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Note

High-performance liquid chromatography of 34 selected flavonoids

D. J. DAIGLE* and E. J. CONKERTON
Southern Regional Research Center/Agricultural Research Science, U.S. Department of Agriculture, P.O. Box 19687, New Orleans, LA (U.S.A.)
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The analysis of flavonoids by high-performance liquid chromatography (HPLC) offers an accurate, sensitive technique which yields results in minutes compared to classical procedures requiring large amounts of material, and days, if not weeks, for analysis. In 1974, Ward and Pelter[1] published the first application of HPLC to flavonoid analysis. Wulf and Nagel[2] later demonstrated, with a dozen flavonoids, the theory and practicality of separating flavone type compounds for identification purposes. Their solvent system of methanol–acetic acid–water (30:5:65) on a C_{18} column was considered adequate for the resolution of a mixture of aglycones containing the same sugar.

In the last five years, researchers have applied HPLC analysis to flavonoids in citrus fruit[3–7], tobacco leaf[8], soybeans[9,10], celery and tomatoes[7], poinsetta[11,12], and species of Larix[13–15] and Cedrus[16]. Attention has also been given to particular flavones such as polymethoxylated flavones[5,7], biflavones[1], and acetylated flavones[7].

The customary column packing was C_{18} but C_8[4,5,7,17] and alkylphenyl columns[10] have been employed. There has been only one report of columns (C_{18}) in series[18]. Most of the work has been reversed-phase and, in addition to methanol, acetonitrile[9,16], ethanol[10,13], and tetrahydrofuran[4,5] have been used.

This note reports the results of HPLC analysis of 34 flavonoids using a methanol–acetic acid–water eluting system on a C_{18} column. Also the mechanism of the HPLC analyses of certain types of flavonoids is interpreted as being related to structure or adsorption qualities.

EXPERIMENTAL

A Waters Assoc. liquid chromatograph equipped with a Model 440 absorbance detector, 254 nm wavelength; two 6000 A pumps and a Model 660 solvent programmer were used. The column was 30 cm × 3.9 mm. I.D. packed with μBondapak C_{18}, 10 μm. Solvents were filtered using a glass Millipore system with a 0.45-μm filter and degassed at room temperature under vacuum with magnetic stirring. Samples were obtained from commercial sources (C) or isolated at Southern Regional Research Center (S) and were used as received. Working solutions contained 1 mg of sample per 2 ml of methanol filtered through a Swinny stainless unit with a 0.45-μm filter. The elution solvent was water–acetic acid (495:5) from pump A and methanol

from pump B. Flow-rate was 2 ml/min with pump A providing 70% and pump B 30% of the solvent mixture for 28 of the compounds studied. The other six used the same solvent system and flow-rate but with each pump providing 50% of the solvent mixture.

The retention times were measured to calculate two chromatographic parameters: the capacity factor, k', and the relative retention, α. These parameters were calculated by the equations[19]:

$$k' = \frac{t_r - t_0}{t_0} \text{ and } \alpha = \frac{k'_2}{k'_1}$$

where t_r = retention time of compound, t_0 = time of the non-retained solvent peak; k'_2 = capacity factor of component 2, and k'_1 = capacity factor of component 1.

RESULTS AND DISCUSSION

Table I lists the common name, descriptive name, capacity factor, and relative retention times of the 34 flavonoids selected for the study.

These data confirmed and extended the findings of Wulf and Nagel[2]. The solvent system used here allowed for greater separation, *e.g.*, quercetin–quercetrin α = 1.90 (Wulf and Nagel α = 1.67).

TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF FLAVONOIDS

Column, μBondapak C_{18}; solvent system, methanol–(acetic acid–water, 5:495) (30:70 for compounds 1–28; 50:50 for compounds 29–34); flow-rate 2 ml/min.

	Common name	*IUPAC name*	t_r *(min:sec)*	k'
1	D-Catechin	3,3′,4′,5,7-Flavanpentol (C)	2:14	0.43
2	Epeatechin	3,3′,4′,5,7-Flavanpentol (C)	2:49	0.80
3	Fustin	3,3′,4′,7-Tetrahydroxyflavanone (C)	3:50	1.45
4	Dihydroquercetin	3,3′,4′,5,7-Pentahydroxyflavanone (C)	4:59	2.18
5	Eriodictyol-7-glucoside	3,4′,5,7-Tetrahydroxyflavanone-7-D-glucoside (S)	5:09	2.29
6	Galongin	3,5,7-Trihydroxyflavone (C)	5:17	2.37
7	Isohesperidin	3′,5,7-Trihydroxy-4[1]-methoxyflavanone-3′--rhamnoglucoside (C)	9:38	5.15
8	Phloridzin	4,6-Dihydroxy-2(β-D-glucosido)-β-(*p*-hydroxyphenyl)-propeophenone (C)	10:25	5.65
9	Myricitrin	3′,4′,5,5′,7-Hexahydroxyflavone-3-rhamnioside (C)	10:25	5.65
10	Quercetin-3-neohesperidoside	3,3′,4′,5,7-Pentahydroxyflavone-3-(2-O-α-L-rhamnosyl-D-glucoside) (S)	10:39	5.80
11	Hesperidin	3,5,7-Trihydroxy-4′-methoxyflavanone-7-(6-O-α-L-rhamnosyl-D-glucoside) (C)	10:44	5.85
12	Quercetin-3-glucoglucoside	3′,4′,5,7-Tetrahydroxyflavone-3-O-glucoglucoside (S)	11:00	6.02
13	Isoquercitrin	3,3′,4′,5,7-Pentahydroxyflavone-3-glucoside (C)	13:00	7.30
14	Rutin	3,3′,4′,5,7-Pentahydroxyflavone-3-(6-O-α-L-rhaminosyl-D-glucoside) (C)	13:14	7.45
15	Quercetin-3-robinoside	3,3′,4′,5,7-Pentahydroxyflavone-3-O-D-galacatose-1-rhamnoside (S)	13:20	7.51

(Continued on p. 204)

TABLE I *(continued)*

	Common name	*IUPAC name*	t_r *(min:sec)*	k'
16	Kaemferol-3-neohesperidoside	3,4′,5,7-Tetrahydroxyflavone-3-(2-O-α-L-rhamnosyl-D-glucoside (C)	15:32	8.91
17	Kaemferol-3-glucoglucoside	3,4′,5,7-Tetrahydroxyflavone-3-glucoglucoside (S)	15:49	9.10
18	Myricetin	3,3′,4′,5,5′,-7-Hexahydroxyflavone (C)	16:30	9.53
19	Robinin	3,4′,5,7-Tetrahydroxyflavone-3-O-D-galactose-1-rhamnoside-7-O-rhamnoside (C)	16:31	9.54
20	Apigetrin	4,5,7-Trihydroxyflavone-7-D-glucoside (C)	16:48	10.04
21	Morin	2′,2,4′,5,7-Pentahydroxyflavone	19:20	11.34
22	Apiin	4′,5,7-Trihydroxyflavone-7-apoiosylglucoside (C)	19:21	11.35
23	Quercitrin	3,3′,4′,5,7-Pentahydroxyflavone-3-L-rhamnoside (C)	19:46	11.61
24	Naringenin	4′,5,7-Trihydroxyflavanone (C)	23:23	13.93
25	Hespertin	3′,5,7-Trihydroxy-4-methoxyflavanone (C)	32:36	19.81
26	Phloretin	2′,4′,6′-Trihydroxy-3-(*p*-hydroxyphenyl)-propcophenme (C)	34:45	21.18
27	Isorhamnetin	3,4′,5,7-Tetrahydroxy-3′-methoxyflavone (S)	35:36	21.72
28	Quercetin	3,3′,4′,5,7-Pentahydroxyflavone (C)	36:06	22.04
29	Kaemferol	3,4′,5,7-Tetrahydroxyflavone (C)	6:49	–
30	Apigenin	4′,5,7-Trihydroxyflavone (C)	7:33	–
31	Rhamnetin	3,3′,4′,5-Tetrahydroxy-7-methoxyflavone (S)	8:36	–
32	Flavone		12:22	–
33	Acacetin	5,7-Dihydroxy-4′-methoxyflavone	18:46	–
34	Techtochrysin	5-Hydroxy-7-methoxyflavone	36:25	–

The separation of kaemferol (3,4′,5,7 tetrahydroxyflavone) and luteolin (3′,4′,5,7 tetrahydroxyflavone) was attributed to the lesser polarity of the 3-hydroxyl group compared to that group at position 3′. The position of the 3-hydroxyl group in hydrogen bonding with the 4-keto group was given as the reason[2]. Examination of the α-values of several pairs show the hydrogen bonding between 3′ and 4′ hydroxyl groups to be more important for separation purposes than the hydrogen bonding between the 3-hydroxyl and the 4-keto group. The α-value for kaemferol–luteolin is only 1.15 compared to the quercetin–morin pair's 1.45. Although the methylation of a hydroxyl group should reduce a compound's polarity and increase its retention time, the elimination of hydrogen bonding between 3′ and 4′ has a greater effect. Isorhamnetin and quercetin have essentially the same retention times. Rhamnetin, methylated on the 7 hydroxyl group, however, did not even elute in a reasonable time with the methanol–acetic acid–water (30:1:69) system.

This work and Rouseff's[4] with polymethoxylated flavones show that another quality, adsorption, and not size, is operative. Methoxy groups on the B ring reduce an aglycone's retention time, *e.g.*, sinensetin (5,6,7,3′,4′-pentamethoxy flavone) elutes faster than tetramethoxyscutellarein (5,6,7,4′-tetramethoxyflavone). In contrast, a methoxy group on the A ring dramatically increases an aglycone's retention time (rhamnetin *versus* quercetin).

It is possible that the theories of Wulf and Nagel and their extension in this report will enable researchers to predict a known compound's retention time and/or assign a tentative structure to an unknown based on its retention time.

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