Original Article

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Separation of the enantiomers of naringenin and eriodictyol by amylose-based chiral reversed-phase high-performance liquid chromatography

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ABSTRACT: Naringenin and eriodictyol are chiral flavanones widely present in citrus fruits and herbal products. Pharmacological interest in the two flavanones is well known. Due to the chiral carbon atom, the compounds always exist in the racemic form. The present study reported a stereospecific HPLC method for the enantioseparation of naringenin and eriodictyol, which was performed on an amylose-based chiral stationary phase (CSP), Chiralpak AD-RH, in the reversed-phase mode. The effects of the mobile phase on retention, enantioseparation, and elution order were investigated. The different 3',4' substituent pattern of the two compounds affected the enantioselectivity. An online coupling HPLC-CD method was used for elution order determination. Both the CD sign of the eluted peaks at a single wavelength and complete CD spectra of the eluted enantiomers were obtained by the method.

Keywords: Naringenin, eriodictyol, HPLC-CD, enantioseparation

1. Introduction

Naringenin (5,7,4'-trihydroxyflavanone, **1**) and eriodictyol (5,7,3',4'-tetrahydroxyflavanone, **2**) (Figure 1) are chiral flavanones present in citrus fruits and herbal products. Pharmacological interest in the two flavanones is well known. Both compounds have long been realized as antioxidants and chemopreventive agents (*1*). Recent studies have shown that naringenin also possesses activities such as anti-inflammatory (*2*), anti-cancer (*3,4*), anti-metastasis (*5*), normalizing lipids (*6,7*), anti-hyperglycemia (*8*), and anti-hypercholesterolemia (*9*). Eriodictyol can provide a cytoprotective effect in ultraviolet (UV)-irradiated keratinocytes (*10*), induce long-term protection in ARPE-19 cells (*11*), and prevent early retinal and plasma abnormalities in streptozotocin induced diabetic rats (*12*). Deriving from the stereogenic center at C-2, the two flavanones are chiral. It is well known that interactions with enzymes are often stereospecific, so enantiomers should have different behaviors in pharmacological action and metabolic process, but due to the lack of readily available pure flavanone enantiomers, most bioactivity studies were carried out using a racemic mixture. For the separation of enantiomers of compounds **1** and **2**, a couple of methods have been previously reported, such as capillary electrophoresis (*13*), micellar electrokinetic chromatography (*14*), and high performance liquid chromatography (HPLC) under normal-phase conditions (*15*). With respect to normal phase and polar organic mobile phase, the reversed-phase mode is particularly advantageous in the direct analysis of biological matrices without a batch sample preparation step and in coupling with mass spectrometry. There were also reports about the enantioseparation of compounds **1** and **2** using reversed-phase HPLC. The only validated reversed-phase HPLC method reported for the stereospecific separation of **2** was separation on a Chiralpak OJ-RH column (*16*), while **1** could be enantioseparated using a Chiralcel OD-RH column (*17*). Both the Chiralpak OJ-RH column and Chiralcel OD-RH column are cellulose-derived columns. In this article, we report the enantioseparation of compounds **1** and **2** using an isocratic reversed-phase HPLC with two amylose-based chiral stationary phases (CSP), Chiralpak AD-RH and Chiralpak AS-RH. Chiralpak AD-RH provided better enantioseparation of the two analytes.

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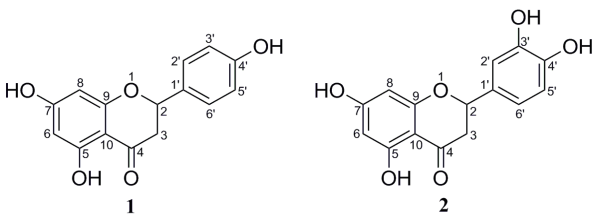

Figure 1. Structures of compounds 1 and 2.

Moreover, the online coupling HPLC-circular dichroism (CD) method makes possible direct absolute configuration assignment of the eluted enantiomers.

2. Materials and Methods

2.1. *Chemicals and reagents*

Racemic naringenin (**1**) and eriodictyol (**2**) were purified from *Dracocephalum rupestre*. The purity was proved to be above 98% by HPLC analysis. The structure identification was carried out using ^{1}H and ^{13}C nuclear magnetic resonance (NMR). HPLC-grade methanol, ethanol and acetonitrile were from Burdick & Jackson (SK Chemicals, Seoul, Korea).

2.2. *Chromatographic system and conditions*

The HPLC-UV was performed on an Agilent 1260 HPLC system, equipped with quaternary pump, diode array detector and an autosampler (Agilent, Palo Alto, LA, USA). The HPLC-CD was performed on a JASCO LC-Net II/ADC HPLC system, equipped with a PU-2089 plus pump, CD-2095 plus CD detector and a 7125 Rheodyne injector with 20 μL sample loop (Jasco, Tokyo, Japan). The columns (150 mm × 4.6 mm) were amylose tris(*S*)-α-methylbenzyl carbamate (Chiralpak AS-RH), amylose tris-3,5-dimethylphenyl carbamate (Chiralpak AD-RH) both coated on 5 μm silica gel. The above columns were obtained from Daicel (Tokyo, Japan). Experiments were performed at ambient temperature. All solvents were degassed in an ultrasonic bath prior to use. To eliminate some unexpected memory effects, a column regeneration procedure according to the vendor's instruction was performed when a new organic modifier was utilized. Once a new chromatographic condition was adopted, the column was equilibrated for at least 1 h before injection. Samples of naringenin and eriodictyol were diluted in methanol to a concentration of 0.1 mg/mL for HPLC-UV and 0.5 mg/mL for HPLC-CD. The prepared HPLC sample solutions were filtered through a nonsterile 0.45 μm PTEE syringe filter. UV and CD detection were performed at 284 nm. The CD spectra of the enantiomers were obtained by stopped-flow scanning at each chromatographic peak by CD detector in the wavelength range of 220-420 nm. Column void volume (t_0) was measured by injection of tri-*tert*-butylbenzene as a non-retained marker. The retention factor (k) was calculated as $k_1 = (t_1 - t_0)/t_0$ and $k_2 = (t_2 - t_0)/t_0$ where t_1 and t_2 are the retention times for the first and second eluting enantiomers, respectively. The separation factor (α) was calculated as $\alpha = k_2/k_1$. 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.

3. Results and Discussion

3.1. *Optimization of chromatographic conditions*

The effect of two amylose-based CSP, Chiralpak AD-RH and Chiralpak AS-RH, on the chiral recognition of compounds **1** and **2** was first studied. The effect of mobile-phase on the separation process was examined by modifying the percentage of water (doped with 0.1% trifluoroacetic acid, TFA) and type of organic cosolvent (methanol, ethanol, or acetonitrile) in the reversed-phase mixtures. The chromatographic parameters, capacity factor (k), separation factor (α), and resolution factor (R_s) for the resolved compounds **1** and **2** are given in Tables 1 and 2 for Chiralpak AD-RH and Chiralpak AS-RH, respectively.

These tables showed that both compounds **1** and **2** could be resolved with good separation factors (α) and resolution factors (R_s) on the Chiralpak AD-RH column by optimizing the mobile phase composition. For the enantioseparation on the Chiralpak AS-RH column, although a variation in the chromatographic parameters was optimized to obtain the best resolution, the two compounds could not be separated very well. Only a partial resolution of compound **1** was achieved using methanol and ethanol as organic modifiers or using acetonitrile as organic modifier for compound **2** (Figure 2). Thus, the results suggested the use of Chiralpak AD-RH to study the enantioseparation of compounds **1** and **2** is better. Chemically, Chiralpak AD-RH is amylose tris (3,5-dimethylphenyl carbamate), while Chiralpak AS-RH is amylose tris (*S*-α-methylbenzyl carbamate). Therefore, it may be concluded that the presence

Table 1. Chromatographic results for enantiomeric resolution of compounds 1 and 2 on Chiralpak AD-RH CSP

Eluent	k_1		k_2		α		R_s	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Methanol-H_2O^a, 95:5	3.14	2.41	4.26	2.86	1.36	1.19	3.76	2.48
Methanol-H_2O^a, 90:10	5.37	3.57	6.91	5.26	1.29	1.47	3.43	4.39
Methanol-H_2O^a, 85:15	9.21	5.73	11.42	9.43	1.24	1.65	2.98	5.90
Ethanol-H_2O^a, 80:20	0.61	1.14	0.61	1.69	1.00	1.48	0	3.30
Ethanol-H_2O^a, 70:30	2.20	2.20	2.35	3.33	1.07	1.65	0.80	4.60
Acetonitrile-H_2O^a, 50:50	1.55	0.94	1.70	1.04	1.10	1.11	1.10	0.82
Acetonitrile-H_2O^a, 35:65	7.02	3.57	8.01	3.95	1.14	1.11	2.86	1.16

[a] H_2O doped with 0.1% TFA.

Table 2. Chromatographic results for enantiomeric resolution of compounds 1 and 2 on Chiralpak AS-RH CSP

Eluent	k_1		k_2		α		R_s	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Methanol-H_2O[a], 80:20	1.73	0.92	1.97	0.92	1.21	1.00	1.47	0
Methanol-H_2O[a], 65:35	6.83	3.34	7.67	3.34	1.12	1.00	1.57	0
Methanol-H_2O[a], 60:40	11.67	-	12.89	-	1.10	-	1.50	-
Ethanol-H_2O[a], 55:45	3.56	1.95	3.86	1.95	1.08	1.00	1.23	0
Ethanol-H_2O[a], 40:60	23.85	9.33	25.29	9.33	1.06	1.00	1.87	0
Acetonitrile-H_2O[a], 25:75	14.17	5.44	14.17	5.76	1.00	1.06	0	1.40
Acetonitrile-H_2O[a], 20:80	-	12.76	-	13.61	-	1.07	-	1.83
Acetonitrile-H_2O[a], 15:85	-	36.88	-	39.81	-	1.08	-	2.96

[a] H_2O doped with 0.1% TFA.

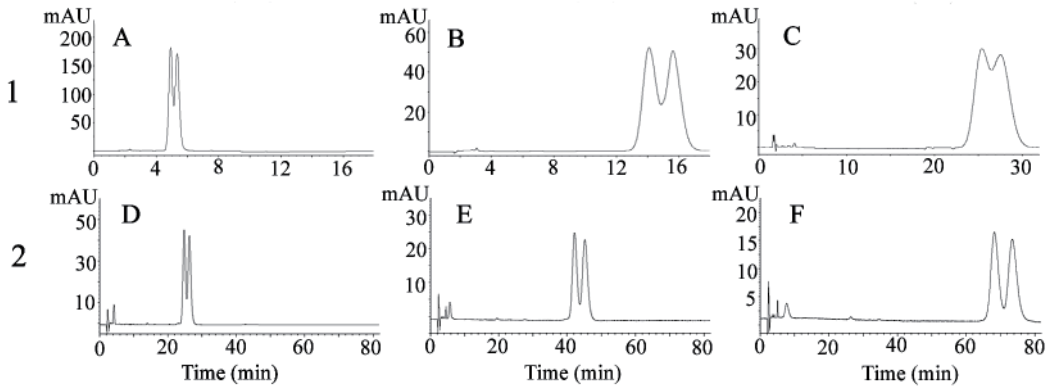

Figure 2. Typical HPLC chromatograms of enantiomeric resolution of compounds 1 and 2 on Chiralpak AS-RH column. Mobile phase: **(A)** methanol-H_2O doped with 0.1% TFA, 80:20 (v/v); **(B)** methanol-H_2O doped with 0.1% TFA, 65:35 (v/v); **(C)** ethanol-H_2O doped with 0.1% TFA, 45:55 (v/v); **(D)** acetonitrile-H_2O doped with 0.1% TFA, 20:80 (v/v); **(E)** acetonitrile-H_2O doped with 0.1% TFA, 17:83 (v/v); **(F)** acetonitrile-H_2O doped with 0.1% TFA, 15:85 (v/v).

of the two methyl groups on the phenyl moieties of Chiralpak AD-RH CSP increases the π basicity of the phenyl moieties, which results in π-π interactions of greater magnitude in comparison to Chiralpak AS-RH, and hence a better resolution occurred on Chiralpak AD-RH in comparison to Chiralpak AS-RH. Typical enantiomeric separations of flavanones **1** and **2** on Chiralpak AD-RH CSP and mobile phase composition are shown in Figure 3.

Several analytical considerations can be made from the results shown in Table 1 and Figure 3. *i*) As expected for the reversed-phase behavior of the Chiralpak AD-RH column, when the organic cosolvent concentration in the mobile phase increased, the *k*-values of the enantiomers were decreased in all cases. *ii*) The use of a different type of organic cosolvent in the mobile phase yielded quite different stereoselectivities for the two enantiomeric pairs. For both compounds **1** and **2**, the use of methanol as organic modifier gave good selectivity factors (α) and resolution factors (R_s). Thus, a mobile phase composition consisting of a simple mixture of methanol-water 90:10 (v/v) achieved an enantioselectivity factor value of 3.43 and 4.39 for compounds **1** and **2** respectively. *iii*) It can also be noted that for compound **2**, both α and R_s increased significantly by decreasing the percentage of methanol in the mobile phase, while for compound **1**, α and R_s only changed slightly by changing the concentration of methanol in the mobile phase. *iv*)The use of ethanol as organic modifier of the eluent could reduce the retention time of the enantiomers of compounds **1** and **2**. The resolution of compound **2** was achieved successfully in the ethanol solvent system, but the resolution of compound **1** was poor when ethanol was used as mobile phase. *v*) Compound **1** could be enantioseparated when acetonitrile was used as organic cosolvent in the mobile phase, but the resolution was poor for compound **2** in the same mobile phase. Thus slight modification in the substitution pattern influences heavily affected the behavior of the flavanones on the same CSP.

3.2. *Online coupling HPLC-CD*

Elution order between a pair of enantiomers is a key theme in the field of chiral HPLC, but until now prediction of elution order remains difficult. An online HPLC-CD method is quite useful to trace the elution order between enantiomers in a given selector system. In addition to obtaining a CD signal at a chosen wave length, the method could also afford the complete CD spectrum of the eluting peak in a stop-flow mode. As shown in Figure 4, the CD signals at 284 nm were obtained for compounds **1** and **2** in a continuous flow mode. Although the use of ethanol for compound **1** and acetonitrile for compound **2** as organic modifier did not afford good resolution as detected by UV (Figure 3),

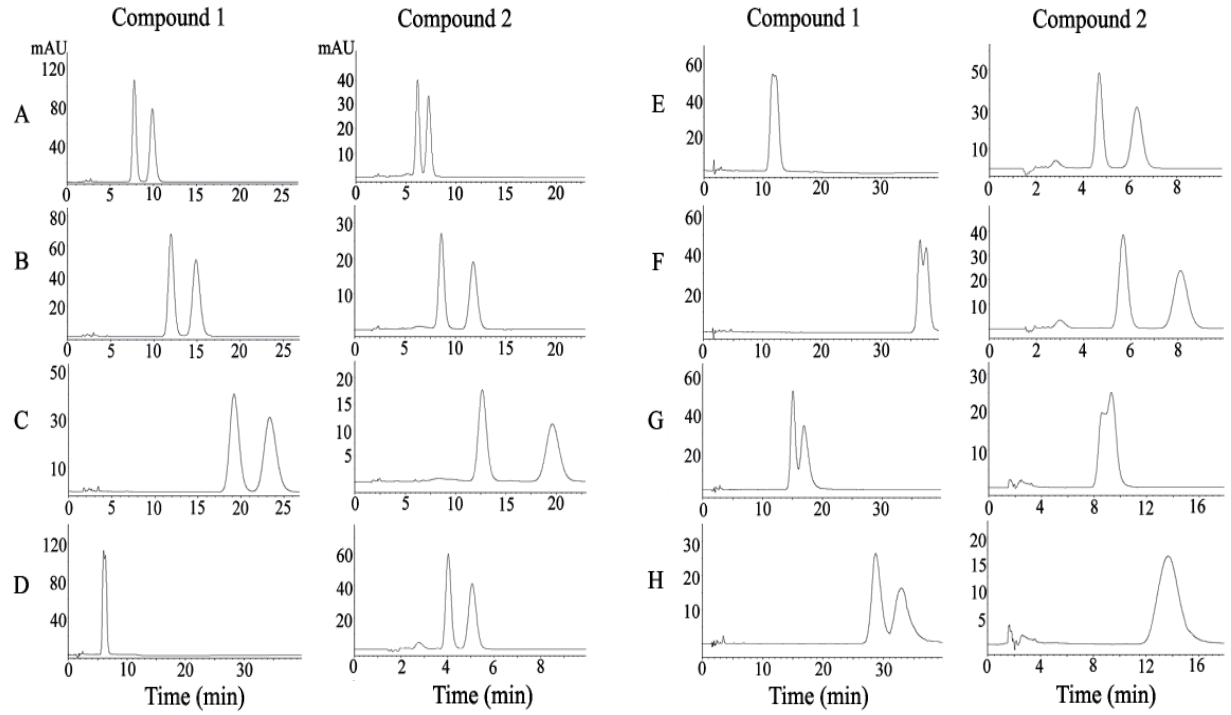

Figure 3. Typical HPLC chromatograms of enantiomeric resolution of compounds 1 and 2 on Chiralpak AD-RH column. Mobile phase: **(A)** methanol-H_2O doped with 0.1% TFA, 95:5 (v/v); **(B)** methanol-H_2O doped with 0.1% TFA, 90:10 (v/v); **(C)** methanol-H_2O doped with 0.1% TFA, 85:15 (v/v); **(D)** ethanol-H_2O doped with 0.1% TFA, 80:20 (v/v); **(E)** ethanol-H_2O doped with 0.1% TFA, 75:25 (v/v); **(F)** ethanol-H_2O doped with 0.1% TFA, 70:30 (v/v); **(G)** acetonitrile-H_2O doped with 0.1% TFA, 35:65 (v/v); **(H)** acetonitrile-H_2O doped with 0.1% TFA, 30:70 (v/v).

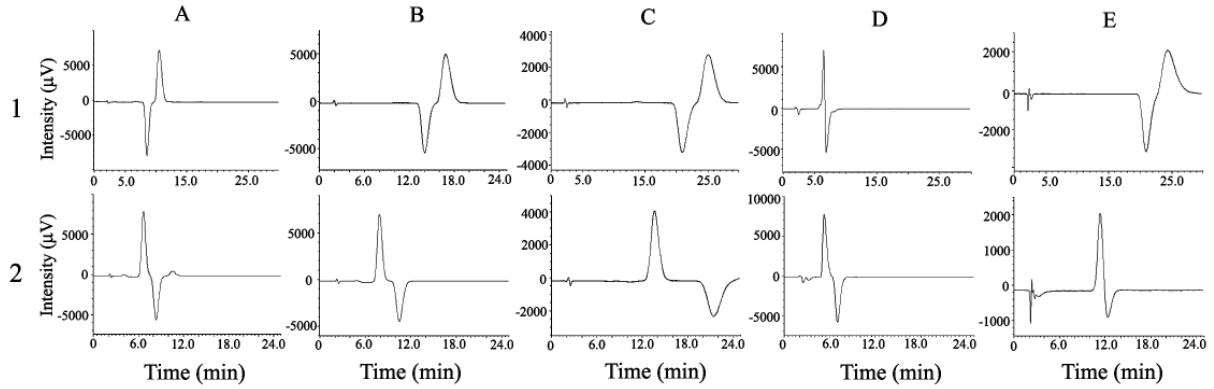

Figure 4. HPLC-CD chromatograms at 284 nm for compounds 1 and 2 on Chiralpak AD-RH. Mobile phase: **(A)** methanol-H_2O doped with 0.1% TFA, 95:5 (v/v); **(B)** methanol-H_2O doped with 0.1% TFA, 90:10 (v/v); **(C)** methanol-H_2O doped with 0.1% TFA, 85:15 (v/v); **(D)** ethanol-H_2O doped with 0.1% TFA, 70:30 (v/v); **(E)** acetonitrile-H_2O doped with 0.1% TFA, 30:70 (v/v).

clear negative signals and positive signals still could be seen in the CD traces.

It has been previously reported that a negative CD signal at 280-290 nm of flavanone is related to the *S*-configuration at C-2, whereas a positive CD signal at 290 nm established an *R*-configuration (*18*). Based on this, the elution order can be easily determined. As evidenced by the positive and negative CD signals at 284 nm, the opposite elution order of compounds **1** and **2** was observed by using methanol and acetonitrile as organic modifiers. For compound **1**, the *S*-enantiomer eluted as the first peak, but eluted as the second for compound **2**. When ethanol was used as organic modifier, the elution order is the same for the two pairs of enantiomers, *i.e.* the first eluted enantiomers are the *R*-configuration and the second eluted are the *S*-configuration. These phenomena indicated that the introduction of OH in position 3' might increase the possibility of additional hydrogen bonding between the compound and the CSP, and this kind of bonding might play a key role in the chiral recognition process. It has been reported that by changing the percentage of polar alcohol in the mobile phase you could induce an elution order reversal (*19,20*). In this experiment, the solvent-induced elution order reversal only took place by changing the type of organic modifier, no enantiomeric

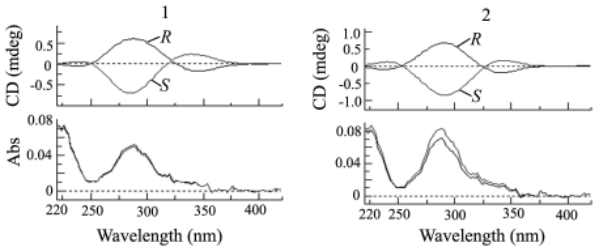

Figure 5. CD spectra of the eluted peaks of compounds 1 and 2 in HPLC-CD online coupling. CSP: Chiralpak AS-RH; mobile phase: methanol-H_2O doped with 0.1% TFA, 90:10 (v/v).

elution order reversal was observed by changing the concentration of organic cosolvent (Figure 4).

The online CD spectra of the enantiomers of compounds **1** and **2** were obtained using the stop-flow mode (Figure 5). The complete CD spectra of the two compounds are very similar, with the typical characteristics of a flavanone, *i.e.* as the signs at 280-290 nm for the $\pi\rightarrow\pi^*$ absorption band and at 330-340 nm for the $n\rightarrow\pi^*$ absorption band are related to the absolute configuration.

4. Conclusion

In summary, two amylose-based CSPs, Chiralpak AD-RH and Chiralpak AS-RH, were used for the enantioseparation of naringenin and eriodictyol. The Chiralpak AD-RH column was found to be more selective for the chiral resolution of the two flavanones. The separation of the enantiomers was optimized by varying the chromatographic parameters. The resolution was found to depend on the nature and concentration of organic modifier in the mobile phase. The 3',4' substituent pattern of the compounds affected the enantioselectivity on the same CSP. The HPLC-CD coupling technique was used for the configuration determination of the enantiomers. Elution order reversal was observed by changing the type of organic modifier in the mobile phase.

Acknowledgements

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