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High-performance liquid chromatographic separation and chiroptical properties of the enantiomers of naringenin and other flavanones

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Abstract

The HPLC enantiomeric separation of naringenin, eriodictyol, hesperetin and pinocembrin was accomplished in the normal-phase mode using two polysaccharide-derived chiral stationary phases (Chiralcel OD-H and Chiralpak AS-H) and various *n*-hexane/alcohol mobile phases. The 3′,4′ substituents pattern affect the enantioselectivity of these phases. Single enantiomers of naringenin were isolated by semipreparative HPLC and their CD spectra were measured and related to the absolute configuration by the exciton-coupling method. Online coupling HPLC/spectropolarimeter afforded the CD sign of the eluted peaks at a single wavelength, and the complete CD spectra of the eluted enantiomers were obtained by trapping them in the spectropolarimeter cell through a switching valve.

Keywords: Pharmacologically active flavanones; *Citrus* lipophilic flavanoids; Polysaccharide chiral stationary phases; Circular dichroism detector; Absolute configuration

1. Introduction

Flavanone glycosides occur in large amount in *Citrus* species and their aglycones, flavanones, are formed in the intestinal epithelia by the enzymatic hydrolysis of the glycosides. The formation of the aglycones is the crucial step in the metabolism of glycosides [1]. Thus, an accurate dosage of the enantiomeric ratio of the flavanones is significant to establish if there is any enantioselectivity in the pharmacokinetics of them. The pharmacological interest of the flavanones reported in Fig. 1 is well known. Naringenin (**1**) possesses chemopreventive potential toward mutagenesis of heterocyclic amines mediated by an isoform of cytochrome P450 [2], naringenin and hesperetin (**3**) inhibit a human breast carcinoma cancer cell line, especially when paired with quercetin [3], and selectively inhibit arachinodate 5-lipoxygenase compared with cyclooxygenase in stimulated rat peritoneal leukocytes [4]. Eriodictyol (**2**) exhibits an antioxidative activity in the rabbit erythrocyte membrane ghost system higher than α-tocopherol [5]. On the other hand, in studies with other enzymatic systems more modest activities were observed and this concerns with the multifaceted concept of cancer chemoprevention that encompasses inhibiting, retarding and blocking agents [6,7].

Nevertheless, the relevance of the stereochemistry at the C-2 stereogenic center was not considered in these studies as well as in other ones, although it is well known that interactions of an enzymatic system leading to a functionalization of a substrate are stereospecific [8].

The chiral separation of compounds **1**–**4** was first achieved by Krause and Galensa using microcrystalline cellulose triacetate as chiral stationary phase (CSP) and methanol as mobile phase and obtaining separation factors α in the range 1.80–1.55 [9]. The same authors used cellulose triacetate coated on silica gel diol as CSP but lower α values were obtained [10,11]. Subsequently, the enantioseparation of compounds **1**–**3**, among other flavanones, was studied using three polysaccharide derived CSP but very low α were obtained (1.00–1.08) [12]. Naringenin (**1**) was separated in its enantiomers in post-administrative urine of volunteers

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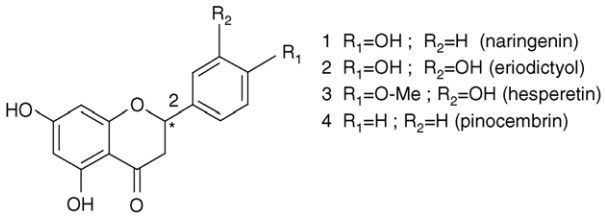

Fig. 1. Structures of compounds **1**–**4**.

taking traditional Chinese medicines, using a Chiralcel OD column and optimizing the mobile phase composition [13].

We had previously reported the normal-phase chiral HPLC separation of (2*R*) and (2*S*)-naringin (the 7-*O*-neohesperidoside of **1**) and we used this method to evaluate the stereochemistry at C-2 of naringin during the maturation of the grapefruits [14].

In this article we report the direct separation of the enantiomers of compounds **1**–**4** using an isocratic normal-phase HPLC with two polysaccharide-derived CSP (Chiralpak AS-H and Chiralcel OD-H) and various *n*-hexane/alcohol mobile phases. We isolated the single enantiomers of naringenin and we measured their circular dichroism (CD) spectra. Moreover, the online coupling HPLC/CD afforded to obtain directly the CD spectra of the eluted peaks of the enantiomeric pairs of compounds **1**–**4**.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Jasco pump PU 980 with Rheodyne 20 or 100 μl sample loops, a low pressure mixer LG-1580-02 and a line degasser 1550-54 (all from Jasco), a Varian DU-50 spectrophotometer operating at 292 nm and a Varian data Jet integrator or Houston Oniscribe recorder for fraction collecting. CD spectra were recorded on a Jasco 810 spectropolarimeter, using 1 mm cell. Online coupling HPLC/CD was realized using the HPLC Jasco components described above with a 20 μl Rheodyne loop, a Rheodyne switching valve receiving the HPLC column eluate before entering a continuous flow cell (8 μl volume, 10 mm optical path) inserted between the photomultiplier of the spectropolarimeter and the usual allocation (empty) of the spectropolarimeter cell. Molecular modeling conformation of *S*-naringenin was obtained using a Chem 3D Ultra.

2.2. Enantioselective columns and chemicals

The polysaccharide-derived columns (250 mm × 4.6 mm) were cellulose tris-3,5-dimethylphenylcarbamate (Chiralcel OD-H), tris(*S*)-1-phenylethylcarbamate (Chiralpak AS-H) both coated on 5 μm silica gel, cellulose tris-3,5-dimethylphenylcarbamate (Chiralcel OD) and amylose tris-3,5-dimethylphenylcarbamate (Chiralpak AD) both coated on 10 μm silica gel. All the above columns were obtained from Daicel (Tokyo). The non polysaccharide-derived columns (250 mm × 4.6 mm) used for the experiments reported in Table 1 were (*R*,*R*) DACH DNB packed with a 3,5-dinitrobenzoyl derivative of (*R*,*R*)-1,2-diaminocyclohexane covalently bonded to silica gel, Whelk (3*S*,4*R*) O-1 packed with 4-(3,5dinitrobenzamido)-tetrahydrophenanthrene covalently bonded to 5 μm 3-propylsilica, both from Regis (Morton Grove, IL, USA), and Kromasil CHI-DMB packed with a polymeric network based on 2*R*,3*R*-*N*,*N*′-diallyl-L-tartardiamide from EKA Nobel AB (Bohus, Sweden). A column in-line filter with a 0.5 μm stainless steel frit of 3 mm diameter from Rheodyne was used to protect the HPLC columns. Disposable PTFE filters of 0.2 μm pore size were used for filtration of sample solutions. Column void volume (t_0) was measured by injection of tri-*tert*-butylbenzene as a non-retained marker [15]. The resolution factor was evaluated according to $R_s = 2(t_2 - t_1)/(w_1 + w_2)$, i.e. the peak separation divided by the mean value of the baseline widths. Retention times (t) were mean values of two replicate determinations. Other HPLC chromatographic parameters were those typically employed [16]. Experiments were performed at ambient temperature.

(±)-Naringenin (**1**) was purchased from Sigma (St. Louis, MO) and from Fluka (Buchs, Switzerland), hesperetin (**3**) from Sigma, eriodictyol (**2**) was from Fluka and from Extrasynthèse (Genay, France), pinocembrin (**4**) from Extrasynthèse.

Table 1
Enantioselective HPLC resolution of naringenin on various CSP

Entry	CSP	*A* (%)[a]	k_1'[b]	t_1	t_2	α	R_s
1	AD	20	2.05	8.6	10.8	1.37	< 0.7
2	AS-H	20	6.90	22.9	25.7	1.13	1.0
3	OD	20	2.13	10.5	11.6	1.16	0.7
4	OD-H	15	4.41	18.4	22.6	1.28	2.0
5	DACH	20	8.86	26.8		1.00	
6	Kromasil	25	2.49	8.33		1.00	
7	Whelk	20	4.30	15.02[c]		1.00	

[a] Percentage of 2-propanol doped with 0.1% of TFA in *n*-hexane at a flow rate of 1 mL/min; t_0, min = 2.8 (AD), 2.9. (AS-H), 3.3 (OD), 3.4 (OD-H), 2.7 (DACH), 2.4 (Kromasil), 2.8 (Whelk).
[b] Retention factor of the first eluted enantiomer.
[c] Peak with large tail.

3. Results and discussion

3.1. Chiral separation with various CSPs

The effect of the type of CSP on the chiral recognition of naringenin was first studied and it is shown in Table 1. The chiral selectors derived from helical polysaccharide CSPs (entries 1–4) are much more efficient than those based on the complementary functionality approach between the CSP and the analyte (entries 5 and 7) and on a C_2-symmetric unit anchored into a network polymer (entry 6). Indeed, enantio-

selectivity (α) and resolution factor (R_s) range from 1.28 and 2.0 using Chiralcel OD-H to 1.16 and 0.7, respectively, using Chiralcel OD. This is expected since the first CSP is coated on a silica gel support of a finer particle size (5 μm). The CSP based on the amylose (Chiralpak AD), although giving a good α, gave a poor R_s (entry 1), the presence of an additional stereocenter in the pending moiety of the amylose-derived CSP improves significantly the R_s (entry 2).

Thus, the results shown in Table 1 suggest the use of Chiralpak AS-H and Chiralcel OD-H to study the enantioseparation of all four flavanones **1**–**4**, although it cannot directly inferred that these CSP are the best also for compounds **2**–**4**. Several analytical considerations can be made from the results: (i) as expected for the normal-phase behavior of both AS-H and OD-H columns, the resolution factor R_s improves significantly by decreasing the polarity of the mobile phase, as shown by comparison of entries 1, 2, 4; 3, 6; 18, 20, although the separation factor α is almost unaffected by the percentage of 2-propanol in the mobile phase. Remarkably, the enantiomers of pinocembrin (**4**) can be separated on AS-H column only decreasing the polarity of the mobile phase (entries 25 and 26). (ii) Resolution (R_s) and separation (α) factors are almost unaffected by increasing the flow rate of the mobile phase (entries 2, 3; 7, 9; 17, 18). (iii) For naringenin (**1**) the chiral discrimination is much better on the OD-H column in terms of both α and R_s for the same mobile phase composition and flow rate, as shown by comparison of entries 2, 7 and 3, 9. On the contrary, for eriodictyol (**2**) and hesperetin (**3**) the chiral discrimination is better, particularly with regard to R_s, on the AS-H column, as shown in entries 10, 12 and 15, 17. For pinocembrin (**4**) the chiral discrimination is almost equivalent on both CSPs, giving an α value of 1.08–1.09. On AS-H column, however, the R_s increases, as shown by comparison of entries 22 and 26. (iv) The use of ethanol as polar modifier of the eluent reduces the retention time of the enantiomers of flavanones **1**–**3** using the Chiralpak AS-H column, but it is very detrimental on the enantioseparation of **1** and **2**, as shown by comparison of entries 7 and 8, and of 12 and 13, respectively and, to a minor extent, on the enantioseparation of compound 3 (entries 18 and 19). Thus, a slight decrease in the percentage of ethanol in the mobile phase (from 20 to 10%) has a profound effect on the enantioseparation, differently than for 2-propanol.

Fig. 2 shows the influence of the different volume of the helical groove of a cellulose derivative (OD-H) and an amylose derivative (AS-H) with an additional stereogenic center on the chiral discrimination of the flavanones **1**–**4**, at the same polarity and flow rate of the mobile phase. The binding of the solutes is achieved through hydrogen bonding interaction with the polar carbamate groups of the CSPs and through insertion of the solute in the helical cavity, and it is well known that amylose derivatives possess a wider and more compact helix [17]. The retention factor k'_1 is much higher on AS-H column for all flavanones as a consequence of a better interaction, as shown by comparison of k'_1 values in Fig. 2a. However, the flavanones **2** and **3**, bearing OH, OH and OH, OCH_3 in positions 3′,4′, respectively, form additional hydrogen bonding with the CSP. Thus, the chiral discrimination is better for **2** and **3** on AS-H column than on OD-H column, as shown by comparison of the α values in Fig. 2b. The use of ethanol further disturbs the hydrogen bonding formation through the OH substituents in positions 3′,4′ and the carbamate groups of the CSP and reduces the discrimination process. Ethanol has in fact an higher polarity index and viscosity than 2-propanol [18] and competes in the interactions with the 3′,4′ substituents. Thus slight modification in the substitution pattern influences heavily the behaviour of the various flavanones on the same CSP.

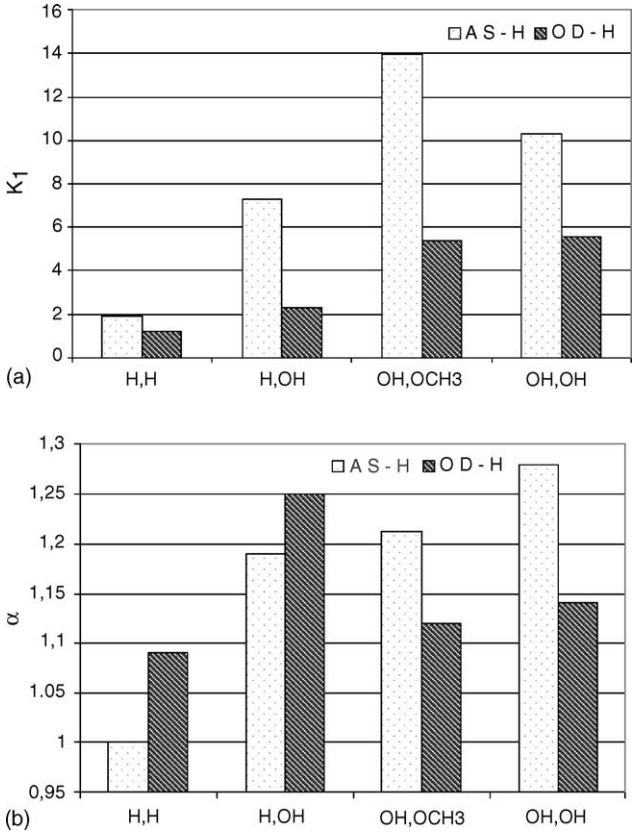

Fig. 2. Retention factors of the first eluted peak (a) and separation factors (b) as a function of the 3′,4′ substituents, from left to right: **4**, **1**, **2**, **3**. Conditions: Chiralpak AS-H and Chiralcel OD-H columns; mobile phase *n*-hexane/2-propanol doped with 0.1% TFA, 80:20 at 1 mL/min.

Typical enantiomeric separations of the flavanones **1**–**4** as a function of CSP and mobile phase composition are shown in Fig. 3. The HPLC traces show that the compounds (from commercial sources) contain other enantiomeric pairs as impurities and that the flavanones are almost racemic as calculated from the integrated areas of the two major peaks.

This also includes the eriodictyol sample from Fluka which is indicated in the catalogue as (*S*)-3′,4′,5,7-tetrahydroxyflavanone. The *R*, *S* composition, as well as the presence of impurities, is reasonably due to the extraction and hydrolysis procedure from *Citrus* fruits where other flavanone glycosides are present.

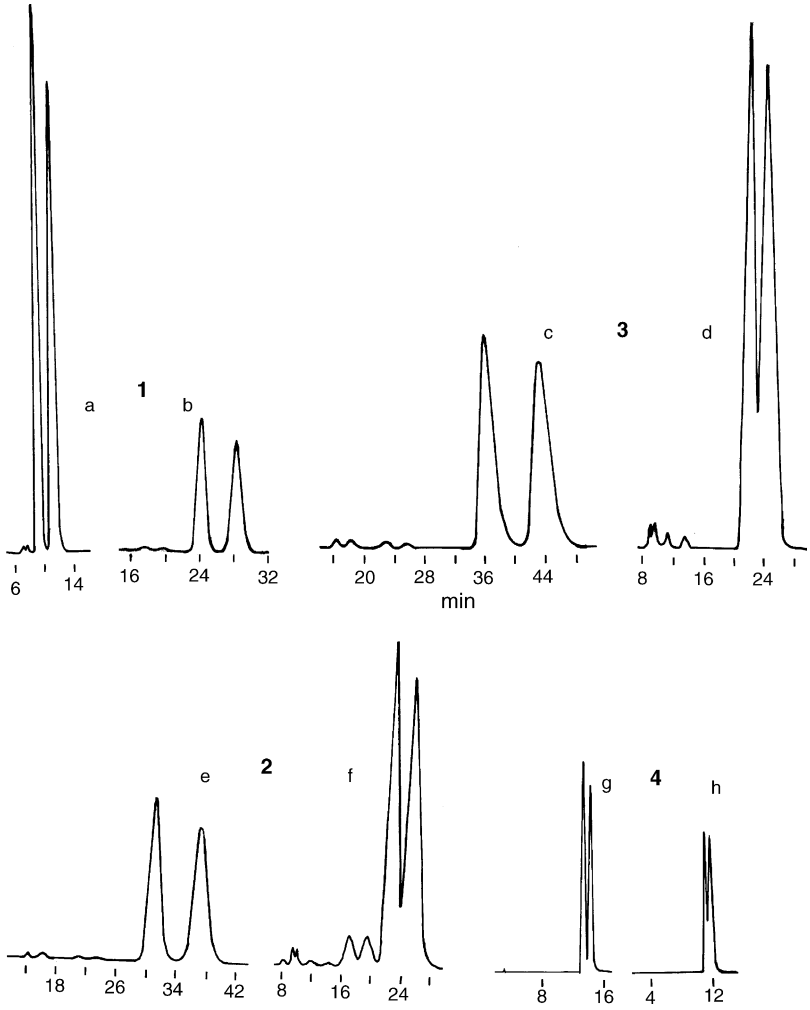

Fig. 3. Typical HPLC separation of compounds **1**–**4** as a function of CSP and mobile phase composition. Conditions: compound **1**: OD-H (a), AS-H (b); compound **2**: AS-H (e), OD-H (f); compound **3**: AS-H (c), OD-H (d); compound **4**: AS-H (g), OD-H (h). Mobile phase *n*-hexane/2-propanol doped with 0.1% TFA, 80:20 at 1 mL/min (a–f), 90:10 (g and h), at 1 mL/min in all cases.

3.2. Isolation of the enantiomers of naringenin and their circular dichroism spectra

Based on the chromatographic results in Table 2, we resorted to the experimental conditions in entry 3, that are a compromise between acceptable elution times and still good resolution factor, to perform the isolation of the single enantiomers of naringenin (**1**). This was accomplished by 100 μl repeated injections (0.3–0.4 mg) of a solution of (±)-naringenin (40 mg in 10 ml *n*-hexane/2-propanol 1:1). Collection of the eluates corresponding to the two major chromatographic peaks gave, after filtration and rotoevaporation 5 mg of each compound.

The CD spectra of both compounds were measured and they were mirror images of each other, as shown in Fig. 4, indicating their enantiomeric nature. Analytical HPLC reruns of the eluates indicated an enantiomeric excess (ee) of 100% for the first peak and 98.7% for the second one. The molecular ellipticity (θ) of naringenin at 287 nm was already reported as −42 100 [19]. However, the θ value measured by us is considerably lower (−27 000). In our opinion, this is due to the hydrolysis of "unfractionated samples of (*S*)-naringin" from the albedo of *Citrus paradisi* [19] and thus to the presence of other CD-active flavanones. The θ value obtained by us after chiral HPLC separation of the enantiomer is instead devoid

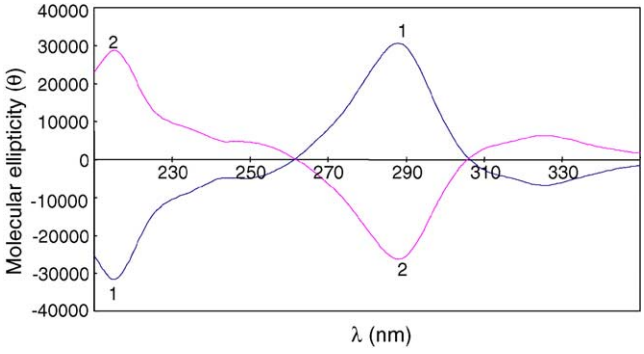

Fig. 4. CD spectra (ethanol, 22 °C) of the enantiomers of naringenin obtained from the first (1) and the second (2) HPLC eluted peaks.

Table 2
Enantioselective HPLC resolution of flavanones **1**–**4** on Chiralcel OD-H and Chiralpak AS-H

Entry	Compound	CSP	*A* (%)[a]	Flow	k'_1	t_1	t_2	α	R_s
1	**1**	OD-H	25	1	1.15	7.3	8.1	1.25	0.7
2	**1**	OD-H	20	1	2.29	11.2	13.2	1.25	1.3
3	**1**	OD-H	20[b]	1.3	2.12	8.6	10.1	1.26	1.3
4	**1**	OD-H	15	1	4.41	18.4	22.6	1.28	2.0
5	**1**	OD-H	15[c]	1	2.94	13.4	14.9	1.15	1.3
6	**1**	OD-H	15	1.3	4.12	14.1	17.1	1.26	1.6
7	**1**	AS-H	20	1	7.27	24.0	28.0	1.19	1.2
8	**1**	AS-H	20[c]	1	2.87	13.2		1.00	
9	**1**	AS-H	20	1.3	6.30	16.7	18.6	1.13	0.9
10	**2**	OD-H	20	1	5.52	22.1	24.8	1.14	0.8
11	**2**	OD-H	15	1.3	11.04	33.1	38.2	1.16	0.9
12	**2**	AS-H	20	1	10.27	32.7	38.8	1.28	1.8
13	**2**	AS-H	20[c]	1	4.18	15.0		1.00	
14	**2**	AS-H	30	1.3	3.70	10.5	12.2	1.21	1.4
15	**3**	OD-H	20	1	5.38	21.7	24.0	1.12	<0.7
16	**3**	OD-H	15	1.3	9.16	27.9	31.3	1.12	0.8
17	**3**	AS-H	20	1	13.97	36.3	43.4	1.21	1.8
18	**3**	AS-H	20	1.3	12.30	29.6	36.4	1.24	1.8
19	**3**	AS-H	20[c]	1.3	4.44	12.1	12.9	1.08	0.6
20	**3**	AS-H	30	1.3	5.77	12.7	15.1	1.22	1.6
21	**4**	OD-H	20	1	1.22	7.6	7.9	1.09	<0.5
22	**4**	OD-H	10	1	2.23	11.0	11.7	1.09	<0.5
23	**4**	OD-H	10[c]	1	2.32	11.3	12.0	1.09	<0.5
24	**4**	OD-H	15	1.3	1.73	7.5	8.0	1.09	<0.5
25	**4**	AS-H	20	1	1.91	8.5		1.00	
26	**4**	AS-H	10	1	3.81	14.0	14.9	1.08	0.8
27	**4**	AS-H	10[c]	1	3.62	13.4	14.3	1.08	0.8

[a] Percentage of 2-propanol doped with 0.1% of TFA in *n*-hexane; t_0, min = 3.40 (OD-H at 1 mL/min), 2.75 (OD-H at 1.3. mL/min), 2.90 (AS-H at 1 mL/min), 2.23 (AS-H at 1.3 mL/min).
[b] Experimental conditions used for semipreparative isolation.
[c] Percentage of ethanol doped with 0.1% of TFA in *n*-hexane.

of this impurities effect. The configurational assignment (−)-(*S*) was empirically suggested by Gaffield by an extension of the Snatzke rule [20]. The CD spectrum of (−)-naringenin exhibits a split CD pattern typical for exciton chirality with two opposite Cotton effects, one due to the strong absorption of the trisubstituted acetophenone chromophore centered at about 285 nm while the other one at lower λ (below 240 nm) is partially due to the methylphenol chromophore.

We built the molecular model of the lowest energy conformation of (*S*)-naringenin by means of Chem 3D Ultra calculation and this is reported in Fig. 5. Applying simple chirality rules and the very versatile exciton-coupling method [21,22] the absolute sense of twist, defined by the methylphenol chromophore (in the front) and the 2,4,6-trihydroxyacetophenone chromophore (in the back), gives the negative chirality observed in the CD couplet thus confirming the correlation (−)-(*S*). The *non* empirical assignment of the absolute configuration of (−)-naringenin by quantitative ab initio calculation of the CD spectrum has been recently achieved [23].

The individual enantiomers of naringenin were recovered from the solution used for the chiroptical measurements and were tested for the inhibition of the cyclosporin A oxidase activity in human liver microsomes. This is in fact an important CYP P3A4-dependent activity. However, neither (*R*)- or (*S*)-naringenin exhibited significant inhibitory effect on this activity and no enantioselectivity was observed. The lack of activity is somewhat surprising since racemic naringenin was reported to be in vitro effective in the inhibition of the nifedipine oxidation and of aflatoxin B_1 activation in human liver microsomes [24].

3.3. Online coupling HPLC/CD

Once the absolute configuration at C-2 of the eluates of naringenin has been assigned by interpreting the Cotton effects in the CD spectra of the enantiomers isolated by HPLC, we resorted to a simpler, quick and reliable procedure to obtain the absolute configuration of the eluting peaks of compounds **1**–**4**. This is online coupling HPLC/CD that, beside to obtain a CD signal at a chosen λ, affords also the complete CD spectrum of the eluting peak by trapping it in the spectropolarimeter cell through a switching valve. HPLC/CD detectors are relatively new and not inexpensive [25]. Some applications and useful comments on the subject are discussed in a recent review [26].

Fig. 6 shows the CD signal at 292 nm (upper trace) and UV absorbance (lower trace) in continuous flow mode. Since the sign of the CD band at lower energy is diagnostic of the

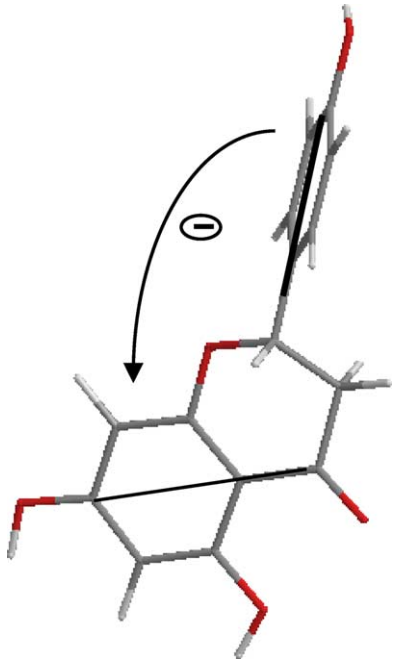

Fig. 5. Absolute sense of twist defined by methylphenol chromophore (in the front) and 2,4,6-trihydroxyacetophenone chromophore (in the back) for the lowest energy conformation of (*S*)-naringenin calculated by Chem 3D Ultra.

absolute configuration, as discussed above, a negative CD signal at 292 nm is related to the (*S*)-configuration. This is observed for compounds **1**–**3**. Remarkably, for pinocembrin (**4**) the opposite elution order of the enantiomers on the AS-H and OD-H columns was observed, as evidenced by the positive and negative CD signals. The (*S*)-enantiomer eluted as the second peak on the amylose-derived column (AS-H), but eluted as first on the cellulose-derived column (OD-H). The reversal of elution order using cellulose and amylose derived CSPs have been also reported in some cases [17]. The online CD spectra of the enantiomers of compounds **1**–**4** are shown in Fig. 7. They were obtained by trapping the eluate of the peak at its absorbance maximum, switching the valve. The pump was also stopped during the acquisition of the CD spectrum of the solution trapped in the cell of the spectropolarimeter. This device avoided to overlap the acquisition of the CD spectrum of the first peak with the elution of the second peak in case the pump was running. Thus, in an unique HPLC run, the CD spectra of both enantiomers were obtained. They were recorded from 245 nm to higher wavelengths to avoid severe interference from the mobile phase at lower wavelengths. A background CD spectrum of the mobile phase was recorded at the end of the HPLC run and subtracted from the spectrum of each peak. The quality of the spectrum is related to the signal-to-noise ratio and thus to the dilution of the solution trapped into the cell. In this respect, the enantiomers of naringenin and pinocembrin (this eluted on AS-H column) exhibit the best and the worst spectra, respectively, as shown in Figs. 6 and 7.

The CD spectra of compounds **1**–**4** are very similar. In particular, the second peak of pinocenbrin shows a negative CD band centered at about 285 nm and attributable to the (*S*)-enantiomer, using the Chiralpak AS-H column, while this enantiomer eluted first on the OD-H column. The band is centered around 8 nm at lower wavelengths with respect to the other flavanones **1**–**3** and this can be

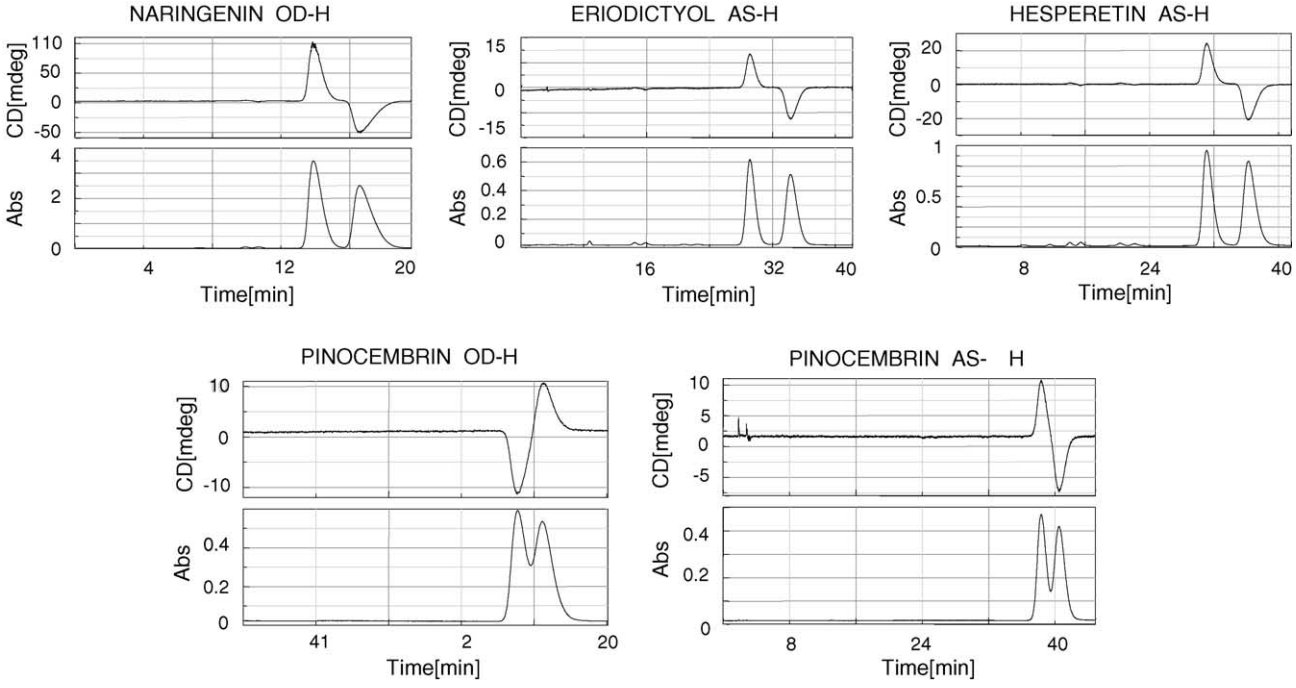

Fig. 6. HPLC/CD chromatograms on the top and HPLC/UV chromatograms on the bottom (at 292 nm) for flavanones **1**–**4**. Conditions: CSP, as indicated; mobile phase *n*-hexane/2-propanol doped with 0.1% TFA: 85:15 naringenin; 80:20 eriodictyol and hesperetin; 95:5 pinocembrin, at 1 mL/min in all cases.

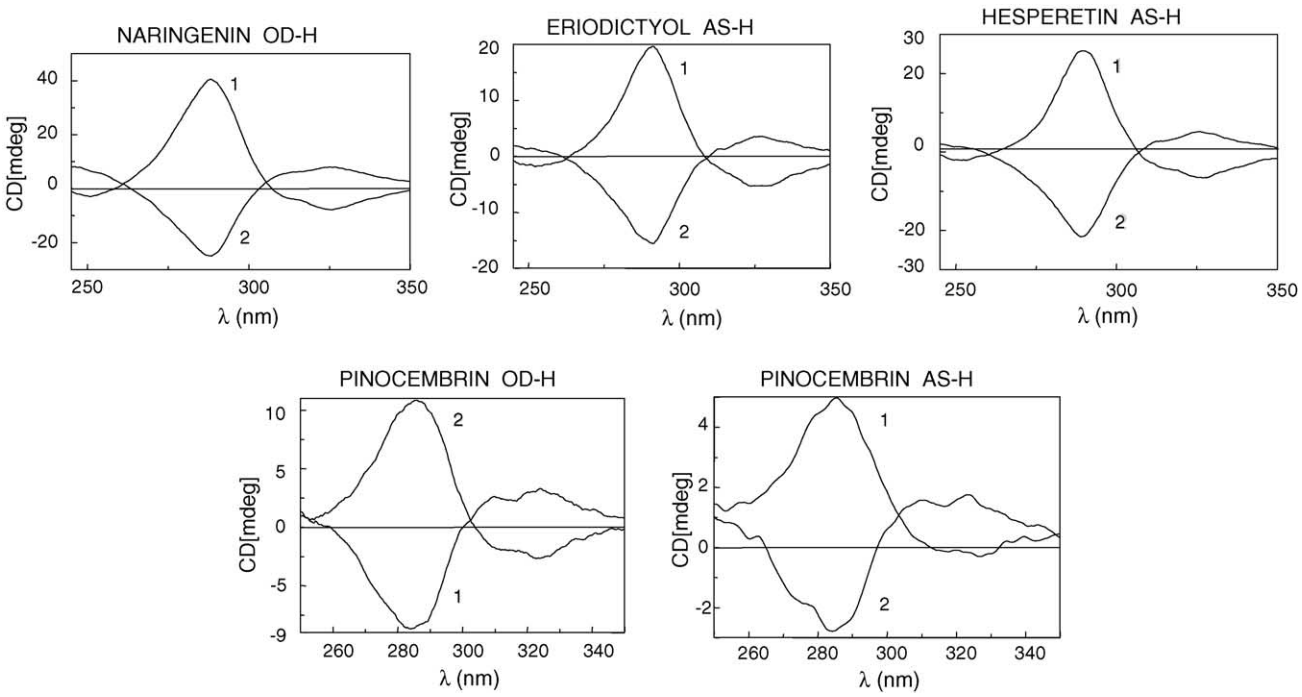

Fig. 7. CD spectra of the eluted peaks of flavanones **1**–**4** in HPLC/CD online coupling. Conditions: CSP, as indicated; mobile phase *n*-hexane/2-propanol doped with 0.1% TFA: 85:15 naringenin; 80:20 eriodictyol and hesperetin; 95:5 pinocembrin, at 1 mL/min in all cases.

due to the minor polarity of solvent (*n*-hexane/2-propanol 95:5).

The approach used by us enable to obtain the complete CD spectrum of an eluted peak with higher sensitivity with respect to that obtainable in dedicated CD detectors. Examples of this kind of application in natural and pharmaceutical products are rare in the literature [14,27–32] and the usefulness of online HPLC/CD coupling is still underestimated.

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