Short Communication

Gas chromatography–mass spectrometry of flavonoid aglycones II[*]. Structure–retention relationships and a possibility of differentiation between isomeric 6- and 8-methoxyflavones

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Abstract

Gas chromatographic analysis of 51 flavonoid aglycones allowed a quantification of structural influences on retention time. Well-reproducible structure–retention increments were calculated. These can be valuable for the analysis of complex flavonoid mixtures in plant extracts. In addition to the results published in Part I, gas chromatographic behaviour and mass spectrometric fragmentation of 8-methoxylated flavonoid aglycones have been studied.

1. Introduction

Recently, we reported on the identification of a great number of flavonoid aglycones by means of GC and coupled GC–MS without derivatization [1]. This method is particularly valuable in analysis of flavonoid mixtures and allows a time-saving and effective analysis of large sample numbers as required in chemotaxonomic studies. As we stated [1], substitution patterns have strong influence on the retention times allowing separation even of isomeric pairs of 3'-hydroxy-4'-methoxy- and 4'-hydroxy-3'-methoxyflavones and -flavonols. The present report is directed towards the quantification of GC structure–retention relationships in order to allow predictions about the retention behaviour of unknown

components in mixtures of flavonoids. More-over, we have extended our studies to the analysis of isomeric pairs of 6- and 8-methoxylated flavones. Discrimination between these isomers is a problem which has received special attention [2].

2. Experimental

2.1. Methods

GC analyses were carried out with a HP 5890 capillary gas chromatograph equipped with a split injector and flame ionization detector. Column: OV-1 DF (Macherey–Nagel) 25 m × 0.25 mm I.D., 0.25 μm film-thickness. Temperatures: injector and detector 300°C, column temperature 270°C isothermal. Carrier gas: nitrogen at 65

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[*] For Part I, see ref. 1.

ml/min total flow; (column flow 1.3 ml/min, split ratio 1:50).

GC–MS analyses were performed with a Varian MAT CH7 A mass spectrometer coupled to a Varian 1700 capillary gas chromatograph. Electron impact ionisation at 70 eV. Mass intensities (Table 1) are expressed as relative values in % of base ions. GC: Column OV-1 DF as above, 0.35 μm film. Temperatures as above. Carrier gas helium. Flow-rates as above.

2.2. Materials

Hispidulin (5,7,4′-trihydroxy-6-methoxyflavone, **1**) and jaceosidin (5,7,4′-trihydroxy-6,3′-dimethoxyflavone, **3**) were isolated from *Arnica* species. Samples of the 8-methoxylated aglycones 5,7,4′-trihydroxy-8-methoxyflavone (**2**) and 5,7,4′-trihydroxy-8,3′-dimethoxyflavone (**4**) were provided by Professor J. Reynaud, Lyon, France (**2** and **4**) and Professor T.J. Mabry, Austin, TX, USA (**4**). For the other flavonoid aglycones see ref. 1.

The flavonoid fraction (Fig. 1) was obtained from flowers of an *Arnica alpina* subsp. *attenuata* population by gel chromatography (Sephadex LH-20, methanol) of the methanol-soluble part of the dichloromethane extract.

Retention times (t_R) are expressed as relative values (RRT) based on the t_R of hispidulin [1], calculated after subtraction of the dead time.

Structure–retention increments i as given in Table 2 represent arithmetic means of t_R ratios $t_R(I)/t_R(II)$ of n pairs differing only in one particular structural element.
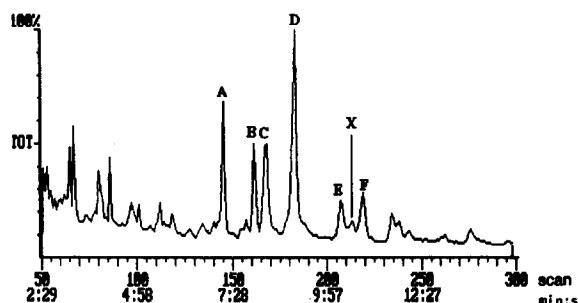

Fig. 1. Gas chromatogram (MS detected) of a flavonoid-containing fraction from a plant extract. Peaks identified by direct comparison of t_R and MS of authentic samples: A = apigenin-7,4′-dimethyl ether; B = pectolinarigenin; C = acacetin; D = betuletol; E = 6-methoxykaempferol; F = salvigenin; X = unidentified peak, RRT = 1.12. For identification of peak X see Figs. 2 and 3.

$$i = \sum_n [t_R(I)/t_R(II)]/n$$

Thus, unknown $t_R(X)$ of a flavonoid aglycone X can be calculated by multiplication (or division, respectively) of the known $t_R(Y)$ of a compound Y differing from X in a particular structure element with $i \pm$ S.D. given for this structural element.

3. Results and discussion

3.1. Differentiation of isomeric 6- and 8-methoxyflavones

Two isomeric pairs, **1 + 2** and **3 + 4**, were analysed by GC and GC–MS. Both pairs turned out to be well separable under the GC conditions
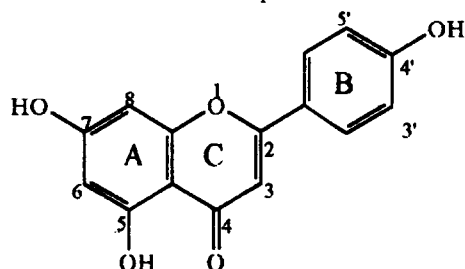
Table 1

Signal intensities in mass spectra of isomeric 6- and 8-methoxyflavones under GC–MS conditions

Compound	Relative intensities			
	$[M]^+$	$[M - CH_3]^+$	$[M - H_2O]^+$	$[M - 43]^+$
1 (6-OCH$_3$)	63	56	42	70
2 (8-OCH$_3$)	40	90	<1	40
3 (6-OCH$_3$)	35	32	29	39
4 (8-OCH$_3$)	38	100	<1	30

Table 2

Influences of substitution patterns on GC retention times of flavonoid aglycones

I	II	$i \pm$ S.D.	n
Strucure of C ring			
2,3-Dihydro	$\Delta 2,3$	0.590 ± 0.013	7
3-OH	3 unsubstituted	1.099 ± 0.021	10
3-OH	3-OCH$_3$	1.028 ± 0.022	2
Methylation of OH groups			
4'-OH	4'-OCH$_3$	1.121 ± 0.049	9
3'-OCH$_3$, 4'-OH	3',4'-OCH$_3$	0.910 ± 0.044	4
7-OH	7-OCH$_3$	1.145 ± 0.018	9
6-OCH$_3$, 7-OH	6,7-OCH$_3$	0.763 ± 0.009	4
Introduction of additional substituents			
6-unsubstituted	6-OCH$_3$	1.027 ± 0.039	6
6-unsubstituted, 7-OCH$_3$	6,7-OCH$_3$	0.653 ± 0.046	3
6-unsubstituted, 3',5'-OCH$_3$	6-OCH$_3$, 3',5'-OCH$_3$	0.54	1
3'-unsubstituted, 4'-OH	3'-OCH3, 4'-OH	0.829 ± 0.003	9
3'-unsubstituted, 4'-OCH$_3$	3'-OH, 4'-OCH$_3$	0.665 ± 0.036	7
3'-unsubstituted, 4'-OCH$_3$	3',4'-OCH$_3$	0.689 ± 0.011	5
Pairs of isomers			
3'-OCH$_3$, 4'-OH	3'-OH, 4'-OCH$_3$	0.861 ± 0.012	7
6-OCH$_3$, 8-unsubstituted	6-unsubstituted, 8-OCH$_3$	0.895 ± 0.004	2

n = Number of pairs compared for a single structural change; i = arithmetic mean of retention ratios t_R(I)/t_R(II) of n pairs; S.D. = mean error of a single measurement (standard deviation)

chosen. The RRT values of the 8-methoxy derivatives are 1.123 (2) and 1.310 (4), relative to hispidulin (1).

The characteristic fragmentation patterns observed in mass spectra in the direct-inlet mode [2,3] are reproduced also under GC–MS coupling. Slight differences with direct-inlet spectra occur in peak intensities. However, 6-methox-

ylated aglycones still exhibit intensive fragments at both $[M - CH_3]^+$ and $[M - H_2O]^+$, whereas in the case of 8-methoxy derivatives $[M - CH_3]^+$ ions have higher intensities than $[M]^+$ while $[M - H_2O]^+$ fragments show only very small intensities or are not present at all (compare Table 1).

Hence, combination of the information pro-

vided by GC retention and mass spectra represents a simple and rapid solution for the identification of such isomers without time-consuming isolation.

3.2. Structure–retention relationships

The retention ratios $t_R(I)/t_R(II)$ (Table 2) of pairs differing in the same structural element turned out to be fairly well reproducible. Thus, for example, the 6-methoxylated compounds (1 and 3) yield retention times a factor 0.895 ± 0.004 lower than those of their 8-OCH$_3$ isomers (2 and 4). In a similar way, the t_R ratio for 4'-hydroxy-3'-methoxy- and 3'-hydroxy-4'-methoxy isomers is consistently very close to 0.86. Similar relations were found for other structural elements.

These findings led to the calculation of several structural increments i, given in Table 2, which —in combination with the experimental data published in ref. 1— allow prediction of retention times for a great number of flavonoids. Hence, they can be helpful in the assignment of compounds in complex mixtures, even if no samples of pure compounds are available for direct chromatographic comparison.

Fig. 1, for example, shows the gas chromatogram (MS detected) of a fraction of flavonoid aglycones from a plant extract. Most of the flavonoid peaks (A–F) could be readily identified by comparison of their mass spectra and RRT with those of authentic samples. Between the peaks of salvigenin (RRT = 1.09) and 6-methoxy-kaempferol (RRT = 1.15), a small peak of an aglycone (X, RRT = 1.12) could be localized. Its mass spectrum (see Fig. 2) indicates the presence of quercetin-7,3'-dimethyl ether (rhamnazin) [4] or its 7,4'-dimethyl isomer (ombuin), which cannot be distinguished by MS because such pairs yield identical fragmentation patterns and could —until now— only be distinguished in mixtures by proton NMR [5]. In order to decide which isomer is present, theoretical RRT values can be calculated for both compounds (cf. Fig. 3) e.g. from the experimental RRT values of isorhamnetin and tamarixetin [1] by using the increment for methylation of the hydroxyl func-
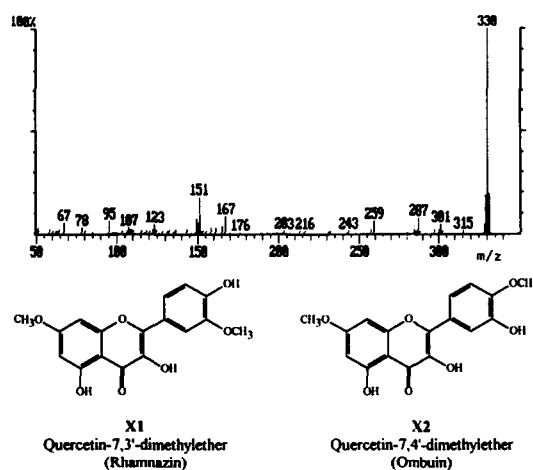

Fig. 2. Mass spectrum of peak X (see Fig. 1) and possible structures X1 and X2.

tion at C-7. In the same way, based upon the increment for an additional hydroxylation at C-3, RRT can be calculated from the corresponding flavones, velutin and pilloin, which leads to very similar values.

The results of these calculations (Fig. 3) clearly illustrate, that the unknown compound must be quercetin-7,3'-dimethyl ether (rhamnazin) [4], as the retention time should be much higher in the case of the 4'-methylated isomer.

The method described here —due to its low requirement of time and material— may play a valuable part in routine analysis of flavonoid
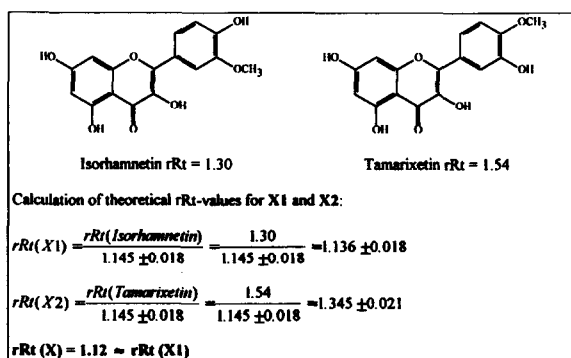

Fig. 3. Calculation of theoretical RRT (in the figure: rRt) for structures X1 and X2 (see Fig. 3) on the basis of RRT of isorhamnetin and tamarixetin [1] using the increment for methylation of the C-7-OH function (see Table II: *Methylation of OH groups*).

aglycone mixtures. Presently, its application is being validated in a chemotaxonomic investigation of different *Arnica alpina* populations which will be the subject of a subsequent publication.

4. Acknowledgements

5. References

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