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Note

Separation of glycoflavones and their glycosides by high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) is considered to be one of the most promising methods for the analysis of flavonoid glycosides[1,2]. However very few papers dealing with separation of flavonoids by HPLC have been published. Complex mixtures of flavonoid glycosides have not been well resolved[3,4], but with different flavonoid classes[3,5] or with combinations of isomeric glycosides[6,7] good separation has been achieved. Two types of column packing, chemically bonded silica gel with either C_8 (or C_{18}) or NH_2, developed with methanol– (or ethanol–) water or acetonitrile–water mixtures, often slightly acidified, have been successfully used. In this paper we describe the influence of isomerization and the glycosylation pattern of the mono-C-glucoside of apigenin on the retention time.

EXPERIMENTAL

The various naturally occurring glycosides of the isomer isovitexin were available from previous work on mutants of *Silene dioica* and *S. alba* (*Melandrium dioicum* and *M. album*)[8], and the vitexin glycosides from previous work on *Larix* needles[9]. Isovitexin and isovitexin 7-O-glucoside were obtained from the collection of Dr. M. Seikel, and vitexin was purchased from C. Roth, Karlsruhe, G.F.R.

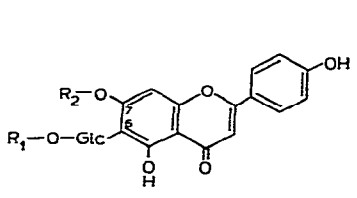
The chromatography was performed on a Dupont 830 chromatograph, with a 24 cm × 2.1 mm I.D. Zorbax-ODS column and double UV detection at 254 and 335 or 360 nm. A concave(2) gradient of ethanol–water with 0.1 *M* phosphoric acid was employed, programmed from 20% to 100%, at a flow-rate of 3%/min, pressure 3000 p.s.i., temperature 50°.

Vitexin was used as internal standard. Absolute retention times (t_r) were measured and used to calculate both the ratio $\alpha = t_r/t_r$ (internal standard), and the retention time difference (RTD) $= t_r - t_r$ (internal standard).

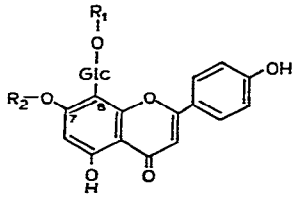
The RTD of 11 isovitexin and 3 vitexin derivatives are summarized in Table I. Apart from compounds 6 and 12, and 10 and 13, all the derivatives differ in their t_r values. As expected from paper chromatography, the two isomers vitexin and isovitexin are well separated. A complete resolution of all 16 compounds was not achieved, but combinations of smaller numbers of glycosides were separated. Fig. 1 illustrates such a separation for a mixture of five isovitexin glycosides.

TABLE I

STRUCTURES AND RETENTION TIMES (RELATED TO VITEXIN) OF THE (6)-O''- AND 7-O-GLYCOSIDES OF ISOVITEXIN AND VITEXIN



A



B

No.	R_1	R_2	α	*RTD (min)*	*a**	*b***
A: Isovitexin and derivatives						
1	H	H	1.17	1.7		
2	Arabinose	H	1.17	1.9	+0.2	
3	Rhamnose	H	1.18	2.1	+0.4	
4	Glucose	H	1.10	1.0	−0.7	
5	H	glucose	0.88	−1.2		−2.9
6	Arabinose	glucose	0.91	−0.9	+0.3	−2.8
7	Rhamnose	glucose	0.93	−0.7	+0.5	−2.8
8	Glucose	glucose	0.83	−1.7	−0.5	−2.7
9	H	xylose	0.98	−0.2		−1.9
10	Arabinose	xylose	1.0	0	+0.2	−1.9
11	Rhamnose	xylose	1.01	0.1	+0.3	−2.0
12	Glucose	xylose	0.91	−0.9	−0.7	−1.9
B: Vitexin and its derivatives						
13	H	H	1.0	0		
14	xylose	H	1.03	0.3	+0.3	
15	Rhamnose	H	1.04	0.4	+0.4	
16	Xylose	glucose	0.97	−0.4	—	−0.7

* *a* gives the difference in t_r between the (6)-O''- or (8)-O''-substituted compound and the unsubstituted derivative with the same R_2 substituent.

** *b* gives the difference in t_r between the 7-O-glycoside and the corresponding aglycone with the same R_1.

The shift in t_r obtained on the addition of one or more sugar substituents after the introduction of the first C-glucosyl unit in the apigenin structure (RTD of apigenin itself = 9.3) is relativily small. However, one of the most prominent aspects of the influence of the introduction of glycosyl substituents is seen when differences in t_r are compared (Table I, columns *a* and *b*). It appears that addition of a sugar at both a (6)-O''- or (8)-O''- and a 7-O-position brings about a change in t_r that is almost independent of the other substituents. The introduction of a (6)-O''-arabinosyl unit, for example, results in a small shift of about +0.2 in the t_r of isovitexin, its 7-O-glucoside and its 7-O-xyloside (compounds 2, 6 and 10). Similarly for rhamnose, a slightly higher change of about +0.4 (compounds 3, 7, 11 and 15) is found, whereas the introduction of glucose at the (6)-O''-position results in a change of −0.5 to −0.7 (compounds 4, 8 and 12). The same holds for substitution at the 7-O position, resulting in a

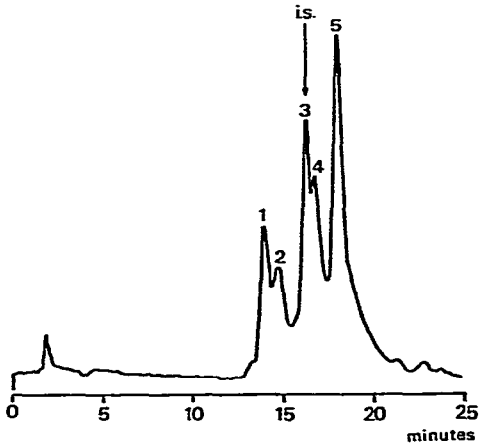


Fig. 1. Separation of five isovitexin derivatives on Zorbax-ODS: 1 = 7-O-glucosyl-(6)-O''-glucosyl-isovitexin (8); 2 = 7-O-glucosyl-(6)-O''-arabinosylisovitexin (6); 3 = 7-O-xylosylisovitexin (9); 4 = (6)-O''-glucosylisovitexin (4); 5 = (6)-O''-rhamnosylisovitexin (3); i.s. marks the position of the internal standard, vitexin.

shift of −2.7 to −2.9 for isovitexin 7-O-glucosides and of −1.9 to −2.0 for isovitexin 7-O-xylosides (Table I, column *b*).

Table II summarizes the derived R_F values of the isovitexin derivatives obtained by subtraction of the R_F value of vitexin in an average of 50–100 paper chromatographic estimations. It can be seen that: (i) the changes in R_F depend more on the other substituent(s), and (ii) introduction of glucose at the (6)-O''-position results in a much smaller change in the R_F value when compared with that of rhamnose and arabinose.

The complete structure of the investigated glycosides is not known as yet, though work on the elucidation of the structure of the isovitexin glycosides is in progress[10]. If the common[11] pyranosyl ring structure for all the sugars is assumed, t_r shifts can still be explained both from differences in the monosaccharides and from differences in the disaccharide linkage. Only for compound 15 (8-O''-rhamnosylvitexin) isolated from needles of *Larix gmelinii*, can a probable structure (apigenin, 8-C-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside) be given, because the latter compound was isolated and completely identified from needles of the same species (*L.dahurica* Turcz. = *L.gmelinii* (Rupr.) Kuz.)[12]. The very close genetical relation between the different Silene species, from which the isovitexin derivatives were isolated, most probably implies a similar disaccharide linkage. It thus seems very likely that the systematic differences in t_r found for 6-O''-glucosides, when compared with the corresponding arabinosides and rhamnosides, can be attributed to the extra hydroxy group in the molecule. The rhamnosyl methyl group shows much less influence.

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TABLE II

R_F VALUES (RELATED TO VITEXIN*, ×100) OF (6)-O''- AND 7-O-GLYCOSIDES OF ISOVITEXIN

BAW = butanol-acetic acid-water (4:1:5, upper phase), TBA = *tert.*-butanol-acetic acid-water (3:1:1), 15HAc = 15% acetic acid in water. For *a* and *b* see notes to Table I.

	Compound	*BAW*			*1% HCl*			*TBA*			*15HAc*		
		R_F	*a*	*b*	R_F	*a*	*b*	R_F	*a*	*b*	R_F	*a*	*b*
1	Isovitexin	+20			+10			+19			+20		
2	(6)-O''-Arabinosylisovitexin	+11	−9		+47	+37		+18	−1		+43	+23	
3	(6)-O''-Rhamnosylisovitexin	+8	−12		+50	+40		+18	−1		+52	+32	
4	(6)-O''-Glucosylisovitexin	+2	−18		+50	+40		+13	−6		+52	+32	
5	7-O-Glucosylisovitexin	−10		−30	+24		+14	−3		−22	+31		+11
6	7-O-Glucosyl-(6)-O''-arabinosylisovitexin	−16	−6	−27	+55	+31	+8	+3	+6	−15	+49	+18	+6
7	7-O-Glucosyl-(6)-O''-rhamnosylisovitexin	−17	−7	−25	+58	+34	+8	−5	−2	−23	+52	+21	0
8	7-O-Glucosyl-(6)-O''-glucosylisovitexin	−22	−12	−24	+59	+35	+9	−6	−3	−19	+54	+23	+2
9	7-O-Xylosylisovitexin	−4		−24	+20		+10	+1		−18	+31		+11
10	7-O-Xylosyl-(6)-O''-arabinosylisovitexin	−12	−8	−23	+56	+36	+9	+3	+2	−15	+56	+25	+13
11	7-O-Xylosyl-(6)-O''-rhamnosylisovitexin	−15	−11	−23	+60	+40	+10	+2	+1	−16	+54	+23	+2
12	7-O-Xylosyl-(6)-O''-glucosylisovitexin	−19	−15	−21	+60	+40	+10	−4	−5	−17	+55	+24	+3
13	Vitexin (abs. R_F)	+56			+7			+45			+18		

* Obtained from absolute R_F values by subtraction of that of vitexin.

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