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Ordered mesoporous silica functionalized with β-cyclodextrin derivative for stereoisomer separation of flavanones and flavanone glycosides by nano-liquid chromatography and capillary electrochromatography.

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Highlights

- A novel mesoporous silica modified with a β-cyclodextrin derivative was synthesized.
- The CSP material was packed into capillaries and used in nano-LC and CEC.

- Flavanones and flavanone glycosides optical isomers were studied and separated.

ABSTRACT

In this paper a chiral stationary phase (CSP) was prepared by the immobilization of a β-CD derivative (3,5-dimethylphenylcarbamoylated β-CD) onto the surface of amino-functionalized spherical ordered mesoporous silica (denoted as SM) via a urea linkage using the Staudinger reaction. The CSP was packed into fused silica capillaries 100 μm I.D. and evaluated by means of nano-liquid chromatography (nano-LC) and capillary electrochromatography (CEC) using model compounds for the enantio- and the diastereomeric separation. The compounds flavanone, 2′-hydroxyflavanone, 4′-hydroxyflavanone, 6-hydroxyflavanone, 4′-methoxyflavanone, 7-methoxyflavanone, hesperetin, hesperidin, naringenin, and naringin were studied using reversed and polar organic elution modes. Baseline stereoisomer resolution and good results in terms of peak efficiency and short analysis time of all studied flavonoids and flavanones glycosides were achieved in reversed phase mode, using as mobile phase a mixture of MeOH/H_2O, 10 mM ammonium acetate pH 4.5 at different ratios. For the polar organic mode using 100% of MeOH as mobile phase, the CSP showed better performances and the baseline chiral separation of several studied compounds occurred in an analysis time of less than 10 min. Good results were also achieved by CEC employing two different mobile phases. The use of MeOH/H_2O, 5 mM ammonium acetate buffer pH 6.0 (90/10, v/v) was very effective for the chiral resolution of flavanone and its methoxy and hydroxy derivatives.

Keywords: Nano-liquid chromatography; Capillary electrochromatography; Ordered mesoporous silica; Chiral separations; β-Cyclodextrin stationary phase; Flavanones

1. Introduction

The separation and analysis of chiral compounds is an important research topic in different fields also including analytical chemistry, and the separation of chiral compounds is increasingly in demand in various application fields such as, pharmaceutical, agrochemical, biomedical, environmental and nutraceutical areas [1]. Therefore, to meet the requirements related to the resolution and quantification of enantiomers, several stereoselective separation methods have been developed [2 - 7].

Different analytical techniques have been used for separation of enantiomers [2, 5 - 11]. In high-performance liquid chromatography (HPLC), the direct resolution method employing chiral stationary phases (CSPs) is very popular, and numerous chiral selectors (CSs) have been used for this purpose [2, 5 – 8, 12 - 14]. In that respect, in the last years, thanks to recent achievements in the field of materials science, chromatographic methods for separation of enantiomers utilizing other CSPs have been developed [15]. In addition, the recent progress of miniaturized analytical techniques such as nano-/capillary liquid chromatography (nano-LC/CLC) or capillary electrochromatography (CEC) has opened new horizons in the field of separation science. These techniques can be considered complementary or alternative to HPLC or CE and offer good separation efficiency and resolution, shorter analysis time and rapid optimization of experimental conditions [3, 4, 16, 17]. Developments in the preparation of nanoparticles and monoliths as stationary phases for miniaturised liquid phase separation techniques have been reviewed recently [18].

The discovery of ordered mesoporous silicas (OMSs) in the early nineties marked the beginning of research in the development of high surface area materials with controlled porosity [19]. The dramatically higher surface area of OMSs in comparison to commercially available chromatographic grade silica enhances resolution of

molecules by increasing retention factors to allow effective separations of analytes. Up to date, a variety of OMSs have been proposed as stationary phases or supports to prepare stationary phases for solid phase extraction [20, 21] and chromatography [22].

Among the CSs used to develop CSPs, β-cyclodextrin (β-CD) and its derivatives have been extensively used for chiral separations employing different chromatographic modes [11, 23 - 31]. Although many CSPs based on CDs are commercially available nowadays, there is a need to develop new packing materials offering high enantioselectivity in short analysis time. In some studies, the use of OMSs as supports for CSPs preparation have improved enantioselectivity and resolution with respect to conventional silica. Some of these articles have been discussed recently in a review paper [15] but, to the best of our knowledge, papers dealing with the use of CSPs based on OMSs for capillary/nano-LC have not been reported yet. In CEC only one paper has demonstrated the application of submicron OMS modified with phenylcarbamoylated-β-CD as CSP [32].

The first high coverage stable bonded phase CD-based CSP was developed by Armstrong and DeMond in 1984 [33]. β-Cyclodextrin was bonded to silica gel via an ether linkage and the resulting CSP could separate many compounds in reverse phase mode [33]. Thereafter, the application of CSPs based on chemically anchored CDs and the understandings of their properties have been broaden tremendously. Thus, numerous publications about synthethic routes and/or immobilization strategies have emerged during the last years, in order to develop well-defined CD-based CSPs. These strategies have been reviewed recently by Xiao et al. [34] and after this review other interesting strategies have also been published [35, 36].

In our paper, a spherical mesoporous silica (denoted SM) with 3-D wormhole-like porous framework was used for the first time as support to prepare a CD-CSP. For

this purpose, mono(6-azido-6-deoxy)perfunctionalized β-CD was first synthesized and purified, and then this derivative was immobilized onto the surface of amino-functionalized SM (SM-NH_2). The Staudinger reaction, which was applied for the first time by Zhang et al. [37] for inmobilization of CD onto aminised silica gel, was used. Under optimized conditions, the chemical anchoring of the β-CD derivative onto the SM support was effective via the hydrolytically stable urethane linkage (Fig. 1) and the current procedure afforded a structurally well-defined β-CD based CSP. In this regard, the objective of this work was to investigate the enantioselectivity of the prepared CD-CSP, by using nano-LC and CEC, for the enantiomeric and diastereoisomeric separation of some selected flavanones as model analytes.

2. Experimental

2.1 Chemicals and samples

All chemicals were of analytical reagent grade and used as received. Tetraethylorthosilicate 98% (M = 208.33 g/mol, d = 0.934 Kg/m^3), poly(ethylene glycol)-block-poly(propylene glycol)-blockpolyethylene glycol, Pluronic 123 (M_{av} = 5800 g/mol, d = 1.019 Kg/m^3), cetyltrimethylammonium bromide (CTAB) 98%, (M = 364.46 g/mol), β-CD, ethyl acetate, pyridine, 3,5-dimethylphenyl isocyanate 99%, sodium azide and 3-aminopropyltriethoxysilane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid 37%, acetone, ethyl ether, ethanol and anhydrous sodium sulphate were purchased from Scharlau (Barcelona, Spain). Acetonitrile (AcN), methanol (MeOH), ethanol (EtOH), isopropanol (i-PrOH), acetone and n-hexane (n-Hex) were purchased from Carlo Erba (Milan, Italy). A Milli-Q system (Millipore Waters, Milford, MA, USA) was employed to purify water. Ammonium

acetate (500 mM), utilized for the chromatographic runs, was obtained by titrating the appropriate volume of acetic acid with concentrated ammonia solution to pH 4.5. Mobile phases employed for the nano-LC experiments were daily prepared by mixing suitable volumes of buffer solution, water and organic solvents (AcN or MeOH). LiChrospher 100 RP-C_{18}, 5 μm particle diameter, was from Merck (Darmstadt, Germany). Fused silica capillaries, 100 μm I.D. x 360 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). The selected flavonoids (flavanone, 2′-hydroxyflavanone, 4′-hydroxyflavanone, 6-hydroxyflavanone, 4′-methoxyflavanone, 7-methoxyflavanone, hesperetin, hesperidin, naringenin, and naringin) were from Sigma–Aldrich. Stock standard solutions of each flavonoid (1 mg/mL) were prepared in MeOH, and stored at 4ºC. Further dilutions were daily done with water and AcN (60/40, v/v) to obtain the final concentration of 100 μg/mL. Fig 1S. shows the chemical structure of the studied compounds (supplementary material).

2.2 Instrumentation

A BASIC 20 pH meter (Crison, Barcelona, Spain) was employed for accurate pH measurements in aqueous buffer solution. A Series 10 LC HPLC pump (Perkin Elmer, Palo Alto, CA, USA) a Decon model FS 100b (Hove, UK) ultrasonic bath and a Stereozoom 4 optical microscope (Cambridge Instruments, Vienna, Austria) were employed for the capillary packing process.

The nano-LC experiments were carried out with a laboratory-assembled instrumentation utilizing a LC10 HPLC pump (Perkin Elmer, Palo Alto, CA, USA), a modified injection valve (Enantiosep GmbH, Münster, Germany), and an UV–vis on-column detector (Spectra Focus PC1000, Thermo Separation Products, San Jose, CA, USA), set at 200 nm. The HPLC pump, delivering continuously MeOH isocratically,

and the injector were connected so as to obtain a passive split-flow system needed to reduce the flow rate at nL/min levels. The capillary column was directly inserted into the modified injector equipped with 50 μL loop. Both pump and injection valve were joined to a stainless steel T piece (Vici, Valco, Houston, TX, USA) by means of 500 μm I.D. stainless steel tubes with lengths of 70 and 5 cm, respectively. The third entrance of the T was connected to the MeOH reservoir of the pump, through a fused silica capillary (50 μm I.D. × 50 cm) achieving a continuous recycling of the organic solvent.

Sample and mobile phase were introduced into the nano- LC system through the injection valve. Injections were done by filling the loop with the sample solutions, switching the valve for appropriate time and then flushing the loop with the mobile phase. The flow rate was estimated by connecting a 10 μL syringe (Hamilton, Reno, NV, USA) to the capillary column outlet through a Teflon tube (TF-350; LC-Packing, CA, USA) and by measuring the volume of mobile phase accumulated over 5 min. Data were collected using Clarity™ Advanced Chromatography Software (DataApex Ltd., Prague, Czech Republic).

CEC experiments were performed on a HP^{3D} CE apparatus (Agilent Technologies, Waldbronn, Germany) with on-column UV-diode array detector operating at 214 nm. The same capillary column employed in nano-LC containing the packed CSP with different length of 23 cm (total length, 33.5 cm) was used for the separation of enantiomers. During runs the two electrode compartments were pressurized at 10 bar to avoid bubble formation. A voltage of -15 kV was applied during CEC experiments. Samples were injected by hydrodynamic method (8 bar x 0.5 min). Data were recorded with Chemstation software (Rev. A.09.01, Agilent Technologies).

2.3 Preparation of stationary phases

2.3.1 Preparation of 3,5-dimethylphenylcarbamoylated β-CD

Firstly, monotosyl-β-CD (compound **1**) and azido-β-CD (compound **2**) were prepared according to previously reported methods (see Fig. 1 and suplementary material for details). Then, 1 mmol of dried compound **2** was dissolved in 20 mL of anhydrous pyridine and 25 mmol of 3, 5-dimethylphenyl isocyanate was added and allowed to stir overnight at room temperature. The pyridine was removed by vacuumed distillation. The solid was dissolved in 100 mL of ethyl acetate and filtered to eliminate rest of unsolved solid. The mixture was washed three times with 100 mL of brine (10% , w/v) and the solid was dried in anhydrous sodium sulphate. The ethyl acetate solution was concentrated to about 25 mL and 100 mL of hexane was added to the concentrate, the suspension was filtrated to eliminate rest of solid. Finally the ethyl acetate was evaporated and the obtained solid (3,5-dimethylphenyl carbamoylated β-CD, compound **3**) was dried for 8 h at 65°C (Fig. 1). Characterization data for compound **3**: FT-IR (cm^{-1}, ATR): 3393-3309 (amide N-H str), 3015 (arom C-H str), 2919 (C-H str), 2108.7 (N_3 str), 1714 (esther C=O), 1613, 1538, 1437 (arom C=C), 1212, 1042 (C-O-C). ^{1}H NMR ($CDCl_3$, TMS) δ (ppm) 6.92-6.42 (m, C_6H_3); 5.96-5.55, 5.16-4.67, 4.12-3.94 (m, β-CD); 2.04-1.26 (m, CH_3).

2.3.2 Functionalization of SM-NH_2 with 3,5-dimethylphenylcarbamoylated β-CD

Derivatization of SM-NH_2 (for details of SM-NH_2 preparation see supplementary material) with compound **3** was carried out according as follows: 2.3 g of SM-NH_2 were stirred in 25 mL of anhydrous THF under a CO_2 atmosphere (flow 2 bar). After 10 min, a solution of compound **3** in 20 mL of anhydrous THF was added and stirring with CO_2

bubbled for 5 min. Then was added 1 g of triphenylphosphine dissolved in 20 mL of THF. The mixture was stirred with constant bubbling of CO_2 for 21 h. After this time the solid was filtered and washed with 100 mL of THF. Finally, in order to purify the solid, Soxhlet extraction with acetone was used for 48 h to remove the triphenylphosphine oxide and any unreacted CD by-products. The solid obtained (denoted as SM-β-CD) was dried for 72 h at 60 °C (Fig. 1). Mesoporous silicas were characterized using conventional characterization techniques (see supplementary materials for details)

2.4 Preparation of capillary columns

The capillary columns (100 μm I.D. x 375 μm O.D. x 50 cm) were packed following a previous reported procedure [2, 38]. 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 (50 mm × 4.1 mm I.D.), which was used as reservoir of the slurry. A series LC10 pump, PerkinElmer (Palo Alto, CA, USA) was used for the packing procedure.

The capillary was firstly packed to 10 cm with LiChrospher 100 RP-C_{18} stationary phase suspended in MeOH (3-5 mg in 1 mL) for the preparation of the first frit. Then the slurry was removed from the reservoir and the capillary flushed with water (about 15 min) in order to completely eliminate the organic solvent. In order to prepare the frit, the RP-C_{18} was sinthered for 8 s at about 700 ºC with a heating wire (laboratory made apparatus), flushing continuously with water. Then the temporary frit was removed and, the capillary was flushed with MeOH followed by i-PrOH to eliminate the excess of packing material. Afterwards the column was packed with the CSP suspended in i-PrOH to 20 cm. Finally, the other extreme of the column was

packed again with the RP-C_{18} particles to prepare the second frit following the same procedure applied for the first one. The detection window was prepared at 2 cm to the outlet frit by removing the outside polyimide layer with a razor.

3. Results and discussion

3.1 Synthesis and characterization of the mesoporous silicas

The XRD patterns of the obtained SM material (Fig. 2A) exhibited a single (100) diffraction peak at low angle region (0.93º). In this material the d_{100} spacing, assigned to the pore-to-pore centre correlation distance, was 94.9 Å. This pattern is typical of materials with uniform pores in the mesoporous range and non-symmetrical 3-D wormhole-like porous framework, with a pore structure lacking long-range order. XRD pattern of the modified SM-β-CD (Fig. 2B) indicates that the basic pore structure of the material remains unchanged after functionalization, and the decrease in XRD signal intensity can be attributed to the presence of the β-CD groups inside the pore channels of the material [39]. In addition, the increase in the wall thickness, from 15.04 Å in SM to 57 Å in SM-β-CD, confirmed that the functionalization occurred also inside the mesopore channels.

Fig. 2C shows N_2 adsorption-desorption isotherms for SM, SM-NH_2 and SM-β-CD materials. All the isotherms are type IV according to the IUPAC classification with hysteresis loops type H1, which are representative of mesoporous materials. At low relative pressures P/P_0, between 0 and 0.3 the nitrogen adsorption is produced in monolayer. At pressure upper 0.3 capillarity condensation and pores filling occur. The isotherms of the modified materials (SM-NH_2 and SM-β-CD) were of the same type as than the non-modified material (SM), with a reduction in the adsorbed volume and in the hysteresis loop due to the decrease in the pore size that takes place when the ligand

is attached into the pores. These results showed an important decrease in the surface area (from 660.66 to 389.36 m^2/g), pore diameter (from 74.5 to 45.6 Å) and pore volume (1.2 to 0.54 cm^3/g) after the functionalization with the β-CD derivative (see Table 1S in supplementary information). Thus, it can be inferred that the β-CD groups were grafted not only to the external surface area of the mesoporous silica particles, but also inside the mesostructured pore channels. This fact agrees with the results obtained by other authors who indicated that only with pore diameters > 60 Å the β-CD moieties can react with the anchoring sites ($-NH_2$) inside the pore channels [39]. Fig. 2D shows the pore size distribution of the materials after and before the functionalization procedure.

NMR spectroscopy is one of the most powerful techniques for verifying the incorporation of functional groups by enabling the simultaneous identification of multiple functionalities, as well as, the different types of silanol groups and the effectiveness of the covalent bonding of the ligand to the silica framework. The ^{29}Si MAS NMR and ^{13}C MAS NMR spectra of these new materials corroborated the presence of the β-CD derivative groups. ^{29}Si MAS NMR spectra of SM and SM-β-CD are shown in Fig. 3A and 3B. In these spectra, resonances around -110 ppm, -105 ppm and -92 ppm can be assigned as Q^4 [$(SiO)_4$***Si***], Q^3 [$(SiO)_3$***Si***-(OH)] and Q^2 [$(SiO)_2$***Si***-$(OH)_2$] sites, respectively. Clearly, Q^4 is the dominant peak because it is the most abundant site. In addition, the two other peaks that appeared at -55 and -65 ppm in the SM-β-CD spectrum (Fig. 3B) were assigned to T^2 ($(SiO)_2$***Si***OH-R) and T^3 ($(SiO)_3$***Si***-R) sites, respectively, and corroborated the covalent attachment of the organic ligand in this material. The ^{13}C CP-MAS NMR spectrum of SM-β-CD (see Fig. 2S in supplementary material) shows peaks in the central spectral region (ca. δ = 98.8 - 58.3 ppm) typical of cyclodextrin units. The peaks for the triehoxysilane unit

(**C**H_2**C**H_2**C**H_2Si) are observed at ca. δ = 10 – 42 ppm and the urethane linkage (O**C**ONH) was observed at ca. δ = 160 ppm. Finally, signals observed at ca. δ = 110 - 140 ppm are assigned to the carbon atoms of phenylcarbamoyl groups. Results of NMR spectroscopy are in agreement with other works [34, 40, 41] and confirmed the presence of β-CD derivative into the SM structure and, therefore, the right functionalization of the material.

The morphology, shape and size of the particles of the prepared materials were studied by SEM. SEM micrographs showed that the particles had a spherical morphology (Fig. 4A), with a very good particle circularity factor centered at 0.87 (Fig. 4B). In addition, a reasonable distribution of particle size (dispersity) to have good column packing quality was achieved, with the vast majority of the particles in the range of 4 – 6 μm. This particle size distribution is in agreement with previous synthesis of mesoporous materials type SM [36]. SEM images for the SM-β-CD material show that the morphology and size of the particle remains similar after the funtionalization process. Fig. 4B shows a SEM image of cutting section of 100 μm I.D. capillary column, where the column bed formed by the spherical SM-β-CD particles can be observed. On the other hand, TEM images of these silicas show irregularly aligned mesopores throughout the materials with relatively uniform pore sizes (wormhole-like pore arrangement). These results are in good agreement with the related XRD patterns and N_2 adsorption–desorption isotherms.

The amount of aminopropyl groups and β-CD derivative attached onto the silica surface was calculated by the % N and % C obtained by elemental analysis in both SM-NH_2 and SM-β-CD materials. Results obtained indicated that the SM-NH_2 material had a functionalization degree of 1.38 mmol/g. After the immobilization of the β-CD derivative (compound **3**) onto its surface, via the Staudinger reaction, the residual

aminopropyl groups were estimated in 1.27 mmol/g. These results confirmed that due to steric hindrance it was not possible to functionalize all the amino groups of the material. On the other hand, functionalization degree of the SM-β-CD was 32 µmol/g with a CD surface coverage of 8.22 x 10^{-8} mol/m^2. TGA curves of the prepared silicas (see Fig. 3S in supplementary material) showed that exothermic degradation processes occur in the range of 200 – 600ºC, with weight loss of 7 and 20% in SM-NH_2 and SM-β-CD, respectively, that demonstrated good thermal stability of these modified materials. These characterization results confirmed that the SM-β-CD material was successfully prepared, in order to evaluated as CSP for the enantiomeric and diastereoisomeric separation of some selected flavanones as model analytes.

3.2 Evaluation of the CSP using reversed-phase conditions on nano-LC

The prepared CSPs was packed into fused silica capillaries and the enantioresolution capability was firstly evaluated by means of nano-LC for chiral separation of several flavanones (Fig. 1S in supplementary material). As CSP studied contained a modified β-CD, the enantioresolution mechanism expected was an inclusion-complexation one, but also other additional mechanisms based on adsorption have also to be considered. Therefore, the composition of mobile phase must be carefully controlled to achieve optimum separation of enantiomers. For this purpose, the enantioselectivity was firstly evaluated under reversed elution mode.

3.2.1 Influence of acetonitrile content in the mobile phase

Based on our experience and on data reported in literature, racemic model mixtures of compounds of nutraceutical interest have been chosen (see Fig 1S in suplementary material). Their enantiomeric separation was studied by nano-LC using

the SM-β-CD as CSP. Mixtures of AcN/H_2O, 10 mM ammonium acetate pH 4.5 at different ratio (40 - 90% of AcN, v/v) were used as mobile phase. Flow rates in the range 225-281 nL/min were applied.

The plot of *ln k* vs AcN concentration of the first eluted enantiomer (when resolved) showed a linear behaviour for all studied compounds. Therefore, from these experiments, it can be deduced that the chromatographic mechanism is of reversed-phase type. Fig. 5 gives an idea about the linear relationship observed for the representative compounds 7-methoxyflavanone and flavanone. As it can be seen, the increase of the organic solvent concentration in the mobile phase caused a reduction of retention factor (*k*). This was mainly due to the competition enantiomers/AcN with the hydrophobic CD cavity. The organic solvent had higher affinity to the analyte, having been observed similar trend for the other compounds [42].

Retention times (t_r) and enantioresolution factors (R_s) also decreased by increasing the AcN concentration for all analyzed compounds. For example, eluting with AcN/H_2O, 10 mM ammonium acetate pH 4.5 (40/60, v/v), t_r and R_s of 7-methoxyflavanone (first eluted enantiomer) were 33.0 min and 2.0, respectively. The two parameters decreased to 4.4 min and 0 increasing the content of organic solvent to 90%. Baseline enantioresolution of flavanone in its enantiomers was achieved using AcN/H_2O, 10 mM ammonium acetate pH 4.5 (40/60, v/v) (Rs = 1.80, t_r = 38.4 min), whereas employing 90% of AcN the enantioresolution was completely lost (t_r = 4.3 min).

Table 1 reports the chromatographic parameters, t_r, *k*, selectivity (α) and R_s for all compounds under optimal experimental conditions. As it can be observed, good resolution was achieved for flavanone and 7-methoxyflavanone, for the other compounds poor enantioresolution was obtained with R_s values in the range 0.00 - 0.8.

These values are pobably due to the position of the substituent groups in the structure of the other flavanones that is not favourable and the interaction with the CSP is not enough to show good R_s. In addition, AcN could hidden the formation of stereoselective interaction between the stereogenic centre and the functional groups of the CSP. The chromatograms of some flavanones enantiomers resolved under reversed-phase conditions by using a mixture of AcN/H_2O, 10 mM ammonium acetate pH 4.5 (40/60, v/v) as mobile phase are showed in suplementary material (Fig. 4S).

3.2.2 Influence of methanol content in the mobile phase

Further experiments were carried out using the same capillary column and a mobile phase containing MeOH at concentration range of 70 - 95% (v/v). The organic solvent was mixed with 10 mM ammonium acetate buffer at pH 4.5. Lower concentrations of MeOH were not studied because by working in this range the separation of all compounds was obtained. In addition, at lower MeOH concentration, the t_r began to be higher without increasing chiral resolution.

The increase of MeOH in the mobile phase caused a decrease of *k*. The plot of *ln k* vs organic solvent concentration was linear for all studied compounds, confirming, also with this solvent, a typical reversed phase mechanism at least at the concentrations studied. The use of MeOH instead of AcN gave better results for analysis times and enantioresolution. For example, at 95 % MeOH concentration all analyzed compounds were resolved in their enantiomers or diastereoisomers with the exception of naringin.

Table 2 summarizes the chromatographic data, t_r, *k*, α, *N/m* and R_s of all flavanones tested under the optimized separation conditions. As can be seen, the highest R_s values were achieved for flavanone and for all methoxyflavanones. Lower R_s values were achieved for 2´-hydroxyflavanone, naringin, naringenin and hesperidin. Hesperetin

showed a higher R_s value relative to its correspondent glycosidic compound hesperidin. These results 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 [3]. Similar effect was observed for naringenin and naringin. Fig. 6 shows the nano-LC separation of the studied enantiomers or diastereoisomers achieved under the optimal experimental conditions. As it can be observed, the peaks obtained show very good symmetry. This fact can be due to the high surface area and ordered pore structure of the mesoporous silica, which offers quick mass transfer kinetics during separation.

The better results with MeOH than AcN were due to the weaker displacing effect of MeOH that allowed a greater inclusion complex formation between the analyte and the CD cavity [43]. In addition, MeOH has an amphiprotic nature and the polar interactions give a contribution to the chiral recognition [44]. Experiments performed using EtOH or i-PrOH instead of MeOH revealed not satisfactory results concerning enantioresolution.

Comparing the results here obtained with those reported in the work published in reference [3] in reversed chromatografic mode, using MeOH as organic modifier, it can be observed that in this study, the chiral separations were obtained with shorter analysis times and resolution values similar or in some cases higher. By using AcN instead of MeOH in the mobile phase, Si-Ahmed *et al.* achieved the complete enantioresolution for all the compounds with long analysis times (more than 45 min for the chiral separation of flavanone).

3.2.3 Influence of the buffer added to the mobile phase

Ammonium acetate buffer in the range 0 - 20 mM was investigated, keeping constant the organic modifier content. When buffer concentration was increased, no significant effect on enantioresolution was observed. The addition of a buffer system to the mobile phase did not affect the enantioresolution of flavanones significantly.

3.3 Evaluation of the CSP using organic polar-phase conditions on nano-LC

The SM-β-CD CSP was finally studied in polar organic mode by using 100% AcN or MeOH as mobile phase, without any additives. With 100% AcN as mobile phase, no enantioseparation was achieved for all the studied compounds. Since the C-H bonds of AcN have a very high pKa value, the solvent will be aprotic [45], but with MeOH 100%, all analysed compounds were resolved in their enantiomers or diastereoisomers with the exception of naringin, naringenine and hesperidine (Table 3 and Fig. 5S in suplementary material).

3.4. Evaluation of the CSP on capillary electrochromatography

The novel capillary CSP column was also studied analyzing the same model standard racemic mixtures used in nano-LC with CEC. As reported in literature, the driving force of analytes and mobile phase into the column is the electroosmotic flow (EOF) generated by the application of a relatively high electric field. The EOF can move in the direction of either cathode or anode depending on some experimental conditions such as, the charge of the stationary phase, the composition of the mobile phase, the applied electric field, etc. Therefore, these parameters must be carefully selected and controlled. First of all, we selected a reversed polarity mode (EOF to anodic direction) just considering the chemical structure of the CSP (see Fig. 1) where

positively charged amino groups close to the CDs are present. Just to compare CEC data with the ones achieved by nano-LC, the same mobile phase was tested. MeOH and/or AcN with water also containing ammonium acetate were selected. The presence of the buffer was necessary in order to have sufficient electric conductivity during the runs.

Table 4 shows the CEC data achieved analyzing flavanone and its derivative enantiomers employing two different mobile phases. The use of MeOH/H_2O, 5 mM ammonium acetate buffer pH 6.0 (90/10, v/v) was very effective for the chiral resolution of flavanone and its methoxy and hydroxy derivatives. R_s values in the range 1.34 - 6.24 were obtained, while relatively long retention times were achieved (16.9 - 25.5 min, second eluted enantiomer).

The composition of the mobile phase was modified keeping constant the content of MeOH and the ionic strength, decreasing water concentration to 5% and adding 5% of AcN. As can be observed in Table 4 retention times decreased due to both increase of EOF and decrease of interactions with the CSP. This is documented by the decrease of retention factors. In addition, resolution factors decreased with poor enantioresolution of 2'-hydroxyflavanone. No peaks were detected for hesperetin, hesperedin, naringin and naringenin. As an example of the good performace of CEC, Fig. 7 shows some representative electrochromatograms achieved using the two mobile phases with and without AcN in the mixtures. As we have indicated previously, ordered mesoporous silicas possess larger surface/volume ratio than conventional silica gel, which is important in chromatography for achieving favourable mass transfer. In that respect, considering the high surface area and special pore structure of ordered mesoporous silicas, symmetric peaks with little peak broadening could be achieved with the new CSP developed.

In Tables 2 and 4, efficiency of both analytical methods, in terms of theoretical plates number per meter (*N/m*) was also evaluated. The *N/m* values obtained with the CEC and nano-LC methods, obtained in the same experimental conditions, were in the range between 39580 - 27010 and 19175 - 10789, respectively. As exprected higher values were obtained with CEC than with nano-LC, due to the well known presence of EOF.

4. Conclusions

A chiral stationary phase (CSP) has been successfully prepared and characterized, using spherical ordered mesoporous silica (SM) as support. Amino-functionalized SM was modified with 3,5-dimethylphenylcarbamoylated β-CD (SM-β-CD) and its potential of application as CSP, in nano-liquid chromatography (nano-LC) and capillary electrochromatography (CEC), was evaluated for the first time. Flavanones and flavanone glycosides were used as model compounds for the enantioseparation and the diastereomeric separation. The analyses were carried out in capillary columns of 100 μm I.D. packed with the SM-β-CD. In nano-LC, the chiral separation of all studied compounds was obtained in reversed phase mode using as mobile phase a mixture of MeOH/H_2O, 10 mM ammonium acetate pH 4.5, at different ratios. Although good results were achieved, polar organic phases (100% AcN or MeOH) were not as effective as the reverse phase conditions for the separation of these compounds. On the other hand, in CEC, the use of MeOH/H_2O, 5 mM ammonium acetate buffer pH 6.0 (90/10, v/v) was very effective for the chiral resolution of flavanone and its methoxy and hydroxy derivatives.

The use of the SM-β-CD CSP in the nano-LC system allowed to achieve the separation of enantiomers and diastereoisomers of flavanones with good results.

Comparing the data about chiral resolution of these compounds with CSPs based on commercial silica gel derivatized with different β-CD derivatives in HPLC [43, 45] and nano-LC [3], it can be concluded that, in general, higher enantioresolution and shorter analysis time can be obtained with the CSP prepared and evaluated in this paper. Moreover, due to the high surface area and ordered pore structure of the OMSs, symmetric peaks with little peak broadening could be achieved with the CSP developed. In summary, these studies demonstrated that OMSs possess a promising potential as supports to develop CSPs for nano-LC and CEC applications.

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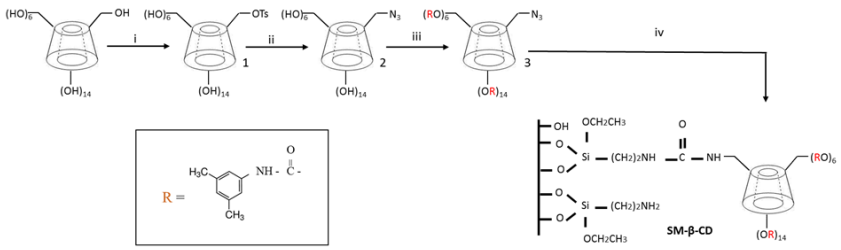
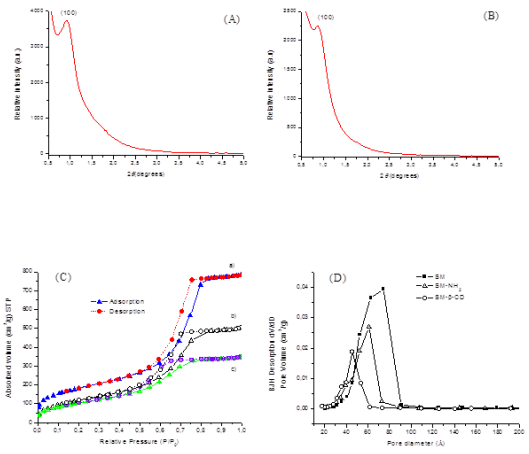
Legend

Figure 1 - Preparation of SM-β-CD. (i) Tosyl chloride/pyridine/18h room T[a]; (ii) NaN_3/water/18h 86ºC; (iii) 3,5-dimethyl phenyl isocyanate/pyridine/8h room T[a]; (iv) amino functionalized SM/PPh_3/THF/CO_2.



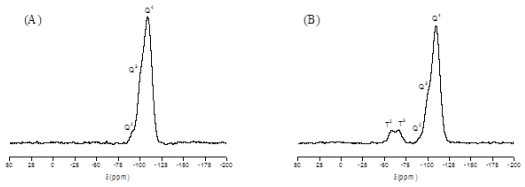
Fanali, Figure 1

Figure 2 A, B, C and D - (A) XRD pattern of SM. (B) XRD pattern of SM-β-CD. (C) N_2 adsorption-desorption isotherms of a) SM, b) SM-NH_2, c) SM-β-CD. (D) Pore size distribution of the mesoporous silicas.



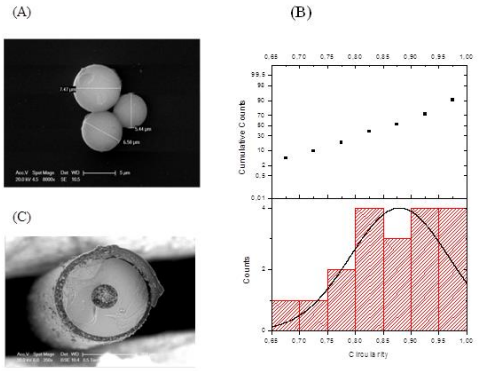
Fanali, Figure 2

Figure 3 - ^{29}Si MAS NMR of (A) SM and (B) SM-β-CD.



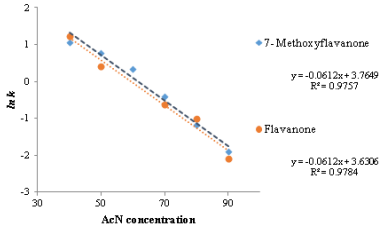
Fanali, Figure 3

Figure 4 A and B - (A) SEM images of SM. (B) Particle circularity factor of SM. (C) SEM image of cutting section of 100 µm I.D. capillary column packed with SM-β-CD.



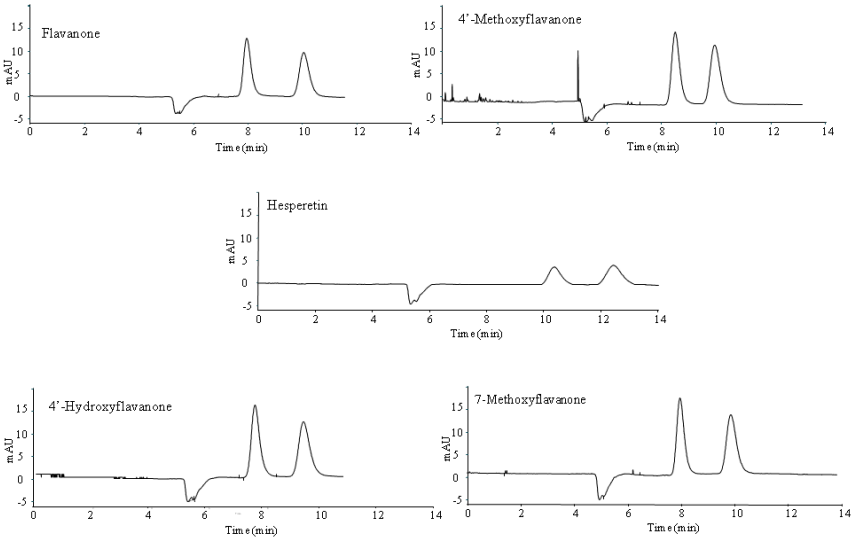
Fanali, Figure 4

Figure 5 - Linear relation recorded plotting AcN concentration *vs ln k* for 7-methoxyflavanone and flavanone. Analyzed by nano-LC employing a capillary column packed with SM-β-CD under reversed phase conditions (mobile phase AcN/H_2O,10 mM ammonium acetate pH 4.5).



Fanali, Figure 5

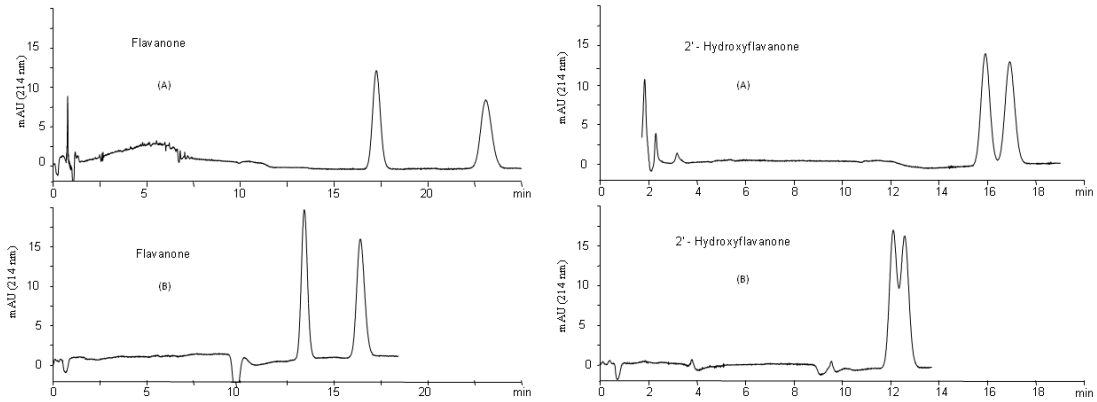
Figure 6 - Enantioseparation of selected flavanones analyzed by nano-LC employing a capillary column packed with SM-β-CD under reversed phase conditions (mobile phase MeOH/H_2O, 10 mM ammonium acetate pH 4.5 (90/10, v/v)). For other experimental conditions see Table 1.



Fanali, Figure 6

Figure 7 - Enantioseparation of selected flavanones analyzed by CEC employing a capillary column packed with SM-β-CD (A) MeOH/H_2O, 5 mM ammonium acetate pH

6.0 (90/10, v/v). (B) MeOH/AcN/H_2O, 5 mM ammonium acetate pH 6.0 (90/5/5, v/v/v). For other experimental conditions see Table 4.



Fanali, Figure 7

Table 1

Nano-LC enantiomeric separations of flavanones under reversed-phase condition using a mobile phase based on buffer and acetonitrile.

Compounds	t_{r1}	k_1	t_{r2}	k_2	R_s	α
Flavanone	38.40	3.41	42.30	3.86	1.80	1.10
4´- Methoxyflavanone	22.72	1.83	23.58	1.94	0.60	1.04
7- Methoxyflavanone	33.00	2.93	37.20	3.43	2.00	1.13
2´-Hydroxyflavanone	21.02	1.60	21.89	1.71	0.60	1.04
4´- Hydroxyflavanone	18.00	1.31	19.50	1.50	0.80	1.08
6- Hydroxyflavanone	16.00	0.98	-	-	0	
Hesperetin	13.24	1.11	13.78	1.19	0.50	1.04
Naringenin	15.70	1.57	-	-	0	

Nano-LC experimental conditions:

Capillary column packed with SM-β-CD, 100 μm I.D. x 20 cm packed length; Mobile phase: AcN/H_2O, 10 mM ammonium acetate pH 4.5 (40/60, v/v); flow rate, 281 nL/min; samples concentration, 100 μg/mL.

Table 2

Nano-LC chiral enantiomeric and diastereoisomeric resolutions of flavanones under reversed-phase mode using a mobile phase based on buffer and methanol*.

Compounds	t_{r1}	k_1	N_1/m	t_{r2}	k_2	N_2/m	R_s	α
Flavanone [a]	7.95	0.50	19025	10.04	0.89	16895	3.50	1.26
4´- Methoxyflavanone [a]	8.5	0.63	19175	9.94	0.91	16825	2.30	1.17
7- Methoxyflavanone [a]	8.64	0.61	17900	10.72	1.00	16195	3.1	1.24
2´-Hydroxyflavanone [b]	10.8	0.58	18030	11.64	0.7	16300	1.1	1.08
4´- Hydroxyflavanone [a]	7.52	0.47	16300	9.16	0.80	13835	2.7	1.22
6- Hydroxyflavanone [a]	7.52	0.37	13550	8.60	0.56	14190	1.6	1.14
Hesperetin [a]	10.4	0.96	12480	12.44	1.35	11900	2.2	1.20
Naringenin [c]	38.8	1.98	16970	43.50	2.35	18290	1.6	1.12
Hesperidin [c]	17.6	0.89	11380	20.00	1.15	10789	1.3	1.14
Naringin [c]	17.73	0.91	12579	19.47	1.10	11470	1.00	1.10

* Other experimental conditions as Table 1.

[a] Mobile phase: MeOH/H_2O, 10 mM amonium acetate pH 4.5 (90/10, v/v).

[b] Mobile phase: MeOH/ H_2O, 10 mM amonium acetate pH 4.5 (80/20, v/v).

[c] Mobile phase: MeOH/ H_2O, 10 mM amonium acetate pH 4.5 (70/30, v/v).

Table 3

Nano-LC chiral enantiomeric and diastereoisomeric resolutions of flavanones under polar-phase mode using a mobile phase based on methanol*.

Compounds	t_{r1}	k_1	t_{r2}	k_2	R_s	α
Flavanone	5.43	0.33	6.43	0.57	1.50	1.18
4´- Methoxyflavanone	6.31	0.37	7.05	0.53	1.70	1.12
7- Methoxyflavanone	5.74	0.37	6.73	0.60	1.20	1.17
2´-Hydroxyflavanone	4.92	0.23	5.04	0.26	0.45	1.02
4´- Hydroxyflavanone	5.98	0.33	7.07	0.57	2.00	1.18
6- Hydroxyflavanone	5.72	0.27	6.44	0.43	1.50	1.13
Hesperetin	16.59	2.77	18.23	3.14	1.00	1.10
Naringenin	16.21	2.77			0	
Hesperidin	7.63	0.62			0	
Naringin	6.70	0.52			0	

* Other experimental conditions as Table 1.

Table 4 Chiral separations by CEC

Compounds	MP: MeOH/H_2O, 5 mM ammonium acetate pH 6 (90/10, v/v)								MP: MeOH/ACN/H_2O, 5 mM ammonium acetate pH 6 (90/5/5, v/v/v)							
	tr_1	k_1	N_1/m	tr_2	k_2	N_2/m	Rs	α	tr_1	k_1	N_1/m	tr_2	k_2	N_2/m	Rs	α
Flavanone	17.24	0.66	39580	23.08	1.22	35990	6.24	1.34	13.40	0.41	37540	16.39	0.72	33550	4.29	1.22
4-Methoxyflavanone	19.29	0.86	37930	23.48	1.27	34360	4.14	1.22	15.75	0.71	34500	17.89	0.88	32010	2.63	1.14
7-Methoxyflavanone	19.31	0.86	36795	25.52	1.46	32080	5.67	1.32	15.13	0.60	33395	18.22	0.94	31955	3.75	1.20
2´-Hydroxyflavanone	15.91	0.53	38069	16.90	0.63	37850	1.34	1.06	12.08	0.28	24080	12.57	0.33	22785	0.67	1.04
4-Hydroxyflavanone	17.48	0.69	30158	22.48	1.17	27010	4.80	1.29	12.61	0.34	27550	14.98	0.59	25535	3.12	1.19
6-Hydroxyflavanone	17.40	0.67	38460	19.94	0.92	37320	3.00	1.15	12.60	0.34	26590	14.05	0.50	25495	1.96	1.12

CEC experimental conditions:

Capillary column, 100 µm I.D. packed for 23.0 cm with SM-β-CD, effective and total lengths 23.5 and 33.5 cm, respectively (the packing procedure is the same used for the nano-LC columns) ; MP: mobile phases (see the Table 4), negative polarity, applied voltage, -15 kV; capillary temperature, 25 °C, pressurized column at both ends with 10 bar; injection by pressure: 8 bar x 0.5 min. Samples were diluted in MP at a final concentration of 50 µg/mL.

Hesperetin, naringenin, naringin and hesperidin were also analysed in those conditions without detecting any signal.

A mobile phase of MeOH/H_2O, 5 mM ammonium acetate pH 6 (70/30, v/v) was also tested for the four mentioned compounds but no peaks were detected.