Journal of Chromatography A, 1218 (2011) 5414–5422

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Quick development of an analytical enantioselective high performance liquid chromatography separation and preparative scale-up for the flavonoid Naringenin☆

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ARTICLE INFO

Article history:
Received 12 October 2010
Received in revised form 15 February 2011
Accepted 15 February 2011
Available online 22 February 2011

Keywords:
Enantioselective HPLC
Chiral resolution
Enantiomer separation
Naringenin
Elution order
Amylose and cellulose derived CSPs

ABSTRACT

The HPLC enantioselective separation of (*R*/*S*)-Naringenin, a chiral flavonoid found in several fruits juices and well-known for its beneficial health-related properties, including antioxidant, anti-inflammatory, cancer chemopreventive, immunomodulating and antimicrobial activities, has been performed on both analytical and (semi)-preparative scale using an amylose derived Chiralpak AD chiral stationary phase (CSP). A standard screening protocol for cellulose and amylose based CSPs was firstly applied to analytical Chiralcel OD-H and Chiralpak AD-H, as well as to Lux Cellulose-1, Lux Cellulose-2 and Lux Amylose-2 in order to identify the best experimental condition for the subsequent scaling-up. Using Chiralpak AD-H and eluting with pure methanol (without acidic or basic additives) relatively short retention times, high enantioselectivity and good resolution ($\alpha = 1.49$, $R_s = 3.48$) were observed. Therefore, these experimental conditions were properly scaled-up to (semi)-preparative scale using both a pre-packed Regispack column and a Chiralpak AD column packed in house with bulk CSP. The developed preparative method proved to be superior to previously published methods in terms of elution times, separation and resolution and is suitable for obtaining a quick access to the desired enantiomers with high enantiomeric excess and amounts sufficient for biological investigations. Future scale-up options (enantioselective supercritical fluid chromatography or HPLC in the Simulated Moving Bed mode) were also evaluated. It could be shown that both methodologies have a high potential for future production of Naringenin enantiomers by enantioselective chromatography.

1. Introduction

Naringenin (5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one, Fig. 1) is a chiral flavanone belonging to the flavonoid class, well-known for its beneficial health-related properties. It is present in various natural sources, such as *Citrus* fruits, tomatoes, pears, apples, thyme and almond skins [1–3]. Particularly, grapefruit and sour orange, contain significant amounts of its 7-O-glycoside – called Naringin – which after consumption rapidly undergoes cleavage of the sugar moiety, leaving the free aglycone in the gastrointestinal tract [1]. Additionally, our recent research focused on drug discovery from plants evidenced that Naringenin is one of the principal phyto-components of *Amygdalus lycioides* Spach, an Iranian medicinal plant known as *Badam Talkh kuhi* and commonly used by native people of Iran for managing pain and inflammatory conditions, inflammatory skin disorders and infectious diseases [4,5].

Only few studies have properly assessed the stereospecific distribution of Naringenin in its natural sources [2,6]. (*S*)-Naringenin is the predominant enantiomer in orange, apple and tomato juices; generally, low amounts of (*S*)-Naringenin are present in nature, with the exception of tomato juice [2]. Interestingly the enantioselective disposition of Naringenin in tomato fruit tissues (exocarp, mesocarp and seed cavity) is influenced by genotype as well as maturation stage [6].

The biological properties of (*R*/*S*)-Naringenin have been extensively investigated [7–12]. The first study focused on the evaluation

☆ Presented at the 13th Symposium on Preparative and Industrial Chromatography and Allied Techniques, Stockholm, Sweden, 12–15 September 2010. A part of this work has been presented during the *Conference on Preparative & Process Scale HPLC, MCC and SFC*, Sarö, June 1–2, 2009.

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doi:10.1016/j.chroma.2011.02.038

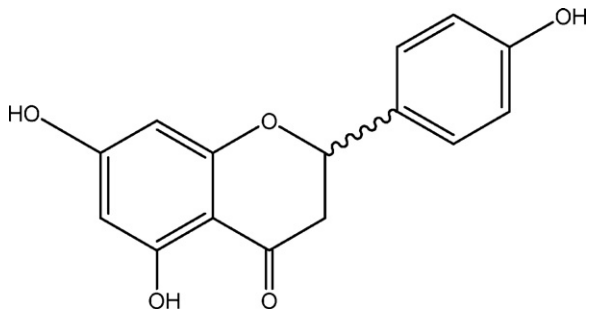


Fig. 1. (*R*/*S*)-Naringenin.

of cytochrome P450 inhibitory effect of the grapefruit-specific flavonoids, including (*R*/*S*)-Naringenin. Results clearly evidenced that (*R*/*S*)-Naringenin is effective in inhibiting the P450IIIA4-mediated oxidation/inactivation of the dihydropyridines cardiodepressive drugs (nifedipine and felodipine) as well as in blocking the P450IIIA4-mediated activation of aflatoxin B1, suggesting the cancer chemopreventive properties of this flavanone [7]. Successively, various papers stated that (*R*/*S*)-Naringenin possesses a chemopreventive effect towards mutagenesis of heterocyclic amines [8], acts as immunomodulator [9] and presents antioxidant and antimicrobial properties, analogously to other polyphenols [10]. Moreover, the anti-inflammatory activity of (*R*/*S*)-Naringenin *via* inhibition of the pro-inflammatory cytokines interleukin-1β and tumor necrosis factor-α was also reported [11]. Recently, it has been demonstrated that (*R*/*S*)-Naringenin intake is associated with reduced risk of several chronic diseases, including vascular risk. This activity seems to be related to an increase of both nitric oxide production and HDL plasma levels combined with a decrease of triacylglycerol, total cholesterol and phospholipid levels in serum [12]. Surprisingly, to the best of our knowledge, the pharmacological profile of Naringenin enantiomers has not been exhaustively investigated yet, even in view of the obvious fact that two enantiomers may exert different biological properties and show distinguished toxicological properties and potential benefits [13]. Only one paper examined the Naringenin enantiomers effect on the inhibition of cyclosporine A oxidase activity in human liver microsomes, but no enantioselectivity was demonstrated [14].

Given the significant pharmacological interest of (*R*/*S*)-Naringenin and the lack of knowledge about the relevance of chirality regarding its biological profile, the aim of the present work was to develop a productive and robust system suitable to isolate pure enantiomers in amount sufficient to support an exhaustive biological investigation. Due to the paucity of Naringenin in natural sources, the resolution of commercially available (*R*/*S*)-Naringenin *via* enantioselective HPLC on a (semi)-preparative scale was selected as a promising strategy for providing a quick and easy access to its enantiomers. In fact, enantioselective HPLC is one of the most rapid and efficient methods for obtaining directly both enantiomers in high optical purity.

2. Experimental

2.1. Chemicals and instruments

(*R*/*S*)-Naringenin (5,7-dihydroxy-2-(4-hydroxyphenyl) chroman-4-one, Fig. 1) was obtained from Sigma–Aldrich (Buchs, Switzerland).

The solvents used as eluents were HPLC grade and were obtained from Merck-VWR (Dietikon, Switzerland or Milan, Italy).

Analytical chiral resolutions were performed on: (a) Agilent HP1100 system (Agilent Technologies Switzerland, Basel, Switzerland) consisting of a quaternary pump, an autosampler and a photo-diode array detector (PAD) (system 1); (b) Jasco system (JASCO Europe, Cremella, LC, Italy) consisting of a PU-2089 plus pump, a AS-2055 plus autosampler, a MD-2010 plus detector and a CD-2095 plus circular dichroism (CD) detector (system 2).

The (semi-)preparative HPLC was produced by Varian Chromatographic Systems (Walnut Creek, CA, USA) and consists of two Rainin SD-1 pumps with 500 mL/min pump heads, a 410 Varian autosampler, a 320 Varian Prostar UV-detector and a 320 Varian fraction collection module (system 3).

SFC experiments were performed on a semi-preparative Thar (Pittsburgh, PA, USA) SFC Method Station (system 4).

The DIP 1000 photoelectric polarimeter from Jasco (JASCO Europe, Cremella, LC, Italy) was used for [α] measurements that were recorded at room temperature using a 1 dm cell and a sodium lamp.

The MS analyses were performed on Finnigan LCQ Fleet system, controlled by Xcalibur software 1.4 (Thermo Finnigan, San Jose, CA, USA). Mass spectra were generated in negative ion mode under constant instrumental conditions: ion spray voltage 5 kV, capillary voltage −49 V, capillary temperature 220 °C, and tube lens voltage −100 V.

2.2. Chiral chromatographic resolution

2.2.1. Analytical screening of amylose and cellulose based chiral stationary phases (CSPs)

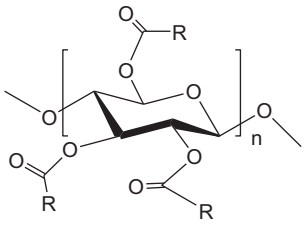
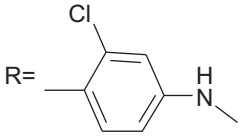
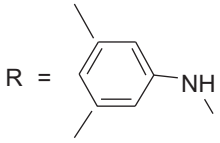
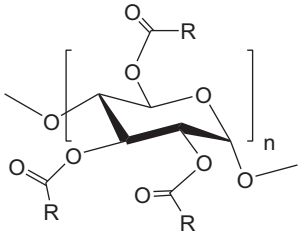
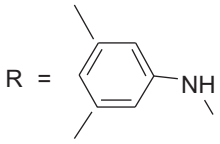
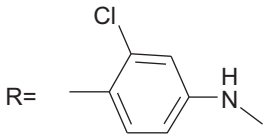
Analytical chiral resolution was carried out on system 1 using the following columns: Chiralcel OD-H (250 mm × 4.6 mm I.D., d_p = 5 μm) and Chiralpak AD-H (250 mm × 4.6 mm I.D., d_p = 5 μm), that are products of Daicel Industries Ltd. (Japan) and can be obtained from Chiral Technologies Europe (Illkirch, France); Lux Cellulose-1 (150 mm × 4.6 mm I.D., d_p = 5 μm), Lux Cellulose-2 (150 mm × 4.6 mm I.D., d_p = 5 μm) and Lux Amylose-2 (150 mm × 4.6 mm I.D., d_p = 5 μm) that are produced by Phenomenex (Torrance, CA, USA) and were obtained from Brechbühler (Schlieren, Switzerland). Structures of the chiral selectors used in the screening are reported in Table 1. The mobile phases employed are reported in Table 2 and include mixtures of *n*-heptane and polar modifiers, alcohols, acetonitrile as well as mixtures of alcohols and acetonitrile.

Sample solutions were prepared dissolving analytes at ~2 mg/mL in ethanol and filtered through 0.45 μm PTFE membranes before analysis. The injection volume was 10 μL and the flow rate was 1 mL/min unless otherwise specified. Analytes were detected photometrically at 290 nm. HPLC analyses were performed at 20 °C. Retention factors (κ_1 or κ_2), separation factor (α) and resolution (R_s) were calculated according to international recommendations [15,16]. The dead time (t_0) was considered to be equal to the peak of the solvent front and taken from each particular run.

2.2.2. (Semi-) preparative enantioselective chromatography

The (semi-)preparative enantioselective chromatography was carried out on system 1 using: (a) a pre-packed Regispack column (250 mm × 10 mm I.D., d_p = 5 μm), produced by Regis (Morton Grove, IL, USA) and purchased directly from the supplier, eluting with pure methanol at room temperature with a flow rate of 4 mL/min; (b) system 3 employing a Chiralpak AD column packed and tested in-house with bulk CSP. The CSP was obtained from Chiral Technologies Europe (Illkirch, France) as 20 μm bulk packing. An amount of 100 g of Chiralpak AD, d_p = 20 μm, was packed into a 41.4 mm (I.D.) Dynamax RamRak Column Packing station (Varian Chromatographic Systems, Walnut Creek, CA, USA) and then tested by pulse injections of racemic Naringenin at various flow rates. The (semi-)preparative enantiomer separation runs were performed with pure methanol at room temperature (23 ± 2 °C) with a flow rate of 30 mL/min. The eluate was properly partitioned according

Table 1
CSPs employed in the analytical screening protocol.

Structure	Substituent	Chiral selector	Trade name
		Cellulose tris(3-chlor-4-methylphenylcarbamate)	Lux Cellulose-2
		Cellulose tris(3,5-dimethylphenylcarbamate)	Lux Cellulose-1: Chiralcel OD
		Amylose tris(3,5-dimethylphenylcarbamate)	Chiralpak AD(-H): Regispack[a]
		Amylose tris(3-chlor-4-methylphenylcarbamate)	Lux Amylose-2

[a] Not included in the initial screening.

Table 2
Screening results for the enantiomer separation of (*R*/*S*)-Naringenin on five CSPs derived from cellulose and amylose.

Eluent[a]	Cellulose based CSP										
	Chiralcel OD-H				Lux Cellulose-1			Lux Cellulose-2			
	κ_1	κ_2	α	R_s	κ	α	R_s	κ_1	κ_2	α	R_s
A	11.46	13.44	1.17	1.51	n.e.[c]	–	–	47.15	57.18	1.21	0.91
B	4.60	5.20	1.13	1.20	13.59	–	–	11.64	14.05	1.21	1.01
C	24.88	31.86	1.28	1.63	>90	–	–	<80		–	–
D	6.10	7.43	1.22	1.40	29.09	–	–	16.55	19.46	1.18	0.4
E[b]	1.31		–	–	8.31	–	–	2.31		–	–
F	1.77		–	–	10.64	–	–	2.31		–	–
G	2.85		–	–	7.63	–	–	3.21		–	–
H	1.41		–	–	46.65	–	–	1.31		–	–
I	1.23		–	–	3.91	–	–	1.61		–	–
J	1.17		–	–	3.31	–	–	2.11		–	–

Eluent[a]	Amylose based CSP						
	Chiralpak AD-H				Lux Amylose-2[b]		
	κ_1	κ_2	α	R_s	κ	α	R_s
A	n.e.[c]		–	–	>65	–	–
B	n.e.[c]		–	–	>27	–	–
C	24.52	36.31	1.48	7.18	>65	–	–
D	5.32	7.43	1.40	5.13	>23	–	–
E[b]	5.98	6.22	1.04	6.12	4.21	–	–
F	1.23		–	–	17.30	–	–
G	1.47		–	–		–	–
H	4.36	6.40	**1.47**	**3.48**	3.16	–	–
I	2.55		–	–	1.71	–	–
J	1.47		–	–	1.57	–	–

Bold type indicates best result obtained.

[a] Eluent compositions: A: *n*-heptane/ethanol (90:10, v/v); B: *n*-heptane/ethanol (80:20, v/v) C: *n*-heptane/2-propanol (90:10, v/v); D: *n*-heptane/2-propanol (80:20, v/v); E: 100% 2-propanol; F: acetonitrile/2-propanol (90:10, v/v); G: 100% acetonitrile; H: 100% methanol; I: ethanol/methanol: (50:50, v/v); J: 100% ethanol.

[b] Flow rate 0.8 mL/min.

[c] n.e.: not eluted.

to the UV profile (detection preformed at 290 nm, see Section 3); analytical in process control of collected fractions was performed on system 1 using a Chiralpak AD-H column (250 mm × 4.6 mm I.D.; d_p = 5 μm) and a mobile phase consisting of pure methanol (flow rate 1 mL/min, UV detector at 290 nm). Dilution with the eluent was employed when necessary. The obtained fractions containing the enantiomers were evaporated at 334 mbar and 40 °C and dried in a vacuum oven at 0.1 mbar and 25 °C.

2.2.3. Elution order of Naringenin enantiomers

The study of the elution order of Naringenin enantiomers was performed on system 2 using Chiralcel OD-H (150 mm × 4.6 mm I.D., d_p = 5 μm) and Chiralpak AD-H (150 mm × 4.6 mm I.D., d_p = 5 μm), produced by Daicel Industries Ltd. (Japan) and obtained from Merck-VWR (Milan, Italy), eluting with *n*-heptane/2-propanol (containing 0.1% of trifluoro acetic acid) (85:15, v/v) and pure methanol, respectively. Sample solutions were prepared dissolving analytes at ∼ 2 mg/mL in ethanol and filtered through a 0.45 μm PTFE membrane before analysis. The injection volume was 10 μL, the flow rate 1 mL/min and detection preformed at 290 nm HPLC analyses were carried out at room temperature.

3. Results and discussion

3.1. Development of an analytical enantiomeric assay and screening for preparative scale-up

In order to obtain both Naringenin enantiomers in amounts sufficient for an exhaustive biological investigation, a preparative enantioselective HPLC separation of commercially available (*R*/*S*)-Naringenin was developed. Several enantioselective HPLC separations on analytical and semi-preparative scale have been described during the recent years for (*R*/*S*)-Naringenin using a variety of CSPs derived from cellulose, among them cellulose triacetate [17], a CSP consisting of methylated β-cyclodextrin covalently bonded to silica [18], Chiralcel OD [14], Chiralcel OD-H [14,19], Chiralcel OD-RH [20], Chiralpak IB [21], as well as amylose derived CSPs [14].

Among the most important prerequisites for an economic and productive preparative enantiomer separation are retention times as short as possible, a high solubility of the racemate and the enantiomers in the eluent/injection solvent and the use of a mobile phase consisting of a pure low-cost solvent, facilitating workup and re-use of mobile phase. Since the enantioselective HPLC separations described until now do not satisfy these requirements, a standard screening protocol for cellulose and amylose derived CSPs [22] was applied to Chiralcel OD-H and Chiralpak AD-H, as well as to Lux Cellulose-1, Lux Cellulose-2 and Lux Amylose-2, which have become commercially available during the last year (Table 1 for structures of the chiral selectors). Elution conditions experimented include mixtures of *n*-heptane and polar modifiers (ethanol or 2-propanol), alcohols (methanol, ethanol and 2-propanol), acetonitrile as well as mixtures of acetonitrile and 2-propanol. Results of the screening protocol are reported in Table 2 as capacity factor (κ'), selectivity (α) and resolution (R_s) factors.

Surprisingly, in our hands the results for Chiralcel OD and Lux Cellulose-1 are not comparable when using pure solvents, despite the fact that both columns contain the same chiral selector. Only after addition of an acidic modifier, e.g. 0.1% formic acid, Naringenin is resolved on Lux Cellulose-1 [23]. The reason for this difference in retention behavior on two cellulose tris(3,5-dimethylphenylcarbamate) derived CSPs remains unknown. It can be based on the use of different silica in the manufacturing process, variations in origin, chain length, and branching of the derivatized cellulose, the degree of substitution, carbon load and coating solvent used in the production process.

Table 3
Scale-up calculations for the enantiomer separation of (*R*/*S*)-Naringenin based on loading study on a Chiralpak AD-H column (250 mm × 4.6 mm I.D.).[a]

Column I.D. [mm]	4.6	10.0	41.0
Injected amount [g]	0.0012	0.0057	0.0549
Run time [min]	16		
Equilibration time [min]	1		
Column length [mm]	250	250	144
Column volume [L]	0.004	0.020	0.19
Flow rate [L/min]	0.001	0.005	0.03
Injections per hour	3.53		
Injections per shift	28.2		
Injections per day	84		
Productivity per hour [g/h]	0.004	0.02	0.21
Productivity per shift [g/8 h]	0.03	0.16	1.55
Productivity per day [g/24 h]	**0.10**	**0.48**	**4.65**
Solvent consumption per hour [L/h]	0.06	0.28	1.8
Solvent consumption per shift [L/8 h]	0.48	2.27	14.40

Bold type indicates key feature productivity.
[a] Comparable properties of amylose tris(3,5-dimethylphenylcarbamate) derived stationary phase (e.g. Chiralpak AD or Regispack) are assumed.

The retention times of Naringenin enantiomers on Lux Cellulose-2 are quite long and do not give grounds for a productive scale-up. No enantiomer separation was observed on Lux Amylose-2, while the results on Chiralpak AD-H turned out to be quite promising. Using pure methanol as eluent (without acidic or basic additives) relatively short retention times (8.9 min for the first eluted enantiomer and 12.3 min for the second), high enantioselectivity and good resolution (α = 1.49, R_s = 3.48 at 20 °C) could be observed [24]. Accordingly, these experimental conditions were selected for the scale-up to (semi)-preparative scale. Our experimental results are in good agreement with the study by Vander Heyden and co-workers, who recently presented a screening strategy for cellulose and amylose-based CSPs focused on the use of polar eluents and found a R_s = 3.00 at 20 °C for the enantiomer separation of Naringenin on Chiralpak AD-H [25].

3.2. Determination of loading capacity and scale-up conditions

In order to determine the best conditions for a (semi)-preparative scale-up the loading behavior of the CSP Chiralpak AD-H was determined by injecting increasing amounts of (*R*/*S*)-Naringenin. A maximum of 1.2 mg could be separated in one single injection within 16 min, which allows predicting a scale-up to the g scale.

Based on simple scale-up calculations [22] and assuming a column I.D. of either 1.0 cm or 4.1 cm the separation of 5–10 g of (*R*/*S*)-Naringenin should be possible within a few days (Table 3) on any amylose tris(3,5-dimethylphenylcarbamate) based CSP (e.g. Chiralpak AD or Regispack (*cf.* Table 1)), assuming comparable enantioselectivity, loading capacity and retention behavior.

3.3. Stability of Naringenin enantiomers in solution

Several reports describe the racemisation of Naringin in its natural sources [26] as well as under basic conditions [27]. For Naringenin enantiomers, racemisation under aqueous conditions [28] as well as enantiomerisation during micellar electrokinetic chromatography under basic conditions [29] have been reported. In details, Krause and Galensa observed a small racemisation tendency of Naringenin enantiomers in methanol at 70 °C [28], while significant conversion of Naringenin enantiomers *via* non-enzymatic cyclisation of the corresponding Naringenin-chalcone was detected during use of a polar gradient (methanol/acetic acid) [28]. Therefore, the chemical stability of Naringenin in methanol,

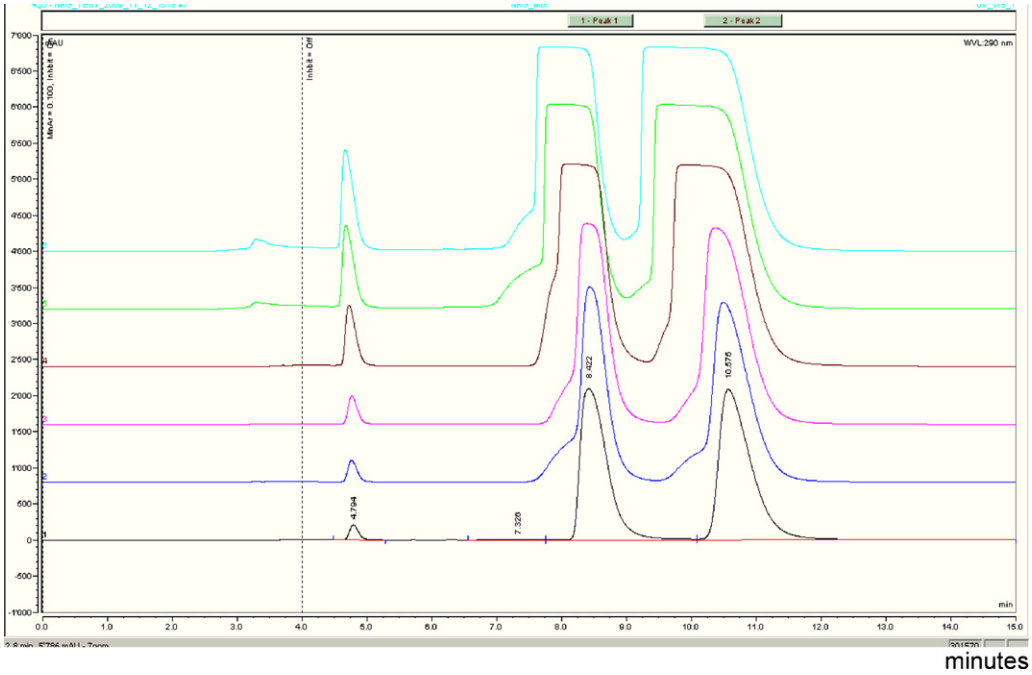


Fig. 2. Semi-preparative enantiomer separation of (*R*/*S*)-Naringenin on Regispack (250 mm × 10 mm I.D.) employing system 1; eluent: 100% methanol, flow: 4.00 mL/min, T = 25 °C, detection at 290 nm, injected amounts 0.35 mg (bottom trace), 0.53 mg, 0.70 mg, 1.75 mg, 2.63 mg, 3.5 mg (top trace).

the eluent used in the chromatographic separation, was evaluated by ESI/MS analysis (flow injection analysis, FIA) operating in negative ion mode. The mass spectra acquired before and after three months storage (at room temperature) did not evidenced any change in the mass profiles, confirming the chemical stability of Naringenin. Moreover, no significant racemisation of enantiomers dissolved in methanol and stored for three months at ambient temperature in clear glass bottles was observed, as evidenced by enantioselective HPLC–UV–CD analysis using an AD-H column. We conclude that methanol (without acidic or basic additives) is a suitable eluent for enantioselective preparative chromatography and subsequent isolation of enantiomers if elevated temperatures are avoided during work-up (see Section 2.2.2).

3.4. Linear scale-up

Scaling-up analytical separation to (semi-)preparative scale can be done using either pre-packed columns of appropriate size or by packing columns in-house with bulk stationary phase. The former approach is to be preferred when no column packing equipment is available or when resources or knowledge for column packing are not accessible, the latter approach is advisable for multi-purpose units requiring high throughput at reasonable cost. It is obvious that column packing and testing in-house is more time consuming [30] than installing a pre-packed column, but bulk stationary phases are often cheaper and available "from the shelf" in a shorter time frame.

For the envisaged scale of our project (separation of 5–10 g of (*R*/*S*)-Naringenin) we decided to compare both options, using a pre-packed 1.0 cm I.D. column (d_p = 5 μm) and packing a 4.1 cm I.D. dynamically axial compressed (DAC) column available in our laboratories (Table 3) with 100 g bulk Chiralpak AD CSP (d_p = 20 μm).

Pre-packed columns containing the CSP amylose tris(3,5-dimethylphenylcarbamate) are currently available from various suppliers. Fig. 2 shows the enantiomer separation of (*R*/*S*)-Naringenin on a 250 mm × 10 mm I.D. Regispack column.

The column shows a good loading behavior and would allow for a daily productivity of approximately 0.25–0.5 g/24 h. Interestingly, we observed a peak shapes implying a Lagmuiran adsorption isotherm for both enantiomers, although a slight distortion in the peaks seems to indicate the presence of one or two impurities co-eluting with the two enantiomers.

The results obtained on the Regispack column were compared to those found using a DAC-Rampack-column packed with 100 g of bulk Chiralpak AD CSP (d_p = 20 μm).

Our calculations for the scale-up (Table 3) predict a maximum loading of 55 mg for a single run on a Chiralpak AD column (144 mm × 41 mm I.D.) with a flow rate of 30 mL/min under the assumption of identical HETP and loading capacity of the chiral polymer coated onto the silica core compared to the screening column. In view of the fact that 5 μm material of amylose tris(3,5-dimethylphenylcarbamate) is not available at reasonable cost we decided to use Chiralpak AD as 20 μm material, a standard CSP for large scale enantiomer separations [31], for our purposes. Actually, at a flow rate of 30 mL/min 30 mg of (*R*/*S*)-Naringenin were processed in a single run (17 min) (Fig. 3); however, not unexpectedly, the shorter column length and the lower HETP led to a peak overlap of first and second eluting enantiomer necessitating the collection of an intermediate fraction, which had to be reprocessed. Cut-points (see dashes in Fig. 3) were determined after fraction analysis of test runs with manual collection.

Summarizing, using the conditions described above, 2.46 g of (*R*/*S*)-Naringenin were processed in 28 h with an eluent consumption of 51 L. This data is equivalent to a specific productivity of 21 g racemate separated per 24 h on 1 kg of CSP.

In this way two batches of racemic Naringenin were processed. Overall, 430 mg (ee = 81%, yield 17.4%) and 214 mg (ee = 93%, yield 8.7%) of the first enantiomer as well as 171 mg (ee = 90%, yield 6.9%) and 430 mg (ee = 92, yield 17.4%) of the second one were obtained together with an intermediate fraction of 79 mg (yield 3.2%) as a mixture of the two enantiomers. The optical rotatory power of the obtained enantiomers and their enantiomeric excess, determined by HPLC/PAD/CD analysis (Chiralpak AD-H columns, HPLC system 2, see Section 2), are summarized in Table 4, while the UV (upper) and CD (lower) chromatograms are reported in Fig. 4. The differences in enantiomeric excess for the isolated enantiomers are based on

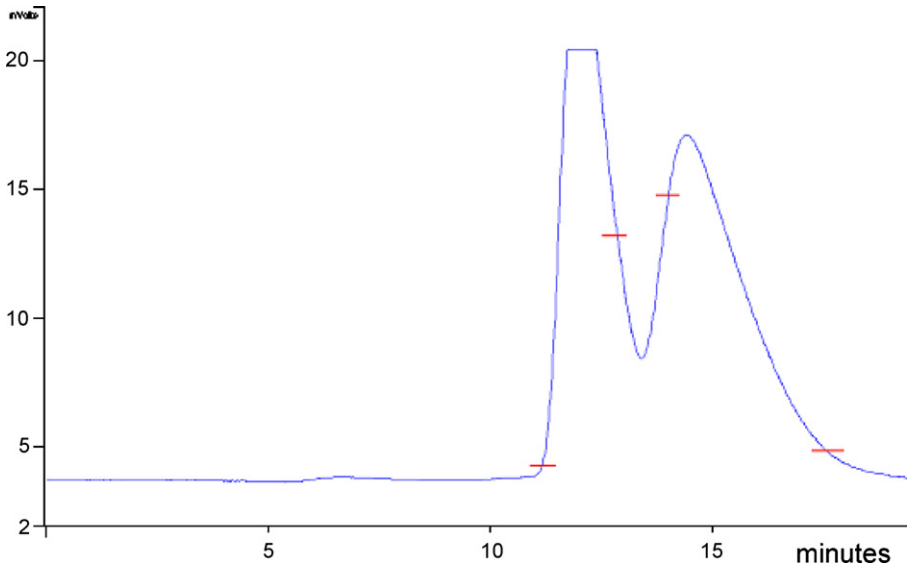


Fig. 3. Semi-preparative enantiomer separation of (*R*/*S*)-Naringenin on Chiralpak AD (144 mm × 41 mm I.D.) employing system 3, eluent: 100% methanol, flow: 30.00 mL/min, $T = 23 \pm 2\,^{\circ}C$, detection at 290 nm, injected amount 30 mg, cut points given by dashes (—).

slightly different cut-points for fraction collection (not shown in Fig. 3).

We decided to explore the online coupling of HPLC/CD in order to get more insight into the chiroptical properties of resolved enantiomers. The online coupling of HPLC/CD yielded directly the CD signal of the resolved peaks, which was used by us as a useful tool for the assignment of the absolute configuration. Particularly, the configuration assignment was performed basing on either optical rotatory power (measured in ethanol) or CD signal (λ: 290 nm) of the isolated enantiomers, in accordance with Caccamese et al. [14,19]. In more details, in previous papers Caccamese clearly stated that a negative optical rotatory power and a negative CD signal at 292 nm are related to (*S*)-configuration [14,19]. Since (−)-Naringenin ($[\alpha]_{589}^{25} = -28.7^{\circ}$, $c = 0.36\%$ in ethanol) gave rise to a negative peak in the HPLC–CD chromatogram, as shown in Fig. 4, the (*S*)-absolute configuration was assigned to the first eluted

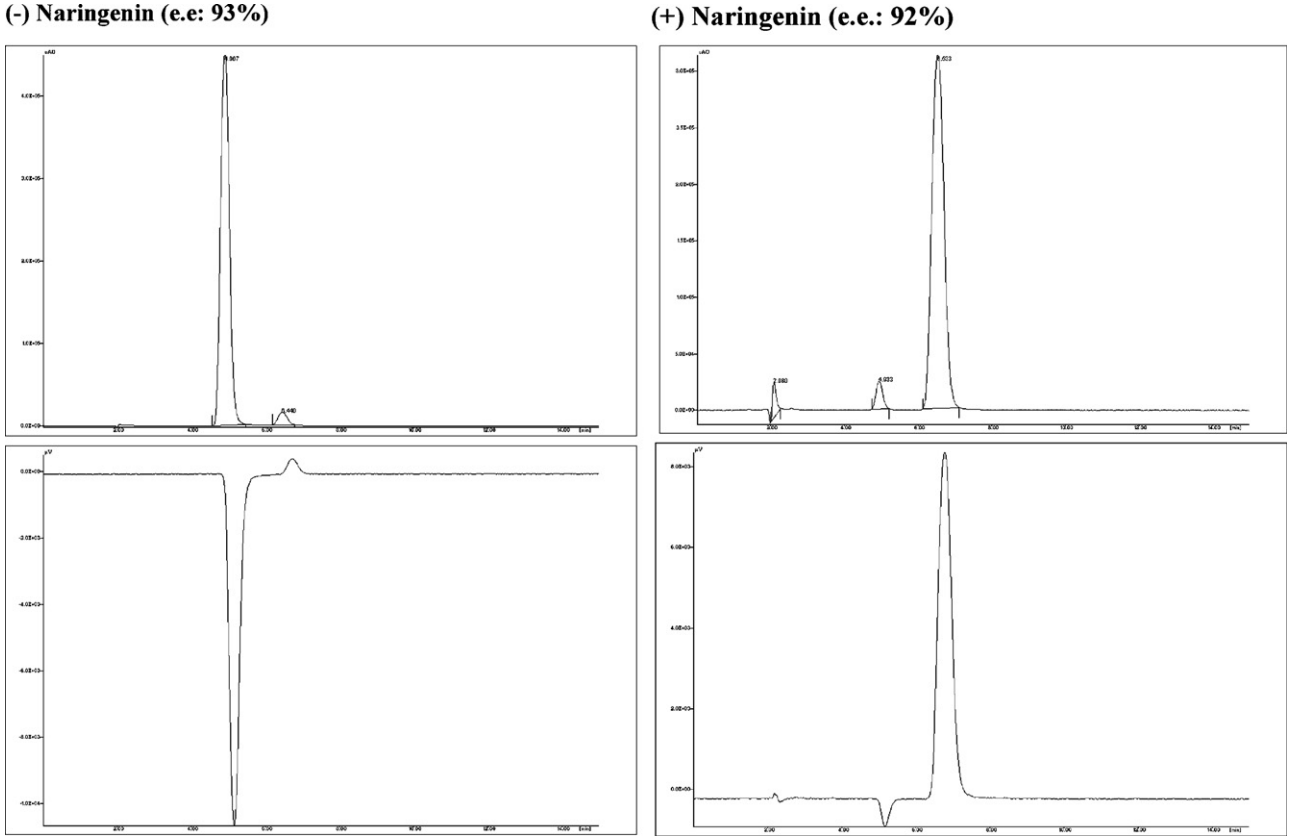


Fig. 4. UV (upper) and CD (lower) chromatograms (recorded at 290 nm) of Naringenin enantiomers on Chiralpak AD-H (150 mm × 4.6 mm I.D., $d_p = 5$ μm;), HPLC system 2, eluent: 100% methanol, flow: 1 mL/min.

Table 4
Naringenin fractions obtained after preparative HPLC on Chiralpak AD (144 mm × 41 mm I.D.) starting from 2.46 g racemate.

Compounds	$[\alpha]_{589}^{25}$	ee[a]	Amount	Yield
(−)-Naringenin	−23.5° (*c* = 0.36%; EtOH)	93%	214 mg	8.7%
(−)-Naringenin	−20.4° (*c* = 0.36%; EtOH)	81%	430 mg	17.4%
(+)-Naringenin	+22.8° (*c* = 0.30%; EtOH)	92%	430 mg	17.4%
(+)-Naringenin	+22.5° (*c* = 0.30%; EtOH)	90%	171 mg	6.9%
(+/−)-Naringenin	n.a.	n.a.	79 mg	3.2%
			1.324 mg	53.6%

[a] Determined on Chiralpak AD-H (150 mm × 4.6 mm I.D., d_p = 5 μm;) using HPLC system 2; eluent: 100% methanol, flow rate: 1 mL/min, detection at 290 nm.

enantiomer; on the other hand, (+)-Naringenin ($[\alpha]_{589}^{25} = +22.8°$, *c* = 0.30% in ethanol) appeared as a positive peak, and hence the (*R*)-absolute configuration was assigned (Fig. 4).

Remarkably, an opposite elution order of Naringenin enantiomers was observed by us using the amylose derived Chiralpak AD-H with respect to the one evidenced by Caccamese et al. using the cellulose derived Chiralcel OD-H [14,19]. To further investigate this issue, and also to gain an additional direct proof for the configuration assignment, the HPLC/PAD/CD analysis (HPLC system 2, see Section 2) of the racemate as well as resolved enantiomers were carried out by us on Chiralcel OD-H (see Section 2). The (*S*)-enantiomer, eluted as the first peak on the Chiralpak AD-H column, was eluted as the second one on the Chiralcel OD-H column (Fig. 5), in accordance with results of Caccamese et al., confirming the reversal elution order using cellulose and amylose based CSPs. An analogous chromatographic behavior has been also reported for other flavonoids [14].

3.5. Future scale-up options

The successful isolation of the Naringenin enantiomers in high ee and yield can be seen as a first step in the development of a larger scale separation using either enantioselective super critical fluid (SFC) chromatography or transferring the separation to a continuous high pressure liquid chromatographic system, like Steady State Recycling [34] or Simulated Moving Bed (SMB) chromatography [33]

Due to lower viscosities SFC allows running chromatographic separations at faster flow rates and often gives the opportunity to use less solvent in the final fraction. First scouting experiments using SFC show a high selectivity for (*R*/*S*)-Naringenin on Regispack (250 mm × 4.6 mm I.D.) using CO_2/methanol (75:25; v/v) as eluent at a 4 mL/min for analytical and overloaded conditions (Fig. 6).

Based on a loading study a maximum amount of 7.0 mg could be injected. The injection cycle time was 6.5 min, which is equivalent to 9.2 injections per hour (64.4 mg/h). Based on these experiments a specific productivity of 55 g racemate separated per 24 h on 1 kg of CSP can be assumed. The specific productivity that can be expected by using SFC is about 2.6 times higher than using a preparative HPLC unit, while the overall eluent consumption is only 18% of the amount needed by preparative HPLC. In regard to the organic modifier (methanol), the solvent savings of SFC are even more pronounced: <5% of methanol would be needed to separate the same amount of racemic Naringenin (Table 5).

The loading study data (previously performed) can be used to predict by software simulation calculations a SMB separation. Using e.g. a simulation software called "softSMB" [32] it is possible to estimate competitive adsorption isotherm for the two enantiomers based on overload injections and to predict and optimize the oper-

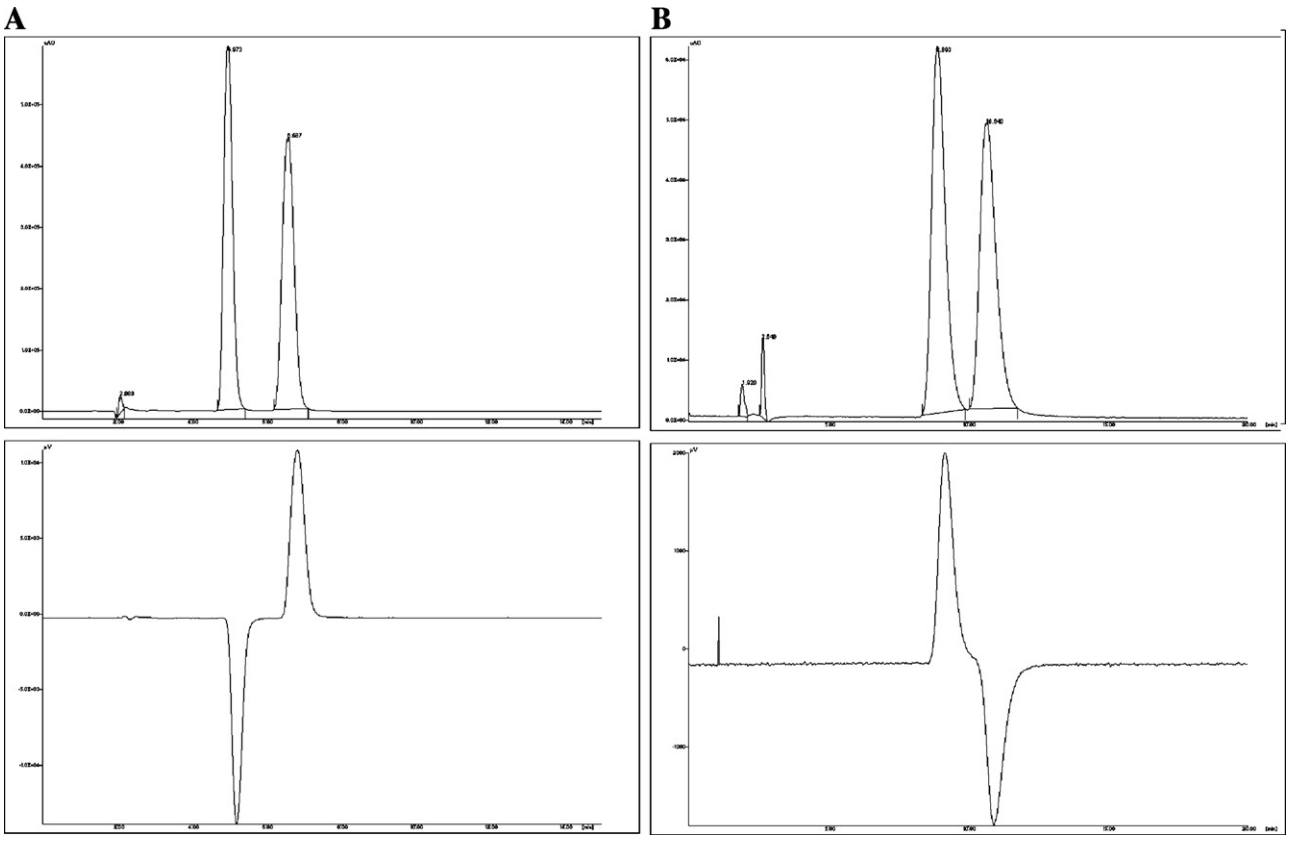


Fig. 5. UV (upper) and CD (lower) chromatograms (recorded at 290 nm; HPLC system 2) of (*R*/*S*)-Naringenin on: (A) Chiralpak AD-H (150 mm× 4.6 mm I.D., d_p = 5 μm;), eluent: 100% methanol, flow: 1 mL/min; (B) Chiralcel OD-H (150 mm× 4.6 mm I.D., d_p = 5 μm), eluent: with *n*-heptane/2-propanol (doped with 0.1% of trifluoro acetic acid) (85:15, v/v), flow 1 mL/min.

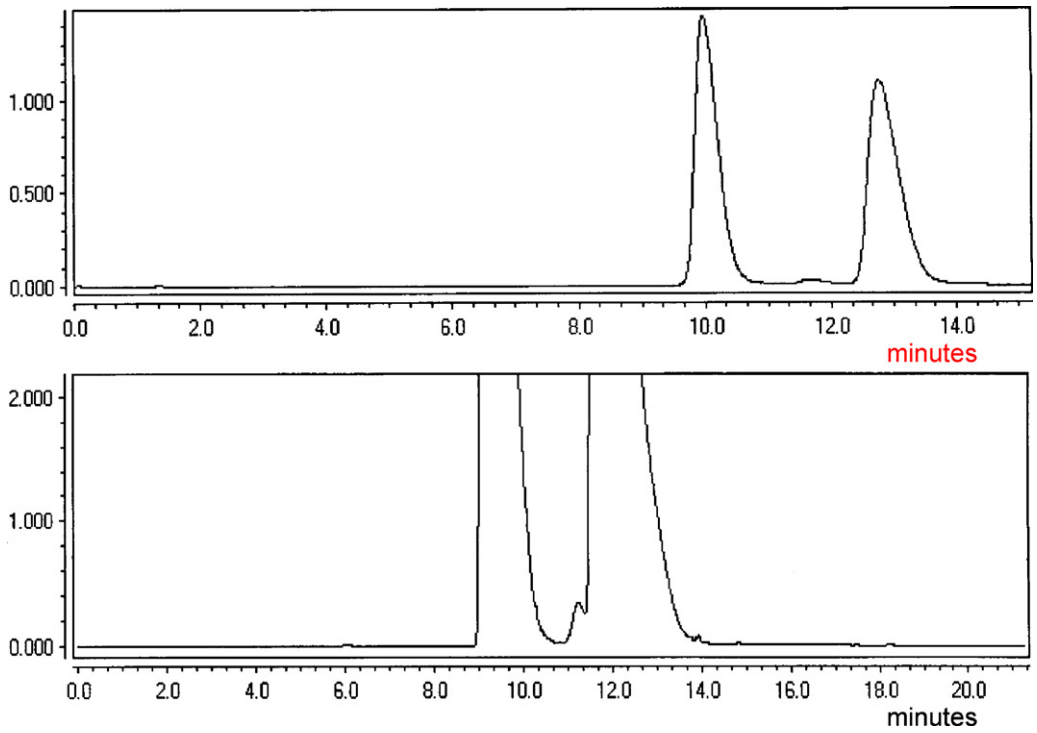


Fig. 6. SFC enantiomer separation of (*R*/*S*)-Naringenin on Regispack (250 mm × 4.6 mm I.D.), eluent CO_2/methanol (75:25, v/v), flow: 4.00 mL/min, T = 30 °C, detection at 290 nm. Top: analytical injection, bottom: injected volume: 20 μL (c = 35 mg/mL). Chromatograms by courtesy of T. Szczerba, Regis.

ating parameters before starting the unit itself. For our calculations a standard pilot SMB system (8 columns 110 mm × 48 mm I.D., i.e. 880 g CSP) was assumed. The estimated isotherm parameters were: $\lambda = 1.5$, $NK_1 = 1.423$, $NK_2 = 2.647$, $\bar{N}_i = 6$. The calculations resulted in the following values: $Q_{recycling}$ = 402.4 mL/min Q_{feed} = 14.2 mL/min; Q_{eluent} = 181.7 mL/min; $Q_{extract}$ = 142.9 mL/min; $Q_{raffinate}$ = 53.00 mL/min with a unit switch time of 1.41 min at a feed concentration of 6 g/L. The simulation calculations imply a modest loading capacity which will result in a moderate productivity employing the SMB technique (Table 5). However, it should be noted that the specific productivity that can be expected by employing a SMB unit is about 6.5 times higher than using a preparative HPLC unit, while solvent consumption will be decreased by approximately 90%. Industrial SMB processes allow recycling up to 99.9% of the solvent inventory, therefore the use of a pure solvent (methanol) in the chromatographic system will have a positive impact on the overall separation cost on large scale.

A comparison of the three techniques HPLC, SFC and SMB (Table 5) highlights the advantages and problems of the methods for the chromatographic enantiomer separation of racemic Naringenin. Preparative HPLC gives a quick access to the desired enantiomers in g-amounts and follows a straightforward scale-up protocol; however, the solvent consumption is considerable. A further scale-up of the separation of (*R*/*S*)-Naringenin seems to be possible using either SFC or HPLC in the SMB mode. Preparative SFC seems to be better suited for the production of kg amounts of Naringenin enantiomers and will open the door to substantial solvent savings, but will require the use of supercritical fluid handing systems. Currently, these systems are not yet suited for large scale, i.e. multi-*t*-scale production purposes. A SMB process, although, it will necessitate a higher solvent consumption in comparison to SFC, can lead to an industrial approach for producing Naringenin enantiomers in *t*-quantities when needed.

Table 5
Predicted productivity for the enantiomer separation of (*R*/*S*)-Naringenin on amylose tris(3,5-dimethylphenylcarbamate) derived chiral stationary phases employing either HPLC, SFC or a SMB unit.

Parameters	Experimentally determined HPLC results	Predicted SFC[a] results	Predicted SMB results
Productivity [kg_{rac}/kg CSP/24 h]	0.021	0.055	0.141
Eluent use [L MeOH/kg racemate]	20,731	3728[a]	2001

[a] SFC: CO_2/methanol (75:25; v/v), i.e. 932 L methanol/kg racemate.

4. Conclusion

A systematic and straightforward screening protocol for enantioselective HPLC-separations was established for (*R*/*S*)-Naringenin, which led to a rapid and easy-to-use chiral HPLC separation suitable for a further preparative scale-up. The developed preparative method using a pure commodity solvent as eluent proved to be suitable for obtaining a quick access to the desired enantiomers with high enantiomeric excess and amounts sufficient for biological assays. Neither chlorinated solvents nor acidic additives are needed for the enantioselective preparative HPLC. The chromatographic method proved to be superior to previously published methods in terms of elution times, separation and resolution; in solution no degradation or racemisation of the enantiomers was observed. The isolated compounds will be used for an in-deep examination and comparison of the pharmacological profile of the two enantiomers.

Experiments employing Supercritical Fluid Chromatography and software simulations for a continuous Simulated Moving Bed process show a route for future scale-up of the chromatographic enantiomer separation with reduced solvent usage and increased productivity. Both of these methods have the proven potential for production on pilot and commercial scale and can not only give quick and reliable access to g or kg amounts of pure enantiomers

needed for clinical studies, but can also provide *t*-amounts needed for commercialization of Naringenin-enantiomers in pharmaceutical and nutritional applications. Enantioselective chromatography might be an alternative to complex extractions of Naringenin enantiomers from natural sources, also on large scale.

Acknowledgments

MJ thanks P. Scheidegger, Brechbühler AG, G. Krautz, Phenomenex, J. Kocergin, Z. Ali and T. Szczerba, Regis, and D. Frühauf, B. Katrinski, SIL, DSM Nutritional Products for their support of this work. SC thanks Stazione Sperimentale per la Seta, Milan, Italy, for the financial support and JASCO Europe, Cremella, LC, Italy, for technical support with the CD detector. We thank Dr. S. Abel, CarboGen-Amcis, Aarau, Switzerland for her professional support regarding scale-up calculations using the SMB technology. We acknowledge especially the support of Dr. A. Gaa and Dr. B. Achermann, DSM Nutritional Products, who made this collaboration possible.

References

[1] J.A. Yanez, P.K. Andrews, N.M. Davies, J. Chromatogr. B 848 (2007) 159.
[2] J.A. Yanez, C.R. Remsberg, N.D. Miranda, K.R. Vega-Villa, P.K. Andrews, N.M. Davies, Biopharm. Drug Dispos. 29 (2008) 63.
[3] I. Fecka, S. Turek, Food Chem. 108 (2008) 1039.
[4] R. Gaggeri, D. Rossi, N. Hajikarimian, E. Martino, F. Bracco, P. Grisoli, C. Dacarro, F. Leoni, G. Mascheroni, S. Collina, O. Azzolina, Open Nat. Prod. J. 3 (2010) 20.
[5] R. Gaggeri, D. Rossi, D. Passarella, D. Zampieri, E. Laurini, F. Leoni, G. Mascheroni, S. Collina, O. Azzolina, Phytochemical fingerprint and anti-inflammatory properties of crude extract and naringenin from Amygdalus lycioides Spach, SCI 2009, 5–10 July, Sorrento, Italy, 2009.
[6] C.A. Torres, N.M. Davies, J.A. Yanez, P.K. Andrews, J. Agric. Food Chem. 53 (2005) 9536.
[7] F.P. Guengerich, D.H. Kim, Carcinogenesis 11 (1990) 2275.
[8] W.L. Bear, R.W. Teel, Anticancer Res. 20 (2000) 3609.
[9] L. Du Jin, X. Han, Z. Song, H. Zhang, W. Liang, Cancer Res. 69 (2009) 3205.
[10] G. Galati, O. Sabzevari, J.X. Wilson, P.J. O'Brien, Toxicology 177 (2002) 91.
[11] C. Bodet, V.D. La, F. Epifanio, G. Grenier, J. Periodont. Res. 43 (2008) 400.
[12] S. Shinkaruk, V. Lamothe, J.M. Schmitter, C. Manach, C. Morand, A. Berard, B. Bennetau, C. Pelissero, Food Chem. 118 (2010) 472.
[13] N.M. Maier, P. Franco, W. Lindner, J. Chromatogr. A 906 (2001) 3.
[14] S. Caccamese, C. Caruso, N. Parrinello, A. Savarino, J. Chromatogr. A 1076 (2005) 155.
[15] L.S. Ettre, Pure Appl. Chem. 65 (1993) 819.
[16] L.S. Ettre, Chromatographia 38 (1994) 521.
[17] M. Krause, R. Galensa, J. Chromatogr. 441 (1988) 417.
[18] S. Ng, T.T. Ong, P. Fu, C.B. Ching, J. Chromatogr. A 968 (2002) 31.
[19] E. Giorgio, N. Parrinello, S. Caccamese, C. Rosini, Org. Biomol. Chem. 2 (2004) 3602.
[20] J.A. Yáñez, N.M. Davies, J. Pharm. Biomed. Anal. 39 (2005) 164.
[21] S. Abbate, L.F. Burgi, E. Castiglioni, F. Lebon, G. Longhi, E. Toscano, S. Caccamese, Chirality 21 (2009) 436.
[22] M. Juza, in: S. Kromidas (Ed.), HPLC Made to Measure – A Practical Handbook for Optimization, Wiley-VCH, Weinheim, 2006, p. 427.
[23] Lux Cellulose-1 *vs.* Chiralcel OD-H: $R_s = 2.10$ ($\alpha = 1.11$) vs. 3.37 ($\alpha = 1.21$) for hexane/ethanol/formic acid (v.v:v; 90:10:0.1) and $R_s = 1.35$ ($\alpha = 1.09$) vs. 2.08 ($\alpha = 1.19$) for hexane/ethanol/formic acid (v.v:v; 80:20:0.1) at 20 °C.
[24] This result is in good agreement with experiments presented by Knauer, Berlin, Germany on the webpage (www.knauer.com) for the enantiomer separation of Naringenin employing Europak 01 as CSP. Europak-1 contains the same chiral amylose derivative as Chiralpak AD, but is currently not commercially available.
[25] H. Ates, D. Mangelings, Y. Vander Heyden, J. Chromatogr. B 875 (2008) 57.
[26] S. Caccamese, R. Chillemi, J. Chromatogr. A 1217 (2010) 1089.
[27] C.O. Miles, L. Main, J. Chem. Soc. Perkin Trans. II (1998) 195.
[28] M. Krause, R. Galensa, Chromatographia 32 (1991) 69.
[29] D. Wistuba, O. Trapp, N. Gel-Moreto, R. Galensa, V. Schurig, Anal. Chem. 78 (2006) 3424.
[30] Packing and testing a preparative column takes about 6 to 8 hours in the hands of an experienced worker.(up-to a column ID of about 20 cm).
[31] S. Abel, M. Juza, in: G. Subramanian (Ed.), Chiral Separation Techniques – A Practical Approach, Third, Completely Revised and Updated Edition, Wiley-VCH, Weinheim, 2006, p. 203.
[32] M. Juza, M. Mazzotti, M. Morbidelli, TIBTECH 18 (2000) 108.
[33] C.M. Grill, J. Chromatogr. A 796 (1998) 101.
[34] E. Huthmann, M. Juza, J. Chromatogr. A 908 (2001) 185.