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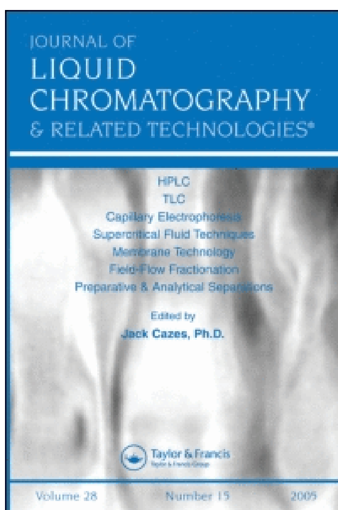

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María I. Gil[a]; Federico Ferreres[a]; Francisco A. Tomás-Barberán[a]
[a] Laboratorio de Fitoquímica Departamento de Ciencia y Tecnología de Alimentos CEBAS (CSIC), Murcia, Spain

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MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY OF METHYLATED FLAVONE AGLYCONES

MARÍA I. GIL, FEDERICO FERRERES, AND FRANCISCO A. TOMÁS-BARBERÁN*

Laboratorio de Fitoquímica
Departamento de Ciencia y Tecnología de Alimentos
CEBAS (CSIC)
P.O. Box 4195
Murcia 30080, Spain

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Abstract

Thirty four flavone aglycones isolated from herbs and spices were analysed by MECC on fused silica capillaries with sodium borate buffers and SDS micelles. Addition of organic solvents was necessary to improve the separation. However, when methanol was used, either as a sample solvent or as a constituent of the buffer, the most hydrophobic flavones appeared as double or triple peaks in the electropherograms. These double peaks disappeared when acetonitrile was used instead of methanol. The best separation was obtained with buffers containing acetonitrile when samples were dissolved in the same running buffer. In the separation of these flavones, in addition to the hydrophobic interaction with micelles, the ionization of phenolic hydroxyls and the borate complexation had some effect in the migration order of the molecules. A correlation between the migration order in MECC and the elution order previously reported for reversed-phase HPLC analyses was generally observed.

* Address for correspondence.

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INTRODUCTION

Analysis of plant flavonoids by Capillary Electrophoresis has been developed in the last few years since the first work published by Pietta and coworkers in 1991 [1]. Capillary Zone Electrophoresis (CZE) has been used in the analysis of flavonol O-glycosides [2-4], flavone O-glycosides [5] and flavonoid C-glycosides [6]. In addition, Micellar Electrokinetic Capillary Chromatography (MECC) has been used in the analysis of flavone O-glycosides [5] and flavonol O-glycosides [1,7,8]. However, analysis of flavonoid aglycones by these techniques has not been studied to the same extent, and only a few works on the MECC analysis of polyhydroxylated flavonol aglycones [7,9], and on the flavonoid aglycones (flavonols, flavones and flavanones) from honey [10,11] have been published. To date no analysis of lipophilic methylated flavones by Capillary Electrophoresis has been reported. These flavonoids are constitutive of many herbs and spices, and are partly responsible for their food antioxidative properties [12]. In addition, these lipophilic flavonoids have pharmacological [13] and ecological effects [14]. The aim of the present work is to establish conditions for the analysis of these flavonoid aglycones and to study the effect of the structures on their electrophoretic mobility.

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EXPERIMENTAL

Materials

The flavonoid aglycones listed in table I were used as standards. All these compounds had previously been isolated and identified from Labiatae herbs [15] and these are deposited in the collection of the Phytochemical Laboratory (CEBAS, CSIC, Murcia).

Sample preparation

The different standard aglycones were dissolved in methanol, acetonitrile or running buffer to be analysed by CE. The available flavonoid aglycones were grouped into six different groups (**1-6**) (table 1) according to their oxygenation pattern in order to study the influence of increasing methylation on CE behaviour.

Table 1. MECC analysis of different lipophilic methylated flavones.

Groups	Aglycone names	Structures	OMe/OH	Buffers		
				A	B	C
	sideritoflavone	5,3',4'-OH 6,7,8-OMe	3/3	8.01	11.26	6.70
	thymonin	5,6,4'-OH 7,8,3'-OMe	3/3	10.18	12.27	7.10
	menthoflavone	5,6-OH 7,8,3',4'-OMe	4/2	18.85 / 19.90	12.75	7.28
1	8-methoxycirsilineol	5,4'-OH 6,7,8,3'-OMe	4/2	18.94	20.62	15.69
	gardenin D	5,3'-OH 6,7,8,4'-OMe	4/2	19.85	22.31	17.28
	nobiletin	5,6,7,8,3',4'- OMe	6/0	19.13 / 19.53*	22.31/22.55*	17.60
	5-desmethilnobiletin	5-OH 6,7,8,3',4'-OMe	5/1	19.15 / 19.47*	25.06/25.83*	18.38
	scutellarein	5,6,7,4'-OH	0/4	7.03	12.57	7.17
	isoscutellarein	5,7,8,4'-OH	0/4	7.56	12.82	7.37
2	hispidulin	5,7,4'-OH 6-OMe	1/3	10.46	13.81	9.22
	cirsimaritin	5,4'-OH 6,7-OMe	2/2	14.36	21.44	16.30
	tetramethoxyflavone	5,6,7,4'-OMe	4/0	19.23/19.42*	28.71/29.74*	18.12
	salvigenin	5-OH 6,7,4'-OMe	3/1	19.32/19.70*	30.91/31.39*	18.80
	apigenin	5,7,4'-OH	0/3	12.54	14.68	10.51
3	acacetin	5,7-OH 4'-OMe	1/2	14.01	18.04	14.58
	genkwanin	5,4'-OH 7-OMe	1/2	14.69	18.22	14.80
	apigenin 7,4'-methyl ether	5-OH 7,4'-OMe	2/1	16.70 /17.44 *	25.63*	25.07
	cirsiliol	5,3',4'-OH 6,7-OMe	2/3	7.90	12.52	6.80
	nodifloretin	5,6,7,4'-OH-3'-OMe	1/4	8.63	13.01	8.70
	6-OH luteolin	5,6,7,3',4'-OH	0/5	10.12	14.27	9.28
	hypolaetin	5,7,8,3',4'-OH	0/5	10.42	14.43	9.66
4	nepetin	5,7,3',4'-OH 6-OMe	1/4	10.49	16.58	11.70
	cirsilineol	5,4'-OH 6,7,3'-OMe	3/2	17.16	21.79	15.73
	sinensetin	5,6,7,3',4'-OMe	5/0	17.25 *	22.02/22.29*	16.18
	eupatorin	5,3'-OH 6,7,4'-OMe	3/2	17.60*	24.13/24.42*	18.02
	5-demethylsinensetin	5-OH 6,7,3',4'-OMe	4/1	18.90*	27.30/27.78*	19.18
	thymusin	5,6,4'-OH 7,8-OMe	2/3	9.92	13.13	10.18
5	xanthomicrol	5,4'-OH 6,7,8-OMe	2/3	14.08	20.49	21.36
	tangeretin	5,6,7,8,4'-OMe	5/0	14.52/14.80*	24.64/25.52*	24.46
	gardenin B	5-OH 6,7,8,4'-OMe	4/1	14.90*	28.39/29.31*	26.16
	luteolin 7-methyl ether	5,3',4'-OH 7-OMe	1/3	7.76	9.92	8.17
6	luteolin	5,7,4',3'-OH	0/4	8.54	10.66	9.92
	chrysoeriol	5,7,4'-OH 3'-OMe	1/3	11.65	15.10	10.95
	diosmetin	5,7,3'-OH 4'-OMe	1/3	14.38	16.26	11.62

Values are migration times in minutes. Buffer conditions: buffer **A** 0.2M sodium borate (pH 8) 50 mM SDS and 10% MeOH; buffer **B** 0.1M sodium borate (pH 8) 30 mM SDS and 25 % MeOH; buffer **C** 0.1 M sodium borate (pH 8) 50 mM SDS and 10% ACN. The samples were dissolved in methanol in the separations with buffers **A** and **B** and in the running buffer in separation with buffer **C**. *Double or triple peaks.

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Micellar electrokinetic capillary chromatography (MECC).

MECC separations were carried out using a Beckman P/ACE System 5000 apparatus equipped with a 87 cm X 75 µm I.D. (80 cm to detector) fused-silica capillary and a Diode Array Detector. The daily conditioning started by rinsing the column with methanol (5 minutes), then with 1M NaOH solution (5 minutes), 0.1M NaOH (5 minutes), distilled water (5 minutes) and ending with the running buffer (3 minutes). Between consecutive runs the capillary was flushed with 0.1M NaOH (3 minutes), water (2 minutes) and running buffer (3 minutes). All buffers were freshly prepared after 2 injections. The following running buffers were used for the analyses of the different flavonoid aglycones: (**A**) 0.2M sodium borate (pH 8) 50 mM SDS and 10% MeOH; (**B**) 0.1M sodium borate (pH 8) 30 mM SDS and 25 % MeOH; (**C**) 0.1M sodium borate (pH 8) 50 mM SDS and 10% ACN (SDS, Sigma). The voltage was 20 kV, with an average current of 55µA, 32 µA and 65 µA respectively for buffers **A**, **B** and **C**, and the samples were injected by hydrodynamic injection for 2 seconds. All electropherograms were recorded on a Merck-Hitachi (Darmstadt, Germany) integrator. The on-column detector was operated at 280 nm, and the UV spectra recorded with the diode array detector.

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RESULTS AND DISCUSSION

Separation of lipophilic methylated flavone aglycones by CE.

Due to the lipophilic nature of these flavone aglycones, and to the reduced number of ionizable phenolic hydroxyls in many of their molecules, MECC was considered the technique of choice for the separation of these substances by CE. In fact, some preliminary assays were achieved with CZE, using 0.1 M sodium borate buffer (pH 9.5 and 10.5), and under these conditions no separations were obtained for the more methylated flavones, which eluted together very close to the electroosmotic flow (data not shown).

The first approach to the analysis of these flavone aglycones by MECC was attempted using the conditions recently described for the separation of honey flavonoid aglycones [11]. In this case 0.2 M sodium borate buffer (pH 8) with 50 mM SDS and 10% MeOH was used (buffer **A**). The migration times (MTs) for the different flavonoids analysed under these conditions are shown in table 1. In order to test the influence of increasing

methylation on the electrophoretic behaviour of these molecules, the available flavonoid aglycones were arranged, according to their oxygenation pattern, in six differents groups (table 1, groups **1-6**). With buffer **A**, the flavones with a methoxyl/hydroxyl ratio lower than 1, showed a good resolution, but the most lipophilic flavones (methoxy/hydroxyl ratio higher than 1) migrated together and showed broad peaks remaining unresolved. In addition, an unexpected effect was observed. The highly methoxylated flavones 5,6,7,4'-tetramethoxyflavone, gardenin B, tangeretin, salvigenin, nobiletin, 5-desmethylnobiletin, 5-hydroxy-6,7,3',4'-tetramethoxyflavone and sinensetin showed double and sometimes triple peaks (Figure 1). The double peaks observed for each lipophilic flavone showed the same UV spectra (recorded with the diode array detector) and demonstrated that all the peaks observed were produced by the same substance.

Increasing the percentage of methanol in the running buffer has been considered a convenient and useful procedure in order to improve the separation both in CZE and MECC [6,11,16-20]. By this reason the percentage of methanol added to the separation buffer was increased gradually to reach 25%. As the MTs became too large when increasing methanol concentration, the ionic strength of the buffer was reduced to 0.1 M sodium borate, in order to increase the electroosmotic flow, and therefore reduce the MTs of the different flavonoids. The SDS concentration was also reduced to 30 mM to contribute to this MTs reduction. Under these new conditions the aglycones were much better separated. The effect observed when increasing the methanol concentration is shown in figure 1. All structural groups were quite well resolved with buffer **B** (Table 1), but the most hydrophobic (methylated) flavones still showed double or triple peaks as it happened with 10% methanol .

In order to determinate which was the origin of these double peaks, the flavone salvigenin (5-hydroxy-6,7,4'-trimethoxyflavone) was analysed using different solvents to dissolve and inject the sample (methanol, isopropanol, acetonitrile, and buffers **B** and **C**), and three different buffers were assayed to achieve the separations (buffers **A**, **B** and **C**). The results obtained are shown in Table 2, and indicate that the use of methanol to dissolve the sample is one of the factors leading to double peak formation, since double peaks are observed in separations achieved with the three buffers when salvigenin is dissolved in methanol. Isopropanol gives the same results as methanol, as well as acetonitrile when buffers containing methanol are used (buffers **A** or

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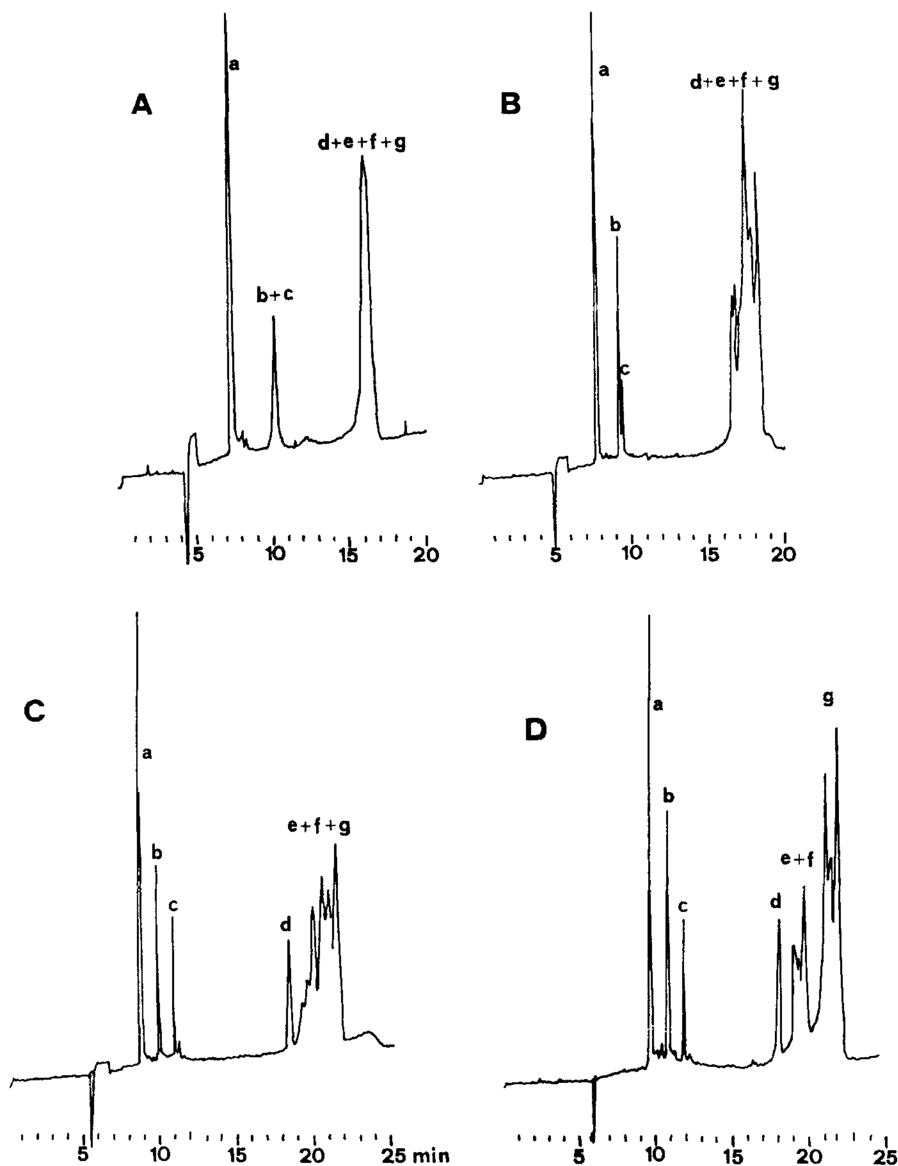

Fig. 1. Electropherograms of flavones included in group **1**. Influence of addtition of methanol to the buffer. (**A**) 0.2M sodium borate (pH 8), 50 mM SDS, 5% methanol; (**B**) 0.2M sodium borate (pH 8), 30 mM SDS, 10% methanol; (**C**) 0.2M sodium borate (pH 8), 30 mM SDS, 15% methanol; (**D**) 0.1M sodium borate, 30 mM SDS, 20% methanol; (**E**) 0.1M sodium borate, 30 mM SDS, 25% methanol. (**a**) sideritoflavone = 5,3',4'-OH-6,7,8-OMe; (**b**) thymonin = 5,6,4'-OH-7,8,3'-OMe; (**c**) 5,6-dihydroxy-6,7,3',4'-tetramethoxyflavone; (**d**) 8-methoxycirsilineol = 5,4'-OH-6,7,8,3'-OMe; (**e**) gardenin-D = 5,3'-OH-6,7,8,4'-OMe; (**f**) nobiletin = 5,6,7,8,3',4'-OMe; (**g**) 5-desmethylnobiletin = 5-OH-6,7,8,3',4'-OMe

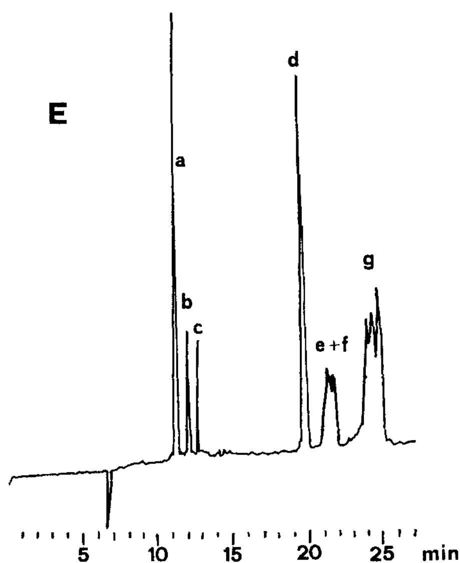

Fig. 1 (continued)

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Table 2. Analysis of the flavone salvigenin (5-hydroxy-6,7,4'-trimethoxyflavone) under different conditions.

Sample solution	Separation buffer	Result
Methanol	Buffer A	Double peaks
Methanol	Buffer B	Double peaks
Methanol	Buffer C	Double peaks
Isopropanol	Buffer B	Double peaks
Acetonitrile	Buffer B	Double peaks
Buffer B	Buffer B	Double peaks
Acetonitrile	Buffer C	Single broad peaks
Buffer C	Buffer C	Single peaks with good resolution

B). When the sample was dissolved in buffer **B** and the analysis was achieved with the same buffer, double peaks were still observed.

These results show that when methanol, is present either as a sample solvent, or as an additive of the running buffer, the peak of salvigenin appears as a double peak. However, the use of buffers with acetonitrile (buffer **C**), dissolving the sample either with acetonitrile or with the running buffer **C**, produced single peaks, although very broad peaks were observed when the samples were dissolved in acetonitrile.

Multiple peaks formation could be explained by the partial precipitation of the lipophilic flavonoids in the running buffers, when the samples dissolved in methanol are injected in the buffer. In fact, if salvigenin is dissolved in methanol, and this sample is added to the different buffers used, a precipitation occurs. However, when acetonitrile is utilized as a sample solvent or as an organic modifier of the buffer, the solubility of the lipophilic flavones is higher.

As a summary, we can conclude that the best separations of these lipophilic flavonoids were observed when dissolving the samples in the acetonitrile containing buffer **C**, and using the same buffer to run the electrophoresis.

These conditions were then applied to the different flavone standards groups and the same effect was observed, leading to electropherograms with single sharp peaks as it can be seen in fig. 2.

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Structure of flavonoid aglycones and electrophoretic migration in MECC

When analysing flavonoids by MECC, three different factors affect the electrophoretic migration; the hydrophobic interaction with micelles, which is the main factor, the ionization of free hydroxyls providing a negative charge to the molecule, and the complex formation with borate.

1- Hydrophobic interaction with micelles.

The flavone aglycones were analysed in the three buffers (**A**, **B** and **C**) to test the influence of different buffer compositions on their migration behaviour and as a general rule, as could be expected for a MECC separation, the higher the hydrophobic character of a flavone, the higher its MTs, since there is more interaction with the SDS micelles. This can be easily observed when comparing the methoxyl/hydroxyl ratio of the different

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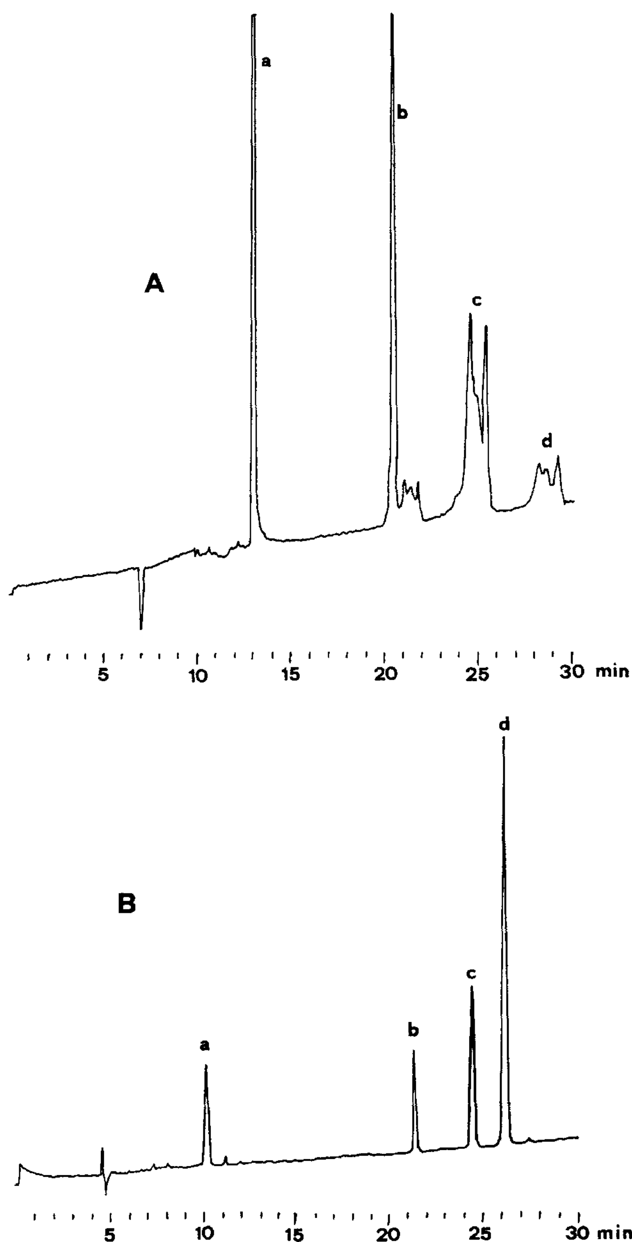

Fig. 2. Electropherograms of flavones included in group **5**. Influence of methanol on double peak formation. (**A**) running buffer: 0.1 M sodium borate (pH 8), 30 mM SDS, 25% methanol. Sample dissolved in methanol. (**B**) running buffer: 0.1 M sodium borate (pH 8), 50 mM SDS, 10% acetonitrile. Sample dissolved in running buffer. (**a**) thymusin = 5,6,4'-OH-7,8-OMe; (**b**) xanthomicrol = 5,4'-OH-6,7,8-OMe; (**c**) tangeretin = 5,6,7,8,4'-OMe; (**d**) gardenin-B = 5-OH-6,7,8,4'-OMe.

flavones with their MTs. In the different groups, when increasing this ratio the MTs increase. For instance, in group **2**, the substitution of one hydroxyl by one methoxyl in the molecule of scutellarein (5,6,7,4'-tetrahydroxyflavone) to give hispidulin (5,7,4'-trihydroxy-6-methoxyflavone) increases the MT in nearly 2 minutes (buffer **C**), and the further substitution of another hydroxyl to give cirsimaritin (5,4'-dihydroxy-6,7-dimethoxyflavone) increases the MT by 7 minutes. The introduction of another methyl ether at C-4' position to give salvigenin (5-hydroxy-6,7,4'-trimethoxyflavone) increases the MT by another 3 minutes. However, the substitution of the last hydroxyl to produce a completely methylated flavonoid (5,6,7,4'-tetramethoxyflavone) decreases its MT by nearly one minute in buffer **C**. The same effect is observed in groups **1**, **4** and **5**. The fully methylated nobiletin (group **1**), sinensetin (group **4**) and tangeretin (group **5**), migrate with shorter MTs than the corresponding flavones with a free hydroxyl in 5 position. A similar effect has previously been reported in the HPLC analysis of this type of compounds on reversed-phase columns [21]. In this case it was suggested that when a hydroxy group is present at position 5 (as in most of the flavones studied in this work), a strong internal hydrogen bond is formed between this group and the carbonyl group at position 4, and therefore the carbonyl, which is the strongest bond acceptor in a flavone, can no longer interact with the buffer [21-23]. Thus, the flavones with a methyl ether on the hydroxyl at 5, prevent this internal hydrogen bonding and allow the hydrophylic interaction of the carbonyl with the buffer, decreasing its MT. The same effect could explain why the 6-hydroxyflavone isomers migrate with shorter MTs than the 8-hydroxy isomers. For instance, the pair of isomers scutellarein (5,6,7,4'-tetrahydroxyflavone) and isoscutellarein (5,7,8,4'-tetrahydroxyflavone) and the isomers 6-hydroxyluteolin (5,6,7,3',4'-pentahydroxyflavone) and hypolaetin (5,7,8,3',4'-pentahydroxyflavone). This could be explained by an internal hydrogen bonding between the hydroxy groups at C-6 and C-5, which decrease the interaction described above between the latter hydroxy and the 4-keto group, and therefore decreasing MTs.

Isomers which are difficult to be separated by HPLC, are nicely separated by MECC due to the higher resolution of this technique. For instance the isomers showing the ring B substitution 3'-methoxy-4'-hydroxy and the isomeric forms 3'-hydroxy-4'-methoxy are very well separated. In all the compounds analysed, the isomers with a free hydroxyl in C-4' position (8-methoxycirsilineol, cirsilineol, and chrysoeriol) migrate with shorter MTs than

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the corresponding isomers with free hydroxyl at C-3' position (gardenin-D, eupatorin and diosmetin respectively) (Table 1).

2- Electric effects

The migration order observed in the different groups is generally similar to that observed when analysing these substances in HPLC with reverse-phase columns [20]. However, some significant differences are observed, and could be explained by the electric effects, since this is an electrodriven separation. Therefore, flavones with free hydroxyls which are susceptible of ionization at the pH of the running buffer, will have a negative charge and therefore an electrophoretic migration to the anode (injection end of the capillary) and will show higher MTs. In this case the acidity (the pKa) of the different phenolic hydroxyls plays a crucial role in the separation. Phenolic hydroxyls with pKa values below 8 (the pH of the buffer) will be ionized and will suffer electrophoretic migration. For instance, the 7-methyl ether of luteolin (5,3',4'-trihydroxy-7-methoxyflavone) migrates with shorter MTs than luteolin (5,7,3',4'-tetrahydroxyflavone). This does not agree with the lipophilicity of the flavonoid, but in this case an electric effect is working in the separation. The most acidic phenolic hydroxyl in the flavonoid molecule is the hydroxyl at 7-position. The pKa for this hydroxyl in flavonoids with two free hydroxyls on ring B at C-3' and C-4', is generally below 8 (7.3 for quercetin), while in flavonoids with only one hydroxyl on ring B at C-4' the pKa values for the hydroxyl at 7 position is usually higher than 8 (8.2 for kaempferol) [4]. At pH 8 (the pH of the running buffer in these separations), when a free hydroxyl is present in C-7 of the flavone luteolin, this hydroxyl is ionized, and therefore has a negative charge which is not present in the compound with a methyl ether at this position, and this explains the higher MTs of luteolin with respect to its 7-methyl ether. The same effect could explain why cirsiliol (5,3',4'-trihydroxy-6,7-dimethoxyflavone) migrates with shorter MTs than 6-hydroxyluteolin (5,6,7,3',4'-pentahydroxyflavone).

However, when regarding the pair apigenin (5,7,4'-trihydroxyflavone) and genkwanin (5,4'-dihydroxy-7-methoxyflavone), the same effect is not observed, since in this type of compounds (flavonoids with a mono-oxigenated ring B), the pKa of the hydroxyl at C-7 is higher than 8 [4], and the hydroxyl at C-7 in apigenin is not ionized at pH 8, and therefore, the behaviour is similar to that observed in reversed-phase HPLC, and the MTs of apigenin 7-methyl ether (genkwanin) and the 4'-methyl ether (acacetin) are similar.

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3- Borate complexation

Another possible factor which could affect the separation of flavonoids by MECC is the possibility of borate complexation. If a complex is formed with ortho-dihydroxyl groups, a negative charge is introduced in the molecule producing a retarding effect on the MT. Some examples illustrate this effect. For instance, *nodifloretin* (5,6,7,4'-tetrahydroxy-3'-methoxyflavone) migrates at 8.70 minutes in buffer C, while 6-hydroxyluteolin (5,6,7,3',4'-pentahydroxyflavone), which is more hydrophylic than nodifloretin and should interact less with the micelles eluting first, shows a migration time of 9.28 min. This could be explained by the formation of a borate complexation with the hydroxyls *at* C-3' *and* C-4', which provide additional negative charge, and therefore an increase in the MT for 6-hydroxyluteolin.

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