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Note

Capillary column gas chromatography of methyl and trimethylsilyl derivatives of some naturally occurring flavonoid aglycones and other phenolics

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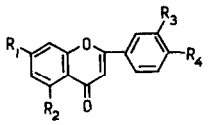
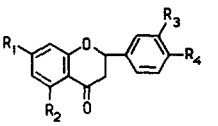
Flavonoids and related phenolic compounds are widespread components in all parts of higher plants, and are important as flower pigments, growth regulators, phytoalexins and animal toxins[1,2]. They are active in the control of legume root nodulation and are implicated in the induction of *Rhizobium* nod gene expression[3–5]. In the course of investigations aimed at the identification of these plant intermediary metabolites, we have examined the application of capillary gas chromatography (GC) for the separation and identification of some flavones (I), flavanones (II), flavonols (III) and isoflavones (IV). The sensitivity and resolving power of capillary GC make this technique particularly suitable for application to natural product mixtures.



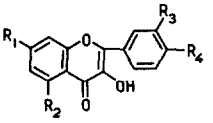
I

I_a : R_1, R_2 = OH; R_3, R_4 = H
I_b : R_1, R_2, R_4 = OH; R_3 = H
I_c : R_1, R_3, R_4 = OH; R_2 = H
I_d : R_1, R_2, R_3, R_4 = OH



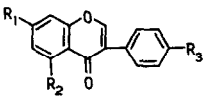
II

II_a : R_1, R_2, R_4 = OH; R_3 = H
II_b : R_1, R_2, R_3 = OH; R_4 = OMe
II_c : R_1, R_2, R_3, R_4 = OH



III

III_a : R_1 = OMe; R_2, R_3, R_4 = OH
III_b : R_1, R_2, R_4 = OH; R_3 = H



IV

IV_a : R_1, R_3 = OH; R_2 = H
IV_b : R_1, R_2, R_3 = OH

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Paper chromatography[7–9], column chromatography[7,10–12], thin-layer chromatography (TLC)[7,13,14] and spectrophotometric methods[6,7], have all previously been applied to the separation and identification of flavonoid and phenolic compounds, but these methods are time consuming or limited in separation power[15]. The methyl[16,17] and trimethylsilyl (TMS)[18–20] derivatives of these compounds have been prepared for GC analysis on non-polar packed columns. The GC of methyl and TMS derivatives of hydroxyflavones on mixed (OV-1 + OV-17) liquid phases has also been reported[21]. Hemingway and Hillis[22] applied packed column GC of TMS derivatives to the determination of dihydroquercetin, a flavanone aglycone, in wood. Nine flavonoid aglycone TMS derivatives have been separated on a GC column, packed with OV-17, by Vanhaelen and Vanhaelen-Fastré[23] who concluded that the conditions required to elute flavonoids are incompatible with the use of capillary columns.

We report for the first time the successful use of capillary column GC for the separation of methyl and TMS derivatives of flavonoid aglycones and other phenolic compounds.

EXPERIMENTAL

Flavonoid and phenolic compounds were gifts from the AFRC Institute of Plant Science Research and John Innes Institute, Norwich, U.K. These compounds were used without further purification. MethElute, 0.2 *M* trimethylanilinium hydroxide (TMAH) in anhydrous methanol, was purchased from Pierce. Pyridine, 99% (anhydrous), 1,1,1,3,3,3-hexamethyldisilazane, 98% (HMDS) and trimethylchlorosilane (TMCS) were obtained from Aldrich.

Methylation

MethElute (0.1 ml) was added to approximately 1 mg of each flavonoid or phenolic compound in a screw-cap vial. The mixture was shaken vigorously for 1 min and then maintained at 50°C for 45 min using a heating block. A 0.5–2-μl volume of the solution was used for injection into the gas chromatograph.

Trimethylsilylation

A 1–2-mg amount of each flavonoid or phenolic compound was dissolved in 0.1 ml of anhydrous pyridine in a screw-cap vial. A 0.1-ml volume of HMDS and 0.05 ml of TMCS were added. The mixture was shaken vigorously for 1 min and allowed to stand at room temperature for 30 min for phenols or heated at 60°C overnight for the flavonoids. After centrifugation 0.5 to 2 μl of supernatant solution was used for injection into the gas chromatograph.

Gas chromatography

GC analysis was carried out on a Pye Unicam series 104 gas chromatograph equipped with flame ionisation detection (FID) and a modified capillary inlet injector [SGE (UK)]. The FID output was recorded using a Phillips Analytical Chromate PC data system. Two non-polar bonded-phase fused-silica capillary columns were used, a 25 m × 0.22 mm I.D. × 0.25 μm film thickness BP-5 column [SGE (UK)] for methyl derivatives of phenolic compounds and a 50 m × 0.25 mm I.D. × 0.2 μm film thickness RSL 200 BP column (Alltech U.K.) for methyl and TMS derivatives of

flavonoids and phenolics. The linear velocity of oxygen-free nitrogen carrier gas was 17.5 cm s^{-1} for both columns and the split flow-rate was 30 ml min^{-1}. The following oven temperature programmes were used: 180°C isothermal (phenolics), 280°C isothermal and 235°C (for 2 min) to 290°C at 1°C min^{-1} (flavonoids).

RESULTS AND DISCUSSION

Volatile derivatives were readily prepared for the phenolic compounds and the di- and trihydroxyflavones (I), flavanones (II) and isoflavones (IV), by reaction with MethElute under mild conditions (45 min at 50°C). However, attempts to methylate the tetrahydroxyflavanone, eriodictyol (II_c) and the tetrahydroxyflavonols rhamnetin (III_a) and kaempferol (III_b) were unsuccessful even after heating overnight at 60°C. The only tetrahydroxyflavonoid which gave a permethylated product was the flavone, luteolin (I_d). The poor reactivity of the flavonols has been previously reported and is attributed to the presence of the 3-hydroxyl group[21]. In contrast to the unsuccessful methylation of some flavonoids, all the flavonoids investigated were successfully converted into TMS ethers by HMDS and TMCS.

The chromatograms obtained for the methyl derivatives of the trihydroxyflavanones (II_a and II_b) and the TMS ethers of the tri- and tetrahydroxyflavanones (II_a, II_b and II_c) showed a minor peak followed by a major peak after derivatisation under mild conditions (30 min at room temperature). A few small additional peaks were also observed for the methyl derivatives of the flavanones. The methylation reaction of hesperetin (II_b), a flavanone, resulted in a single chromatographic peak after heating overnight at 60°C while the two peaks observed for naringenin (II_a) remained unchanged. TMS derivatives of the di- and trihydroxyflavones (I_a, I_b, I_c), isoflavones (IV_a, IV_b) and the tetrahydroxyflavonol (III_a) prepared under mild condition (30 min at room temperature) gave a single chromatographic peak, while a seconday peak was observed for TMS derivatives of the tetrahydroxyflavone, luteolin (I_d) and the flavonol, kaempferol (III_b) under these conditions. Luteolin gave a single peak after heating overnight at 60°C while the secondary peak for kaempferol remained even after reaction overnight. Heating the TMS ethers of the flavanones (II_a, II_b and II_c) caused the growth of the minor peak in the chromatogram at the expense of the major peak. After overnight heating, the TMS ethers of the flavanones, hesperetin (II_b) and eriodictyol (II_c) showed a single peak at the retention time of the original minor peak, but, naringenin (II_a) still exhibited two peaks.

The presence of two peaks for the methyl and TMS derivatives of the flavanones (II) has been reported before in packed-column GC and was discussed in terms of an interconversion between flavanones and the corresponding chalcones[16,18]. On further investigation of the TMS derivatives of the flavanones (II) we have found that temperature, derivatisation time and capillary column injection technique all have an influence on the relative heights of the chromatographic peaks[24]. The relative intensities of the multiple peaks being particularly susceptible to small variations in capillary column injection technique. The sensitivity of some of the chromatographic results to the experimental conditions employed indicates that the derivitisation chemistry is not always straightforward. However, under the conditions reported here for the formation of TMS derivatives (60°C, overnight) characteristic and reproducible major peaks were obtained for each of the flavonoid compounds investigated.

The retention times for some typical di-, tri- and tetrahydroxyflavonoid and other phenolic derivatives with a variety of subsitution patterns are given in Tables I and II. These results demonstrate the potential of high-resolution capillary GC as an alternative to the packed-column methods for such separations reported by other workers[15–23]. The phenol derivatives were easily prepared and chromatographed as expected on the non-polar BP-5 and RSL 200 BP capillary colums. Although attempts to improve the separation of methyl and TMS derivatives of the flavonoid compounds by temperature-programmed packed-column chromatography met with little success[21], this work presents a successful separation of several flavonoid derivatives by capillary GC. Fig. 1 shows the capillary gas chromatograms obtained for mixtures of TMS or methyl derivatives of several dihydroxy-, trihydroxy- and tetrahydroxyflavonoids. Both isothermal and temperature-programming conditions were investigated to obtain the optimum separation. As expected, the retention times of the compounds in a particular group of flavonoids (flavones, flavanones and isoflavones) increase with the number of methyl or TMS ether groups. For example, the elution order for the flavones is: chrysin (5,7-dihydroxyflavone, I_a) < apigenin (4′,5,7-trihydroxyflavone, I_b), 3′,4′,7-trihydroxyflavone (I_c) < luteolin (3′,4′,5,7-tetrahydroxyflavone, I_d). When the substitution pattern of the flavonoid skeleton remains constant, the observed elution order is: flavanone, isoflavone and flavone in all the cases examined. Thus, for 4′, 5, 7-substituted flavonoids, the elution order is naringenin (flavanone), genistein (isoflavone) and apigenin (flavone). A comparison of the retention times of the flavones, chrysin (two hydroxyl groups) and apigenin (three hydroxyl groups), with those of the isoflavones, daidzein (two hydroxyl groups) and genistein (three hydroxyl groups), suggests that the increase in retention times with increasing number of hydroxyl groups is more marked with the flavones than the isoflavones for both the methyl and more particularly the TMS derivatives.

TABLE I

ADJUSTED RETENTION TIMES OF METHYL AND TRIMETHYLSILYL DERIVATIVES OF SOME NATURALLY OCCURRING PHENOLIC COMPOUNDS

GC conditions: capillary columns, 25 m × 0.22 mm I.D. BP-5 for methyl and a 50 m × 0.25 mm I.D. RSL 200 BP for TMS derivatives; column temperature, 180°C isothermal, linear velocity of nitrogen carrier gas 17.5 cm s^{-1} for both columns. s = Minor peak; m = main peak.

Compound	*Adjusted retention time (min)*	
	Methyl	*TMS*
Carboxylic acids		
Syringic acid	8.1	21.2
Sinapic acid	17(s), 26.7(m)	41.3
Aldehyde		
Syringaldehyde	5.1	9.9
Acetophenones		
Acetovanillone	4.3	7.1
Acetosyringone	7.1	13.0
4-Hydroxyacetophenone	1.9	4.1

TABLE II

ADJUSTED RETENTION TIMES OF METHYL AND TRIMETHYLSILYL DERIVATIVES OF SOME NATURALLY OCCURRING FLAVONOID AGLYCONES

GC conditions: 50 m × 0.25 mm I.D. RSL 200 BP capillary column for both methyl and TMS derivatives, temperature programme, A: 235°C (for 2 min) to 290°C at 1°C min^{-1}; B: 280°C isothermal. Other conditions as for Table I. s = Minor peak; m = main peak.

Compound	*Number of OH Groups*	*Adjusted retention time (min)*			
		Methyl		*TMS*	
		A	*B*	*A*	*B*
Flavones					
I_a Chrysin	2	21.21	6.38	18.58	5.31
I_b Apigenin	3	36.32	12.82	36.84	12.49
I_c 3′,4′,7-Trihydroxyflavone	3	34.53	11.32	39.40	13.63
I_d Luteolin	4	46.15	18.79	44.70	16.98
Flavanones					
II_a Naringenin	3	25.12(s), 26.00(m)	7.32(s), 7.62(m)	23.60(m), 24.06(s)	6.35(m), 6.58(s)
II_b Hesperetin	3	33.40	10.53(s), 11.07(m)	27.64	8.18
II_c Eriodictyol	4	–	–	29.16	8.28
Flavonols					
III_a Rhamnetin	4	–	–	40.89	14.32
III_b Kaempferol	4	–	–	33.37(s), 34.14(m)	10.42(s), 10.69(m)
Isoflavones					
IV_a Daidzein	2	19.56	5.27	26.23	7.71
IV_b Genistein	3	29.43	9.40	27.81	8.28

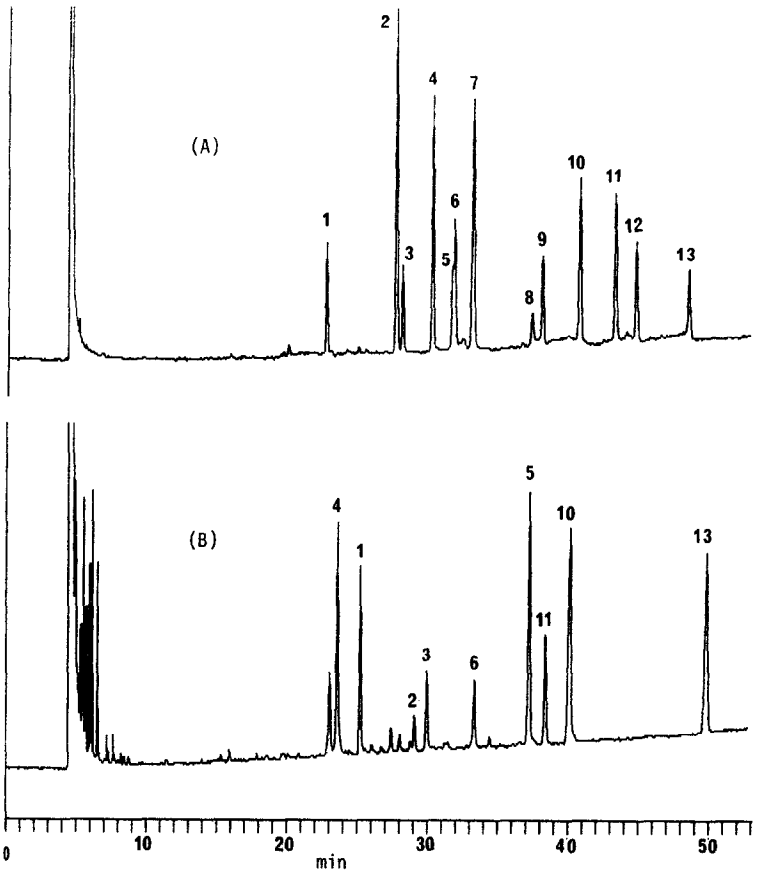

Fig. 1. Capillary gas chromatograms of trimethylsilyl (A) and methyl (B) derivatives of some naturally occurring flavonoid aglycones. GC conditions: column, bonded-phase fused-silica capillary RSL 200 BP (50 m × 0.25 mm I.D. × 0.2 μm film thickness); column temperature programme, 235°C (for 2 min) to 290°C at 1°C min^{-1}; linear velocity for nitrogen carrier gas, 17.5 cm s^{-1}. Peaks: 1 = chrysin; 2, 3 = naringenin; 4 = daidzein; 5 = hesperetin; 6 = genistein; 7 = eriodictyol; 8, 9 = kaempferol; 10 = apigenin; 11 = 3′,4′,7-trihydroxyflavone; 12 = rhamnetin; 13 = luteolin.

Capillary GC offers an attractive method for the separation and identification of flavonoid aglycones in complex mixtures. In general, TMS derivatives of the flavonoid aglycones have been found to be more suitable for GC than methyl derivatives, because of the superior derivitisation reaction with the tetrahydroxy flavonoids. The conditions for TMS derivatives reported here overcome the complexity arising from the occurrence, in certain cases, of multiple GC peaks, and provide a basis for the high-resolution separation of flavonoid aglycones.

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