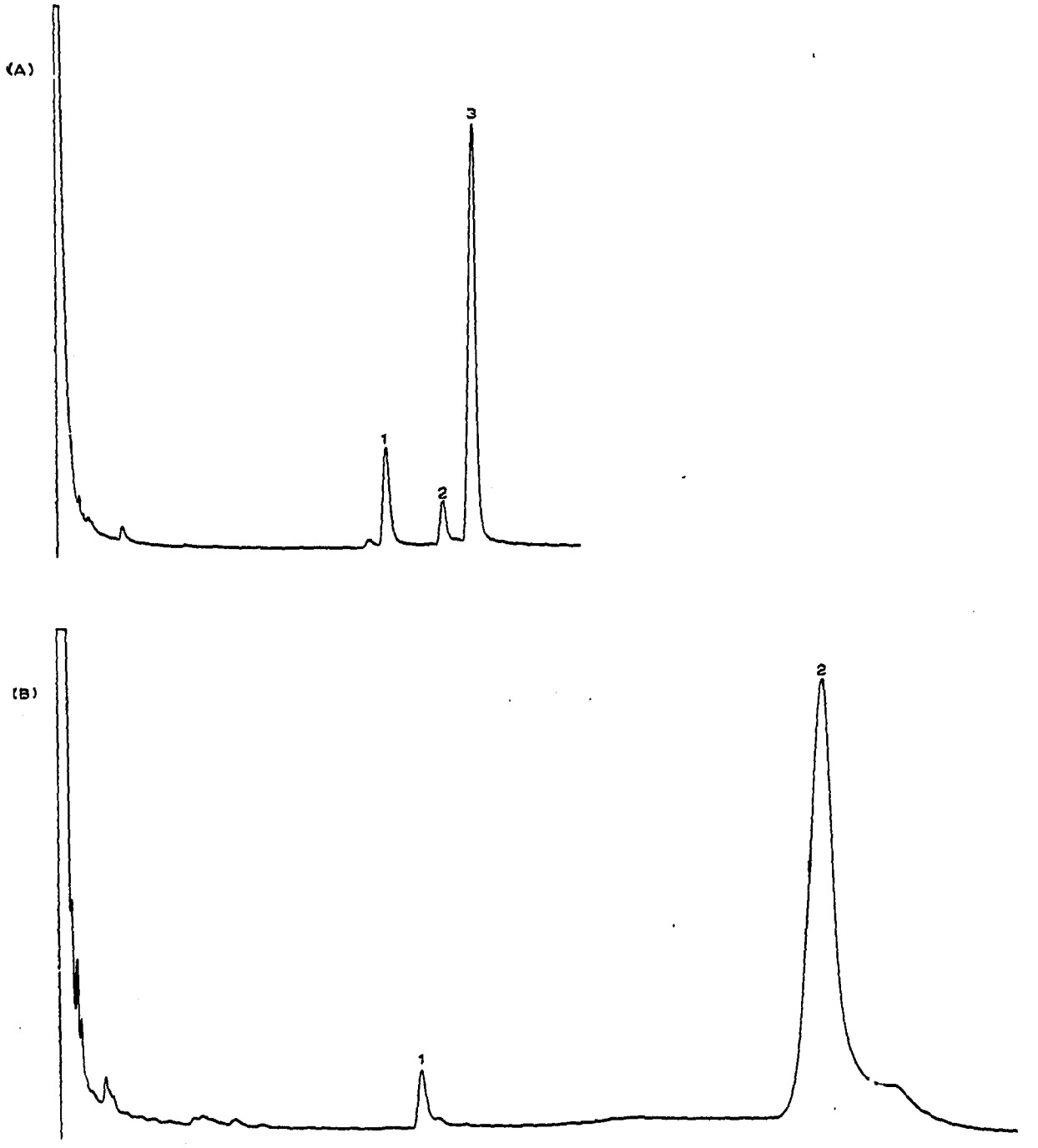


Fig. 3. Gas chromatograms of the extracts obtained from crude drugs. (A) Seeds of *Alpinia japonica*. Temperature programming from 150 to 270° at 5°/min. (1) Alpinon; (2) izalpinin; (3) 3-acetylalpinon. Scale: 4.00 cm = 10 min. (B) Buds of *Sophora japonica*. Temperature programming from 170 to 270° at 5°/min. (1) Quercetin; (2) rutin. Scale: 3.71 cm = 10 min.

HMDS–TMCS, the reaction rate reached a maximum after 1.5 h, but it unaccountably decreased after 2 h. By using TMSDMA–TMCS reagent, which has a low reactivity in the derivatization of steroids[15], rutin was rapidly converted into its TMS ether, while TMSDMA alone, which is the most effective reagent for the silylation of amino acids[16], reacted only slowly and even after 5 h the proportion of the TMS ether of rutin formed was approximately 50 %. This observation suggested that TMCS is necessary as a catalyst to achieve the complete silylation of glycosides. BSTFA[17], which is a volatile and powerful reagent for silylation, is more reactive than BSA in the derivatization of steroids[15], and is also used for the silylation of amino acids[18–20], but in the present work this reagent, even with TMCS, did not give the maximum yield of the TMS derivative of rutin. When the trimethylsilylation was carried out with BSA–TMCS at room temperature, a long reaction time (*ca.* 5 h) was required to complete the derivatization of rutin. The best results were obtained when the combination of BSA–TSIM–TMCS, with which all hindered hydroxyl groups of steroids are converted into TMS ethers[15,21], BSA–TMCS or TMSDMA–TMCS was used, and the optimum temperature and time to achieve maximum response were 60° and 1.5 h. In a sealed vessel, the TMS ether of rutin in pyridine was stable for 2 days at room temperature, but after 10 days the pyridine solution was coloured and the detectable amount of rutin derivative decreased to about 85 % of the initial amount owing to decomposition.

From studies of the column temperature, column length and concentration of different liquid phases, it was found that a column of length 1.0 m and 3 mm I.D. packed with 0.5 % OV-17 on Gas-Chrom Q (80–100 mesh) was adequate for the chromatography of the TMS derivative of rutin, and the column temperature required was more than 255°, as a peak with a small tail was observed at 250°. When a column containing crystalline powder (100–180 mesh) coated with 0.05 % of OV-17, which is inactive towards organic compounds, was used instead of Gas-Chrom Q, the peak area and retention time could not be estimated because the peak was extremely small and broad. A better response with a moderately small error was obtained when a comparatively large amount of rutin (5–10 μg) was injected on to a short column so as

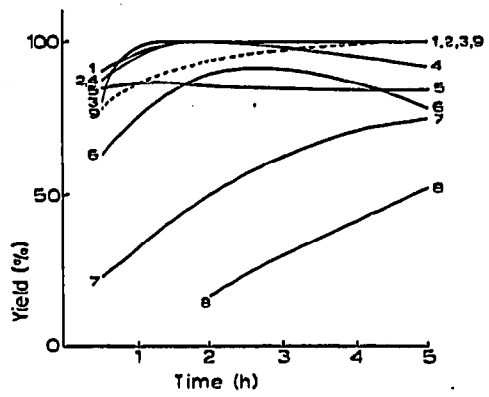


Fig. 4. Rate of derivatization of rutin by trimethylsilylating reagents in pyridine. Reagents: (1) BSA–TSIM–TMCS; (2) BSA–TMCS; (3) TMSDMA–TMCS; (4) BSA–HMDS–TMCS; (5) BSTFA–TMCS; (6) TSIM–TMCS; (7) HMDS–TMCS; (8) TMSDMA, all at 60°; (9) BSA–TMCS at room temperature.

to prevent the variation of the peak ratio which is probably due to the adsorption of rutin on the support for the stationary phase.

From the above information, it was decided that a column of 0.5 % OV-17 on Gas-Chrom Q (1.0 m × 3 mm I.D.), oven temperature 260°, with silylation with BSA–TMCS at 60° for 2 h is preferred. A gas chromatogram of trimethylsilanised rutin with cholesteryl benzoate as the internal standard on 0.5 % OV-17 is shown in Fig. 5.

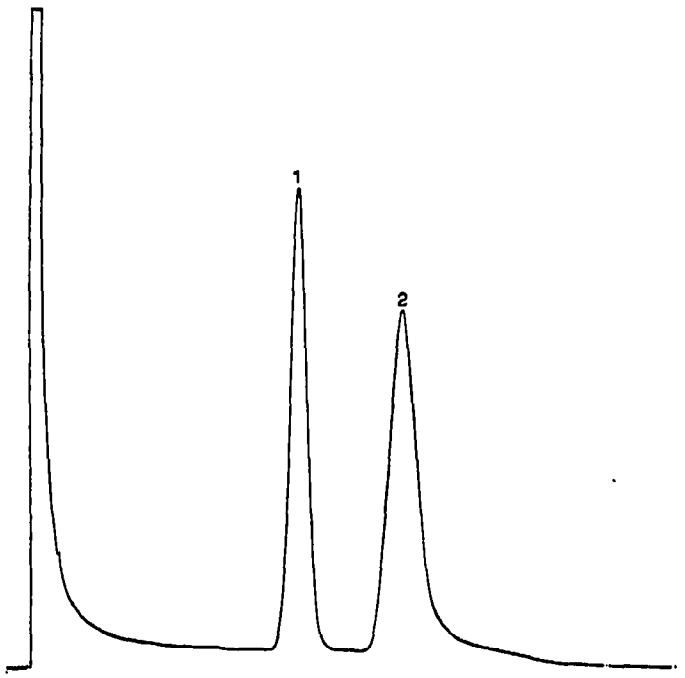


Fig. 5. Gas Chromatogram of TMS derivative of rutin and cholesteryl benzoate on 0.5% OV-17 (1.0 m × 3 mm I.D.). Oven temperature 260°; N_2 flow-rate 40 ml/min. (1) Cholesteryl benzoate (2) rutin. Scale: 3.96 cm = 10 min.

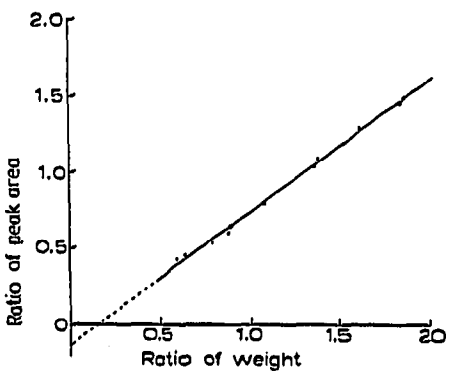


Fig. 6. Calibration graph for the determination of rutin ($y = 0.873x - 0.144$).

TABLE IV

SEMI-QUANTITATIVE ANALYSIS OF RUTIN IN THE BUD OF *Sophora japonica*

Method	*Rutin content*		*Recovery*	
	Range (%)	*Average* (%)	*Range* (%)	*Average* (%)
(A) UV spectrometry	24.2–24.8	24.5	93.8–105.5	97.9
(B) Gas chromatography (by trimethylsilylation of the methanol extract)	26.6–28.1	27.5	91.9–101.8	97.3
(C) Gas chromatography (by treatment of pulverized buds of *Sophora japonica* with BSA in pyridine)	27.9–29.4	28.7		

As shown in Fig. 6, the calibration graph was obtained by plotting the ratio of the peak area of rutin to the peak area of cholesteryl benzoate against the ratio of the weight of rutin to that of cholesteryl benzoate. A variation from linearity was observed, and the regression equation for the determination of rutin is $y = 0.873x - 0.144$.

The methanol extract obtained from the dried pulverised bud of *Sophora japonica* was analyzed five times in order to determine the precision of the determination of rutin. The results and recoveries are shown in Table IV (B).

For comparison of this gas chromatographic determination of rutin in the bud of *Sophora japonica* with another method, the established UV spectrometric method[8], was carried out to determine rutin in the same extract. The two methods gave almost the same results, as shown in Table IV (A and B).

It therefore seems that the gas chromatographic method is superior to some extent to UV spectrometry, as it can avoid the use of complicated and time-consuming procedures.

Furthermore, in order to reduce the error in the extraction from the bud and the time required for analysis, it was attempted to carry out both the extraction and trimethylsilylation simultaneously. The pulverised bud of *Sophora japonica* was heated with BSA in pyridine, and the time course of the extraction and derivatization of rutin was determined, as shown in Fig. 7. The maximum value for the rutin content

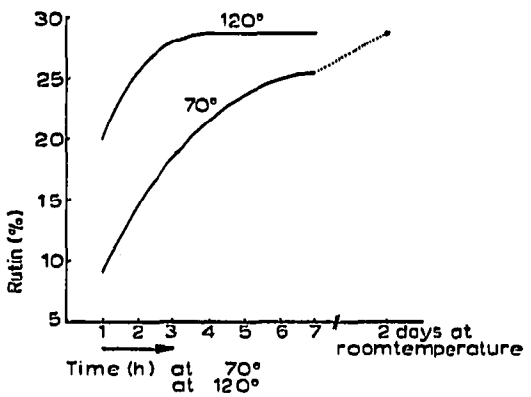


Fig. 7. The time course of the determination of rutin content by gas chromatography. The extraction and trimethylsilylation were carried out simultaneously with BSA in pyridine.

was obtained on heating for about 4 h at 120°, but when the mixture was heated at 70° the extraction and reaction were incomplete even after 7 h and then by allowing the reaction mixture to stand at room temperature for a further 2 days the rutin content reached a maximum value.

The direct determination of rutin in the bud was thus achieved as follows. To an adequate amount of the dried pulverized bud was added an adequate amount of cholesteryl benzoate, pyridine and BSA, and the mixture was heated at 120° for 5 h. An aliquot of the mixture was then injected on to a 0.5 % OV-17 column operated at 260°. The results are identical with those obtained by the above two methods, as shown in Table IV (C).

It is believed that this direct silylation method can be conveniently used for the quantitative determination of rutin in the bud of *Sophora japonica* because it is the simplest and most rapid method.

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