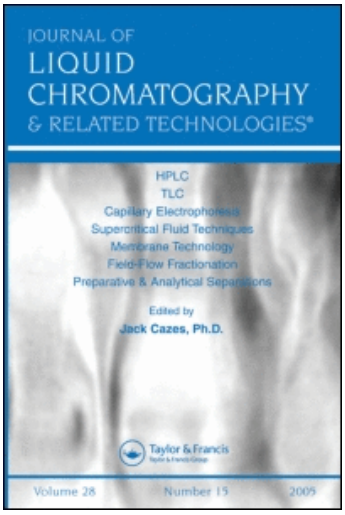

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HPLC SEPARATION OF FLAVONOIDS AND FLAVONOID GLYCOSIDES USING A POLYSTYRENE/DIVINYLBENZENE COLUMN

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ABSTRACT

A polystyrene-divinylbenzene column was investigated for the separation of four different classes of flavonoids and flavonoid glycosides. These analytes were separated using either methanol-water or acetonitrile-water gradients. Acetonitrile-water gradients were judged to be superior for the overall separation of the four classes of flavonoids and flavonoid glycosides. Correlations between chemical structure, and retention and separability are discussed for the various HPLC gradient systems.

INTRODUCTION

Flavonoids are a diverse class of natural products which are essentially ubiquitous in the plant kingdom (1). They display a variety

of interesting antibacterial, antifungal and antitumor activities. Continued interest in the biological activity of the flavonoids has led to the development of a variety of chromatographic techniques for the separation and isolation of flavonoids from plant material. There have been numerous attempts to develop HPLC methods for the separation and quantitation of flavonoids. A few of these methods have been described in the literature. The most successful methods are those based upon reverse phase HPLC (2-5). The analysis of flavonoids and flavonoid glycosides by reversed phase HPLC can be problematic since the compounds are highly polar and tend to tail badly on bonded phase columns. Tailing has generally been avoided by the incorporation of acid or acidic buffers into the mobile phase. There are three major drawbacks to this approach. First, flavonoids are highly unstable to acid and may decompose. Second, repeated use of acidic mobile phases can shorten column life. Third, purification of the compounds on semi-preparative HPLC columns poses the added problem of removing the acid or acidic buffer from the eluent while ensuring that there is no concomitant loss of the flavonoid or flavonoid glycoside.

In this paper, a polymer based PRP-1 column (polystyrene-divinylbenzene) using either methanol-water or acetonitrile-water gradients was investigated for the separation of 18 compounds belonging to the flavone, flavanone, isoflavone and dehydrochalcone

classes. Correlations between chemical structure, retention, and separability are discussed for the various gradients employed.

MATERIALS AND METHODS

Reagents and Chemicals

Acetonitrile and methanol (HPLC grade) were obtained from Fisher Scientific Co. (Pittsburgh, PA.). Table 1 lists systematic names for the four classes of flavonoids studied. Rutin, Rhoifolin, Naringin, Hesperidin, Taxifolin, Phloridzin, Myricetin, Quercetin, Naringenin, Apigenin, Kaempferol, Hesperetin and Chrysin were obtained from Sigma Chemical Company (St. Louis, MO). Phloridzin, Phloretin, Biochanin A, Daidzein, Genistein, Formononetin were purchased from the Indofine Chemical Company (Somerville, NJ). The water used was double distilled in-house.

Chromatographic System

The HPLC system consisted of a Beckman Model 334 gradient liquid chromatograph equipped with a Shimadzu SPD-6AV UV-visible variable wavelength detector and a Shimadzu CR3A integrator. A Hamilton PRP-1 column connected directly to a Altex direct connect PRP guard cartridge was used for analysis. UV spectra were taken on a Beckman DU 7 spectrophotometer. Samples were filtered prior to injection using a nylon-66 membrane filter (0.45 μm, Ranin, Emeryville, CA).

Table 1: Systematic Chemical Names of Flavonoids Studied

FLAVONES	
Rutin	3,3',4',5,7 Pentahydroxy-3-b-rutinoside
Rhoifolin	4',5,7-Trihydroxyflavone-7-O-hesperidoside
Myricetin	3, 3', 4', 5, 5', 7 - Hexahydroxyflavone
Quercetin	3,3',4',5,7-trihydroxyflavone
Apigenin	4',5,7-Tetrahydroxyflavone
Kaempferol	3,4',5,7-Tetrahydroxyflavone
Chrysin	5,7-dihydroxyflavone
FLAVANONES	
Naringin	4',5,7-Trihydroxyflavanone-7-rhamnoglucoside
Hesperidin	3', 5, 7 - Trihydoxy-4'-methoxyflavanone-7 rhamnoglucoside
Taxifolin	3,3',4',5,7-Pentahydroxyflavanone
Naringenin	4', 5, 7- Trihydroxyflavanone
Hesperetin	3', 5,7- Trihydroxy-4-methoxy-flavanone
ISOFLAVONES	
Daidzein	4',7 - Dihydroxyisoflavone
Genistein	4', 5, 7 - Trihydroxyisoflavone
Formononetin	4'-Methoxy-7-hydroxyisoflavone
Biochanin A	5,7-Dihydroxy-4-methoxyisoflavone
DIHYDROCHALONES	
Phloridzin	4,4',6-Trihydroxydihydrochalcone- 2'-glucoside
Phloretin	2',4,4',6'-Tetrahydroxydihydrochalcone

Preparation of Standard Flavonoid and Flavonoid Glysoside Solutions

Each flavonoid or flavonoid glycoside (20 mg) was accurately weighed and dissolved in 100 ml of absolute methanol to give a final concentration of 0.2 mg/ml.

Chromatographic Conditions

A. For methanol/water mobile phase

Mobile phase A consisted of 100% absolute methanol and mobile phase B consisted of 100% water. The analytes were separated using a flow rate of 2.0 ml/min. The UV detector was set at 260 nm. An injection volume of 20 μl was utilized and the chromatographic run time was 35 minutes. The following gradient program was used:

Time, min	% A	% B
0	60	40
15	75	25
25	100	0
35	100	0

B. For acetonitrile/water mobile phase

Mobile phase A consisted of 100% acetonitrile and mobile phase B consisted of 100% water. All other chromatographic conditions were similar to that described above except the run time was 40 minutes.

The following gradient program was used:

Time, min	% A	% B
0	20	80
30	50	50
40	75	25

RESULTS AND DISCUSSION

The initial studies were to determine the optimum wavelength for detection since flavonoids have such diverse structures and hence, different UV maxima. UV spectra for all the flavonoids studied were taken and 260 nm was chosen as the most suitable wavelength for the assay. While the use of 260 nm does not provide the absolute minimum detectable concentration for each compound, it does provide a realistic wavelength to measure each analyte component in complex mixtures of flavonoids. Figure 1 shows typical UV spectra for two of the flavonoids studied, Hesperetin and Biochanin A.

After investigating various methanol-water gradients, the gradient parameters described in the Materials and Methods section (Chromatographic conditions, part A) was found to be suitable for the flavonoid separation. Table 2 lists the analytical figures of merit for flavonoid separations using the methanol-water gradient. It was possible using this gradient to separate flavonoids within a given class, but not

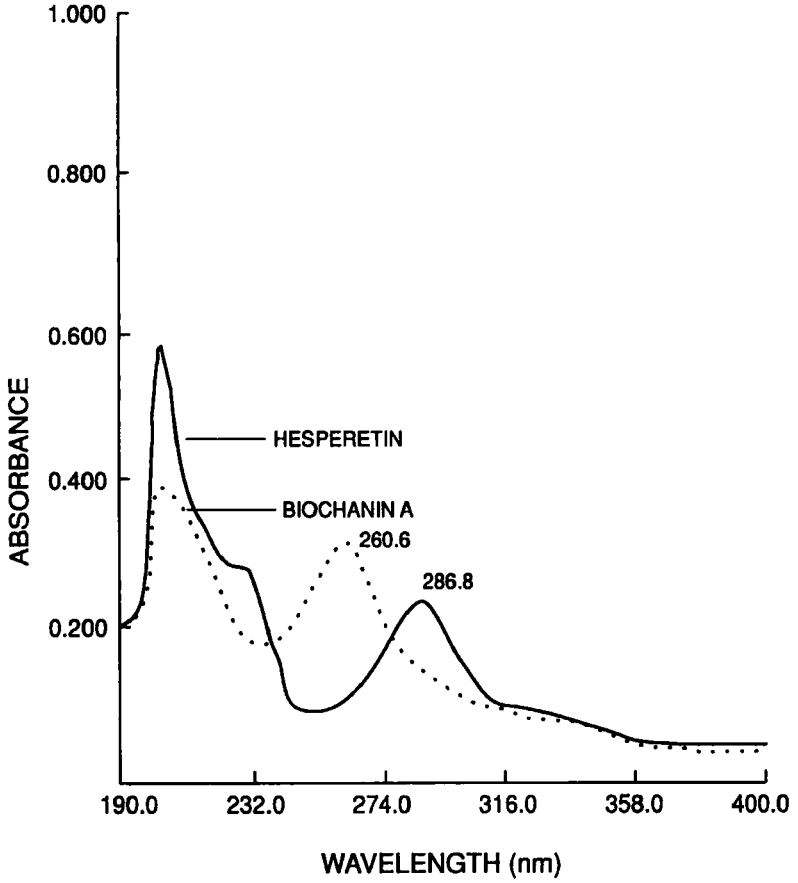

Figure 1 - Comparison of UV spectra of hesperetin and biochanin A

those between classes. However, peak shapes were sharp for all the analytes studied.

Based on these data, acetonitrile was investigated as the organic modifier in the gradient system. After studying different gradients, the acetonitrile-water gradient described in the Materials and Method section

Table 2 - Analytical Figures of Merit Using a Methanol/Water Gradient

Substance	tr (min)	k'	Separation Factor (a)	Tailing Factor (T_f)
Flavones				
Rutin	2.0	2.3	1.0	1.0
Rhoifolin	2.6	3.3	1.4	1.0
Myricetin	4.5	6.5	2.8	1.0
Quercetin	12.1	19.2	8.3	1.1
Kaempferol	22.2	36.0	15.7	1.0
Apigenin	23.0	37.3	16.2	1.0
Chrysin	31.6	51.7	22.5	1.1
Flavonones				
Naringin	2.6	3.3	1.0	1.0
Taxifolin	3.0	4.0	0.3	1.1
Hesperidin	3.3	4.5	1.4	1.0
Naringenin	20.7	33.5	10.2	1.0
Hesperetin	23.7	38.5	11.7	1.0
Isoflavones				
Daidzein	2.5	3.2	1.0	1.0
Genistein	2.9	3.8	1.2	1.1
Formononetin	5.1	7.5	2.4	1.0
Biochanin A	7.7	11.8	3.7	1.1
Dihydrochalcones				
Phloridzin	6.6	10.0	1.0	1.0
Phloretin	2.2	2.7	0.3	1.1

Table 3 - Analytical Figures of Merit Using an Acetonitrile/Water Gradient

Substance	tr (min)	k'	Separation Factor (α)	Tailing Factor (T_f)
Flavones				
Rutin	2.9	3.8	1.0	1.0
Rhoifolin	4.4	6.3	1.7	1.0
Myricetin	8.5	13.2	3.5	1.1
Quercetin	13.1	20.8	5.5	1.1
Apigenin	17.0	27.3	7.2	1.0
Kaempferol	18.1	29.2	7.7	1.1
Chrysin	28.7	46.8	12.3	1.0
Flavanones				
Naringin	5.1	7.5	2.0	1.0
Hesperidin	5.4	8.0	2.1	1.0
Taxifolin	5.6	8.3	2.2	1.1
Naringenin	16.3	26.2	6.9	1.1
Hesperetin	18.9	30.5	8.0	1.0
Isoflavones				
Daidzein	11.3	17.8	4.7	1.0
Genistein	17.7	28.5	7.5	1.0
Formononetin	22.5	36.5	9.6	1.1
Biochanin A	31.3	51.2	13.5	1.1
Dihydrochalcones				
Phloridzin	6.6	10.2	2.7	1.0
Phloretin	15.6	25.7	6.8	1.1

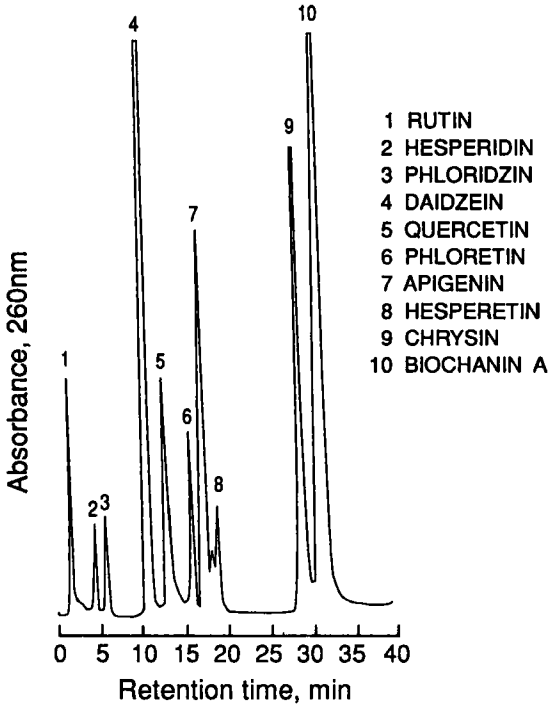

Figure 2 - Typical HPLC chromatogram of mixture of ten flavonoids

(chromatographic conditions, part B) gave the best selectivity. Table 3 lists the analytical figures of merit for each analyte using this gradient. It was observed that there was adequate separation for all of the flavonoid classes studied. Tailing factors were close to 1 compared to experiments performed in these laboratories using a methanol/aqueous pH 4.7 phosphate buffer on a octadecylsilane column where typical tailing factors such as 4.4 for quercetin, 1.4 for taxifolin and 0.4 for rutin were obtained. Figure 2 shows an HPLC separation of 10 different

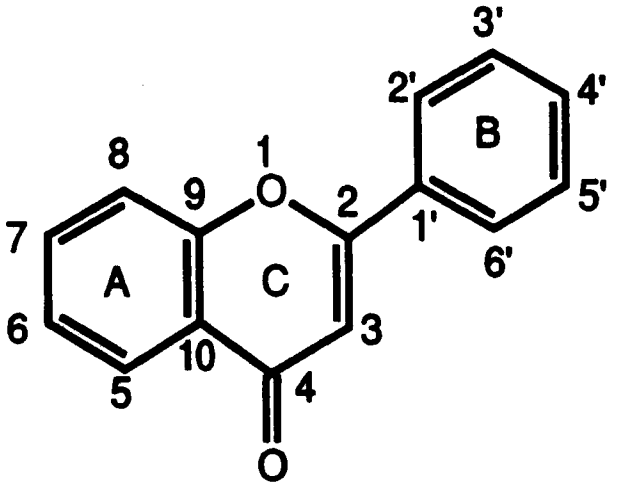

Figure 3 - Basic chemical structure of a flavonoid

flavonoids in a single injection on the PRP-1 column using the acetonitrile-water gradient.

The elution sequence of the individual analytes appears to depend on increased or decreased polarity of the compounds and also on the extent of hydrogen bonding (3). The order of elution is therefore similar to that reported for flavonoids on bonded reversed phase columns (3,4).

Analogous arguments regarding the effect of hydroxyl groups on the retention time of these flavonoids can be made (3,4). Figure 3 shows the basic chemical structure of a flavonoid. Hydroxyl groups at positions other than three and five on rings A or C render a flavonoid more hydrophilic. This is evidenced by the elution profile of myricetin, quercetin and taxifolin and naringenin (See Table 3).

Hydroxyl groups at positions three and five on rings A and C increase retention time, although this effect is not as pronounced with kaempferol and apigenin as it is with daidzein and genistein or formononetin and biochanin A. This can best be explained by the formation of internal hydrogen bonding between a carbonyl group at the position 4 on ring C and the hydroxyl group at position 5 in ring A of these compounds. This is compared to only a slight increase in the retention time of apigenin and kaempferol.

As expected, glycosylation of a flavonoid has a pronounced effect on the retention time of the analyte by reducing the retention by approximately 10 minutes for each of the classes examined (compare rhoifolin-apigenin, naringin-naringenin, hesperidin-hesperetin, rutin-quercetin and phloridzin-phloretin). Interestingly, the effect on retention time is fairly uniform regardless of the position of glycosylation.

Saturation of ring C, (i.e. transformation of a flavone to the corresponding flavonone) decreases the retention time. This is evident comparing Apigenin to Naringenin and Quercetin to Taxifolin, although the effect appears more pronounced for the latter than the former. This is probably due to interruption of conjugation which affects the hydrogen accepting or donating abilities of all the hydroxyl groups. Therefore, the larger the number of hydroxyl groups, the more pronounced is the decrease in retention time.

The acetonitrile-water gradient outlined herein provides a good separation of a diverse group of flavonoid structures without the necessity of using acid or acidic buffers. An added advantage of this method is that both flavonoids and their respective glycosides can be analyzed using the same gradient system. The method thus is an excellent technique for analyzing flavonoids which differ greatly in their polarity. Semi-preparative and preparative isolation of the flavonoids should be possible using commercially available PRP preparative columns and direct scaleup of this procedure.

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