

## Analysis of 5-hydroxy-7-methoxyflavones by normal-phase high-performance liquid chromatography

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### Abstract

The separation and identification of flavones present in a chloroform extract of *Baccharis trinervis* leaves was investigated. The chromatographic system consisted of a amino-bonded column, gradient elution from hexane–chloroform (85:15) to chloroform–acetonitrile (40:60) and detection at 346 nm. Four flavones were found. From NMR and MS data they were identified as 5-hydroxy-7,4'-dimethoxyflavone (I), 5-hydroxy-7,3',4'-trimethoxyflavone (II), 5,3'-dihydroxy-7,4'-dimethoxyflavone (III) and 5,4'-dihydroxy-7-methoxyflavone (IV). Flavone II and III have not been found in *Baccharis trinervis* before. The chromatographic system showed good selectivity for the separation of the flavones. The relation between  $t_R$  and the structure is discussed.

**Keywords:** *Baccharis trinervis*; Flavones; Hydroxymethoxyflavones

### 1. Introduction

Flavones are secondary metabolites found in distinct families and genera in the plant kingdom [1]. They present a significant chemical marker for chemotaxonomic studies [2]. Flavones show several biological properties such as inhibition of protein tyrosine kinase [3], cytotoxicity against cancer cells [4], antiviral [5] and antibacterial activity [6] and antioxidant activity [7].

Wollenweber and Jay reviewed the analysis of flavones by high-performance liquid chromatography (HPLC) and thin-layer chromatography [1]. Analysis of flavonoids has been achieved by capillary zone

electrophoresis [8]. A selective method for the analysis of flavones by gas chromatography with mass spectrometric detection (GC–MS) with low limit of detection, has also been reported [9].

In HPLC, flavones are usually separated with reversed-phase columns [10,11]. Correlation between flavone structure and retention time ( $t_R$ ) have been reported in the case of  $C_{18}$  phases [12]. On the other hand, normal-phase HPLC of flavones has been reported mainly applying silica columns [13]. Pre-acetylated flavonoids have been separated by HPLC on a cyano-silica column [14]. The use of amino-bonded phases in the analysis of flavones by HPLC has not been described.

Amino-bonded phases show good selectivity for polar compounds like sugars [15]. They are used in

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the class separation of polyaromatic hydrocarbons [16] and polyether [17]. Polyhydroxylated steroids have been separated using amino-bonded silica columns [18]. This report presents the results of the application of an amino-bonded column in the HPLC of lipophilic flavones isolated from leaves of *Baccharis trinervis*. The identification of the flavones was carried out by spectroscopic methods.

## 2. Experimental

### 2.1. Materials

For chromatographic separation solvents were HPLC grade whereas analytical grade reagents were used in the extraction of leaves. Chloroform, acetonitrile and hexane were obtained from J.T. Baker (Phillipsburg, NY, USA). Ethanol and lead acetate were obtained from BDH (Poole, UK) and dimethyl sulfoxide from Riedel-de Haën (Hannover, Germany).

### 2.2. Plant material

The leaf samples of *Baccharis trinervis* were collected in Cumaná (Venezuela). They were taxonomically identified by the Department of Botany of the Universidad de Oriente (Venezuela).

### 2.3. Chromatographic system

Isocratic and gradient elutions were performed with a Water Associates HPLC system composed of a Model 660 solvent programmer in conjunction with a Model M6000A and a Model M45 pump and a U6K injector and a Model 484 UV detector set at 346 nm. Fractions were collected with an Isco Foxy fraction collector and an Isco Model 2150 peak separator.

### 2.4. Extraction

The flavones were found in a sesquiterpenic lactone extract which was obtained as previously described [19]. The leaves (2000 g, dry mass) were extracted with chloroform (3×900 ml), extracts were combined and the solvent was removed under re-

duced pressure at 35°C. The residue was dissolved in 95% ethanol (250 ml) and 4% aqueous lead acetate (250 ml). After formation of a precipitate, the solution was filtered. The filtrate was extracted with chloroform (3×150 ml). The chloroform fractions were combined and reduced to 10 ml under reduced pressure. The solution was kept at -10°C to allow precipitation of the flavones, filtered and the precipitate was washed with chloroform to remove terpenoids. A sample of the dry precipitate (64 mg) was dissolved in 1 ml of dimethyl sulfoxide for the chromatographic analysis.

### 2.5. Elution

The flavones were injected onto an amino-bonded column ( $\mu$ Bondapak-carbohydrate, 300×4 mm I.D., 10  $\mu$ m particle diameter, Waters Associates) at room temperature (22°C) and eluted with a gradient, from hexane-chloroform (85:15) (isocratic, 10 min) to chloroform in 12 min (linear) then chloroform (isocratic, 6 min) to chloroform-acetonitrile (40:60) in 25 min (linear). The flow-rate was set at 1 ml min<sup>-1</sup>.

### 2.6. Identification

Separation and collection of the flavones (50.2 mg) was achieved in two steps using the same analytical column. Compounds III and IV were eluted with chloroform-acetonitrile (95:5). The injection volume was 25  $\mu$ l (64 mg ml<sup>-1</sup>). Compounds I and II were chromatographed with hexane-chloroform (90:10). The injection volume was 15  $\mu$ l (45 mg ml<sup>-1</sup>). The solvents were removed from collected fractions in a rotaevaporator under reduced pressure.

### 2.7. GC-MS

The fractions were analyzed by capillary gas chromatography (Hewlett-Packard Model HP58890 Series II) equipped with a split/splitless injector, a cross-linked methyl silicone capillary column (HP Ultra 1, 25 m×0.2 mm I.D., 0.11  $\mu$ m thickness) and a mass detector (Hewlett-Packard Model HP 5971A). Injections were splitless with the oven temperature

programmed from 100°C (1 min) to 300°C (3 min) at 6°C/min. The injector, transfer line and mass detector temperatures were 280, 300 and 186°C, respectively. The ionization voltage applied was 70 eV. Samples of the flavones were dissolved in dimethyl sulfoxide for the GC–MS analysis.

### 2.8. NMR

<sup>1</sup>H NMR was carried out in a 300 MHz Bruker spectrometer, Model AM. The analyses were taken in deuterated chloroform. Tetramethyl silane was the internal standard.

## 3. Results and discussion

Fig. 1 shows a typical chromatogram of the chloroform extract of *Baccharis trinervis* leaves. The amino-bonded column showed adequate affinity for the flavones and gave well-resolved and symmetric peaks. The conditioning of the column was critical for the separation of I and II. Adequate resolution was only achieved after equilibrating with 90 ml of initial mobile phase (equivalent to thirty times the column volume) prior to each separation. Slow column equilibration has been reported for amino-bonded stationary phases [20].

In order to identify the flavones, they were collected using HPLC with the same analytical amino-bonded column. In this case isocratic elution was chosen to avoid the slow equilibration after the gradient. Two mobile phases were necessary since there were two groups of the flavones that differ considerably in their affinity for the stationary phase. With chloroform–acetonitrile (95:5), III ( $t_R=12.9$  min) and IV ( $t_R=16.4$  min) could be separated and collected as pure compound while the less-strongly retained eluted at the same retention time (3.4 min). With hexane–chloroform (90:10), I ( $t_R=9.1$  min) and II ( $t_R=12.5$  min) were then separated and collected. Due to the high selectivity of the chromatographic system, the injections for the collection of the flavones could be run under relatively high overloading conditions. No deterioration of the efficiency of the column was observed in the course of the flavone collection experiments.

The amounts of the collected flavones after remo-

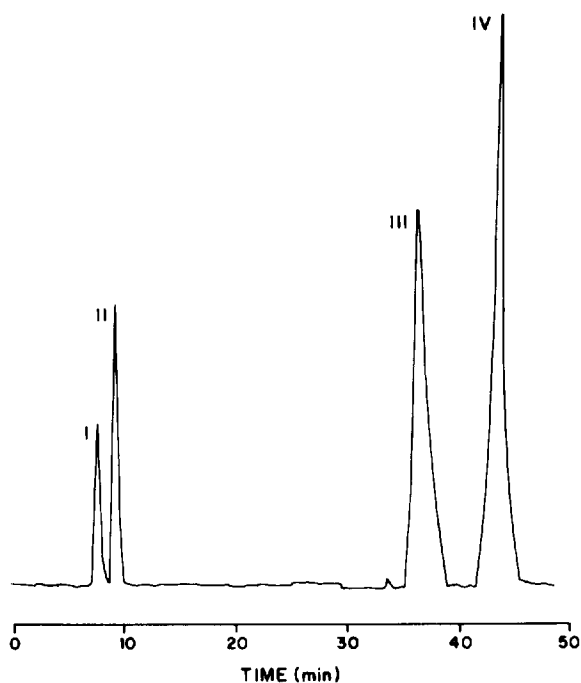


Fig. 1. Separation of the flavones on an amino-bonded column ( $\mu$ Bondapak/carbohydrate, 300 $\times$ 4 mm I.D.). Solvent system: hexane–chloroform (85:15) (isocratic 10 min) to chloroform in 12 min (linear) then chloroform (isocratic 6 min) to chloroform–acetonitrile (40:60) in 25 min (linear). Detection was at 346 nm. Flow-rate 1 ml min<sup>-1</sup>. Peaks: I=5-hydroxy-7,4'-dimethoxyflavone,  $t_R=7.33\pm 0.04$  min; II=5-hydroxy-7,3',4'-trimethoxyflavone,  $t_R=9.0\pm 0.1$  min; III=5,3'-dihydroxy-7,4'-dimethoxyflavone,  $t_R=36.2\pm 0.1$  min; IV=5,4'-dihydroxy-7-methoxyflavone,  $t_R=43.8\pm 0.1$  min.

tion of the mobile phases were 7.4, 9.1, 14.2 and 17.1 mg for I, II, III and IV, respectively. The overall quantity of collected flavones represented 95% of the flavones chromatographed for the identification in the amino-bonded column. The purity of each fraction was confirmed by the GC–MS analysis.

The results of MS analysis of the flavones is shown in Table 1. The ions designated as  $A_1^+$  or  $[A_1+H]^+$  and  $B_1^+$  result from a retro-Diels–Alder cleavage of the flavones. Whereas  $B_2^+$  is a benzoyl type ion which arises from the other common fragmentation of flavones [21,22]. These ions provide information about the groups bound to the A and B rings of the isolated flavones.

The flavones gave an intense molecular ion as is usually the case for aromatic compounds. The  $A_1^+$

Table 1  
Ions in mass spectra of the flavones

Flavone	Mass data, $m/z$ (intensity as % base peak)						
	[M] <sup>+</sup>	[M-CHO] <sup>+</sup>	[A <sub>1</sub> ] <sup>+</sup>	[A <sub>1</sub> +H] <sup>+</sup>	[A <sub>1</sub> -CO] <sup>+</sup>	[B <sub>1</sub> ] <sup>+</sup>	[B <sub>2</sub> ] <sup>+</sup>
I	298 (100)	269 (21)	166 (10)	N.D. <sup>a</sup>	138 (7)	132 (11)	135 (15)
II	328 (100)	299 (15)	N.D. <sup>a</sup>	167 (27)	138 (9)	162 (19)	N.D. <sup>a</sup>
III	314 (100)	285 (18)	N.D. <sup>a</sup>	167 (23)	138 (7)	148 (9)	N.D. <sup>a</sup>
IV	284 (100)	255 (43)	N.D. <sup>a</sup>	167 (18)	138 (19)	118 (11)	121 (8)

<sup>a</sup> Not detected.

( $m/z$  166) or [A<sub>1</sub>+H]<sup>+</sup> ( $m/z$  167) ions were the same in the four cases. Thus, the flavones had their A rings with the same substituents, a hydroxyl and a methoxyl group. The position of these groups was also the same as can be seen from the NMR data (Table 2). Each flavone presented a proton at C-6 (doublets in the range 6.30–6.35 ppm) and C-8 (doublets in the range 6.47–6.62 ppm) with a characteristic coupling constant for the *meta* position

( $J=2.3$  Hz). The hydroxyl group was assigned at C-5 on the basis of the singlets in the range of 12.8–13.0 ppm. These chemical shifts are characteristic of an internal hydrogen bond between the 5-OH and the carbonyl group at C-4. Thus the four compounds isolated are identified as 5-hydroxy-7-methoxy-type flavones.

The structure of the flavones differed in the number and type of substituents in the B rings (Fig.

Table 2  
<sup>1</sup>H NMR data of the flavones

Flavone	Protons <sup>a</sup>								
	H-3 <sup>b</sup>	H-6	H-8	H-2'	H-3'	H-5'	H-6'	Methoxyl H <sup>b</sup>	C-5 OH <sup>b</sup>
I	6.57	6.35d (2.3)	6.47d (2.3)	7.83m (8.97) (2.57) (0.1)	7.00m (8.97) (2.57) (0.1)	7.00m (8.97) (2.57) (0.1)	7.83m (8.97) (2.57) (0.1)	3.87 3.88	12.80
II	6.57	6.34d (2.3)	6.47d (2.3)	7.31d (2.1)	N.A. <sup>d</sup>	6.95d (8.5)	7.50dd (8.5) (2.1)	3.86 3.94 3.96	12.84
III	6.78	6.30 (2.3)	6.62d (2.3)	7.48 <sup>c</sup>	N.A. <sup>d</sup>	6.94d (8.6)	7.50dd (8.6) (2.2)	3.88 3.94	12.93
IV	6.54	6.31d (2.4)	6.54d (2.4)	7.78m (8.4) (2.4) (0.1)	6.96m (8.4) (2.4) (0.1)	6.96m (8.4) (2.4) (0.1)	7.78m (8.4) (2.4) (0.1)	3.89	12.85

<sup>a</sup> Chemical shifts in ppm down field from (CH<sub>3</sub>)<sub>4</sub>Si; CDCl<sub>3</sub> solvent; multiplicity indicated by: d=doublet, dd=doublet of doublets, m=multiplet, s=singlet; coupling constants  $J$  (hertz) given in parentheses.

<sup>b</sup> All singlets.

<sup>c</sup> Overlapped by H-6'.

<sup>d</sup> does not apply.

2). The  $^1\text{H}$  spectra of I and IV (Table 2) showed a *para* substitution pattern on their B rings. Thus, I and IV had their B ring substituted only at C-4'. The  $\text{B}_1^-$  and  $\text{B}_2^+$  ions (Table 1) indicated, in the case of I that the substituent was a methoxyl group whereas in IV it was a hydroxyl group.

Flavone II and III had a disubstituted B ring (two methoxyl groups in II, a hydroxyl and a methoxyl group in III). The  $^1\text{H}$  spectra of these flavones had characteristic signals (7.31–7.50 ppm) for protons attached to 2' and 6'. Also, both flavones presented *ortho* proton–proton coupling between H-5' and H-6' (Table 2). Thus II and III had their B ring substituted at the same position, C-3' and C-4' (Fig. 2). Flavone II and III have not been found previously in *Baccharis trinervis* [23].

For amino-phase systems that do not involve the

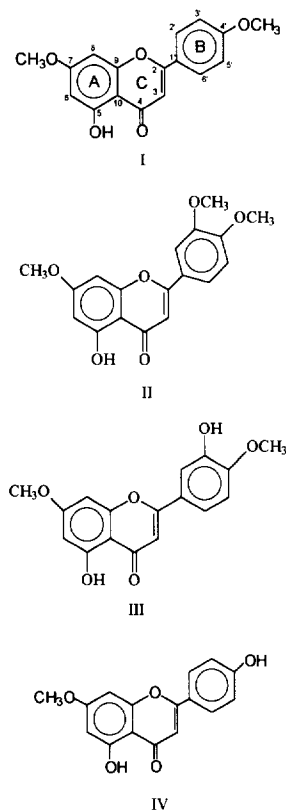


Fig. 2. Structure of the 5-hydroxy-7-methoxyflavones. I=5-Hydroxy-7,4'-dimethoxyflavone; II=5-hydroxy-7,3',4'-trimethoxyflavone; III=5,3'-dihydroxy-7,4'-dimethoxyflavone; IV=5,4'-dihydroxy-7-methoxyflavone.

use of alcohols as mobile phase solvents, the displacement model has been found adequate to describe the retention of solute [20]. This model focuses on adsorption interactions. The variation of  $t_R$  found in this work can be explained on the basis of flavone–amino group interactions.

The values of  $t_R$  indicate that an hydroxyl group in the B ring increased the retention in comparison with methoxyl groups (see Fig. 1). This difference in interaction between these groups and amino-bonded stationary phase has been observed in the case of substituted naphthalene [20]. These results can be explained on the basis of stronger interaction via hydrogen bonds between the hydroxyl groups and the amino-bonded stationary phase.

Flavone III was less strongly retained than IV. Flavone III had the methoxyl and hydroxyl group in *ortho* position so they can form intramolecular hydrogen bonding. This interaction competes with the interactions of either the hydroxyl and methoxyl groups and the stationary phase. In the case of IV, the hydroxyl group is freer to interact with the stationary phase, since it is unable to form intramolecular hydrogen bonding.

The difference in  $t_R$  between flavones I and II can be attributed to the number of localizing groups attached to the B rings enabling more interactions of II with the stationary phase.

Flavones with a hydroxyl group at C-5 have been found to interact strongly with silica phase whereas the substitutions of hydroxyl groups at other positions, e.g., 3 or 7, produced considerably lower interaction [24]. An opposite behavior was observed in this work and has also been found when a cyano column was used [24]. The contribution of the 5-OH to the retention of the flavones in the amino column was lower than the contribution of hydroxyl groups bound to other positions. Thus, amino-bonded stationary phases are expected to give higher selectivity than silica columns for the separation of 5-hydroxyflavones that differ in the OH pattern substitution.

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