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Short Communication

Resolution of complex mixtures of flavonoid aglycones by analysis of gas chromatographic-mass spectrometric data

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ABSTRACT

Forty-nine flavones, flavonols, flavanones and chalcones were analysed without derivatixation by GC and GC-MS using an OV-1 capillary column. Retention times are affected by the number, position and type of the substituents. The mass spectra show the same typical fragmentation pattern as obtained by the direct inlet method. The effectiveness of the proposed method is demonstrated by the GC and GC-MS analysis of a plant extract in which 21 different flavonoid aglycones could be identified.

INTRODUCTION

Flavonoid aglycones are valuable compounds because of their many biological and pharmacological effects [1,2]. Further, they are often used in chemotaxonomic studies [3]. Numerous chromatographic techniques have previously been applied to their separation and identification, e.g., column chromatography, paper chromatography, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). However, these methods are time consuming or limited in separation power [4]. Few reports have been published on the gas chromatography (GC) of methyl or trimethylsilyl ethers of flavonoids, only one of the polymethoxylated flavones [4,5].

In this paper we report the analysis of 49 flavones, flavonols, flavanones and chalcones without derivatization by GC and GC-MS with an OV-1 capillary column. Further, we demonstrate the application of this rapid and sensitive method to a plant extract containing 21 flavonoid aglycones.

EXPERIMENTAL

Materials

Flavonoids were isolated from flowers of *Arnica* species and from *Heterotheca inuloides*

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Cass. [6–8], except for 22, 26, 27, **30**, **42**, **43** and 46, which were purchased from Roth (Karlsruhe, Germany). All products were identified by comparison of UV, mass and ^{1}H NMR-spectra with published data.

The plant extract examined was obtained from flowers of *Arnica alpina* ssp. *attenuata* by gel chromatography (Sephadex LH-20, methanol) of the methanol-soluble part of the methylene chloride extract.

Gas *chromatography*

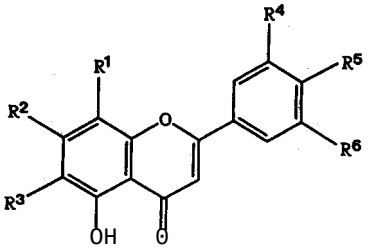
GC analysis was carried out on a Hewlett-Packard HP 5890 gas chromatograph equipped with a Permabond OV-l-capillary column (25 m × *0.25* mm I.D.) (Macherey-Nagel) and a

TABLE I

STRUCTURES OF FLAVONOID AGLYCONES AND THEIR RELATIVE RETENTION TIMES

Values are expressed relative to hispidulin, corrected for the dead time.

Flavones:

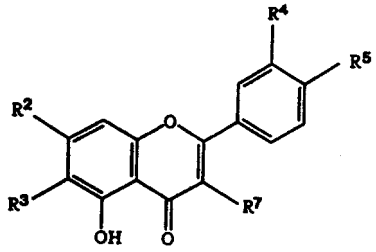


No.	Compound	Substituent						RRT
		R^1	R^2	R^3	R^4	R^5	R^6	
1	Apigenin	H	OH	H	H	OH	H	1.02
2	Genkwanin	H	OCH_3	H	H	OH	H	0.98
3	Acacetin	H	OH	H	H	OCH_3	H	0.88
4	Apigenin 7,4′-di-Me	H	OCH,	H	H	OCH_3	H	0.78
S	Chrysoeriol	H	OH	H	OCH,	OH	H	1.22
6	Diosmetin	H	OH	H	OH	OCH,	H	1.40
7	Velutin	H	OCH,	H	OCH,	OH	H	1.07
8	Pilloin	H	OCH,	H	OH	OCH,	H	1.24
9	Luteolin 7,3′,4′-tri-Me	H	OCH,	H	OCH,	OCH,	H	1.14
10	Hispidulin	H	OH	OCH,	H	OH	H	1.00
11	Pectolinarigenin	H	OH	OCH,	H	OCH,	H	0.87
12	Cirsimaritin	H	OCH,	OCH,	H	OH	H	1.32
13	Salvigenin	H	OCH,	OCH,	H	OCH,	H	1.15
14	Jaceosidin	H	OH	OCH,	OCH,	OH	H	1.19
1s	Desmethoxycentaureidin	H	OH	OCH,	OH	OCH,	H	1.37
16	Eupatilin	H	OH	OCH,	OCH,	OCH,	H	1.27
17	Cirsilineol	H	OCH,	OCH,	OCH,	OH	H	1.55
18	Eupatorin	H	OCH,	OCH,	OH	OCH,	H	1.82
19	Tricin	H	OH	H	OCH,	OH	OCH,	1.06
20	Apigenin 6,3′,5′-Tri-OMe	H	OH	OCH_3	OCH_3	OH	OCH,	1.95
21	Nevadensin	OCH,	OH	OCH_3	H	OCH,	H	1.29

(Continued on p. 352)

TABLE I *(continued)*

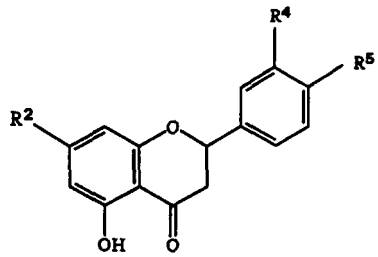
Flavonols:



No.	Compound	Substituent					RRT
		R^2	R^3	R^4	R^5	R^7	
22	Galangin	OH	H	H	H	OH	0.52
23	Kaempferol	OH	H	H	OH	OH	1.10
24	Kaempferid	OH	H	H	OCH,	OH	0.99
25	Kaempferol 3,7-di-Me	OCH_3	H	H	OH	OCH_3	0.83
26	Kaempferol 3,4′-di-Me	OH	H	H	OCH,	OCH_3	0.95
27	Kaempferol 7,4′-di-Me	OCH_3	H	H	OCH,	OH	0.84
28	Kaempferol 3,7,4′-tri-Me	OCH_3	H	H	OCH,	OCH_3	0.83
29	Isorhamnetin	OH	H	OCH_3	OH	OH	1.33
30	Tamarixetin	OH	H	OH	OCH,	OH	1.57
31	Dillenetin	OH	H	OCH_3	OCH,	OH	1.43
32	Quercetin 3,7,3′-tri-Me	OCH_3	H	OCH_3	OH	OCH_3	1.10
33	Quercetin 3,7,4′-tri-Me	OCH_3	H	OH	OCH,	OCH_3	1.30
34	Quercetin 3,7,3′,4′-tetra-Me	OCH_3	H	OCH_3	OCH,	OCH,	1.17
35	6-Methoxykaempferol	OH	OCH_3	H	OH	OH	1.09
36	Betuletol	OH	OCH_3	H	OCH_3	OH	0.98
37	Penduletin	OCH_3	OCH_3	H	OH	OCH,	1.38
38	spinacetin	OH	OCH_3	OCH,	OH	OH	1.31
39	Quercetagetin 6,3′,4′-tri-Me	OH	OCH_3	OCH_3	OCH_3	OH	1.42
40	Veronicafolin	OCH,	OCH_3	OCH_3	OH	OH	1.69
41	Quercetagetin 3,6,7,4′-tetra-Me	OCH_3	OCH_3	OH	OCH,	OCH,	1.92

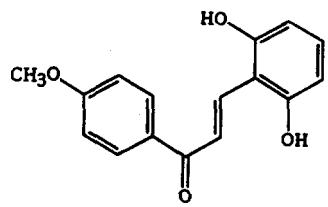
TABLE I *(continued)*

Flavanones:



No.	Compound	Substituent			RRT
		R^2	R^4	R^5	
42	Naringenin	OH	H	OH	0.60
43	Sakuranetin	OCH_3	H	OH	0.53
44	Isosakuranetin	OH	H	OCH_3	0.54
4s	Naringenin 7,4′-di-Me	OCH_3	H	OCH_3	0.47
46	Hesperetin	OH	OH	OCH_3	0.83
47	Eriodictyol 7,3′-di-Me	OCH_3	OCH_3	OH	0.62
48	Persicogenin	OCH_3	OH	OCH_3	0.71

Chalcone:



No.	Compound	RRT
49	2,6-Dihydroxy-4′-methoxychalcone	0.27

flame ionization detector. The carrier gas was nitrogen at a flow-rate of 1.3 ml/min, with a splitting ratio of 150. The injector and detector temperatures were 300°C and the column temperature was 270°C (isothermal).

Relative retention times (RRT) are expressed relative to hispidulin and were calculated after subtraction of the dead time (dead time = 0.94 min).

Gas chromatography-mass spectrometry

GC–MS analysis was carried out on a Varian-MAT CH7 A mass spectrometer in the electron impact mode (70 eV), coupled to a Varian 1700 gas chromatograph with an OV-1 capillary column. The carrier gas was helium and the injector and column temperatures were 270°C.

RESULTS AND DISCUSSION

The various flavonoid aglycones investigated, consisting of 21 flavones, 20 flavonols, 7 flavanones and 1 chalcone, with their relative retention times, expressed relative to hispidulin, are given in Table I.

Gas chromatography

Substitution at the various positions of the flavonoid nucleus affects the retention times characteristically. Conversion of a free hydroxyl group at C-4' or C-7 into the methyl ether decreases the retention time significantly (l-4, **42–45),** but in the latter instance only if C-6 is unsubstituted. The retention times of **6,7-**dimethoxylated flavones and flavonols are much higher than those of compounds unsubstituted at C-6 (compare, e.g., **12/2; 13/4; 17/7; 18/8),** probably because of steric hindrance. Introduction of a methoxyl group causes different effects depending on the position. Methoxylation at C-6 has a negligible effect on retention times (compare, e.g., **1/10; 3/** 11; **5/14; 6/15),** whereas methoxylation at C-3' causes significantly higher values (compare, e.g., **1/5; 10/14; 35/38; 42/46).** The fact that hydroxylation at C-3 (compare, e.g., **1/23; 3/24; 10/35; 11/36)** does not increase the retention times very much indicates that substituents at this position are somehow shielded.

The method is inapplicable to the examination of flavonoid aglycones with o-dihydroxy groups, such as luteolin, quercetin and patuletin, as they decompose under the chosen GC conditions. However, these compounds can easily be identified after TLC by their characteristic fluorescence after detection with diphenylboric acid, ethanolamine complex and polyethylene glycol.

Mass spectrometry

Compared with direct inlet mass spectra, those obtained by coupled GC-MS show the same typical fragmentation pattern but with slight differences in intensities. For **8-methoxyflavones** and **-flavonols** it is typical with the direct inlet method, that the mass spectra show base peaks resulting from the fragment ion [M − Me]+, whereas in the spectra of **6-methoxyflavones** and -flavonols the molecular ion forms the base peak **[9].** Under the chosen GC-MS conditions this differentiation based on mass intensities is not possible. The mass spectra of **6-methoxyflavonols** exhibit base peaks formed by the fragment ion $[M - MeCO]^+$, whereas in those of **6-methoxy-**flavones the base peaks are either the molecular ion, the fragment ion $[M - MeCO]^+$ or ***m/z 69.*** In the mass spectra of **6,7-dimethoxyflavones** a loss of [M − Me]+ leads to the most stable ion.

Applications

Fig. 1 shows the chromatogram obtained for a complex mixture of a plant extract containing 21 flavonoid aglycones belonging to different classes. Closely related compounds were separated and eluted as sharp peaks. In some instances, where the peaks consist of more than one compound, identities could be confirmed by comparison with retention times and mass spectra of the pure compounds. The combination of the separating power of this technique with mass spectrometry, which provides complementary structural information, represents an attractive

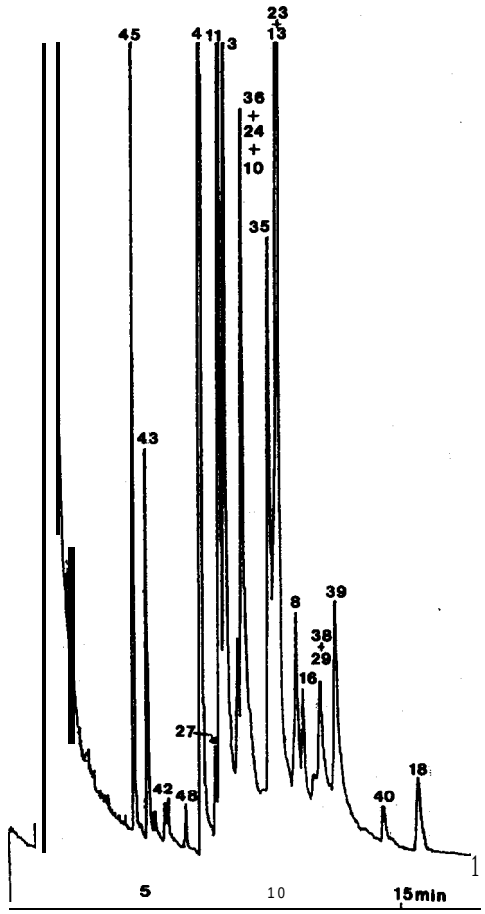


Fig. 1. Gas chromatogram of a crude fraction of flavonoid aglycones from a plant extract. See Table I for peak identification.

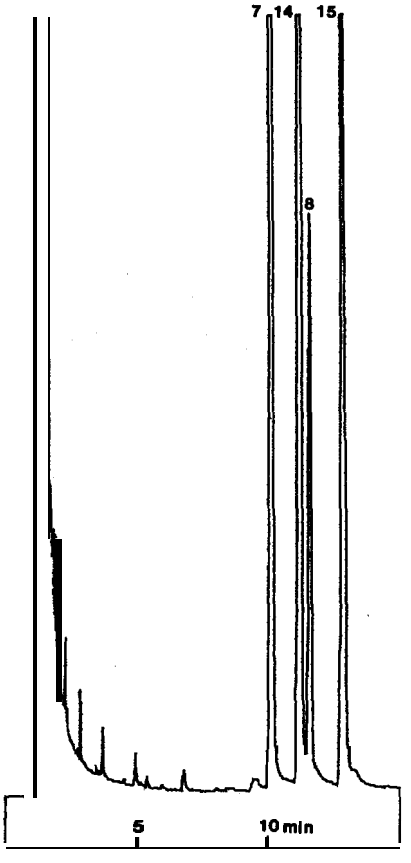


Fig. 2. Gas chromatogram of isomeric flavones. See Table I for peak identification.

and rapid method for the separation and identification of flavonoid aglycones in complex mixtures. Hence the method described here is able to play a valuable part in chemotaxonomic studies.

Another application of the above-described GC and GC-MS analyses is in the identification of isomeric 3′-OH,4′-OCH_3- and 3′-OCH_3,4′-OH-flavones and -flavonols in mixtures. As these pairs yield identical mass spectra and cannot be distinguished by TLC, the only possibility of identifying them in a mixture has, up to now, been by ^1H NMR [7]. Fig. 2 shows the separation of two isomeric pairs of flavones, velutin (7)-pilloin (8) and jaceosidin (14)–desmethoxy-centaureidin (15), by capillary GC. As compounds with a methoxyl group at C-4' have far longer retention times than their C-3' isomers, GC analysis represents a simple solution to the above-mentioned separation problem.

ACKNOWLEDGEMENT

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