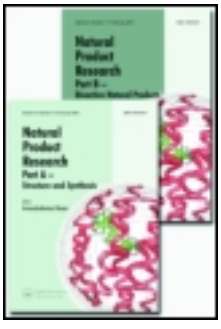

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Chiral separation of hesperidin and naringin and its analysis in a butanol extract of *Launeae arborescens*

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Two flavanone glycosides were isolated from the aerial part of *Launeae arborescens* (Asteraceae), which were identified as hesperidin and naringin. They are the most abundant flavonoids in the edible parts of many species of citrus fruits. In this study, we were interested in the chiral separation and determination of the diastereomerisation barriers of hesperidin and naringin by HPLC methods. The chiral separation HPLC screening of diastereomers of hesperidin and naringin by HPLC methods was accomplished in the normal-phase mode using 11 chiral stationary phases and various *n*-hexane/alcohol mobile phases. The rate constants and activation energy of diastereomerisation ($\Delta G\#$) of flavanones, naringin and hesperidin were determined, respectively, on Chiralpak IC and Chiralpak IA. The analysis of flavanones isolated in butanol extracts of *Launeae arborescens* were confirmed by HPLC on Chiralpak IC.

Keywords: *Launeae arborescens*; Asteraceae; hesperidin; naringin; flavanone; diastereomerisation barriers; enantioselective HPLC

1. Introduction

Flavanone-7-*O*-glycosides, an important class of naturally occurring compounds, are specifically distributed in citrus fruits (Aturki, Brandi, & Sinibaldi, 2004; Harborne, T. Mabry & H. Mabry, 1993). Flavanones are chiral compounds because of the asymmetric C-2 position in their moiety, and thus can exist in two enantiomeric forms. As their glycosides have an additional optically active sugar residue, they appear as a pair of diastereomers. Due to their polyphenolic structure, these compounds have health-related properties, which are based on their antioxidant activity as well as anticancer, antiviral and anti-inflammatory activities (Asztemborska & Zukowski, 2006; Yáñez, Teng, Roupe, & Davies, 2005).

The flavonoid hesperidin is a flavanone glycoside comprising the flavanone hesperitin and the disaccharide rutinose. Hesperidin is the predominant flavonoid

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in lemons and oranges. The peel and membranous parts of these fruits have the highest hesperidin concentrations (Emin, Oliveira, & Lapa 1994). Therefore, orange juice containing pulp is richer in flavonoids than juice without pulp. Sweet oranges (*Citrus sinensis*) and tangelos are the richest dietary sources of hesperidin, classified as a citrus bioflavonoid (Asztemborska & Zukowski, 2006). Hesperidin, in combination with a flavone glycoside called diosmin, is used in Europe for the treatment of venous insufficiency and haemorrhoids (Bok, 1999). Hesperidin, rutin and other flavonoids thought to reduce capillary permeability and to have anti-inflammatory action were collectively known as vitamin P. These substances, however, are not vitamins and are no longer referred to, except in older literature, as vitamin P (Berkarda, Koyuncu, Soybir, & Baykut, 1998).

In a recent study, hesperidin was separated using normal phase HPLC in commercial hesperidin and herbal medicine samples, and although the 2*S* epimer was predominant, there was a significant amount of 2*R* hesperidin present in some samples (Uchiyama, Kim, Kawahara, & Goda, 2005). Finally, the baseline separation of hesperidin by capillary electrophoresis using sulphobutyl ether β-cyclodextrin as the selector was accomplished, and 2*S* hesperidin was found to be predominant in lemon and orange juice (Aturki & Sinibaldi, 2003). Furthermore, the baseline separation of hesperidin by capillary electrophoresis using carboxymethyl-β-cyclodextrin as the selector has also been accomplished (Wistuba, Trapp, Gel-Moreto, Galensa, & Schurig, 2006) (Figure 1).

Naringin is a chiral flavanone-7-*O*-glycoside present in citrus fruits, tomatoes, cherries, oregano, beans and cocoa; naringin gives the characteristic bitter flavour to grapefruit. The processors of grapefruit try to select fruits with a low level of naringin, and often the juices of the mixture use varieties of different grapefruits to get the required degree of bitterness (Wistuba et al., 2006). Naringin can be used in the inhibition of cancer and therefore has potential chemotherapeutic value. Several drugs that are known to be affected by the naringin in grapefruit include blockers of the calcium channel, estrogen, calmatives, medicines for high blood pressure, allergies, AIDS, and cholesterol-lowering drugs (Mouly, Arzouyan, Gaydou, & Estienne, 1994). Caffeine levels and the effects of caffeine can also be spread while consuming grapefruit or juice of the grapefruit. While the effect of naringin on the metabolism of a drug can increase the efficiency of the drug, it can also result in dosages that are accidentally too high. Therefore, it is better not to take any drug

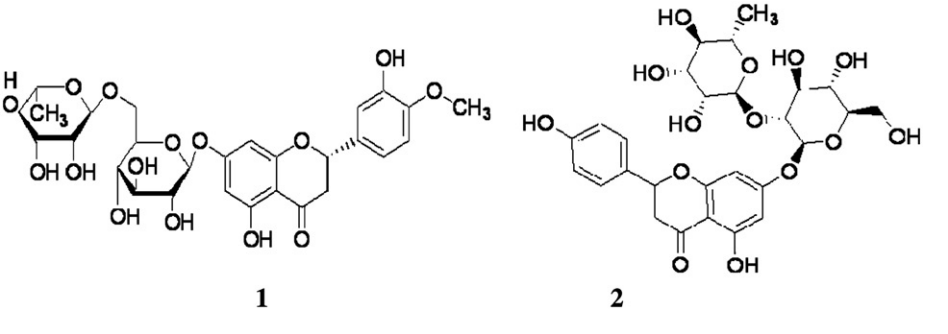

Figure 1. Structures of hesperidin (**1**) and naringin (**2**).

with grapefruit juice unless the interaction with the drug is known. Besides, the effects of drinking grapefruit juice are cumulative, which means, if you drink a glass of grapefruit juice with your medicine daily for a week, the interaction of the drug would be stronger at the end of the week compared to the beginning of the week (Bear & Teel, 2000).

The stereochemistry of naringin changes with the diameter of the fruit, with greater concentrations of *S*-naringin in grapefruits with the smallest diameter (Caccamese, Manna, & Scivoli, 2003). A recent report using β-cyclodextrin, dimethyl-β-cyclodextrin and hydroxypropyl β-cyclodextrin as mobile phase additives in capillary electrophoresis resolved the epimers of naringin, although baseline resolution was not obtained (Gel-Moreto, Streich, & Galensa, 2001). A Chiralpak IA column was also able to separate naringin directly under normal phase isocratic conditions, although the baseline resolution was not obtained (Uchiyama, et al. 2005). A more recent study reported the enantiomeric separation of naringin by capillary electrophoresis using the various cyclodextrins as selectors and demonstrated the separation with the best resolution of $Rs = 4.85$ with hydroxypropyl-γ-cyclodextrin and baseline resolution with methyl-γ-cyclodextrin ($Rs = 3.81$), carboxymethyl-β-cyclodextrin ($Rs = 2.26$) and sulphato-β-cyclodextrin ($Rs = 3.63$) (Wistuba et al., 2006).

These two glycoside flavanones were detected and isolated from the butanol fraction of a water–acetone extract of the aerial part of *Launeae arborescens* (Asteraceae) in minor quantities by two different methods (naringin at 25°C, hesperidin at 65°C reflux) (Belboukhari & Cheriti, 2009).

The present work reports the chiral separation screening on 11 chiral stationary phases (CSPs) and the determination of rate constants and activation energy barriers of diastereomerisation $\Delta G\#$ of naringin and hesperidin using HPLC methods.

2. Experimental

2.1. *Reagents*

Naringin (97%), hesperidin (97%), TEA, TFA, hexan, ethanol, methanol and isopropanol were from Acros Organics (New Jersey, USA).

2.2. *Apparatus and procedures*

The analytical chiral HPLC experiments were performed on a unit composed of a Merck D-7000 system manager, Merck-Lachrom L-7100 pump, Merck-Lachrom L-7360 oven, Merck-Lachrom L-7400 UV-detector and a Jasco OR-1590 polarimeter. The sign given by the online polarimeter for an enantiomer is the sign in the solvent used for the chromatographic separation. Retention times (Rt) in minutes, retention factor, k_i ¼ $(Rt_i - Rt_0)/Rt_0$, and enantioselectivity factor, a ¼ k_2/k_1, are given.

Hexan, 2-PrOH and ethanol were of HPLC grade, degassed and filtered on a 0.45 lm membrane before use. Chiralcel OD-H, OD, OJ, OB-H, Chiralpak AD, AD-H, AD-RH, AS, IA, IC, available from Chiral Technologies Europe (Illkirch, France), and Ulmo (S, S) (250 3 4.6 mm), Sumichiral OA-2500, Kromasil CHI-TBB,

available from Regis (Morton Grove, USA), were used for the analytical separations.

2.3. *Plant materials (extraction and traditional uses)*

Launeae arborescens (vernacular name: Oumlbina) is a herbaceous plant belonging to the Asteraceae family, which is widely distributed in the west Algerian Sahara. The aerial part of this plant is used in folk medicine for treating diarrhoea fever, gastrointestinal tracts and various diseases (Belboukhari & Cheriti, 2006; Ozenda 1967). The whole plants were collected in March 2000 from Bechar, south Algeria. The botanical identification and a voucher specimen are conserved at the Phytochemical Herbarium of the Phytochemical and Organic Synthesis Laboratory under accession number CA99/25 (Cheriti, Belboukhari, & Hacini, 2004).

The aerial parts were separated and dried and the plants were ground into powder using a grinder. Hesperidin and naringin were isolated from a butanol fraction of water : acetone (2 : 1) extract of an aerial part of the plant by reflux for 3 h using a Soxhlet apparatus and by steeping to ambient temperature during 24 h (Belboukhari, Cheriti, & Roussel 2007).

3. Results and discussion

3.1. *Chiral separation screening*

To optimise the conditions for obtaining the separation of the C-2 diastereomers (epimers) of hesperidin and naringin present in butanol extracts of *L. arborescens*, to assess their identity, and to study their C-2 stereochemistry, we used commercialised compounds in the chiral separation screening on 11 CSPs and various (*n*-hexane/ethanol or isopropanol) mobile phases (Table 1). Typical separation of the epimers of compounds **1** and **2** is shown in Figures 2 and 3.

Table 1 shows the chromatographic results for the separation of (2*R*/2*S*)-flavanone glycosides using the Chiralcel OD-H as CSP. Epimer selectivity values (*R*) ranged from 1.81 for naringin to 1.16 for hesperidin, Chiralpak IA ranged in different conditions from 1.25 to 1.13 for naringin and hesperidin and Chiralpak AD-H presented a good chiral separation of naringin and hesperidin with a selectivity factor towards 1.28.

The Chiralpak AD phase presented only the epimer separation of hesperidin with a selectivity towards 1.21. Analogously, the resolution factor (*R*s) ranged from 2.27 for naringin to 0.97 for hesperidin. The values of *R* and *R*s obtained for naringin were much better than those obtained using another polysaccharide-derived CSP (Chiralpak AD) and a very similar mobile phase (1.51 and 0.7, respectively).

The difference in the chiral recognition ability may be due to the different volumes of the helical groove of the cellulose derivative (OD-H) and the amylose derivative (AD), because it is well known that amylose-derived phases possess a wider and more compact helix (Okamoto & Yashima, 1998).

These two compounds were not resolved into their epimers using the composition of the mobile phase and Chiracel OJ, Chiracel OBH, Sumi, Ulmo and Kromasil phases reported in Table 1, as elution times for the epimers of hesperidin on Chiralpak

Table 1. Chromatographic data for the separation of **1** and **2** on 11 CSPs ($T = 25°C$).

Entry	Compound	CSP	Eluent (alcohol%)	FR	k_1	k_2	t_1 (min)	t_2 (min)	*R*s	α
1	**1**	Chiralpak IA	30[a] (0.1TEA)	1	18.70	22.57	61.67	73.07		1.20
2	**1**	Chiralpak IA	30[a] (0.1TFA)	1	13.93	17.21	46.28	56.45		1.24
3	**1**	Chiralpak IA	100[a]	1	0.71	–	5.30	–		–
4	**1**	Chiralpak IA	50[a]	1	4.30	4.83	16.42	18.09		1.13
5	**1**	Chiralpak IA	50[b]	1	4.47	–	16.95	–		–
6	**1**	Chiralpak AS–H	50[a]	1	1.22	–	6.90	–		
7	**1**	Chiralpak AD–H	40[b]	1	6.77	8.66	24.09	29.95	–	1.28
8	**1**	Chiralpak AD–H	40[a]	1	6.78	8.62	24.13	29.83	–	1.27
9	**1**	Chiralpak AD	50[a]	1	4.11	–	15.59	–	–	–
10	**1**	Chiralpak AD	50[b]	1	3.54	4.28	13.83	16.12	0.97	1.21
11	**1**	Chiralpak AD–RH	20[b]	0.5	4.60	–	17.37	–	–	–
12	**1**	Chiralcel OB–H	50[a]	1	0.85	–	5.64	–	–	–
13	**1**	Chiralcel OB–H	30[a]	1	4.26	–	16.04	–	–	–
14	**1**	Chiralcel OD–H	50	1	0.84	–	5.62	–	–	–
15	**1**	Chiralcel OD–H	30[a]	1	3.28	3.80	13.05	14.61	–	1.16
16	**1**	Chiralcel OJ	50[a]	1	2.61	–	11.01	–	–	–
17	**1**	Chiralcel OJ	30[a]	1	2.54	–	10.81	–	–	–
18	**1**	Chiralcel OJ	50[b]	1	0.52	–	4.64	–	–	–
19	**1**	Kromasil CHI–TBB	50[a]	1	0.00	–	2.73	–	–	–
20	**1**	Sumichiral OA–2500	50[a]	1	5.10	–	18.61	–	–	–
21	**1**	Ulmo (S, S)	50[a]	1	0.52	–	9.81	–	–	–
22	**1**	Ulmo (S, S)	30[a]	1	2.22	–	4.73	–	–	–
23	**1**	Chiralpak IC	50[a]	1	1.24	1.66	6.82	8.12	1.13	1.34
24	**1**	Chiralpak IC	30[a]	1	5.93	7.68	21.13	26.47	1.34	1.29

(*Continued*)

Table 1. Continued.

Entry	Compound	CSP	Eluent (alcohol%)	FR	k_1	k_2	t_1 (min)	t_2 (min)	*R*s	α
25	**2**	Chiralpak IA	30[a] (0.1TFA)	1	2.47	–	10.77	–	–	–
26	**2**	Chiralpak IA	30[a] (0.1TFA)	0.4	8.07	–	28.11	–	–	–
27	**2**	Chiralpak IA	50[a]	1	0.50	–	4.66	–	–	–
28	**2**	Chiralpak IA	50[b]	1	0.51	0.64	4.68	5.08		1.25
29	**2**	Chiralpak IA	20[b]	1	8.03	9.65	28.01	33.03		1.20
30	**2**	Chiralpak IA	20[b] (0.1TFA)	1	10.31	12.70	35.09	42.48		1.23
31	**2**	Chiralpak IA	20[b] (0.1TEA)	1	10.01	11.68	34.13	39.31		1.17
32	**2**	Chiralpak AS–H	20[a]	1	29.23	–	93.73	–	–	–
33	**2**	Chiralpak AS–H	50[a]	1	0.21	–	3.75	–	–	–
34	**2**	Chiralpak AD–H	40[b]	1	0.73	0.91	5.38	5.91	–	1.25
35	**2**	Chiralpak AD	50[a]	1	0.55	–	100	–		–
36	**2**	Chiralcel OJ	50[a]	1	0.41	–	100	–	–	–
37	**2**	Kromasil CHI–TBB	50[a]	1	–0.03	–	100	–	–	–
38	**2**	Ulmo (S, S)	50[a]	1	0.21	–	100	–	–	–
39	**2**	Sumichiral OA–2500	50[a]	1	3.07	–	100	–	–	–
40	**2**	Chiralcel OD–H	50[a]	1	0.97	1.76	6.01	8.41	1.62	1.81
41	**2**	Chiralpak IC	50[a]	1	1.12	1.51	6.48	7.64	1.24	1.34
42	**2**	Chiralpak IC	30[a]	1	5.21	7.42	18.95	25.67	2.27	1.42

Note: CSP, chiral stationary phase. [a]Hexan/ethanol. [b]Hexan/isopropanol.

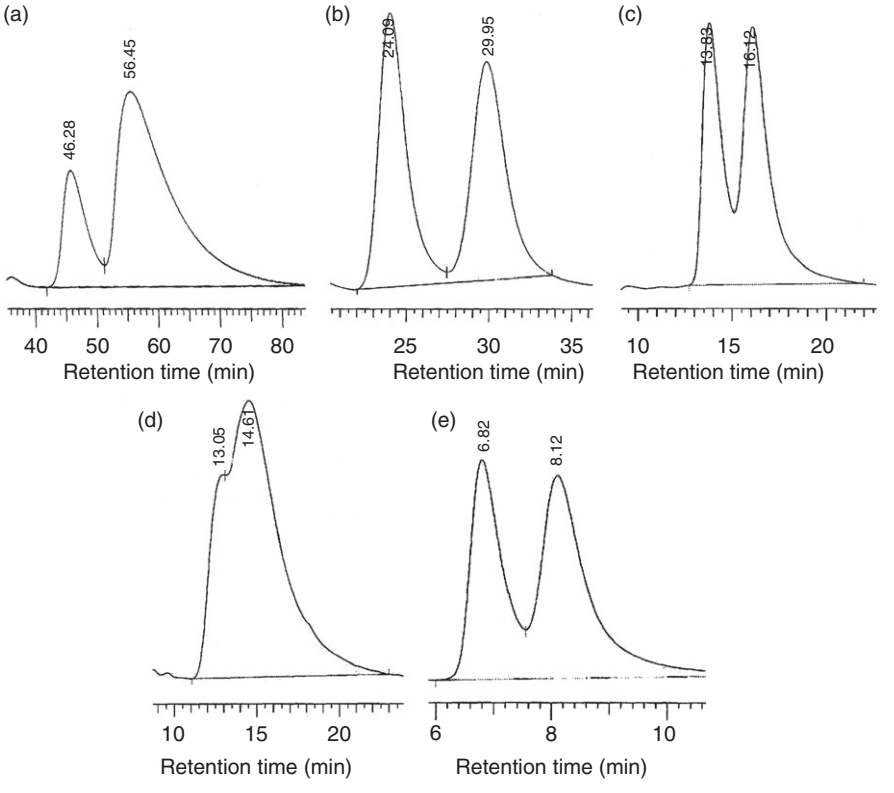

Figure 2. Chromatograms of chiral separation of epimers of hesperidin on (a) Chiralpak IA (Entry 2), (b) Chiralpak AD (Entry 10), (c) Chiralpak AD-H (Entry 7), (d) Chiralpak OD-H (Entry 15), and (e) Chiralpak IC (Entry 23).

IA can be achieved only by using a more polar mobile phase (50% doped ethanol in *n*-hexane). Much higher elution times for very broad peaks (>60 mn) were obtained when using a less polar phase.

The strong interaction of hesperidin and naringin with the CSP results in a very high k_1 due to a more polar mobile phase for reasonable elution, and their presence in the structures of compounds **1** and **2** of two glycosides groups act as hydrogen donor and hydrogen acceptor groups for hydrogen-bonding interaction with the carbamate groups of the phase (Caccamese, Caruso, Parrinello, & Savarino, 2005).

The absolute configuration at C-2 of the first and the second peaks of compounds **1** and **2**, as reported in Table 1, has been assigned by the knowledge of the Cotton effects in the circular dichroism (CD) spectra of flavanone glycosides and their aglycones, because the carbohydrate moiety is CD-inactive (Caccamese et al., 2005; Yáñez, Andrews, & Davies 2007). Moreover, the assignment of the absolute configuration of the eluting peaks is not automatic in the absence of information about the CD spectra or if the authentic samples of C-2 diastereomers are not available. Integration of the peaks shows that the two commercial compounds are richer in the 2*S* epimer, which show a diastereomeric purity (dp) $2S/(2S+2R)$, 79.4% of hesperidin and 75.1% of naringin. However, the isolated compounds

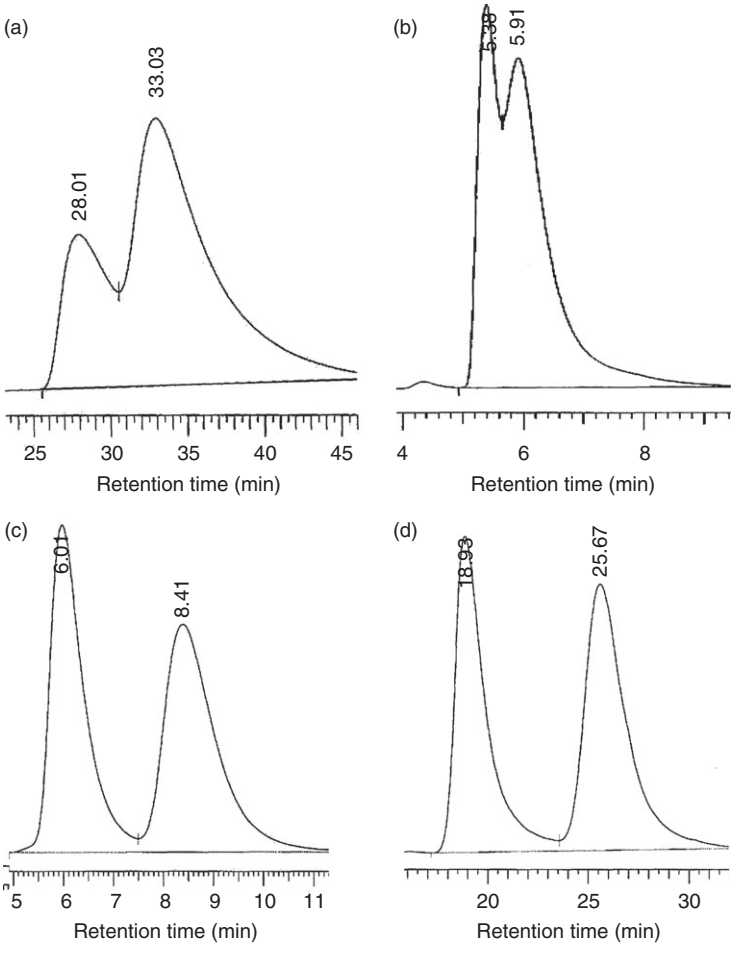

Figure 3. Chromatograms of chiral separation of epimers of naringin on (a) Chiralpak IA (Entry 28), (b) Chiralpak AD-H (Entry 34), (c) Chiralpak OD-H (Entry 40), and (d) Chiralpak IC (Entry 42).

present a diastereomeric purity towards 53% of hesperidin and 68% of naringin, although the stereochemistry at C-2 is not depicted. The predominance of one C-2 diastereomer with respect to another one is undoubtedly due to the extraction of these compounds (Trapp & Schurig, 2001).

3.2. *Chiral separation composition of glucoside flavanones (1 and 2) from* L. arborescens

The two glucoside flavanones isolated from the butanol fraction of the water acetone extract of *L. arborescens* were identified as hesperidin and naringin, and the structures were confirmed by chiral separation on eluted Chiralpak IC (hexane/ethanol, 70 : 30). The results presented in Table 2 demonstrate a racemic composition of hesperidin (46.96 : 53.04) and naringin (68.45 : 31.55) (Figure 4). The results were explained by the protocol condition for the isolation of these two compounds, of which the naringin

Table 2. Chiral separation of hesperidin and naringin on Chiralpak IC.

Compound	Hesperidin	Naringin
CSP	Chiralpak IC	Chiralpak IC
Eluent (%)	70	70
FR	1	1
k_1	5.12	4.55
Rt_1	18.67	16.93
Rt_2	23.51	22.75
Rs	1.55	2.43
α	1.31	1.42
Composition (%)	46.96 : 53.04	68.45 : 31.55

Note: CSP, Chiral stationary phase.

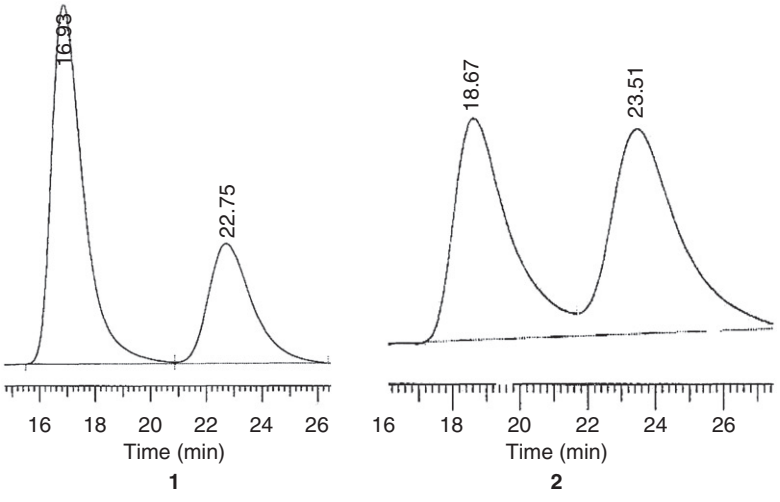

Figure 4. Chiral separation of hesperidin and naringin epimers on Chiralpak IC.

was separated at t.a. but hesperidin was extracted with reflux at 100°C (Trapp & Schurig, 2001).

3.3. *Diastereomerisation of hesperidin and naringin*

The diastereomerisation of two flavanone glycosides was studied by enantioselective HPLC combined with the classical kinetic method, enantiomerisation and calculated from the following equation (Figure 5):

$$k = (1/2t)\ln([A]0 - [A]eq/[A]t - [A]eq). \quad (1)$$

The diastereomerisation barrier $\Delta G^{\#}$ was calculated using the Eyring equation:

$$\Delta G^{\#} = RT\ln(hk/\kappa k_B T), \quad (2)$$

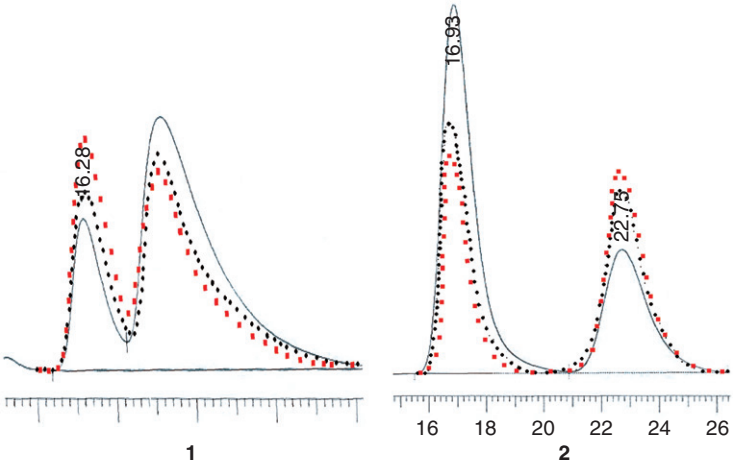

Figure 5. Chromatograms of diastereomerisation of hesperidin (**1**) and naringin (**2**) epimers in MeOH at 40°C by HPLC method.

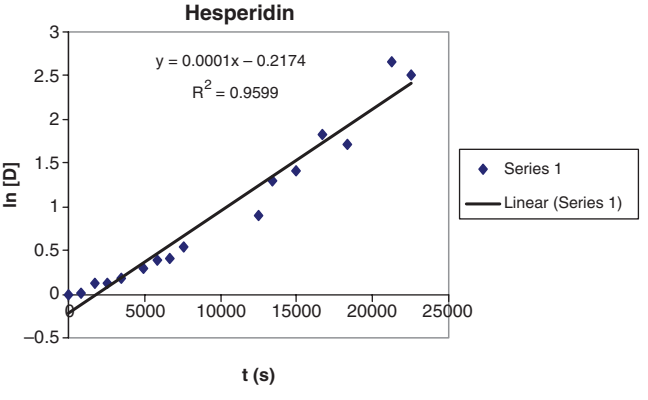

Figure 6. Plot of $\ln[D] = f(t)$ for hesperidin obtained by HPLC experiment.

where T is the temperature of diastereomerisation in k, R is the universal gas constant ($R = 8.31441\,\mathrm{JK^{-1}\,mol^{-1}}$), k_B is the Boltzman constant ($k_B = 1.380662 \times 10^{-23}\,\mathrm{JK^{-1}}$), h is the Planck's constant ($h = 6.626176 \times 10^{-34}\,\mathrm{Js}$) and κ is the transmission coefficient ($\kappa = 0.5$) (Reich, Trapp, & Schurig, 2000).

The plot of $\ln([A]_0 - [A]_{eq})/([A]_t - [A]_{eq})$ as a function of time for hesperidin and naringin are presented, respectively, in Figures 6 and 7, and demonstrate a linear relationship according to Equation (1). The rate constants were determined from the plot by linear regression.

In this method, diastereomerisation is performed outside the separation system at a chosen temperature and time (Asztemborska & Zukowski, 2006). Afterwards, samples are analysed by enantioselective HPLC at the temperature at which diastereomerisation is suppressed. This method was applied to determine the rate constant and Gibbs activation energy of the diastereomerisation of hesperidin and

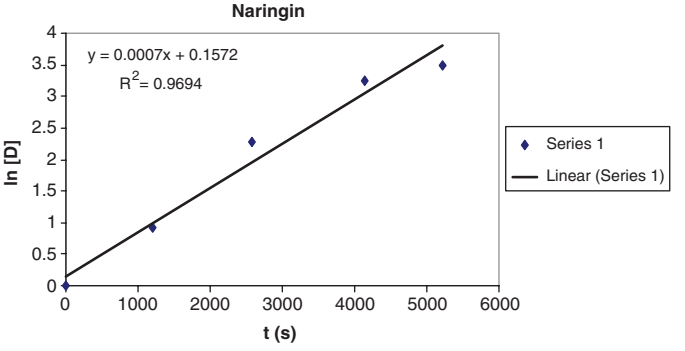

Figure 7. Plot of $\ln[D] = f(t)$ for naringin obtained by HPLC experiment.

Table 3. The rate constants and Gibbs activation energy of diastereomerisation reaction of naringin and hesperidin determined by HPLC methods.

Compounds	Method	T (°C)	Solvent	k (s^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})
Hesperidin	HPLC	40°	MeOH : TEA (10 : 0.1)	10^{-4}	84.00
	HPLC	60°	MeOH	2.4×0^{-6}	115.8
Naringin	HPLC	40°	MeOH	7×10^{-4}	78.93
	D-HPLC	50°	MeOH : H_2O (10 : 90)	9.218×10^{-5}	102.5
	D-HPLC	60°	MeOH : H_2O (10 : 90)	2.078×10^{-4}	103.5

naringin (Table 3). The 0.27 mM solution of hesperidin and naringin in methanol (added 3 μ mL TEA) was stored at 40°C in a thermostat. Every 24 h, samples were taken and analysed directly by enantioselective HPLC. Additionally, it was proved that after a few weeks of storage of the solutions at 40°C, the ratio of 2*R*- to 2*S*-diastereomer reached a value very close to 1, which leads to the conclusion that, for hesperidin as well as for naringin, $k_{eq} \approx 1$.

The plot of $\ln([A]_0 - [A]_{eq})/([A]_t - [A]_{eq})$ as a function of time for hesperidin and naringin presented in Figures 6 and 7 demonstrates a linear relationship according to Equation (3).

4. Conclusion

In summary, the developed HPLC assay is stereospecific, reproducible and accurate. It has been successfully applied to chiral analysis and quantification of diastereomers of flavanone glucosides in different stages of evolution of the plant, and successfully used to study the diastereomeric composition of various plant parts (root, stem, twig, leaf, flower and seed).

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