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Optical isomer separation of flavanones and flavanone glycosides by nano-liquid chromatography using a phenyl-carbamate-propyl-β-cyclodextrin chiral stationary phase

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ABSTRACT

In this paper a phenyl-carbamate-propyl-β-cyclodextrin stationary phase was employed for the enantioseparation of several flavonoids, including flavanones and methoxyflavanones by using nano-liquid chromatography (nano-LC). The same stationary phase was also used for the diastereoisomeric separation of two flavanone glycosides. The compounds: flavanone, 2′-hydroxyflavanone, 4′-hydroxyflavanone, 6-hydroxyflavanone, 7-hydroxyflavanone, 4′-methoxyflavanone, 6-methoxyflavanone, 7-methoxyflavanone, hesperetin, hesperidin, naringenin and naringin were studied using reversed, polar organic and normal elution modes. The effect of the nature and composition of the mobile phase (organic modifier type, buffer and water content in the reversed phase mode) on the enantioresolution (R_s), retention factor (k) and enantioselectivity (α) were investigated. Baseline resolution of all studied flavonoids, with the exception of 2′-hydroxyflavanone and naringin, was achieved in reversed phase mode using a mixture of MeOH/H_2O at different ratios as mobile phase. Good results, in terms of peak efficiency and short analysis time, were obtained adding 1% triethylammonium acetate pH 4.5 buffer to MeOH/H_2O mixture. The separation of the studied compounds was also performed in polar organic mode. By using 100% of MeOH as mobile phase, the resolution was achieved for the studied analytes, except for 7-hydroxyflavanone, 2′-hydroxyflavanone, naringenin, hesperidin and naringin. Normal mode was tested employing a mixture of EtOH/hexane/TFA as mobile phase achieving the enantiomeric and diastereomeric separation of only hesperetin and hesperidin, respectively. The use of nano-LC technique for the resolution of flavanones optical isomers allowed to achieve good resolutions in shorter analysis time compared to the results reported in literature with conventional HPLC.

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1. Introduction

Enantioseparation is a topic of growing importance in pharmaceutical, biomedical, agrochemical and environmental research. Chiral compounds may differ significantly in their biological, pharmacological and toxicological effects. Nowadays a great deal of progress has been made in terms of regulations and requirements from the US Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA) [1]. The increasing demand of the complete separation and quantification of any chiral molecule, especially those of pharmaceutical interest, has been resulted in the development of several stereoselective separation methods [2].

For analytical purposes, chromatographic techniques including gas chromatography (GC) and high performance liquid chromatography (HPLC) have been largely proposed for the separation and purification of chiral compounds [3]. As reported in literature, the majority of chiral separations are performed with the direct method mainly using chiral stationary phases (CSPs) [4,5]. Despite of the robustness of the chromatographic systems, they present some drawbacks, e.g., large volumes of organic solvents employed, high costs of the used chiral columns, etc. For that reason, micro-scale separation techniques such as capillary electrophoresis (CE) and electrochromatography (CEC), capable to achieve rapid chiral separations with high efficiency, have been successfully investigated [6–8].

Recently, miniaturization has also been introduced in liquid chromatography, reducing both the inner diameter of the columns (100 μm < I.D. < 500 μm) and the flow rate (200–1000 nL/min).

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Therefore, capillary and nano-liquid chromatography (CLC and nano-LC) have been applied mainly for analytical purposes.

Nano-LC, firstly introduced by Karlsson and Novotny [9], has been further studied over the past decade developing theory, instrumentation, technology and applications [10,11]. Indeed, many papers reported in literature, have proved that this miniaturized chromatographic technique is very promising in different application fields such as proteomic, metabolomic, pharmaceutical, food and chiral analysis [12–17]. This analytical technique offers several advantages over conventional HPLC such as higher efficiency, shorter analysis time, use of minute volumes of both samples and reagents with consequent lower environmental impact and reduced costs [9,18,19]. Furthermore small amount of stationary phase is required for the packing procedure and this is particularly relevant for expensive chiral stationary phases. Therefore, nano-LC can be complementary or even competitive to HPLC in several analytical applications, also including chiral separations.

Methods so far used to achieve enantioseparations employ chiral selectors such as polysaccharides, proteins, macrocyclic antibiotics and cyclodextrins. Among them β-cyclodextrin (β-CD) and its derivatives have been extensively used for optical isomers separation, employing different chromatographic modes [20,21].

CD enantioselectivity is influenced by several parameters such as analytes' size, CD's cavity dimensions, organic solvent and nature of polar functional groups, especially those capable of hydrogen bonding. For this purpose, derivatized β-CDs were synthesized, functionalising the hydroxyl groups belonging to the two sides of the cavity with hydrophobic (e.g. methyl, propyl) or hydrophilic groups (sulphate, phosphate, quaternary amine). These substituents enable to further enhance the chiral recognition towards the analytes, owing to additional interactions such as hydrogen-bonding, dipolar, steric repulsion, π–π complexation, etc. Currently CD-CSPs are either chemically bonded via spacers [22,23] or adsorbed to silica gel as a cyclodextrin polymer [24]. Recently monolithic columns functionalised with cyclodextrins have also been developed for enantiomeric separations [25].

Using CDs stationary phases, multimodal elution can be applied for the resolution of many classes of stereoisomeric compounds; they include reversed, normal and polar organic-modes [26–28].

Although many CD-CSPs are commercially available, there is a need to develop new packing materials offering high enantioselectivity in short analysis time.

Recently Tazerouti et al. have synthesized different stationary phases based on native and hydroxypropyl-substituted β-CD, obtained by a chemical immobilization of the CD or CD derivative on silica gel via a difunctional spacer [29]. Among them, a phenyl-carbamate-propyl-β-CD was successfully used for the enantioseparation of several racemic compounds, e.g., non-steroidal anti-inflammatory drugs, benzodiazepines, Tröger's base, other compounds of pharmaceutical interest employing both reversed, polar organic or normal phase HPLC modes [30].

Considering the good enantioselective capability of this CSP, we extended the study to micro-scale dimensions. To the best of our knowledge only few papers, dealing with the use of CD-CSPs by capillary/nano-liquid chromatography, have been reported [31–34].

In this work the enantioselectivity of phenyl-carbamate-propyl-β-CD silica stationary phase was investigated by using nano-LC for the enantiomeric and diastereoisomeric separation of some selected flavanones, belonging to the class of polyphenols.

Such compounds usually occur in fruits and vegetables and it has been reported that they possess health-related properties including anticancer, anti-inflammatory, antiviral activities. Despite the potential therapeutic use of chiral flavanones, very few studies concerning the pharmacological activity of the single enantiomers have been carried out [35]. The chiral separation of the selected flavanones was then evaluated by using reversed, polar organic or normal modes. Different chromatographic parameters including composition of the mobile phase, nature of organic solvent and flow rate were optimised to obtain the complete enantioresolution of all studied compounds.

2. Experimental

2.1. Chemicals and samples

All chemicals were of analytical grade and used as received. Acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), propanol (PrOH), isopropanol (i-PrOH), n-hexane (n-Hex) and heptane (n-Hep) were purchased from Carlo Erba (Milan, Italy), triethylamine (TEA) was from Fluka Chemie (Buchs SG, Switzerland). LiChrospher 100 RP-18 silica phase, 5 μm particle diameter, was from Merck (Darmstadt, Germany). The CD-CSP was prepared following the method previously described [30]. As reported in that work, the CHN elemental analysis of CD-CSP gave the following results: C 9.3%, H 1.2% and N 2.5%. These values corresponded to roughly 49 mmol/g of groups chemically bonded to silica. The selected flavonoids (flavanone, 2′-hydroxyflavanone, 4′-hydroxyflavanone, 6-hydroxyflavanone, 7-hydroxyflavanone, 4′-methoxyflavanone, 6-methoxyflavanone, 7-methoxyflavanone, hesperetin, hesperidin, naringenin, and naringin) were from Sigma–Aldrich (St. Louis, MO, USA). Standard solutions of each flavonoid (1 mg/mL) were prepared in MeOH or EtOH, working in reversed or normal chromatographic mode, respectively, and stored at +4 °C. Further dilutions were daily done with water or EtOH to obtain the final concentration of 100 μg/mL.

2.2. Apparatus

The nano-LC experiments were carried out using a laboratory-assembled instrumentation. The system included a micro-LC pump Accela™, Thermo Electron Corporation (San Jose, CA, USA) which was controlled by a software system (Xcalibur 2.0 data system). The flow rate was reduced from μL/min to nL/min using a static splitting device. For this purpose, a stainless steel T piece, Vici Valco (Houston, TX, USA) was connected at one end to the pump through a 50 cm × 130 μm I.D. PEEK tube. The other two ends were connected to the injector valve by means of a stainless steel tube of 7 cm × 500 μm I.D. and to the waste using a 50 cm × 50 μm I.D. fused silica capillary. This arrangement allowed to obtain a flow rate in the range 120–400 nL/min. The flow rate was estimated connecting the end of the capillary column to a micro-syringe through a teflon tube and measuring the mobile phase volume after 5 min.

Sample injection was done by using a four port micro-volume injection valve (100 nL) Vici Valco (Houston, TX, USA). On-column detection was carried out at 205 nm with a Spectra 100 photometric UV–vis detector, Thermo Separation Products (San Jose, CA, USA). Data were collected with a Shimadzu CR5A Chromatopac integrator (Kyoto, Japan). The analysis of racemic compounds was performed in a capillary column (100 μm I.D.) packed with a silica phenyl-carbamate-propyl-β-CD stationary phase (5 μm) for 22.0 cm. The effective and total lengths of the column were 24.0 and 34.0 cm, respectively.

2.3. Preparation of chiral stationary phase

The CD-CSP was synthesized by grafting 2-hydroxypropyl-β-CD onto silica gel (5 μm) following an earlier described method [29,30]. Briefly, the selected spacer 3-iso-cyanatopropyltriethoxysilane (3.0 mM) was added drop wise to an appropriate amount of 2-hydroxypropyl-β-CD (2-HP-β-CD, 2.5 mM) dissolved in a pyridine

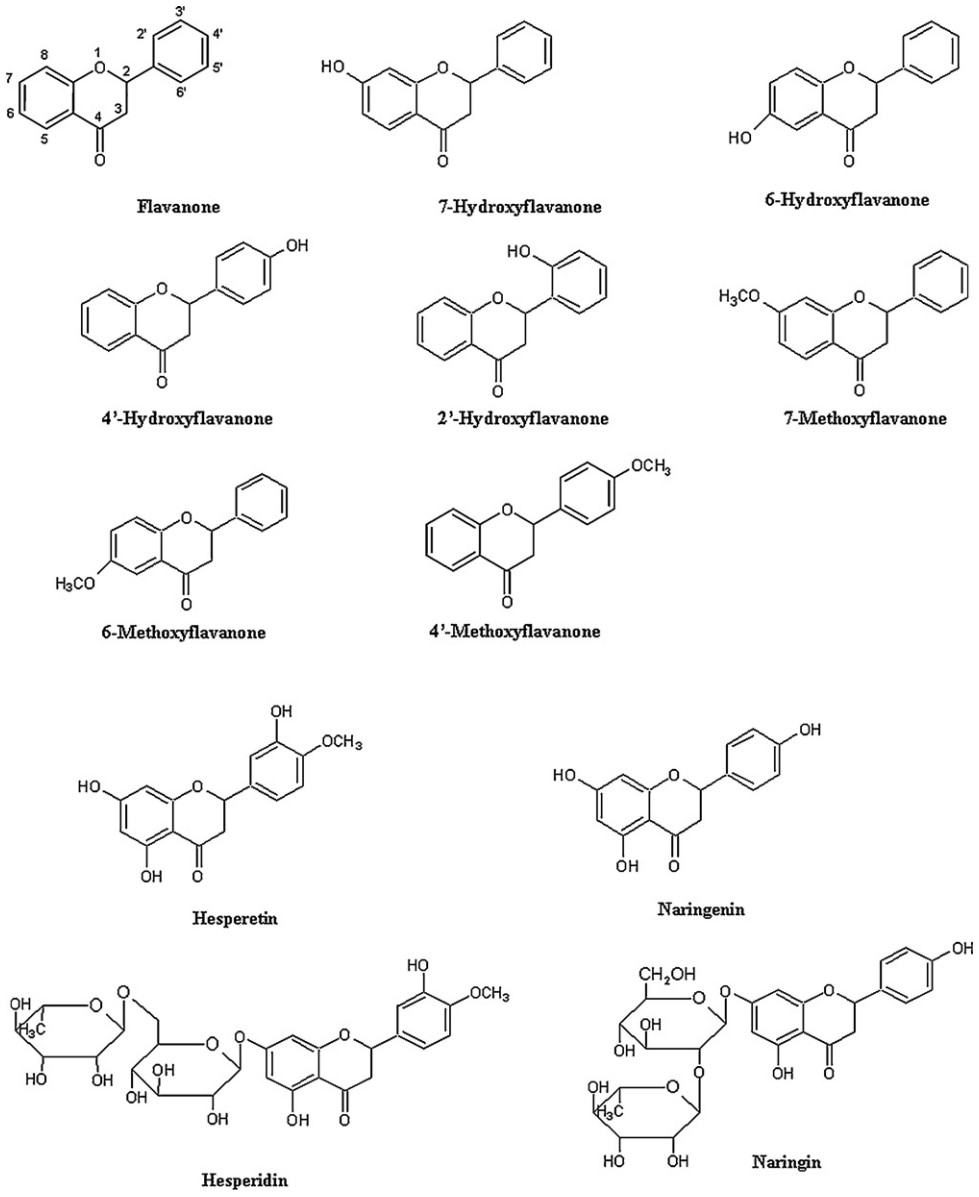

Fig. 1. Chemical structures of studied compounds.

solution (35 mL). The mixture reacted at 80 °C for 6 h under a nitrogen atmosphere. An excess of phenylisocyanate (3.75 mL) was added to the reaction mixture and the reaction was continued for 5 h at 80 °C. To the reaction solution was added 3 g of silica gel which was dried under vacuum at 180 °C for 4 h. The suspension was then refluxed under nitrogen for 24 h. The modified silica material obtained was filtered and washed carefully with pyridine, acetone, methanol, water, tetrahydrofuran, and dichloromethane and then dried under vacuum.

2.4. Capillary column preparation

Fused silica capillaries, 100 μm I.D. × 375 μm O.D. were purchased from Composite Metal Services (Hallow, Worcestershire, UK) and packed with the cyclodextrin chiral stationary phase (CD-CSP). Capillaries were packed in our laboratory following a previous published procedure [36]. Briefly, one end of the capillary was connected to a mechanical temporary frit, Valco (Houston, TX, USA) to retain the packing material and the other end to a stainless steel HPLC pre-column (10 cm × 4.1 mm I.D.) which was used as reservoir for the slurry. A series 10 LC pump, PerkinElmer (Palo Alto, CA, USA) was used for the packing procedure.

The capillary was firstly packed with RP-18 stationary phase (a few mg were suspended in 1 mL of acetone) for about 10 cm. Then the slurry was removed from the reservoir and the capillary flushed with water in order to completely eliminate the organic solvent. The frit was done by sintering the reversed phase particles for 6 s at 700 °C with a heating wire (laboratory made apparatus), flushing continuously with water. The temporary frit was removed and the excess of packing material was eliminated by flushing with water. Afterwards the column was packed with the chiral stationary phase suspended in MeOH–i-PrOH mixture (1:1) for 22.0 cm. Finally the column was packed again with the RP-18 particles to prepare the second frit following the same procedure above discussed. The detection window was prepared by removing the polymide layer with a razor. The capillary was cut at the desired length and equilibrated with the mobile phase using the LC pump at 35 MPa.

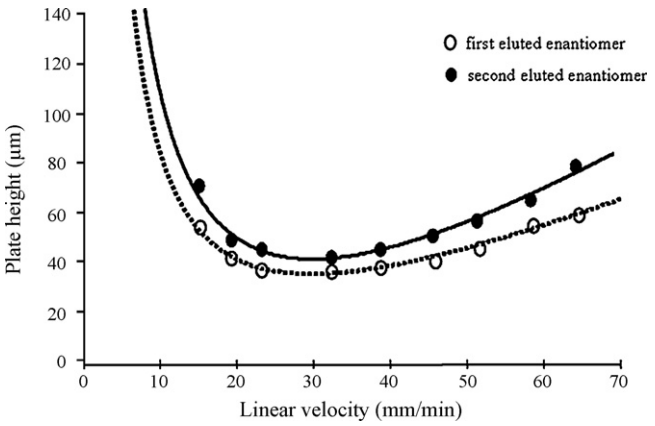

Fig. 2. Dependence of plate heights on linear flow velocity of the mobile phase. *Experimental conditions*: capillary column packed with phenylcarbamate-2-propyl-β-CD, 100 μm I.D. × 22.0 cm packed length, 22.5 cm and 34.0 cm effective and total lengths, respectively; mobile phase, H_2O/ACN, 60/40 (v/v); variable flow rate (120–400 nL/min); flavanone concentration, 100 μg/mL, injected volume 100 nL.

3. Results and discussion

3.1. Chromatographic evaluation

In this work the enantioresolution capability of phenyl-carbamate-propyl-β-CD silica stationary phase was evaluated employing nano-LC for the chiral separation of several flavanones (structures reported in Fig. 1). The CSP was also successfully used for the diastereoisomeric resolution of hesperidin, naringin, two flavanone glycosides. All analysed compounds, with the exception of flavanone-7-*O*-glycosides, possess one chiral centre in position 2 of their chemical structure and therefore, each one, exist as a pair of enantiomers. Instead, due to the presence of the disaccharide moiety at C7 position of the ring, flavanone-7-*O*-glycosides show a couple of diastereoisomers for each compound (Fig. 2).

The β-CD-CSP herein assessed contains in its structure polar aromatic groups such as phenyl-carbamate-propyl ones which enhance chiral discrimination of the stationary phase because they are capable to promote useful hydrogen bonding [37]. Owing to the nature of these substituents, the enantioselectivity of the phenyl-carbamate-propyl-β-CD CSP was evaluated under reversed, polar organic or normal elution modes.

3.2. Evaluation of the chiral stationary phase using reversed-phase conditions

3.2.1. Influence of mobile phase composition and flow rate on enantioresolution

When using cyclodextrins in LC, the chiral molecular recognition mechanism is governed by various interactions between the CSP and the analysed enantiomers, even if inclusion-complexation plays an important role in the process. Therefore, the selection of an appropriate mobile phase has to be done carefully in order to promote useful secondary interactions enhancing the enantioresolution.

Considering the reversed phase elution mode and the data available in literature about the use of CD based CSPs in HPLC [27,30], a mixture of water with a polar organic solvent such as ACN was evaluated for preliminary experiments. Flavanone enantiomers were selected in order to find the optimum flow rate for achieving the highest efficiency. Experiments were carried out using H_2O/ACN, 60/40 (v/v) as the mobile phase at different flow rates in the range 120–400 nL/min.

Racemic flavanone was resolved in its enantiomers at any flow rate used. Fig. 2 shows the plot of plate heights (H) vs. linear velocity (Van Deemter curve) for the two enantiomers. From the comparison of the curves, we can observe a difference in the slope of Van Deemter curves. Due to the fact that enantiomers have the same physico-chemical properties, this behaviour can be attributed to diverse kinetic effects with the cyclodextrin moiety, including van der Waals, H-interactions, inclusion complexation As can be observed, a decrease of H values was obtained by raising the flow rate from 15.5 to 32.5 mm/min. A further increase of the mobile phase velocity caused a reduction of efficiency (higher plate heights) of both enantiomers. From the Van Deemter's plot, a flow rate of 240 nL/min (38.8 mm/min) was selected for further experiments providing the best compromise in terms of peak efficiency and acceptable analysis time.

CD-CSP enantioselectivity was firstly investigated analysing the studied compounds with mobile phases containing different ratios of ACN/water. ACN concentration was varied in the range 20–80% and the effect of the organic modifier on retention factor k, retention time t_r separation factor α, and resolution R_s was evaluated. The increase of ACN concentration caused a reduction of both retention time and enantioselectivity of all studied compounds (data not shown). In fact the aprotic nature of acetonitrile enhances its affinity for the preferred sites in the hydrophobic CD cavity, raising its competition with the enantiomers [20].

The linear relation recorded plotting ACN concentration vs. ln k studied in the range 20–80% of the organic modifier demonstrates a typical reversed phase mechanism for all studied compounds, that takes place using the CD-CSP (R^2 in the range 0.95–0.99 for all the analysed compounds). Table 1 illustrates the chromatographic results obtained by nano-LC under optimal experimental conditions. As can be seen, the analytes were resolved in their enantiomers and diastereoisomers, with the exception of 2′-hydroxyflavanone and naringin that were partially separated (R_s = 0.50). So low resolution values were probably due to the not favourable position of the substituents groups on the analytes' structure that did not lead strong interactions with the CD-CSP. Fig. 3 shows the chromatograms of some flavanones enantiomers and hesperidin diastereoisomers resolved under reversed-phase conditions by using a mixture of water/ACN as mobile phase.

3.2.2. Influence of methanol content in the mobile phase

The resolution capability of the phenyl-carbamate-propyl-β-CD was then studied in reversed-phase mode, using MeOH as the

Table 1
Nano-LC chiral and diastereoisomeric separations of flavanones under reversed-phase mode using a mobile phase based on water and acetonitrile.

Compounds	t_{r1}	k_1	t_{r2}	k_2	α	R_s
Flavanone[a]	45.73	7.79	51.82	8.96	1.15	2.09
7-Hydroxyflavanone[b]	41.22	5.45	44.78	6.01	1.10	1.23
6-Hydroxyflavanone[a]	29.69	4.71	32.82	5.31	1.13	1.36
4′-Hydroxyflavanone[a]	27.16	4.22	29.89	4.75	1.12	1.56
2′-Hydroxyflavanone[a]	28.84	4.54	30.19	4.81	1.06	0.50
7-Methoxyflavanone[c]	43.57	6.21	48.90	7.08	1.14	2.05
6-Methoxyflavanone[c]	40.38	5.68	44.15	6.31	1.11	1.51
4′-Methoxyflavanone[c]	37.67	5.23	43.37	6.17	1.18	2.07
Hesperetin[a]	32.21	5.19	40.77	6.84	1.32	1.78
Naringenin[a]	27.33	4.25	30.96	4.95	1.16	1.17
Hesperidin[b]	21.32	2.34	27.01	3.23	1.38	2.06
Naringin[b]	25.46	2.98	26.73	3.18	1.07	0.51

Capillary column packed with Phenylcarbamate-2-propyl-β-CD, 100 μm ID × 22 cm packed length, 22.5 cm and 34 cm effective and total length, respectively; different ratios of H_2O/ACN(v/v); flow rate 240 nL/min; samples analysed at a concentration of 100 μg/mL, injected volume 100 nL.

[a] Mobile phase: H_2O/ACN (70/30, v/v).
[b] Mobile phase: H_2O/ACN (80/20, v/v).
[c] Mobile phase: H_2O/ACN (60/40, v/v).

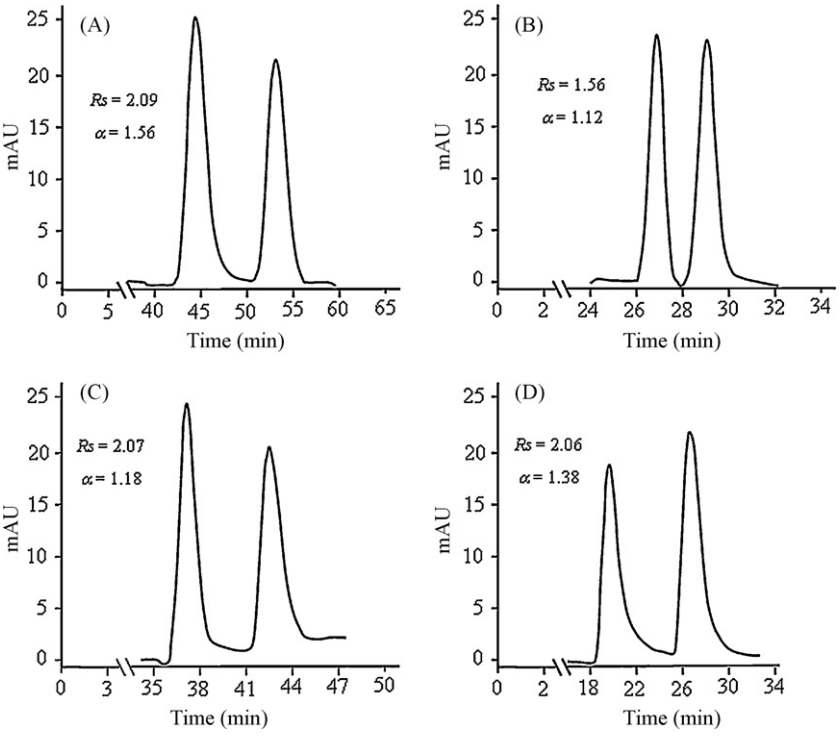

Fig. 3. Chromatograms of chiral separations of (A) flavanone, (B) 4′-hydroxyflavanone, (C) 4′-methoxyflavanone and (D) hesperidin obtained in reversed phase mode, using a mobile phase based on H_2O/ACN. Mobile phase composition: H_2O/ACN (70/30, v/v) for compounds (A) and (B), H_2O/ACN (60/40, v/v) for compound (C) and H_2O/ACN (80/20, v/v) for compound (D); all samples analysed at a concentration of 100 μg/mL, injected volume 100 nL; flow rate, 240 nL/min. For other experimental conditions see Fig. 2.

organic modifier, instead of ACN, in the mobile phase. The effect of the concentration of methanol on retention factors, enantioselectivity and enantioresolution of the compounds was investigated varying the content of MeOH in the mobile phase in the range 50–100%. Table 2 summarizes the chromatographic results for all flavanones tested in the optimised separation conditions. At MeOH concentration in the range 50–70% all analysed compounds were resolved in their enantiomers or diastereoisomers with the exception of 2′-hydroxyflavanone and naringin. As reported in the table, highest resolution values were achieved for methoxyflavanones, especially those bearing a methoxy group in position (4′ or 7). Hesperetin and hesperidin also provided a good resolution probably due to the methoxy group in position 4′. Unlike these results, naringenin and naringin, which have a hydroxyl group in the same position, were less resolved. Lower R_s values were observed for all hydroxyflavanones studied. It seems, that the methoxy group, with respect to the hydroxyl one, offered stronger interactions (hydrogen bonding) with the substituents groups of the CSP. Moreover, comparing the chiral resolution of all injected hydroxyflavanones, 4′ substituted derivative was the best resolved. Afterwards, this position seems to be important in offering the three points of attachment useful for solute–CSP interactions, necessary to achieve enantioresolution in β-CD based CSPs [29]. Hesperetin showed a higher R_s value relative to its correspondent glycosidic compound hesperidin. This result could be explained by the fact that hesperetin exhibited a higher degree of interaction due to the higher hydrophobicity and smaller size of the aglycone molecule, allowing a greater affinity for the CD cavity. A similar behaviour was also obtained with naringenin and naringin. Concerning 2′-hydroxyflavanone, which was the compound less resolved, observing its chemical structure, we can assume an intramolecular hydrogen bonding between the OH in positon 2′ and the O in position 1. This possible bonding can hinder the formation of stereoselective interactions between the stereogenic centre and the functional groups of the CSP, explaining the poor resolution of this compound.

Table 2
Nano-LC enantiomeric and diastereoisomeric resolutions of flavanones under reversed-phase mode using a mobile phase based on water and methanol.

Compounds	t_{r1}	k_1	t_{r2}	k_2	α	R_s
Flavanone[a]	23.13	3.18	29.33	4.31	1.35	2.34
7-Hydroxyflavanone[a]	14.52	1.62	16.05	1.90	1.17	1.40
6-Hydroxyflavanone[a]	14.80	1.67	17.91	2.23	1.34	1.45
4′-Hydroxyflavanone[a]	13.61	1.46	15.92	1.88	1.29	1.49
2′-Hydroxyflavanone[c]	24.35	3.44	25.07	3.57	1.03	0.31
7-Methoxyflavanone[b]	15.55	1.59	18.29	2.05	1.28	2.61
6-Methoxyflavanone[b]	16.87	1.81	19.67	2.28	1.26	1.93
4′-Methoxyflavanone[a]	15.42	1.57	18.64	2.11	1.34	3.28
Hesperetin[a]	16.55	1.99	25.07	3.53	1.77	2.51
Naringenin[c]	17.42	2.17	19.94	2.64	1.21	1.26
Hesperidin[c]	15.68	1.86	20.29	2.70	1.45	1.92
Naringin[c]	12.81	1.34	13.77	1.51	1.13	0.91

Other experimental conditions as Table 1.
[a] Mobile phase: H_2O/MeOH(40/60, v/v).
[b] Mobile phase: H_2O/MeOH (30/70, v/v).
[c] Mobile phase: H_2O/MeOH (50/50, v/v).

The R_s values obtained with 60% of MeOH were higher than the ones observed using the same content of ACN where only three compounds flavanone, 6-methoxyflavanone and hesperetin were partially resolved, $R_s < 1$ (results not shown). Furthermore, although for achieving similar R_s values different concentrations of MeOH or ACN had to be used, shorter analysis times were observed with the first organic solvent, e.g., flavanone enantiomers were separated ($R_s > 2$) in 23 and 45 min using MeOH (60%) and ACN (30%), respectively. In fact, longer retention time does not necessarily produce better resolution. A similar retention behaviour was due to the weaker displacing effect of MeOH [38] than ACN that allowed a greater inclusion complex formation between the analyte and the CD cavity. Thus an increase of retention, enantioselectivity and resolution was observed when methanol was used as organic mod-

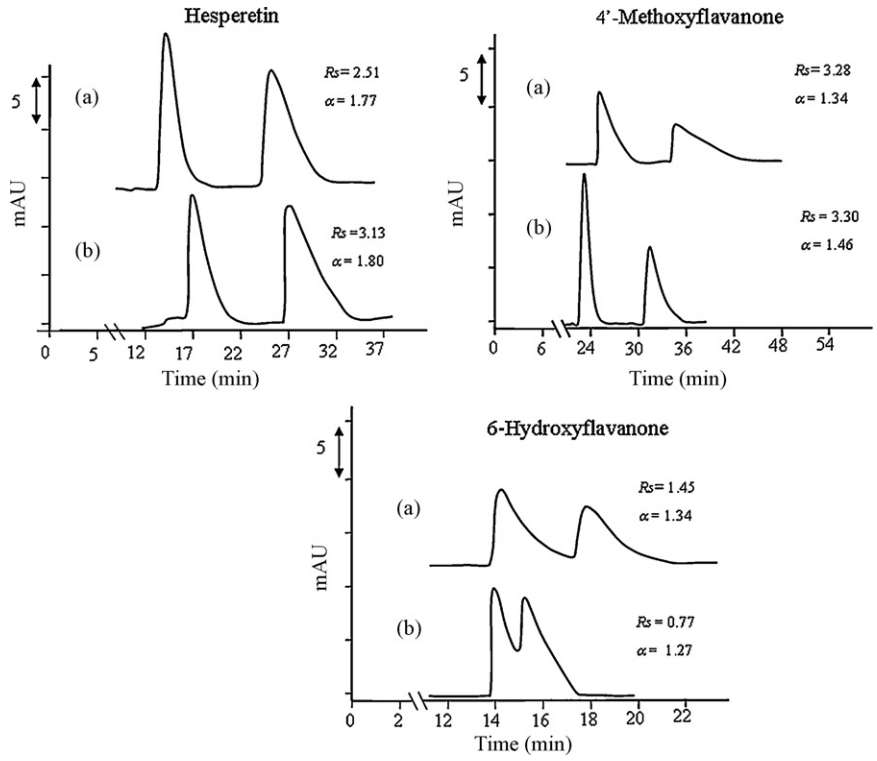

Fig. 4. Chiral separations achieved in reversed phase mode using two different mobile phases. (a) H_2O/MeOH 40/60 (v/v) and (b) H_2O/MeOH/TEAA pH 4.5, 39/60/1 (v/v/v). For other conditions see Figs. 2 and 3.

ifier for the same composition ratio water/organic solvent. Besides, owing to the amphiprotic nature of MeOH [39] polar interactions (hydrogen bonding, ionic interactions, etc.) gave a contribution to the chiral recognition.

Only few papers about chiral separation of flavanones by using HPLC in CSP_s derivatized with (6^A-N-allylamino-6^A-deoxy)permethyl- or methylated-β-CD are reported in literature. In our study almost all compounds were resolved with higher R_s and in shorter analysis time (e.g. R_s values of 2.34 and 2.50 were achieved for flavanone and hesperetin, respectively, in less than 30 min with respect to lower resolution values obtained in 60–110 min by using conventional chromatography) [38,40].

3.2.3. *Influence of buffer added to the mobile phase on nano-LC separation*

The addition of a buffer to the mobile phase, besides modify the ionic strength and pH of the eluent, can also substantially alters the physico-chemical properties of the stationary phase. In fact the residual silanol groups of the stationary phase are masked by the buffer solution, decreasing analyte retention time and sharpening the peaks. Therefore, an improving of efficiency can be obtained [39].

The mobile phase pH can influence the separation of ionisable analytes. The flavanones investigated here are neutral, except hesperetin and naringenin that show a weak acidity, due to the multiple hydroxyl groups present in their chemical structures. Therefore, the pH could not influence significantly the enantioselectivity of the analysed compounds. However, the presence of a buffer system can produce a variation in peak efficiency, enantioresolution and analysis time also for neutral compounds. The buffer composition can modify the hydrophobic nature of the analytes varying the non-covalent interactions (hydrophobic interactions, van der Waals forces, etc.) between analytes and the CD cavity. In fact, the alteration of the solvation shell around the analyte and the cyclodextrin, may influence the enantiorecognition process. Taking into account previous published data about the enantioseparation

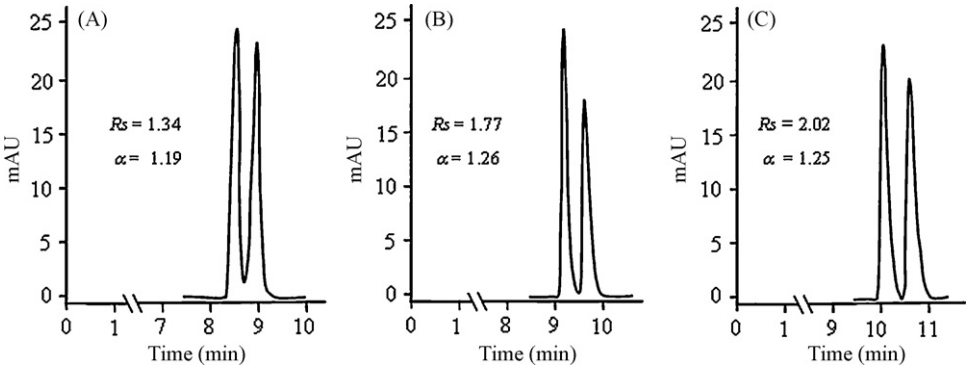

Fig. 5. Chromatograms of chiral separations of (A) flavanone, (B) 7-methoxyflavanone, and (C) 4′-methoxyflavanone obtained in polar organic mode. Mobile phase, 100% MeOH. For other experimental conditions see Figs. 2 and 3.

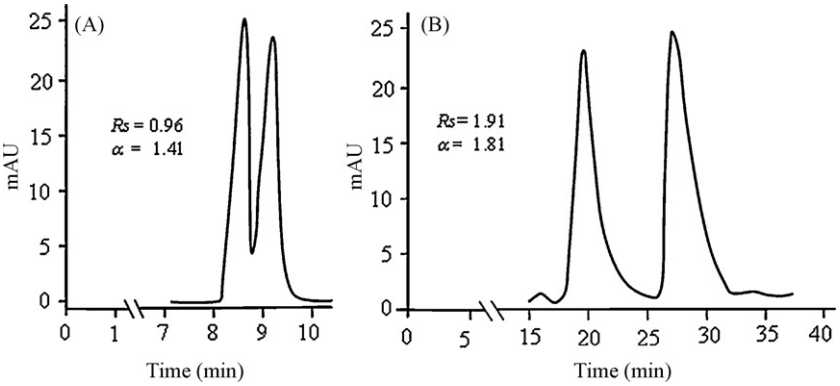

Fig. 6. Chromatograms of chiral separations of (A) 4′-methoxyflavanone and (B) hesperetin achieved in normal phase elution mode. Mobile phase, 0.5% TFA (v/v) in 40/60 and 70/30 hexane/EtOH (v/v) for the separation of the enantiomers of 4′-methoxyflavanone and hesperetin, respectively. For other conditions see Figs. 2 and 3.

of flavanones by HPLC [38], experiments were carried out selecting triethylamine acetate at pH 4.5. As reported in literature, triethylamine acetate buffer (TEAA), masking the residual silanol groups, reduces the interactions of analytes with the acidic sites on the silica support [39] allowing an increase of peak efficiency and enantioresolution.

Experiments carried out using a mixture of H_2O/MeOH (40:60, v/v) supplemented with 1% of TEAA pH 4.5 revealed a general decrease of retention factors with a simultaneous increase of the efficiency and an improvement of the enantioresolution for almost all studied compounds. For 6-hydroxyflavanone, 2′-hydroxyflavanone and naringin, the addition of buffer in the mobile phase seems to have a worst effect. Loss of resolution and peak tailing were observed, probably due to non-enantioselective interactions between the analytes and the chiral stationary phase. Fig. 4 shows, as an example, the effect of the buffer added in the mobile phase on chromatographic separation.

3.3. Evaluation of the chiral stationary phase in polar organic and normal phase conditions

Owing to their particular shape and their ability to form inclusion complexation, cyclodextrins based stationary phases were more effective in reversed phase mode [1]. However, the derivatization of hydroxyl groups on the CD rim with phenylcarbamate substituents has a multimodal behaviour allowing the enantiomeric separation also in polar organic and normal modes [30].

A mobile phase composed by 100% of MeOH was selected and used for the enantiomeric and diastereomeric separation of the flavanones. In those conditions only part of the analysed compounds were resolved. A value of $R_s > 1.23$ was achieved only for flavanone, 7-methoxyflavanone, 6-methoxyflavanone, 4′-methoxyflavanone and hesperetin. Fig. 5 shows the chromatograms of some of the studied compounds.

Unlike the reversed phase mode, most probably, no inclusion complexation takes place owing to the high concentration of methanol which occupies the CD cavity. The mechanism for polar organic separation is based on interactions like, π-complex-hydrogen bonding, dipole–dipole, ion-paring, electrostatic and steric repulsive effects between the analytes and the external CD substituents groups [28,41,42]. The flavanones structures possess aromatic rings and carbonyl moieties that allow π–π interactions with phenyl groups of the CSP, whereas the carbonyl groups of the analytes interact with those of the stationary phase through hydrogen bonding and dipole–dipole interactions. Those effects probably facilitate the formation of diastereomeric complexes in polar organic mode, thus explaining the enantioseparation of the analytes under these conditions.

It is worthwhile to remark that a mobile phase based on pure polar organic solvent, without any additives, enables the enantiomeric discrimination of the analysed compounds.

In order to study this interesting ability, ACN was employed instead of MeOH as the organic modifier. With 100% ACN as mobile phase, no chiral or diastereoisomeric separation was achieved for all studied compounds. Since the C–H bonds of ACN have a very high pKa value [43], this organic modifier is considered an aprotic solvent. For this reason it tends to form solvent clusters instead of an hydrogen bond network (as MeOH) and such phenomenon can justify the observed results.

The chiral stationary phase was finally studied in normal phase mode by using a mixture of hexane/ethanol as mobile phase. As well as in polar organic mode, the CD cavity is occupied by the non-polar solvent of the mobile phase and the inclusion complex formation is hindered. The chiral recognition is mainly due to π–π interactions with the derivatizing groups. Indeed, the phenylcarbamate groups on the CD rim provide hydrogen bonding and dipole stacking interaction sites with the carbamate linkage, which can contribute to enantiodiscrimination [44]. Furthermore, it appears that for chiral discrimination to arise, it is crucial that the analyte contains an aromatic group.

Optimisation of mobile phase composition was performed varying the organic solvents ratio (hexane/ethanol) and adding 0.5% of trifluoroacetic acid (TFA) as an additive. From the data reported in literature, the addition of TFA suppressed peak tailing and reduced acidic compounds retention time [45]. In our study, although the neutral nature of the analysed compounds, the presence of TFA provided sharper peaks and shorter analysis times. In fact without the addition of the acid, the hydroxyl groups of the flavanones interacted with silanol groups of the silica gel substrate through hydrogen bonding, causing peak tailing. The acidic medium present in the mobile phase displaced the solute on silanol sites of the CSP.

In these experimental conditions only methoxyflavanones, hesperetin and hesperidin were resolved achieving R_s values lower than the ones obtained in reversed phase mode (R_s values in the range 0.50–1.91 for the resolved compounds). Fig. 6 shows the chromatograms of the enantioseparation of 4′-methoxyflavanone and hesperetin.

4. Conclusions

Nano-liquid chromatography employing phenyl-carbamate-propyl-β-CD stationary phase has been applied for the enantiomeric or diastereoisomeric separation of several flavanones. The analyses were carried out in capillary columns of 100 μm I.D. packed with the CD-CSP. The chiral separation of all studied compounds was obtained in reversed phase mode using a mixture of

H_2O/MeOH as mobile phase. Although good results were achieved, the normal and polar organic phases were not as effective as the reverse phase conditions for the separation of these compounds.

The use of phenyl-carbamate-propyl-β-CD stationary phase in the nano-LC system allowed us to achieve the separation of enantiomers and diastereoisomers of flavanones with good results. In fact, comparing the data, about chiral resolution of flavanones, achieved with different derivatized β-cyclodextrin based CSP in HPLC [38,40] and those discussed in this communication, it can be concluded that higher enantioresolution and shorter analysis time can be obtained using nano-LC. Moreover, the use of capillary columns permits to work with small amounts of chiral stationary phase and low volumes of organic solvents. These features make this technique a cost-effective analytical tool with a lower environmental impact. These confirm the advantage of using this miniaturized technique over conventional HPLC for the discrimination of optical isomers.

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