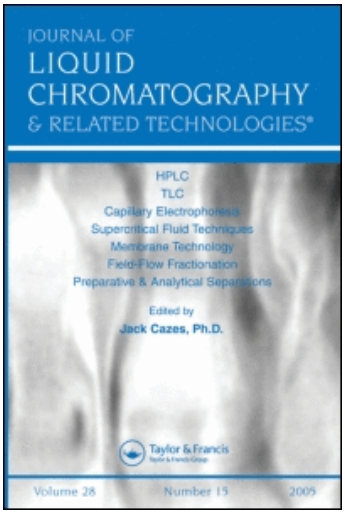

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REVERSED-PHASE PLANAR CHROMATOGRAPHY OF RACEMIC FLAVANONES

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REVERSED-PHASE PLANAR CHROMATOGRAPHY OF RACEMIC FLAVANONES

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ABSTRACT

The direct resolution of nineteen structurally related racemic flavanones was evaluated by reversed-phase planar chromatography using both home-made microcrystalline cellulose triacetate (MCTA) layers and mobile phase modifiers, such as β-cyclodextrin and bovine serum albumin, on commercially available Sil C_{18}-50/UV_{254} plates. Except for the two glycosides, 5-methoxy-, 7-hydroxy- and 5-hydroxy-7-methoxyflavanone, the enantiomers of other flavanones were all resolved by at least one of the three chiral phases tested. Densitograms of racemic flavanones were measured on MCTA layers developed with alcohol-water mixtures and on Sil C_{18}-50/UV_{254} plates after elution with chiral mobile phases.

INTRODUCTION

The preparation of enantiomerically pure compounds and the study of new analytical procedures for the separation of racemates and optical antipodes are of utmost importance since the majority of biologically active compounds are chiral molecules.[1,2]

Flavanones occur in nature, either in the free form or as glycosides; in plants they frequently coexist with the corresponding flavones and were often isolated in an optically active form.[3]

The development of a chromatographic method to determine flavanone enantiomers in plant extract is of primary interest, and the results obtained by Krause and Galensa[4] on cellulose triacetate columns should be mentioned. In addition, flavanone molecules contain only two types of functional groups (-OH and -OCH_3) and differ from one another in the number and/or position of such substituents. Therefore, it is possible to assess the role of the hydroxyl and methoxy groups in chiral interactions which contribute to enantiomeric resolution.

Among the techniques used, thin-layer chromatography has advantages, such as low cost, simplicity of the method and easy control of the results. There are two strategies for the direct separation of the enantiomers; one is the use of chiral stationary phase (CSP) while the second employs a chiral mobile phase (CMP).

Thus, we investigated the use of reversed-phase chromatography for the direct resolution of a large number of structurally related racemic flavanones both on home-made microcrystalline cellulose triacetate (MCTA) plates and by adding chiral selectors, such as β-cyclodextrin (β-CD) and bovine serum albumin (BSA), to the mobile phase while using an achiral stationary phase.

EXPERIMENTAL

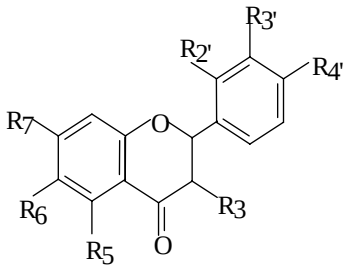
Solutions (2-4 mg/mL) of racemates (Extrasynthése, Genay, France) were prepared in aqueous ethanol (80 %). These solutions (0.5 - 1.5 µL) were applied (Hamilton syringe; Alltech, Deerfield, IL, USA) 1 cm from the bottom and at least 1.5 cm from the sides of the plates.

Chromatography of racemates by adding β-CD or BSA to the mobile phase was performed on 10x10 cm Sil C_{18}-50/UV_{254} plates obtained from Macherey-Nagel (Duren, FRG) [(5-7)]. β-CD and BSA (fraction V, pH=5.2) were supplied by Sigma-Aldrich (Milan, Italy) and Serva (Heidelberg, FRG), respectively.

The plates were chromatographed by ascending development in a Desaga (Heidelberg, FRG) thermostatted chamber (22x22x6 cm), saturated for 1 h at 25°C. The spots were detected by UV illumination at λ = 254 and 364 nm. Densitometric measurements were performed in the reflection mode at λ = 254 nm with a Shimadzu CS-9001 PC scanning densitometer coupled with a 486 IBM-compatible PC.

Table 1

The Structures of the Racemic Flavanones Investigated



R_3	R_5	R_6	R_7	$R_{2'}$	$R_{3'}$	$R_{4'}$	Name
H	H	H	H	H	H	H	Flavanone [a)]
H	OCH_3	H	H	H	H	H	5-Methoxyflavanone
H	H	OH	H	H	H	H	6-Hydroxyflavanone [a)]
H	H	OCH_3	H	H	H	H	6-Methoxyflavanone[a)]
H	H	H	OH	H	H	H	7-Hydroxyflavanone
H	H	H	H	OH	H	H	2'-Hydroxyflavanone
H	H	H	H	H	H	OH	4'-Hydroxyflavanone
H	H	H	H	H	H	OCH_3	4'-Methoxyflavanone
H	OH	H	OH	H	H	H	Pinocembrin
H	OH	H	OCH_3	H	H	H	Pinocembrin-7-methylether
H	OH	H	OH	H	H	OH	Naringenin [a)]
H	OH	H	OH	H	H	OCH_3	Isosakuranetin
H	OH	H	OCH_3	H	H	OH	Sakuranetin
H	OH	H	Gl[b)]	H	H	OH	Naringenin-7-glucoside
H	OH	H	Rh-Gl[c)]	H	H	OH	Naringin
H	OH	H	OH	H	OH	OH	Eriodictyol
H	OH	H	OH	H	OCH_3	OH	Homoeriodictyol
H	OH	H	OH	H	OH	OCH_3	Hesperetin [a)]
OH	OH	H	OH	H	OH	OH	Taxifolin[a)]

[a] These compounds were previously studied on MCTA layers (10).
[b] Gl = Glucoside.
[c] Rh-Gl = Rhamnosidoglucoside.

Plates were scanned in zig-zag form over the sample zones. All functions of the scanner were controlled and the data were processed with TLC-specific software manufactured by Shimadzu. Real-time background correction was automatically performed in the zigzag system.

Home-Made MCTA Plates

As reported in previous studies,[8-10] a slurry of MCTA (HPLC grade, particle size < 10 µm; Fluka, Buchs, Switzerland) with silica gel 60 GF_{254} (particle size 15 µm; Merck, FRG) as binder was prepared by mixing the latter material (3 g) with distilled water (15 mL) for 5 min, then adding MCTA (9 g) and ethanol (35 mL) and shaking the resulting product for 5 min before transferring it to a Chemetron (Camag, Muttens, CH) automatic plate spreader. The layers (10x20 cm and 20x20 cm, thickness 250 µm) were dried at room temperature (≈ 20°C) and used within 2-5 hours.

RESULTS AND DISCUSSION

The structures of the solutes are shown in Table 1. These different compounds contain the same carbon skeleton and differ only in the number and/or position of the substituent groups.

MCTA Layers

Home-made microcrystalline cellulose triacetate plates were found to have excellent chiral properties towards a large number of racemates and optical antipodes,[8-11] including some racemic flavanones.[10] Water-alcohol mixtures were generally used as mobile phases. Consequently, in the present paper, we employed aqueous solutions of methanol, ethanol, and 2-propanol for the resolution of these racemates.

In all instances, there was observed an increased retention of solutes as alcohol percentage decreased, in agreement with the behavior predicted for reversed-phase chromatography. With such mobile phases, the effect of increasing the number of hydroxyl substituents is generally to increase the R_F values in opposition to what observed for methoxy groups.

In the series of flavanones, two trends can be pointed out in the chromatographic properties as examined on MCTA plates. In contrast to the polysubstituted compounds, no chiral discrimination was observed for 2'-hydroxy-, 4'-hydroxy-, and 4'-methoxyflavanone.

Partial resolution was obtained for flavanone, 6-methoxy- and 6-hydroxy-flavanone after development with ethanol-water 80+20 (v/v),[10] but other compounds monosubstituted in the benzene ring fused with the hetero ring, such as 5-methoxy- and 7-hydroxyflavanone, were not resolved at all.

Table 2

Retention (hR_{F1}, hR_{F2})[a] and Resolution (α, R_s)[b] Data for Racemic Flavanones on Microcrystalline Cellulose Triacetate Plates with Silica Gel GF_{254} as Binder (Temperature = 25°C)

Compound	Eluent	hR_{F1}	hR_{F2}	α	R_s
Pinocembrin	70:30[c]	0.54	0.60	1.27	1.8
Sakuranetin	70:30[c]	0.43	0.48	1.22	1.2
Isosakuranetin	80:20[d]	0.18	0.21	1.21	1.3
Homoeriodictyol	80:20[d]	0.23	0.26	1.17	0.8
Eriodictyol	80:20[d]	0.26	0.30	1.21	1.5

[a] R_F x 100. [b] $\alpha = (1/R_{F1}-1)/(1/R_{F2}-1)$; R_s = 2 x (distance between the centers of two adjacent spots)/(sum of the width of the two spots in the direction of development). [c] Ethanol-water; migration distance 14 cm; separation time 2.5 h. [d] Methanol-water; migration distance 16 cm; separation time 2 h.

Successive developments with the same mobile phases effectively improved the resolution of flavanones. For example, the R_S of sakuranetin increased from 1.2 to 1.4 after two successive runs with ethanol-water 70+30 (v/v). No resolution of the racemic flavanones containing sugar residues (naringenin-7-glucoside and naringin) was observed.

Table 2 gives the optimized conditions for the separation of the optical antipodes of five racemic hydroxyflavanones determined by varying the type and composition of the mobile phase. None of these solutes was resolved eluting with 2-propanol-water mixtures. This behavior agrees with the successful resolution of naringenin, hesperetin and taxifolin obtained on MCTA plates developed with methanol-water 80+20 (v/v).[10]

In general, such flavanones have two hydroxyl groups in 5 and 7 positions with the exception of the 7-methoxy substituted sakuranetin. However, the effect of the 7-methoxyl substituent on the enantiomeric discrimination is controversial owing to the lack of resolution for racemic pinocembrin-7-methylether. The chromatographic properties of this series of compounds are in agreement with the results obtained on MCTA columns.[4]

Densitometric measurements were performed after development with ethanol-water 70+30 (v/v) (see Fig. 1) and after two successive developments with methanol-water 80+20 (v/v) (see Fig. 2). The optical isomers of pinocembrin (Fig. 1b), isosakuranetin and eriodictyol (Fig. 2a and 2b) were

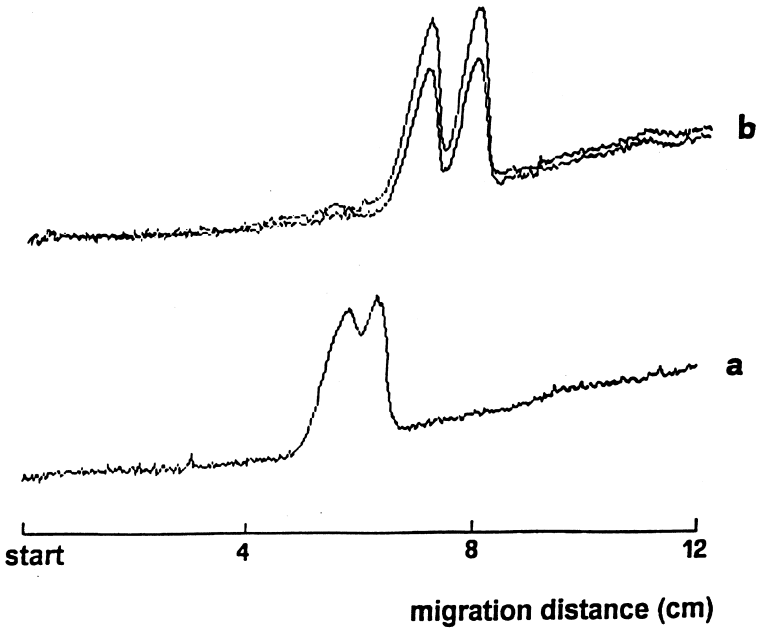


Figure 1. Densitograms of racemic sakuranetin (a) and pinocembrin (b) on MCTA layers developed with ethanol - water 70+30 (v/v). The development distance was 12 cm; the separation time 2 h; 1 µL of (±)-sakuranetin, 1 µL and 1.5 µL of (±)-pinocembrin solution (4 mg/mL) were applied to the plates.

completely resolved to baseline both at 2 and 4 µg. On the contrary, sakuranetin (Fig. 1a) and homoeriodictyol (Fig. 2c) were only partially resolved at 4 µg. These results show that home-made MCTA plates are useful for the quantitative determination of such compounds.

Resolution data are in accord with the observation that compounds bearing an aromatic group in addition to a carbonyl group in the close vicinity of the chiral center have a good chance of enantiomer discrimination.[11] However, only the enantiomers of flavanones with a high extent of substitution differently fit the chiral cavities between the laminae of MCTA leading to separation of the optical antipodes. In this series, an -OH or -OCH_3 group in 3' and/or 4' position seems to have a favorable influence on chiral recognition. Otherwise, monosubstituted flavanones are partially separated or not resolved at all.

The results achieved confirm that the separation of the optical antipodes of flavanones on MCTA plates is mainly governed by the shape and size of solutes and only to a minor extent by electrostatic interactions involving the functional

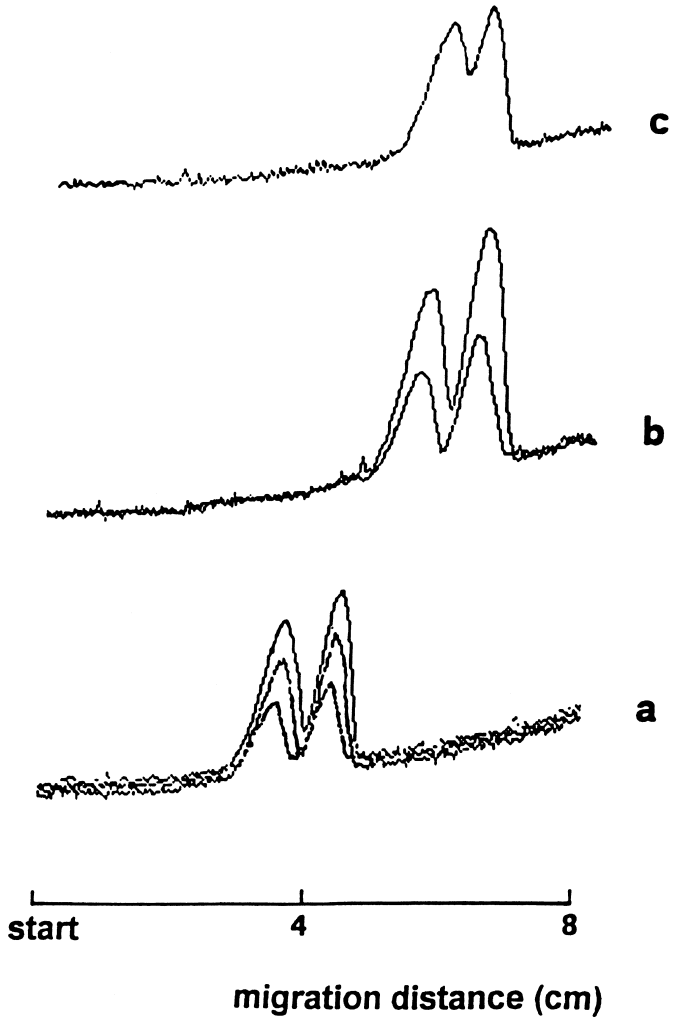


Figure 2. Densitograms of racemic isosakuranetin (a), eriodictyol (b) and homoeriodictyol (c) on MCTA layers after two successive developments with methanol - water 80+20 (v/v). The development distance was 13 cm; the separation time 1.5 h; 0.5 µl, 1 µL and 1.5 µL of (±)-isosakuranetin solution (4 mg/ml) 0.5 µL and 1.5 µL of (±)-eriodictyol solution (4 mg/mL) and 1 µL of (±)-homoeriodictyol solution (4 mg/mL) were applied to the plates.

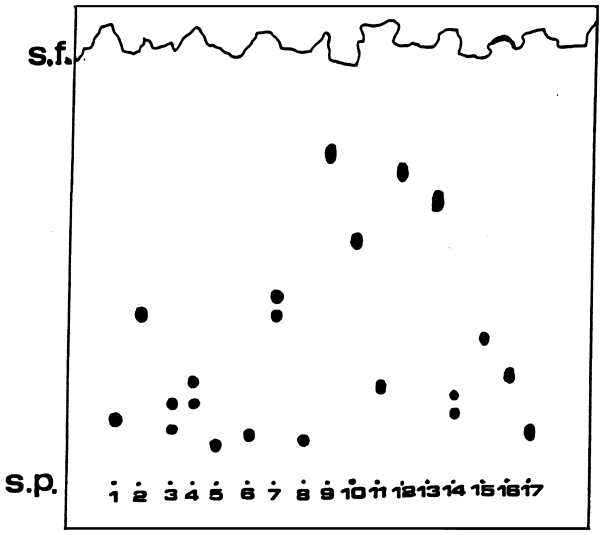


Figure 3. Thin-layer chromatogram of racemic flavanones on Sil C18-50/UV_{254} plates. Eluent: 0.15 M β-cyclodextrin aqueous solution with urea (32 %) and NaCl (2 %) - acetonitrile (80+20 v/v); migration distance 8.5 cm. 1=homoeriodictyol, 2=eriodictyol, 3=4'-methoxyflavanone, 4=2'-hydroxyflavanone, 5=7-hydroxyflavanone, 6=isosakuranetin, 7=4'-hydroxyflavanone, 8=5-methoxyflavanone, 9=naringenin-7-glucoside, 10=naringin, 11=sakuranetin, 12=pinocembrin, 13=pinocembrin-7-methylether, 14=flavanone, 15=naringenin, 16=hesperetin, 17=6-methoxyflavanone.
S.p. = starting point; s.f. = solvent front.

groups of the molecules (i.e. the dipole-dipole interactions among the flavanone carbonyl group and the acetoxy groups of the polymer). The type of alcohol and the composition of the water-alcohol mixtures affect the separation of solutes of different sizes and characteristics because these result in different swelling of MCTA. Probably, multiple chiral sites coexist in the polymer and each might be affected by the above-mentioned parameters, resulting in more chances of successful optical resolution.

β-Cyclodextrin (β-CD)

Reversed-phase systems containing cyclodextrins as mobile phase modifiers were widely used for the thin-layer chromatographic resolution of a variety of molecules on achiral stationary phases.[5,6,12-18] In this investigation, the separation of racemic flavanones reported in Table 1 was examined, for the first time, using β-CD as a chiral mobile phase additive.

Fig. 3 shows the chromatographic behavior of 17 racemates on Sil C_{18}-50/UV_{254} plates developed with 0.15 M β-CD solution containing 32 % urea and 2 % sodium chloride - acetonitrile 80+20 (v/v). The concentration of β-CD is very important for the resolution of the enantiomers.[5] It is thought that each enantiomer forms an inclusion complex on adding a large amount of β-CD to the mobile phase and that the differences in the stability constants of such complexes determine the chromatographic enantioselectivity on silanized silica gel plates. β-CD complexation permits chromatographic separations of the optical antipodes of flavanone, 2'-hydroxyflavanone, 4'hydroxy- and 4'-methoxyflavanone which do not have hydroxyl and/or methoxy groups in the 5,6 and/or 7 position of fused aromatic ring.

Substitution of the 4'- position with a methoxy group significantly increases the enantioselectivity and resolution. In fact, α and R_S values increase from flavanone (1.35 and 1.7, respectively) to 4'-methoxy-flavanone (1.78 and 2.0). Similar resolution data were observed for flavanone and 2'-hydroxyflavanone, but hydroxyl substituent on 4' position slightly increased chiral recognition ($\alpha = 1.39$; $R_S = 1.7$).

Densitometric measurements (Fig. 4) were performed on Sil C_{18}-50/UV_{254} plates developed with the same mobile phase described in Fig. 3. The optical antipodes of the four racemates were fully resolved at 1 μg (flavanone) and 2 μg; the highest sensitivity was shown by 4'-methoxyflavanone (Fig. 4a) and 2'-hydroxyflavanone (Fig. 4b).

It has been stated that factors which increase hydrogen bonding at the mouth of the β-CD cavity tend to benefit chiral recognition.[19] However, the 2' and 4' hydroxyl substituents, which can act as proton donors, enhance chiral recognition to cyclodextrin less than the proton acceptor substituent (i.e., methoxy group) in 4'- position, suggesting that the latter may strongly interact with the hydrogen bonding groups at the mouth of cyclodextrin thereby altering the orientation of the solute in the β-CD cavity.

Probably the observed drop in enantioselectivity for the other flavanones arose from changes in the geometric requirements of the insertion of differently substituted fused aromatic rings in the β-CD cavity.

Bovine Serum Albumin (BSA)

The use of BSA in the mobile phase for the thin-layer chromatographic separation of a wide variety of enantiomers was tested in our laboratory.[7,20-25] In

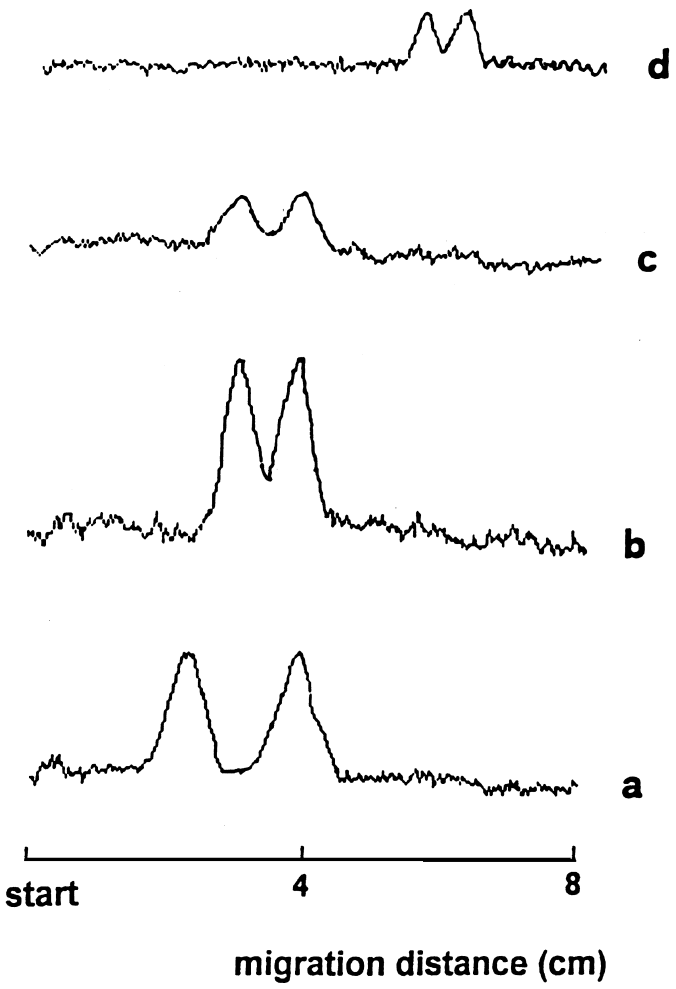


Figure 4. Densitograms of racemic 4'-methoxyflavanone (a), 2'-hydroxyflavanone (b), flavanone (c) and 4'-hydroxyflavanone (d) on Sil C18-50/UV_{254} layers eluted with 0.15 M β-cyclodextrin aqueous solution with urea (32 %) and NaCl (2 %) - acetonitrile (80+20 v/v). The development distance was 8.5 cm; the separation time approximately 70 min. 0.5 µl of (±)-flavanone (2 mg/mL) and of the other racemate solutions (4 mg/mL) was applied to the plates.

addition, chromatographic studies of the binding of commercial pesticides to various albumins were performed on RP-18W/UV_{254} plates.[26] The quality of the results achieved has encouraged us to use BSA for the resolution of racemic flavanones separated elsewhere.

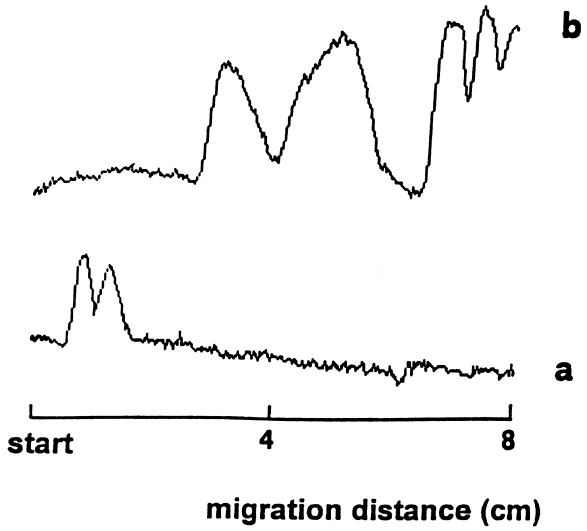


Figure 5. Densitograms of racemic 2'-hydroxyflavanone (a) and homoeriodictyol (b) on Sil C18-50/UV_{254} layers. Eluents: (a) 0.05 M sodium bicarbonate + 0.05 M sodium carbonate solution containing 6 % BSA and 12 % 2-propanol; (b) 0.1 M sodium carbonate solution containing 6 % BSA and 6 % 2-propanol. The development distance was 8 cm and the separation time approximately 2 h. 0.5 µL of (±)-2'-hydroxyflavanone and 1 µl of (±)-homoeriodictyol solutions (4 mg/mL) were applied to the plates.

The best results in terms of the number of flavanones resolved were obtained with alkaline mobile phases in agreement to the results obtained for tryptophanes.[7] The high percentage of BSA (6 %) was chosen according to previous experiments.[7,23] The 2-propanol content is the least amount of organic modifier necessary for development of Sil C_{18}-50/UV_{254} layers with alkaline solutions containing such an high concentration of BSA. The lesser retained flavanones give rise to irregular spots which take the shape of a reversed triangle. Compact, round spots were observed only for 2'-hydroxyflavanone.

The differences in the elution profile of racemic 2'-hydroxyflavanone and homoeriodictyol are reported in Fig. 5. A low thin-layer efficiency is coupled with a large separation factor in the case of homoeriodictyol. Albumin is able to resolve both racemic polysubstituted flavanones containing a methoxyl group in 3' or 4' position such as homoeriodictyol (α=1.95), isosakuranetin (α=1.54) and hesperetin (α=1.62) and, surprisingly, 2'-hydroxyflavanone (α=1.56), probably because of the favorable influence of the hydroxyl substituent in β position with respect to the stereogenic center.

CONCLUSIONS

Chiral stationary phases (MCTA) and chiral mobile phases containing β-CD or BSA are useful for the resolution of racemic flavanones.

Although the first two systems are founded on inclusion processes, the differences in the size of MCTA and β-CD cavities and in the characteristics of the functional groups determine the resolution of different series of racemic flavanones.

In particular MCTA can be used in the separation of highly substituted compounds and only to a minor extent for flavanones monosubstituted in the fused ring while β-CD is able to resolve, in addition to unsubstituted flavanone, the series of compounds substituted in the isolated benzene ring.

BSA enhances its chiral properties and the negative net charge with alkaline mobile phases which are the basis for resolving different substituted racemic flavanones.

The effect of pH on the resolution of such solutes can be attributed to changes in the binding sites of BSA rather than in the charge of flavanones.

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