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Note

Reversed-phase high-performance liquid chromatographic separation of naturally occurring mixtures of flavone derivatives

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Several papers have been published on the high-performance liquid chromatography (HPLC) of phenolic constituents occurring in plant tissues[1–8], but little work has been done on the application of HPLC to the separation of flavone derivatives (2-phenylbenzopyrone lacking a hydroxyl group in the 3-position). Using LiChrosorb NH_2, an excellent separation of isomeric O-glycosides of glycoflavones was achieved by Becker and Wilking[9].

This paper describes the resolution of natural mixtures of flavone derivatives extracted from fronds of *Spirodela* (duckweed) and cotyledons of *Fagopyrum* (buckwheat) on LiChrosorb RP-8 and RP-18.

EXPERIMENTAL

Extraction

For *Spirodela polyrrhiza* Schleiden, 1 g of lyophilized frond material was treated with an Ultra Turrax homogenizer in methanol. For *Fagopyrum esculentum* Moench, 10 g (fresh weight) of 8-day-old cotyledons were treated with boiling water.

Preparation for HPLC

The filtered *Spirodela* extract was diluted with water to give a methanol concentration of 80%, shaken with tetrachloromethane and the upper layer evaporated under reduced pressure at 50° to an aqueous consistency. Both the *Spirodela* and the filtered *Fagopyrum* extracts were fractionated on polyamide CC6 (7 g of CC6, bed dimensions 12 × 2 cm), yielding a water and a methanol fraction. The methanolic fractions were adjusted to a volume of 5 ml with methanol. Before application of HPLC, the fractions were centrifuged at 50,000 *g* for 20 min.

Chromatographic system

The liquid chromatograph used was obtained from Spectra-Physics (Santa Clara, Calif., U.S.A.) and included two Model 740B pumps with 740B pump control units, a 714 pressure monitor, a 744 solvent programmer and a 755 sample injector (loop and syringe injection mode). A Spectra-Physics Model SP 8200 dual-beam UV/visible detector with an SP interference filter kit (254, 312, 365, 436 and 546 nm) was used. The chromatographic columns (25 cm × 4 mm I.D.) were prepacked with

LiChrosorb RP-8 or RP-18 (5 μm) (E. Merck, Darmstadt, G.F.R.) and were run at ambient temperature.

Analytical method

Each flavone derivative was isolated by thin-layer chromatography and separately examined by HPLC in order to determine their retention times and order of elution in our chromatographic system.

Separation was accomplished by gradient elution: solvent A, methanol–water–acetic acid (5:90:5); solvent B, methanol–water–acetic acid (90:5:5); gradient profile, linear from 0 to 35% B in 45 min. The flow-rate was 1.8 ml/min, detection was at 312 nm, 0.04 a.u.f.s., and the sample size was 10 μl.

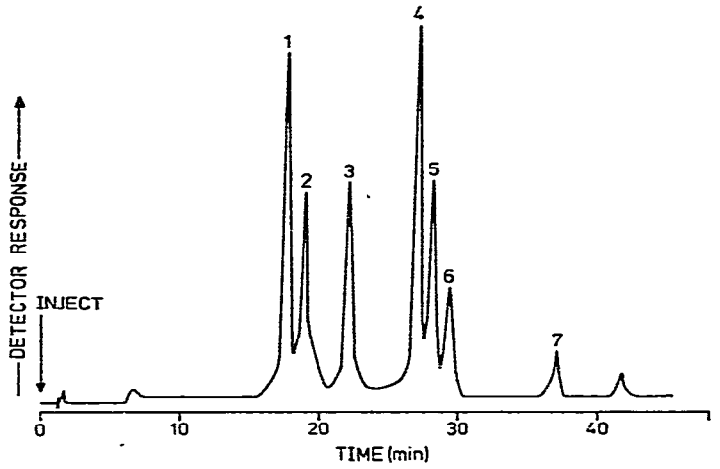

Fig. 1. Separation of flavone derivatives on LiChrosorb RP-8 using a water–methanol gradient. A mixed extract of *Spirodela* and *Fagopyrum* was injected. Peak identifications as in Table I.

TABLE I

CAPACITY FACTORS (k') AND RELATIVE RETENTIONS (α) OF FLAVONE DERIVATIVES ON LICHROSORB RP-8 AND RP-18 USING A WATER–METHANOL GRADIENT

No.	*Compound*	*RP-8*		*RP-18*	
		k'	α	k'	α
1	Orientin	14.83		16.33	
			1.07		1.09
2	Vitexin	15.83		17.83	
			1.16		1.08
3	Isoorientin	18.42		19.33	
			1.22		1.16
4	Isovitexin	22.50		22.33	
			1.04		1.02
5	Luteolin 7-O-glucoside	23.42		22.67	
			1.05		1.02
6	Rutin	24.50		23.17	
			1.26		1.19
7	Apigenin 7-O-glucoside	30.83		27.50	

RESULTS AND DISCUSSION

Fig. 1 demonstrates the separation of six flavone derivatives and one flavonol glycoside (rutin). All components were eluted in less than 40 min. We obtained the same clear resolution when we applied crude extracts. Preliminary cleaning and fractionation is nevertheless preferable in order to avoid loading the column with extraneous components that shorten its lifetime.

Table I gives a comparison of the selectivities of the RP-8 and RP-18 columns. On RP-18 very poor resolution was obtained with isovitexin, luteolin 7-O-glucoside and rutin, precluding their quantitative analysis. On RP-8 these components were clearly resolved.

The results show that in physiological investigations, in which it is desirable to separate rapidly and quantify isomeric C-glycosylflavones from crude extracts, HPLC on RP-8 is an effective and dependable method.

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