Note

Direct enantiomeric separation of racemic flavanones by high-performance liquid chromatography using cellulose triacetate as a chiral stationary phase
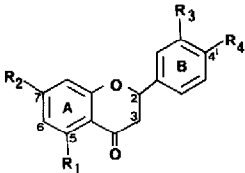
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Many different classes of racemic compounds have been resolved into enantiomers using cellulose triacatate (CTA) as a chiral stationary phase in liquid chromatography[1-3]. In this paper we report the enantiomeric separation of the naturally occurring flavanones Naringenin, Hesperetin, Eriodictyol, Homoeriodictyol, Pinocembrin and Isosakuranetin (Fig. 1) on commercially available high-performance liquid chromatography (HPLC) columns packed with either microcrystalline CTA or "cross-linked acetylcellulose" (which seems to be cellulose 2.5 acetate). Furthermore, a brief comparison between microcrystalline CTA and CTA supported on silica gel is presented for Naringenin.

Okamoto *et al.*[4] successfully resolved the unsubstituted flavanone on cellulose-triphenylcarbamate supported on silica gel. However, we could not achieve a resolution of flavanones with more than two hydroxyl groups on this column without

COMPOUND	R_1	R_2	R_3	R_4
NARINGENIN (I)	OH	OH	H	OH
HESPERETIN (II)	OH	OH	OH	OCH_3
ERIODICTYOL (III)	OH	OH	OH	OH
HOMOERIODICTYOL (IV)	OH	OH	OCH_3	OH
PINOCEMBRIN (V)	OH	OH	H	H
ISOSAKURANETIN (VI)	OH	OH	H	OCH_3
PINOSTROBIN (VII)	OH	OCH_3	H	H
SAKURANETIN (VIII)	OH	OCH_3	H	OH
FLAVANONE (IX)	H	H	H	H

Fig. 1. Substitution pattern of some naturally occurring flavanones.

derivatization. A direct separation of enantiomers is always of great advantage, because further investigations with the isolated compounds are possible.

Since some other flavanones were not or only partially resolved and they all represent a group of closely related compounds, the influence of substitution upon chiral resolution can be studied and the results may contribute to a better understanding of the recognition mechanism of CTA.

EXPERIMENTAL

Materials

Naringenin, Eriodictyol, Sakuranetin, Isosakuranetin, flavanone, 6-hydroxy-, 6-methoxy-, 5-methoxy- and 4'-methoxyflavanone was obtained from Roth (Karlsruhe, F.R.G.), Hesperetin from Senn Chemicals, Homoeriodictyol was synthesized by Wagner (Munich, F.R.G.), Pinocembrin and Pinostrobin were gifts from Wollenweber (Darmstadt, F.R.G.).

Methanol was HPLC-grade, isopropanol, *n*-hexane, 2,4,4-trimethylpentane (isooctane), *tert*.-butyl methyl ether and ethanol were reagent grade. All solvents were obtained from Baker (Gross-Gerau, F.R.G.).

HPLC

The HPLC separation was carried out with a Beckmann (Munich, F.R.G.) HPLC pump, a sampling valve (Altex 210 A, Beckmann) equipped with a 20-μl sample loop and a photodiode-array detector (Pye Unicam PU 4021; Philips, Kassel, F.R.G.) set at 280 nm for compounds I–VIII and at 251 nm for the remaining flavanones. All results were recorded with a video chromatography control center (Pye Unicam PU 4850). Polarimetric detection was performed with a polarimeter detector (PE 241; Perkin-Elmer, Überlingen, F.R.G.), with a micro flow cell of pathlength 100 mm and a volume of 30 μl.

Columns: CTA I, 250 mm × 10 mm, stainless steel, packed with 10-μm microcrystalline cellulose triacetate (Merck, Darmstadt, F.R.G.); CTA II, 250 mm × 4.6 mm, stainless steel, packed with 10-μm microcrystalline cellulose triacetate (Daicel, Baker); CTA III, 250 mm × 4.6 mm, stainless steel, packed with 7-μm crosslinked acetylcellulose with 40% acetyl content (Macherey & Nagel, Düren, F.R.G.); CTA IV, 250 mm × 4.6 mm, stainless steel, packed with 10-μm cellulose triacetate supported on silica gel (Daicel). The flow-rate was 1 ml/min unless stated otherwise; the solutes were dissolved in the mobile phase.

RESULTS AND DISCUSSION

Retention data for the flavanones I–VI (Fig. 1) using methanol as the mobile phase are shown in Table I. Comparable results (CTA II somewhat superior to CTA I) were obtained on CTA I and CTA II with k' values ranging from 1.10 to 0.99 for Eriodictyol (III) to 2.13 to 1.95 for Pinocembrin (V) and α- values between 2.03 (III) and 1.30 (VI). On both stationary phases the enantiomeric flavanones I–VI are baseline-resolved, *e.g.*, Naringenin, Fig. 2A,B. The first flavanone enantiomers eluted were laevorotatory with (2*S*)-configuration[5,6]. Other flavanones were only partially or not at all resolved and will be discussed later.

TABLE I

ENANTIOMERIC SEPARATION OF RACEMIC FLAVANONES: COMPARISON OF RESOLV-
ING POWER BETWEEN THREE DIFFERENT COLUMNS

k'_1 = Capacity factor of the first enantiomer eluted; the calculation of the capacity factors is based on 1,3,5-tri-*tert.*-butylbenzene. α = Separation factor. n.d. = Not detected.

Compound	CTA I*		CTA II**		CTA III*	
	k'_1	α	k'_1	α	k'_1	α
Naringenin	1.13	1.80	1.08	1.99	0.40	1.38
Hesperetin	1.43	1.67	1.32	2.03	0.47	1.38
Eriodictyol	1.10	1.82	0.99	2.03	0.40	1.38
Homoeriodictyol	1.73	1.65	1.54	1.80	0.50	1.33
Pinocembrin	2.13	1.55	1.95	1.68	0.53	1.32
Isosakuranetin	n.d.	n.d.	1.84	1.87	n.d.	n.d.

* Flow-rate 1 ml/min methanol.
** Flow-rate 0.5 ml/min methanol.

Although the capacity factors, k', and α values are in the same range, the retention times using CTA I are very long compared with CTA II, taking into account that the flow-rate of the latter is only 0.5 ml/min as recommended by the producer. This fact is primarily due to the larger diameter of CTA II, which is therefore from the practical point of view more suitable for semipreparative purposes.

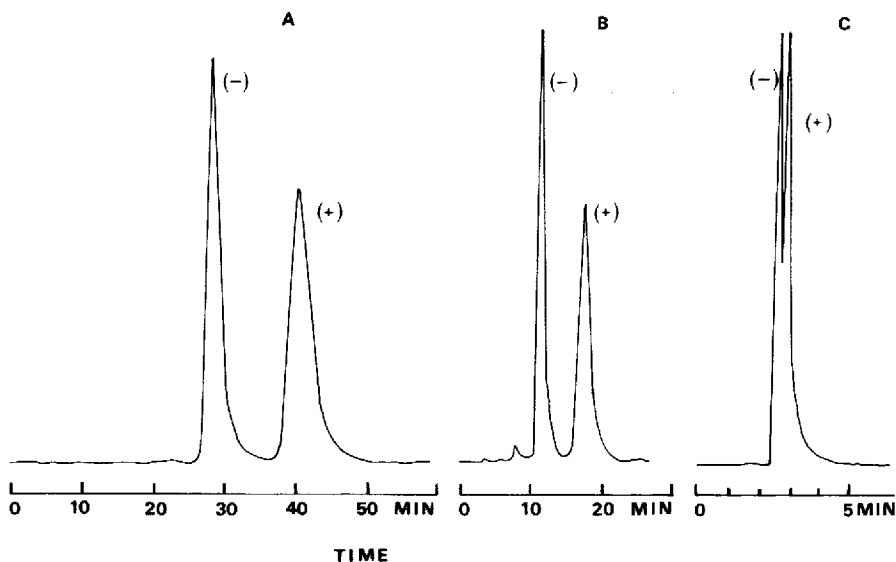Using "cross-linked acetylcellulose" as the chiral stationary phase (CTA III)

Fig. 2. Enantiomeric separation of racemic Naringenin (I) with methanol on three different cellulose triacetate columns. (A) CTA I, 1 ml/min; (B) CTA II, 0.5 ml/min; (C) CTA III, 1 ml/min.

and methanol as the eluent, the capacity factors are reduced to less than 40% under the same conditions. In this case the interaction between the solutes and the stationary phase is less intensive and the α values are below 1.4. Similar observations have been made in a comparative study with cross-linked and non-cross-linked CTA for many pharmaceutical substances[7].

In order to improve the resolution of CTA III, several mobile phase compositions were tested (Table II). The use of methanol–water mixtures increased the capacity factors but the resolution decreased ($k'_1 = 2.3$, $\alpha = 1.15$; 88% methanol). Methanol is much more efficient than ethanol, although the retention is only little altered; with pure or 96% ethanol no peak separation has been observed. Eluents IV and V led to an higher relative retention (k') but the separation coefficients, α, were almost unchanged compared with those using methanol.

Fig. 3 illustrates the profound influence of the temperature upon resolution. The α values increase with decreasing temperature from $\alpha = 1.2$ at 35°C to $\alpha = 1.63$ at 1°C (eluent V). Compared with the situation on the non-cross-linked columns (CTA I and II), the peaks show a stronger tailing and even with an α value of 1.70 (eluent VI, 5°C) no baseline separation was obtained. Substituting isooctane for n-hexane seems to improve the results slightly (eluents V and VI).

Racemic Naringenin remained unresolved with methanol on CTA IV with cellulose triacetate supported on silica gel. The chromatographic and enantioselective behaviour of dissolved and reprecipitated CTA can be completely different to that of microcrystalline CTA and in some cases the elution order of the enantiomers was found to be reversed[8,9]. However, stereoselectivity has been observed too for Naringenin on CTA IV with a mobile phase composition of methanol–n-hexane–isopropanol (20:72:8) (which gave the best results), but the resolution was still unsatisfactory. The observed reversal of the elution order of Naringenin enantiomers seems to

TABLE II

MOBILE PHASE COMPOSITIONS USING "CROSS-LINKED ACETYLCELLULOSE" (CTA III)

Mobile phase*	Naringenin (I)		
	k'_1	α	Temperature** (°C)
I Methanol	0.40	1.38	
	0.50	1.66	5
II Methanol–water (88:12)	2.3	1.15	
III Methanol–ethanol (50:50)	0.40	1.26	
IV Methanol–isooctane–isopropanol (70:27:3)	0.61	1.47	
	0.82	1.69	5
V n-Hexane–methanol–$tert.$-butyl methyl ether (60:24:16)	1.96	1.35	
	2.16	1.57	5
VI Isooctane–methanol–$tert.$-butyl methyl ether (60:24:16)	2.19	1.70	5

* Flow-rate 1 ml/min.
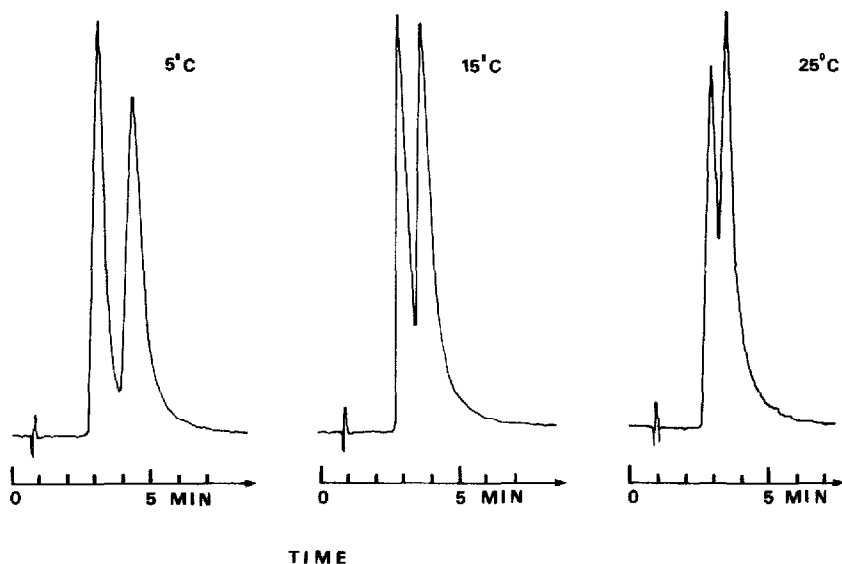** Ambient temperature unless specified otherwise.

Fig. 3. The influence of the temperature upon chiral separation of racemic Naringenin (I) on "cross-linked acetylcellulose" (CTA III) using n-hexane–methanol–tert.-butyl methyl ether (60:24:16) as the mobile phase. Flow-rate 1 ml/min.

be another example indicating that reprecipitated CTA has different recognition properties.

Since the chiral recognition mechanism of CTA (which seems to occur via inclusion) is not understood in detail[3], it is of great interest to study the influence of the substitution pattern of a molecule with respect to chiral recognition. The successful separation of many flavanones is in good accord with the observations of Shibata et al.[3] that compounds bearing an aromatic group in the close vicinity of the chiral centre have a good chance of enantiomeric discrimination. Table III shows the chromatographic results on CTA II of some other flavanones, which were not or only partially resolved. Together with the data from Table I it can be stated that the best results were obtained when two hydroxyl groups in positions 5 and 7 were present (compounds I–VI). In this group, Pinocembrin (V) with $R_3 = R_4 = H$ (Fig. 1) has the lowest α value, which indicates that an OH or OCH_3 group in 4'- or/and 3'-position seems to have a favourable influence upon chiral recognition. Pinostrobin (VII) and Sakuranetin (VIII) [the 7-methyl ethers of Pinocembrin (V) and Naringenin (I)] have high capacity factors ($k' = 11.2$ and $k'_1 = 3.97$), but an apparent peak separation is attained only for Sakuranetin ($\alpha = 1.22$), while Pinostrobin is eluted as a very broad peak and there is only a weak indication of "peak" splitting.

While 6-methoxy- and 6-hydroxyflavanone are not resolved, a partial resolution is observed for flavanone ($\alpha = 1.31$) and 4'-methoxyflavanone ($\alpha = 1.14$), furthermore the weak beginning of peak splitting is seen for 5-methoxyflavanone. All these results indicate that substitution in the A-ring is important for chiral recognition, although these substituents are not in the vicinity of the chiral centre. Probably the molecule is adsorbed in such a way that a discrimination between the two optical antipodes is easier, when either none or a 5-, 7-disubstitution is present in the A-ring,

TABLE III

CHROMATOGRAPHIC DATA OF NOT OR PARTIALLY RESOLVED FLAVANONES ON CTA II WITH METHANOL AS MOBILE PHASE

Flow-rate, 0.5 ml/min.

Compound	k'_1	α
Sakuranetin (VIII)	3.97	1.22
Pinostrobin (VII)	11.18	≈ 1
5-Methoxyflavanone	1.61	≈ 1
6-Methoxyflavanone	2.10	1.00
4'-Methoxyflavanone	2.54	1.14
6-Hydroxyflavanone	1.03	1.00

with the exception of Pinostrobin whose chromatographic behaviour is very different. Further investigations with other flavanones (for example prenylated flavanones) might give more insight into the recognition mechanism, at least for the flavanones.

CONCLUSION

Microcrystalline cellulose triacetate is a useful chiral stationary phase to resolve racemic flavanones with an high degree of hydroxyl substitution, whereas others are not or only partially resolved. Although the enantioselectivity for flavanones I–VI is good, the selectivity between different pairs of enantiomers is not sufficient, for they all appear in a relatively narrow retention zone. "Cross-linked acetylcellulose" is more stable due to the cross-linkage —a greater variety of solvents can be used— but has a lower efficiency.

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