Talanta 85 (2011) 2330–2338

Contents lists available at ScienceDirect

Talanta

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Molecularly bonded chitosan prepared as chiral stationary phases in open-tubular capillary electrochromatography: Comparison with chitosan nanoparticles bonded to the polyacrylamide phase

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ARTICLE INFO

Article history:
Received 19 April 2011
Received in revised form 19 July 2011
Accepted 19 July 2011
Available online 16 August 2011

Keywords:
Capillary electrochromatography
Chiral stationary phase
Chitosan
Open-tubular
Silicon hydride

ABSTRACT

The chiral selector, chitosan (CS), was attached to the silanized capillary via a silane coupling agent, (3-glycidyloxypropyl)trimethoxysilane (GTS), to form the GTS–CS capillary, and results for this capillary were compared with those of a previous study on the copolymerization of CS with methacrylamide (MAA) (forming the MAA–CS capillary). The GTS–CS capillary did not exhibit enantioselectivity for D/L-tryptophan, whereas the GTS–BSA capillary, which was prepared by replacement of CS with bovine serum albumin (BSA), succeeded in the chiral separation with an Rs = 2.4 in Tris buffer (50 mM, pH 8.5). To increase CS attachment, the CS units were crosslinked by succinic acid, and the resulting GTS–CS–s capillary phase improved the resolution to 1.9. Alternatively, the SiH–CS–s capillary was constructed by CS attachment on the silicon hydride phase via stepwise silanization and hydrosilation reactions and crosslinking by succinic acid, but this approach could only achieve a resolution of 1.4 in Tris buffer (50 mM, pH 9.5). Although the GTS–CS–s and SiH–CS–s capillaries were still inferior to the MAA–CS capillary (Rs = 3.8), the enantioselectivities of the three capillaries were all in the range of 1.4–1.6. For the (±)-catechin sample, the plate heights of the GTS–CS–s and SiH–CS–s capillaries conditioned in pH 8.5 Tris buffer with 60% MeOH modifier were 0.9 cm ((−)-catechin) and 6.0 cm ((+)-catechin)) and 2.9 cm (−) and 3.2 cm (+), respectively, and these heights were comparable to the MAA–CS capillary (2.5 cm (−), 6.0 cm (+)) in pH 6.6 phosphate buffer with 80% MeOH. Finally, a racemate of ibuprofen, a weakly acidic anti-inflammatory drug, was successfully baseline resolved by the GTS–CS–s and SiH–CS–s capillaries in the borate buffers, which were 30 mM at pH 7.5 and 10 mM at pH 8.0, respectively.

1. Introduction

Due to the continued development of new chiral stationary phases (CSPs), chiral separation techniques have progressed considerably. Traditionally, the most frequently used chiral selectors among an enormous number of liquid chromatographic CSPs are native or derivatized amino acids [1,2], proteins [3,4], cyclodextrins [5,6], carbohydrates [7,8], macrocyclic antibiotics [9,10], and crown ethers [11,12]. In theory, CSPs or chiral selectors used in HPLC columns could be probably used in capillary electrochromatography (CEC); the packed, open-tubular (OT), and monolithic column technologies in CEC are well suited to the discovery of new phases with the proper column format, and there have been many successful implementations of CEC in chiral separations [13–16].

Due to the lack of phase ratios, the OT column format attracts less attention than the other two CEC column technologies. However, OT is a comparatively straightforward approach that does not require the arduous fabrication of frits, which are required in particulate-packed column construction, or the precise blending of monomer reagents with suitable porogens, which is required for monolith columns. Strategies to increase the loading of chiral selector include physical coating techniques, such as polyelectrolyte multilayer coating [17–19], layer-by-layer assembly [20,21], and high-affinity incorporation into biolayers [22,23], and chemical bonding techniques, such as the sol–gel method [24,25], treatment of the capillary wall with carbosilane dendrimers [26], and bonding of proteins [27,28] and molecular imprinted polymer [29,30].

In our previous study, nano-sized chitosan (CS, poly-β-(1,4)-2-acetamido-2-deoxy-D-glucopyranose, a functional, linear polysaccharide) exhibiting favorable surface-to-volume ratios was copolymerized with methacrylamide monomer (MAA) and bisacrylamide crosslinker to create an OT–CEC column. This MAA–CS capillary exhibited an increasing phase ratio and promising chiral separations of tryptophan, catechin, and α-tocopherol [31]. Because of its specific basic properties, CS and its derivatives have mainly been used as coating reagents that are adsorbed on the bare

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doi:10.1016/j.talanta.2011.07.091

capillaries to separate bioactive molecules using OT–CEC [32,33]. For enantiomeric CEC separations using CS-immobilized CSPs, a monolithic phase composed of sol–gel/organic hybrid materials containing CS and bovine serum albumin (BSA), which both possess chiral selectivity, has been the only successful example studied to date [34]. With regard to HPLC, the CS-immobilized CSPs have been successfully applied in some studies [35–37].

Previously, we showed the superiority of OT–CEC performance on polymeric, porous-layered phases over silica hydride, monolayered phases when using the same achiral functional moiety, such as multi-walled carbon nanotubes [38] and the functional monomer succinyl methacrylate [39]. This result was achieved because the amount of the functional moiety attached to the polymeric phase is higher than that to the monolayered phase. In this study, two monolayered phases were prepared with crosslinking CS units to increase attachment of the functional moiety, and the chiral resolutions of D,L-tryptophan, (±)-catechin, and (±)-α-phenylcarboxylic acids were compared with those of the monolayered phase without CS crosslinking, the BSA-immobilized monolayered phase, and the MAA–CS phase. The electrochromatographic parameters of the chiral samples separated on these capillaries were obtained, and the chromatographic discrimination in CEC was further discussed.

2. Materials and methods

2.1. Reagents and chemicals

Most chemicals were of analytical or chromatographic grades. Chitosan (CS; from shrimp shells, practical grade, ≥75% deacetylated), (3-glycidyloxypropyl)trimethoxysilane (GTS), triethylamine, triethoxysilane (TES), glycidyl methacrylate (GMA), chloroplatinic acid (Spiers catalyst, H_2PtCl_6), bovine serum albumin (BSA), sodium tetraborate, phosphoric acid, sodium dihydrogenphosphate, hydrochloric acid, acetonitrile (ACN), and dimethylsulfoxide (DMSO) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Boric acid, acetic acid, ammonium carbonate, methanol, 1,4-dioxane, tetrahydrofuran (THF), and potassium acetate were obtained from Panreac (Barcelona, Spain). Sodium hydroxide, isopropanol, succinic acid, polyvinyl alcohol (PVA), disodium hydrogenphosphate, trisodium phosphate, citric acid, sodium dihydrogen citrate, disodium hydrogen citrate, and trisodium citrate were supplied by Merck (Darmstadt, Germany). Acetone, toluene, and sodium acetate were obtained from J.T. Baker (Phillipsburg, NJ, USA). Tris(hydroxymethyl)aminomethane (Tris) was obtained from TEDIA (Fairfield, OH, USA). The chemical 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (CDI) was obtained from Acros (Thermo Fisher Scientific, Geel, Belgium).

The stereoisomeric tryptophan samples (L, D, and DL forms), catechins, (+)-(2R,3S)- and (−)-(2S,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol, R(−)- and S(+)-2-phenylpropionic acids, and ibuprofens (S(+)-2-(4-isobutylphenyl)propanoic acid and racemic form) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Sample concentrations were 1.0 mg/mL for tryptophan, 2-phenylpropionic acid, and ibuprofen in H_2O and 25 μg/mL for catechin in MeOH. Purified water (18 MΩ cm) from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare samples and buffer solutions.

2.2. Instrumentation

The laboratory-built electrophoresis apparatus consisted of a ±30 kV high-voltage power supply (TriSep TM-2100, Unimicro Technologies, CA, USA) and a UV–Vis detector (LCD 2083.2 CE, ECOM, Prague, Czech Republic). Electrochromatograms were recorded using a Peak-ABC Chromatography Data Handling System (Kingtech Scientific, Taiwan).

2.3. Preparation of capillary columns

The CS immobilization reactions involved in the preparation of the GTS and the silica hydride derivative stationary phases are illustrated in Fig. 1(A) and (B), respectively. The preparation of the MAA–CS capillary was described previously [31].

2.3.1. Preparation of the GTS–CS, GTS–CS–s, and GTS–BSA capillaries

The preparation of GTS silanized phases proceeded according to previously described protocols [40]. In brief, a new, bare capillary column (Polymicro Technologies, Phoenix, AZ, USA) with a 375-μm O.D. × 75-μm I.D. was treated with 1.0 M NaOH and successively washed with pure water, 0.1 M HCl, pure water, and then acetone. The clean, bare capillary was then continually flushed with a solution composed of 10% (v/v) silanization agent (GTS) and 1% (v/v) triethylamine in dry toluene for 3 h at 35 °C. This capillary was kept at room temperature for 24 h to undergo the silanization, which was completed after a series of rinses with toluene and acetone, and this process prepared the capillary for the epoxide ring-opening reactions, as shown in Fig. 1(A).

A 10-mL aqueous solution containing CS (10 mg), hydrochloric acid (2%, v/v), and PVA (10%, w/v) was filled to the prepared, silanized capillary, and the capillary was sealed with septa at both ends. The reaction proceeded in an oven at 60 °C for 6 h, and the resultant GTS–CS capillary was washed extensively with water until the outlet solution reached pH 7.0; next, the capillary was washed with acetone for 15 min and finally blown dry with nitrogen.

For the synthesis of the GTS–CS–s capillary, the prepared CS–s solution replaced the CS solution, and other modification procedures followed those for the GTS–CS capillary. A 10-mL CS–s solution was prepared by dissolving succinic acid (8 mg) in water and then adjusting the solution pH to 6.5 with 0.1 M sodium hydroxide solution. After the addition of CDI (40 mg) condensation agent, the mixture was stirred at 4 °C for 30 min and subsequently mixed with chitosan (10 mg) at room temperature.

For the GTS–BSA capillary, the silanized capillary was filled with BSA solution (0.4 g/mL) and kept at 37 °C for 24 h. The subsequent preparation procedures were the same as those described for the GTS–CS capillary.

2.3.2. Preparation of SiH–CS–s capillary

Some of the following fabrication processes were employed according to column preparation methods described previously [41–43], and the main steps are shown in Fig. 1(B). In brief, the clean, bare capillary was filled with 1.0 M TES in dioxane and then heated for 1.5 h at 90 °C to attach the silica hydrides to the capillary wall surfaces. Following a series of washings with 50% THF solution, THF, and toluene, the resulting silica hydride capillary was filled with a solution that contained 2 mL of GMA, 4 mL of toluene, and 70 μL of Spiers catalyst (H_2PtCl_6) in isopropanol (10 mM) to undergo the hydrosilation reaction. After standing for 15 min at ambient temperature, the mixture was purged with nitrogen at 30 psi for 30 min to leave a thin layer of GMA solution primed to react with the silica hydride when the capillary was placed in an oven at 100 °C for 16 h. Finally, the reacted capillary was washed successively with toluene, THF, and acetone for 30 min, which prepared it for the next epoxide ring-opening reaction with CS–s solution as described in Section 2.3.1.

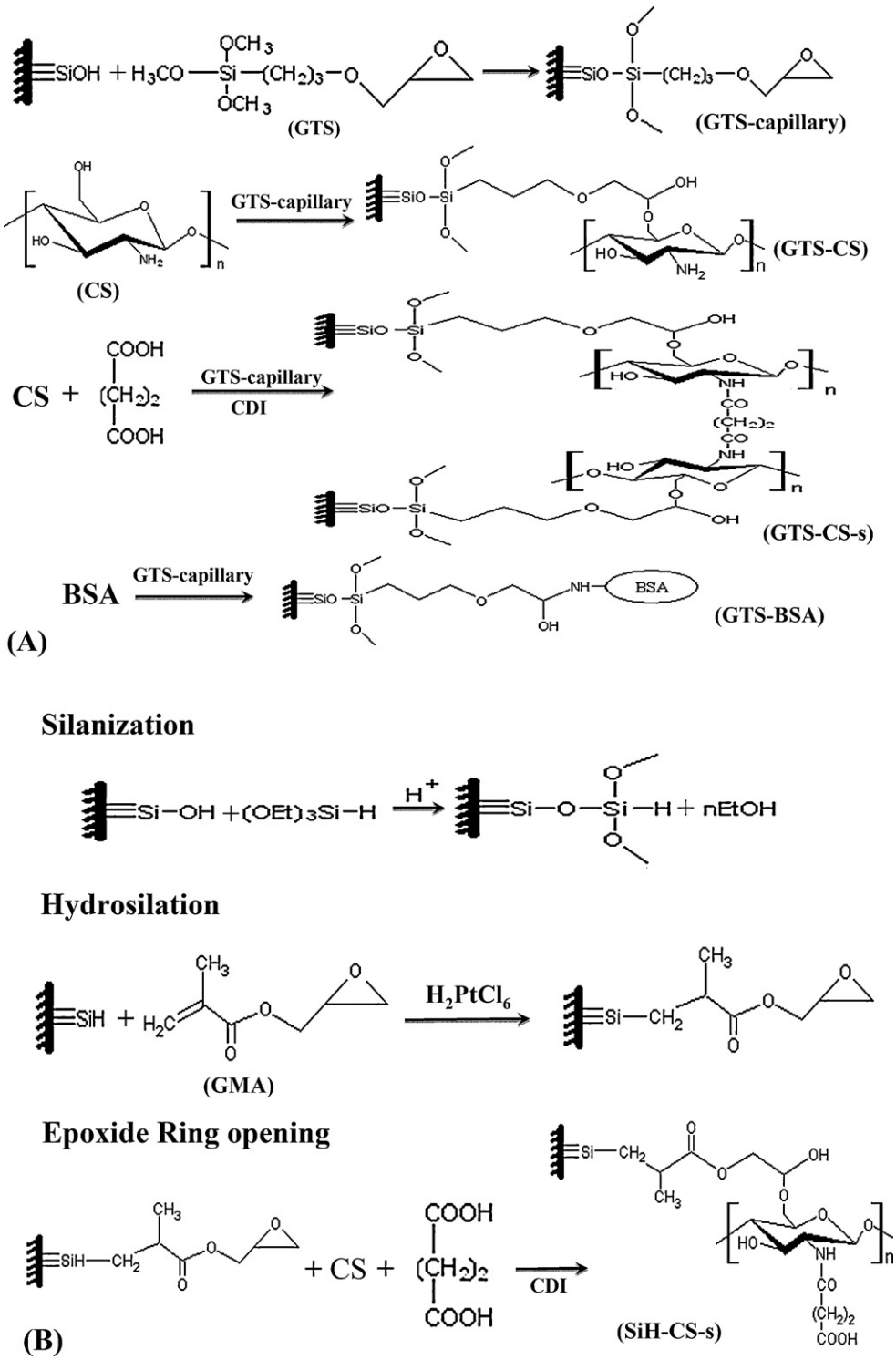

Fig. 1. Schemes to synthesize the GTS–CS, GTS–CS–s, and GTS–BSA capillaries (A); and the SiH–CS–s capillary (B).

2.4. CEC conditions

Most experiments were conducted using the common CZE buffers of Tris, acetate, citrate, phosphate, ammonium carbonate, and borate buffers within a pH range of 5.0–10.5 and an ionic concentration range of 10–300 mM. ACN and MeOH were added to the buffers as organic modifiers. All prepared buffer solutions for CEC analysis were filtered through a 0.45-μm cellulose ester membrane (Adventec MFS, Pleasanton, CA, USA). DMSO and MeOH were used as the neutral markers for the separation of tryptophan and catechin, respectively. The studied capillary was sequentially washed with water (or methanol for catechins) and running buffer between each analysis run. Prior to sample injection, a working voltage was applied for 5 min to condition the charge distribution in the column. The prepared test samples were introduced by siphoning using a height difference. The samples were detected by UV light absorption measurements at 214 nm for DMSO, tryptophan, 2-phenylpropionic acid, and ibuprofen, and 280 nm for catechin.

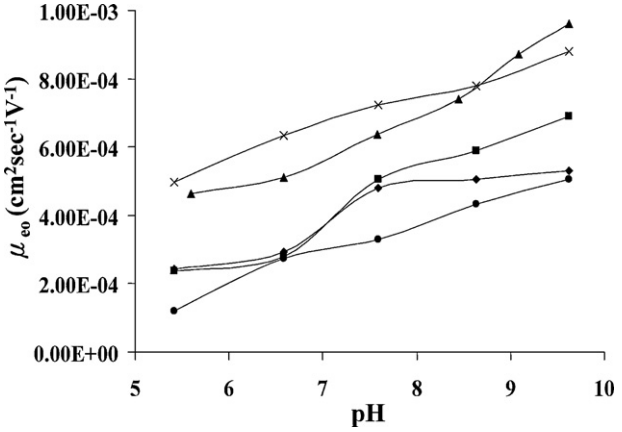

Fig. 2. Dependence of electroosmotic mobility on buffer pH, ionic strength, and the ratio of organic modifier. Columns: (▲) a bare fused-silica capillary; (●) the GTS–CS capillary; (♦) the GTS–CS–s capillary; (■) the SiH–CS–s capillary; and (X) the MAA–CS capillary. Conditions: BGE, phosphate buffer, 50 mM; neutral marker, DMSO; hydrostatic injection, 5 cm, 2 s; applied voltage, 15 kV; detection, 214 nm.

3. Results and discussion

3.1. Measurements of EOF for the monolayered phases

Before the CS-immobilized capillaries were utilized for chiral analyses, the EOF driven by the capillaries under buffers of different pH was characterized to determine the EOF magnitude that contributed to solute migration in the CEC and to examine some of the chemical properties of the modified capillaries. The curves shown in Fig. 2 illustrate the dependence of μ_{eo} on the pH of the phosphate buffer for the bare fused-silica capillary, the GTS–CS capillary, the GTS–CS–s capillary, the SiH–CS–s capillary, and the MAA–CS capillary, which was discussed in a previous study and is used here for comparison [31].

The curve pattern of the GTS–CS capillary was quite similar to that of the MAA–CS capillary. Although the two curves were not identical to that of the bare capillary, the effect of the residual silanol groups on the surface charges of the two capillaries must be considered; this effect is observed in their μ_{eo} increasing values with increasing buffer pH. The silanization of the bare fused-silica capillaries was not completed using γ-MAPS or GTS silane coupling agents before coating with the two CS materials. In addition, the two curves are somewhat humped near pH 6.5, which could be correlated to the deprotonation of primary amine in the CS structure ($pK_a = 6.5$ [44]). Similar cathodic EOF patterns have been observed in CEC columns with immobilized polysaccharides [45,46]. Compared to the MAA–CS capillary, the GTS–CS capillary exhibited low μ_{eo} values in the entire studied pH range. Due to conversion of the amine groups on the nano-CS to amide derivatives upon reaction with *m*-methyl benzoic acid, the positively charged amines that formed on the MAA–CS phase surface could be largely reduced. In contrast, the CS materials were used directly for bonding with the GTS-derivatized capillary without amidation. Another possible reason for the observed difference μ_{eo} is that most of the CS units were embedded in the polyacrylamide network in the MAA–CS capillary, but those units in the GTS–CS capillary were free molecularly bonded on the capillary wall surface to greatly increase the surface charges.

The EOF curve pattern of the GTS–CS–s capillary was totally unlike that of the GTS–CS capillary. The major difference in the synthesis processes between the two columns was the inclusion of succinic acid in the CS solution prepared for the attachment of CS to the GTS-treated surface through the epoxide-ring opening reaction. Succinic acid is characterized by dicarboxylic acid moiety, which could act as a crosslinking agent between CS units through amidation. The crosslinked product is illustrated in Fig. 1(A). The crosslinking reaction might result in a layer of CS units over the capillary surface that shielded the residual silanol groups. As shown in Fig. 2, the GTS–CS–s capillary reaches an asymptotic value at higher pH levels (above 7.5). Here, the sheltered silanols could not affect the μ_{eo} values, but some of the CS moieties bearing carboxylic acid groups, which are derived from succinic acid via an incomplete crosslinking reaction, dominate the EOF. Examination of the entire curve pattern reveals that the dissociation of the carboxylic acid groups in the GTS–CS–s phase seems to start near pH 6.5 and cease above pH 7.5.

In comparison with the GTS–CS–s capillary, the EOF curve of the SiH–CS–s capillary exhibits a similar dependence of the μ_{eo} values on the buffer pH below 7.5. Succinic acid would also react with the primary amines of the CS molecules in the SiH–CS–s phase and yield the product shown in Fig. 1(B). However, the curve in the region above pH 7.5 indicates that the residual silanols still influence the μ_{eo} values, which increase with increasing pH, as previously observed for the GTS–CS and MAA–CS capillaries. The amount of CS loading on the SiH–CS–s capillary could be so low that the products of the crosslinking reaction between the CS units was not sufficient to shield the silanols. There should be a difference in the amount of CS loading between the SiH–CS–s and GTS–CS–s capillaries as a result of the divergent schemes for synthesizing them. Obviously, two derivation steps, silanization and hydrosilation, were needed to introduce GMA onto the SiH–CS–s capillary, but only one step was needed to introduce GTS on the GTS–CS–s capillary. Consequently, fewer epoxide rings would be attached to the SiH–CS–s capillary than the GTS–CS–s capillary. In spite of the reactivity difference of the epoxide-ring opening between GMA and GTS, the hydrophilic CS molecules prefer to approach a silicon oxide-based phase to react with the epoxide rings rather than the less-polar silica hydride-based phase.

The reproducibility of the capillary fabrication was evaluated using the μ_{eo} values measured at pH 7.6 for five runs of the GTS–CS, GTS–CS–s, and SiH–CS–s capillaries. The RSD values were 4.4 (±0.5), 3.6 (±0.3), and 4.2 (±0.5)%, respectively, for three replicate capillaries. At the 95% confidence level, no significant differences between the replicate columns were observed by Student's *t*-test.

3.2. Separation of tryptophan enantiomers

Tryptophan enantiomers were used as chiral probes to assess the CEC enantioselectivity of all of the modified capillaries. After testing several types of buffers (described in Section 2.4), the best peak shape and resolution of the racemic tryptophans were achieved with a Tris buffer system. For the GTS–CS–s capillary, the resulting electropherograms shown in Fig. 3(A1), (B1), and (C1) used Tris running buffers at a fixed concentration (50 mM) with pH values of 7.5, 8.5, and 9.5, respectively. Fig. 3(B1) shows that the buffer at pH 8.5 yielded a much more satisfactory resolution than that at pH 7.5 or pH 9.5. Changing the buffer concentration to 10 and 100 mM verified the suitability of using 50 mM buffer concentration. Thus, we observed that CS–s conjugated with GTS is a potential component for CSP as long as proper buffer conditions are used.

Based on the same silanization reaction with the GTS reagent as the synthesis of the GTS–CS–s capillary, the GTS–BSA capillary was prepared using BSA as a replacement for CS–s in the epoxide-ring opening reaction. The GTS–BSA capillary was also capable of chiral separation of tryptophans, as shown in Fig. 3(A2) and (B2). In addition to direct addition to BGE as a chiral pseudo stationary phase [47], BSA had been attached to CEC phases via several methods, such as adsorption on silica-based ion exchangers [48], phospholipids [23], and Au nanoparticles [49], sol–gel encapsulation [34], and silanization [24] that used 3-aminopropyltrimethoxysilane and glutaraldehyde reagents. Compared to the

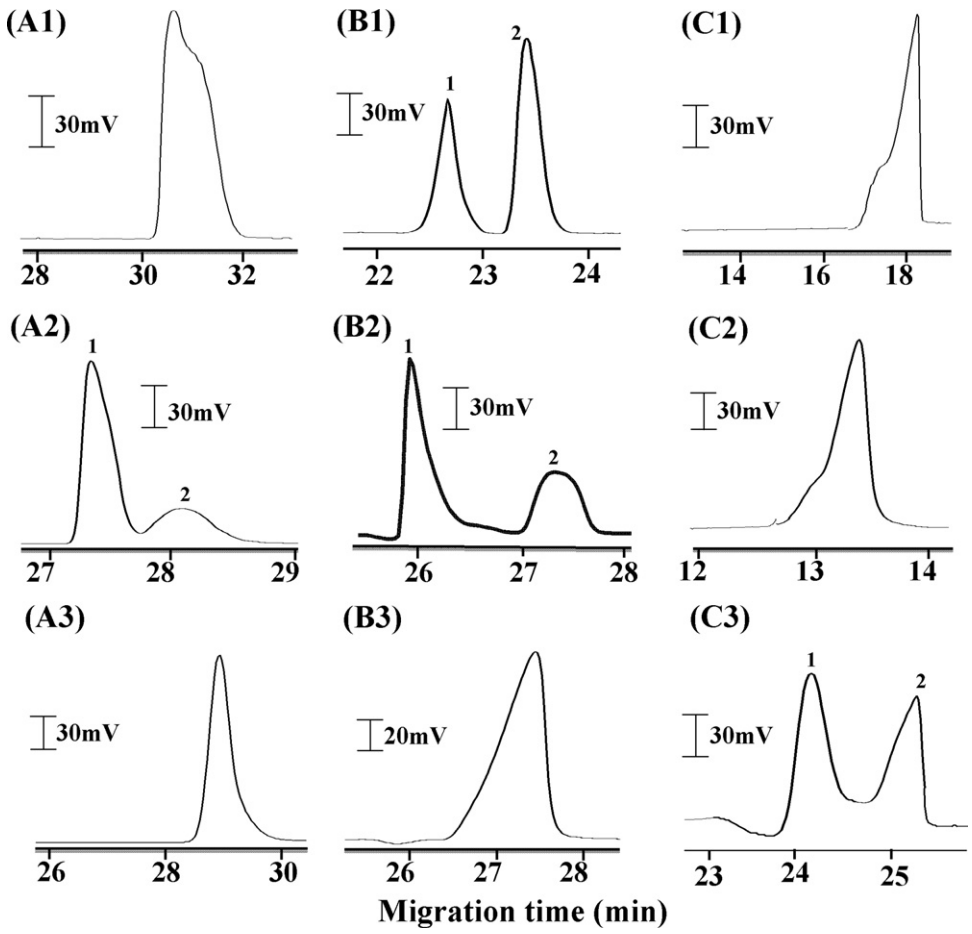

Fig. 3. Enantioseparations of the tryptophans in the GTS–CS–s, GTS–BSA, and SiH–CS–s capillaries. Columns: (1) GTS–CS–s (60 cm (55 cm) × 75 μm I.D.); (2) GTS–BSA capillary (60 cm (55 cm) × 75 μm I.D.); and (3) SiH–CS–s capillary (65 cm (60 cm) × 75 μm I.D.). BGE: Tris buffer (A) 50 mM, pH 7.5; (B) 50 mM, pH 8.5; and (C) 50 mM, pH 9.5. The applied voltage was 15 kV. Samples: hydrostatic injection of 15 cm for 10 s and detection at 214 nm. Peaks correspond to (1) D-tryptophan and (2) L-tryptophan.

two-step aldehyde-terminated silanization [24], this method based on one-step epoxide-terminated silanization using GTS reagent was easier and more efficient for BSA attachment onto the silanized capillaries. The chiral resolution for tryptophan in the reference capillary, Rs = 0.71, is less than that in the GTS–BSA capillary, Rs = 2.4. It is believed that CS and its derivatives could also be efficiently attached to the GTS-modified capillary.

In contrast to the GTS–CS–s capillary, the electropherograms of the GTS–CS capillary did not show any enantioselectivity. As discussed in Section 3.1, the addition of succinic acid to the CS solution would cause the amidation of CS amine groups prior to the crosslinking reaction. Through the crosslinking reaction with the bridging reagent, succinic acid, more chiral CS units were incorporated into the GTS–CS–s capillary than into the GTS–CS capillary. The lack of CS loading would cause a great reduction in chiral resolution in the GTS–CS capillary. Without the addition of succinic acid, the C-2 carbons of the D-glucosamine repeat unit in the CS structures were simply attached with primary amines. The functional groups attached to the C-2 position are distinctly different between the GTS–CS and GTS–CS–s capillaries and could also be responsible for the divergent resolution.

The enantioselectivity of the SiH–CS–s capillary for tryptophan was determined at pH 9.5 in Tris buffer, as shown in Fig. 3(C3). Tryptophan, which has a pI of 5.9, was ionized either at pH 8.5 or at pH 9.5. The difference in optimum pH levels between the SiH–CS–s and GTS–CS–s capillaries might be caused by the difference in phase matrices, where the attached CS molecules were treated identically with succinic acid. As discussed in Section 3.1, the amount of CS attached to the SiH–CS–s capillary was less than that attached to the GTS–CS–s capillary. Accordingly, the SiH–CS–s capillary exhibited poorer resolution (Rs = 1.4, Fig. 3(C3)) than the GTS–CS–s capillary (Rs = 1.9, Fig. 3(B1)).

Differentiating between the electrophoretic and chromatographic contributions to the CEC separation is essential, particularly in this study, which focuses on the chiral selectivity induced by the fixed CS molecules. Adopting the definition formulated by Rathore and Horváth, measurements of electrophoretic migration and chromatographic retention in CEC can be described by a velocity factor (k_e'') and a retention factor (k''), respectively [50,51]; these terms are expressed in Eqs. (1) and (2):

$$k_e'' = \frac{\mu_{ep}}{\mu_{eo2}} \quad (1)$$

$$k'' = \frac{t_{M2} \times 1 + k'' - t_{02}}{t_{02}} \quad (2)$$

where μ_{ep} and μ_{eo2} are the electrophoretic and electroosmotic mobilities. These mobilities can be obtained from open-tubular CE experiments on a bare capillary (column 1) and from the CEC experiments on the Cs-immobilized capillary (column 2), respectively, as follows:

$$\mu_{ep} = \frac{L_1 \times L_{d1}}{V_1} \times \left(\frac{1}{t_{M1}} - \frac{1}{t_{01}}\right)$$

$$\mu_{eo2} = \frac{L_2 \times L_{d2}}{t_{02} \times V_2}$$

Table 1
Electrochromatographic parameters for enantiomeric tryptophans separated in CS- and BSA-immobilized capillaries.

Compounds	t_M (min)	k_e''	k''	α (= k_L''/k_D'')	N ($\times 10^4$)	H ($\times 10^{-3}$ cm)	Resolution
In the GTS–CS–s capillary conditioned as in Fig. 3(B1)							
D-Tryptophan	22.651	−0.63	0.07	1.6	3.8	1.4	1.9
L-Tryptophan	23.514	−0.63	0.11		4.4	1.3	
In the GPT–BSA capillary conditioned as in Fig. 3(B2)							
D-Tryptophan	25.919	−0.67	0.02	4.0	4.4	1.3	2.4
L-Tryptophan	27.463	−0.67	0.08		1.8	3.1	
In the SiH–CS–s capillary conditioned as in Fig. 3(C3)							
D-Tryptophan	24.246	−0.65	0.09	1.6	1.3	4.6	1.4
L-Tryptophan	25.388	−0.65	0.14		1.5	4.0	
In the MAA–CS capillary[a]							
D-Tryptophan	10.109	−0.44	0.22	1.4	12	0.46	3.8
L-Tryptophan	10.777	−0.44	0.30		19	0.29	

[a] CEC conditions: Tris buffer, 70 mM, pH 9.5, with 10% (v/v) MeOH; 15 kV; 60 cm (55 cm) capillary [31].

where L is the total column length, L_d is the distance between the inlet and the detection point, V is the applied voltage, t_M is the migration time of the solute, and t_0 is the migration time of neutral marker. The electrochromatographic parameters for the enantiomeric tryptophans analyzed in Fig. 3 and in the MAA–CS capillary are summarized in Table 1. As shown in Table 1, the k_e'' and k'' values indicate that the chromatographic selectivity with significant α values, defined as k_L''/k_D'', contributed to the enantioseparation, but the electrophoretic action with identical k_e'' values did not contribute to the enantioseparation. The α values observed for the CS-immobilized capillaries, including the GTS–CS–s, SiH–CS–s, and MAA–CS capillaries, ranged between 1.4 and 1.6 while the higher α value, 4.0, was observed for the GTS–BSA capillary. Thus, the chiral recognition of tryptophan exhibited by BSA seemed stronger than that exhibited by CS. Among the CS-immobilized capillaries, the MAA–CS capillary possessed the highest column efficiency and resolution while the SiH–CS–s capillary presented the lowest efficiency and resolution. Specifically, the greater the amount of CS units immobilized in the column, the faster the rate of stationary-phase mass-transfer and the higher the resolution.

3.3. Chiral separation of (±)-catechin

(+)-(2R,3S)-catechin and (−)-(2S,3R)-catechin belong to the flavonoid group and have different bioavailability and bioactivity [52,53]. Their separation can be achieved using cyclodextrin chiral selectors with HPLC, CE, and MEKC methods [54]. Using a CEC method with the GTS–CS–s capillary, the effect of the Tris buffer pH (100 mM, 60% (v/v) MeOH) on the chiral separation of these catechins was evaluated; the electrochromatogram collected at pH 8.5 (Fig. 4(B1)) showed better selectivity than those collected at pH 7.5 (Fig. 4(A1)) and pH 9.5 (Fig. 4(C1)). The migration times of the solvent and sample peaks decreased when the EOF magnitude was increased by increasing the pH of the running buffer. The sample

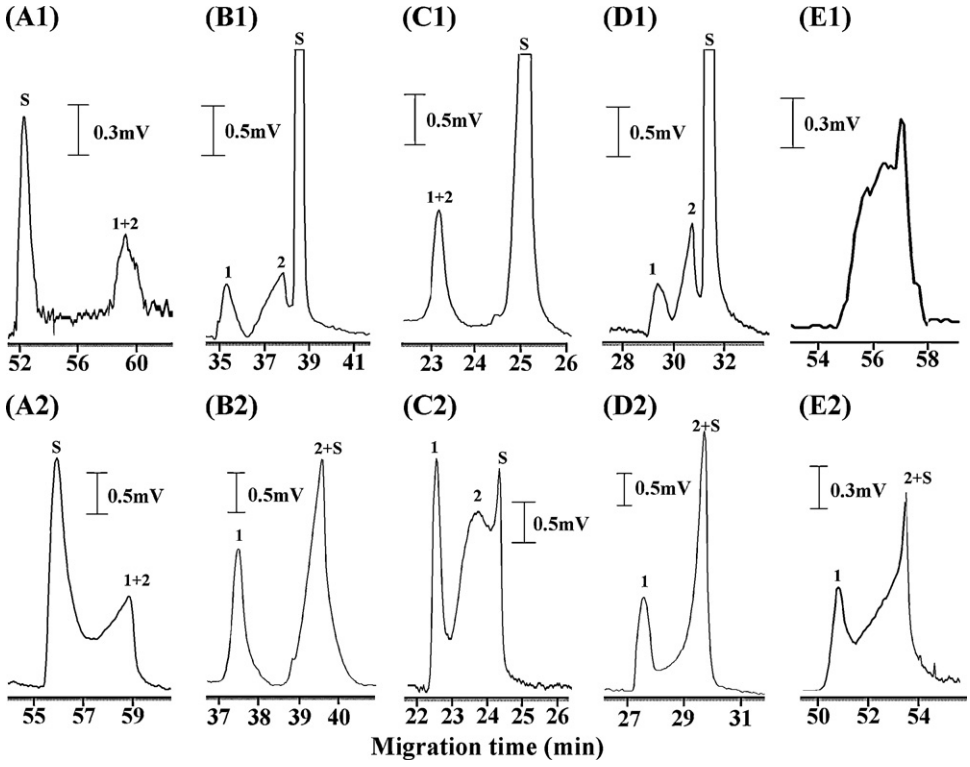

Fig. 4. Enantioseparations of the catechins in the GTS–CS–s and SiH–CS–s capillaries. (1) BGE used in the GTS–CS–s capillary (65 cm (60 cm) × 75 μm I.D.): MeOH (60%, v/v) mixed with Tris buffers, (A1) 100 mM, pH 7.5; (B1) 100 mM, pH 8.5; (C1) 100 mM, pH 9.5; (D1) 50 mM, pH 8.5; and (E1) 150 mM, pH 8.5. (2) BGE used in the SiH–CS–s capillary (60 cm (55 cm) × 75 μm I.D.): MeOH (60%, v/v) mixed with Tris buffers, (A2) 50 mM, pH 7.5; (B2) 50 mM, pH 8.5; (C2) 50 mM, pH 9.5; (D2) 30 mM, pH 8.5; and (E2) 100 mM, pH 8.5. The applied voltage was 15 kV. Samples: hydrostatic injection of 15 cm for 10 s and detection at 214 nm. Peaks correspond to (S) MeOH solvent, (−) catechin, and (+) catechin.

Table 2
Electrochromatographic parameters for stereoisomeric (±)-catechins separated in CS-immobilized capillaries.

Compounds	t_M (min)	k_e''	k''	α	N ($\times 10^4$)	H ($\times 10^{-3}$ cm)	Resolution
In the GTS–CS–s capillary conditioned as in Fig. 4(B1)							
(−)-Catechin	35.119	−0.02	−0.11	2.8 (k_-''/k_+'')	6.7	0.9	2.6
(+)-Catechin	37.781	−0.02	−0.04		1.0	6.0	
In the SiH–CS–s capillary conditioned as in Fig. 4(B2)							
(−)-Catechin	37.537	−0.07	−0.09	3.0 (k_-''/k_+'')	1.9	2.9	2.0
(+)-Catechin	39.835	−0.07	−0.03		1.7	3.2	
In the MAA–CS capillary[a]							
(−)-Catechin	17.642	0.01	0.19	1.8 (k_+''/k_-'')	1.7	2.5	3.0
(+)-Catechin	19.841	0.01	0.34		0.7	6.0	

[a] CEC conditions: phosphate buffer, 70 mM, pH 6.6, with 80% (v/v) MeOH; 15 kV; 45 cm (42 cm) capillary [31].

peak migrated slower than the solvent peak at pH 7.5 and migrated faster than the solvent peak at higher pH (pH 8.5 and 9.5). This behavior means that high pH levels could introduce significant electrostatic repulsions between anionic solutes and the negatively charged GTS–CS–s phase, and thus, chromatographic retention was likely involved in the separation mechanism. To resolve the overlapping peaks of solvent and (+)-catechin in Fig. 4(B2), adjustment of the ionic strength of the Tris running buffer to 50 and 150 mM was tested, as shown in Fig. 4(D1) and Fig. 4(E1), respectively, but this change failed to resolve the peaks. Here, the migration times for the solvent and sample peaks increased because the increase in the buffer concentration resulted in a decrease in the EOF due to compression of the double layer. In addition, a high buffer concentration would reduce electrostatic repulsion, and the migration of samples would consequently lag.

The effects of the pH and ionic strength of the Tris buffer on the chiral separation of the catechins were also studied for the SiH–CS–s capillary. As shown in Fig. 4(A2)–(E2), the issues concerning the effects of the buffer conditions are similar to those for the GTS–CS–s capillary, and the CEC conditions in Fig. 4(B2) were optimal although the solvent and (+)-catechin peaks were still not separated.

The electrochromatographic parameters for the (±)-catechin analyses in the GTS–CS–s, SiH–CS–s, and MAA–CS capillaries under their respective optimum conditions are collected in Table 2. For these runs, the electrophoretic action with identical k_e'' values did not contribute to the chiral separation while the chromatographic selectivity with discriminative k'' values did, as discussion in Section 3.2. Notably, in Table 2, negative values of k'' and k_e'' are observed for the GTS–CS–s and SiH–CS–s capillaries but positive values are found for the MAA–CS capillary. In the GTS–CS–s and SiH–CS–s capillaries, some of the carboxylic acid groups, which were derived from the reaction of succinic acid with CS, were dissociated to form the negatively charged phases and generate electrostatic repulsion with the anionic catechin (pK_{a1} = 8.16, pK_{a2} = 9.2, [55]) in the pH 8.5 buffers. The electrostatic repulsion results in negative k'' values, and the anodic electrophoretic migration counter to the cathodic electroosmotic flow results in negative k_e'' values. In contrast, the MAA–CS capillary possessed greater hydrophobicity due to the amidized amino end groups of the nano-sized CS, and the deprotonation of catechin was more restricted in the pH 6.6 buffers. Furthermore, the five hydroxyl groups of catechin could participate in hydrogen bonding with the amide groups of the polyacrylamide backbone to increase the chromatographic retention and the k'' values. The retention via hydrogen-bonding neither increased the chiral selectivity nor the column efficiency because the polyacrylamide backbone is not a chiral selector and would cause eddy diffusion and slow the stationary-phase mass-transfer rate. However, the high CS loading in the MAA–CS phase would compensate for these drawbacks to maintain a high resolution.

The effect of different volume ratios of MeOH in the Tris buffers on the chiral selectivity between the (+)- and (−)-catechins in the GTS–CS–s and SiH–CS–s capillaries was notable; the presence of the organic modifier not only altered the electrophoretic and electroosmotic flows, but it also affected the chromatographic partitioning between the catechin molecules and the stationary phases. The plots of the k'' values versus the percentage of MeOH modifier are shown in Fig. 5. The k'' values are observed to increase with the increase in the MeOH percentage within the BGE from 50 to 80%, and the optimum selectivity appears around 60% MeOH. The electrostatic repulsions can be weakened by the addition of MeOH modifier, which causes an increase in the pK_a values of the analytes and the ionizable groups on the phase and keeps the molecules in a more neutral form. Thus, the catechin molecules could be further retained by a normal-phase chromatographic mode, which is in agreement with similar results found for the MAA–CS capillary and in the polysaccharide CSPs [56].

3.4. Chiral separations of α-phenylcarboxylic acids

In addition to the samples of tryptophan and catechin, each of the two α-phenylcarboxylic acids, 2-phenylpropionic acid (PPA) and ibuprofen, were separately tested in the OT–CEC systems to increase the sample variety. The optimal results respective to the separations of PPA racemate in the GTS–CS–s and SiH–CS–s capillaries are shown in Fig. 6(A1) and (B1). With the aid of the cathodic EOF generated by a voltage of +25 kV, the migration of the anionic PPA (pK_a = 4.3) could be achieved within reasonable times in borate buffers (pH 7.5/30 mM in Fig. 6(A1) and pH 8.0/10 mM in Fig. 6(B1)). As discussed in Section 3.2, the SiH–CS–s capillary

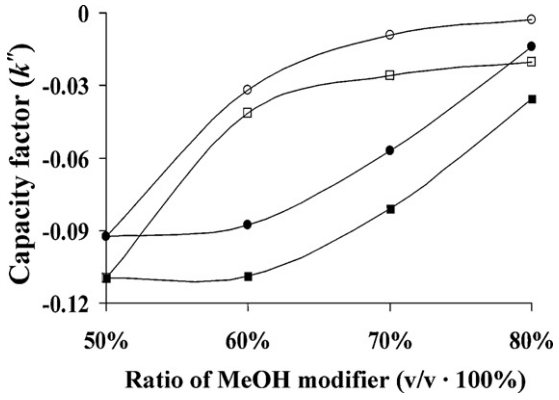

Fig. 5. Effect of the addition of MeOH into the Tris buffer on the retention factor (k'') of catechin enantiomers in the GTS–CS–s and SiH–CS–s capillaries. (■) and (□) represent the k'' values of (−)-catechin and (+)-catechin, respectively, observed under the conditions in Fig. 4(B1) in the GTS–CS–s capillary. (●) and (○) represent the k'' values of (−)-catechin and (+)-catechin, respectively, observed under the conditions in Fig. 4(B2) in the SiH–CS–s capillary.

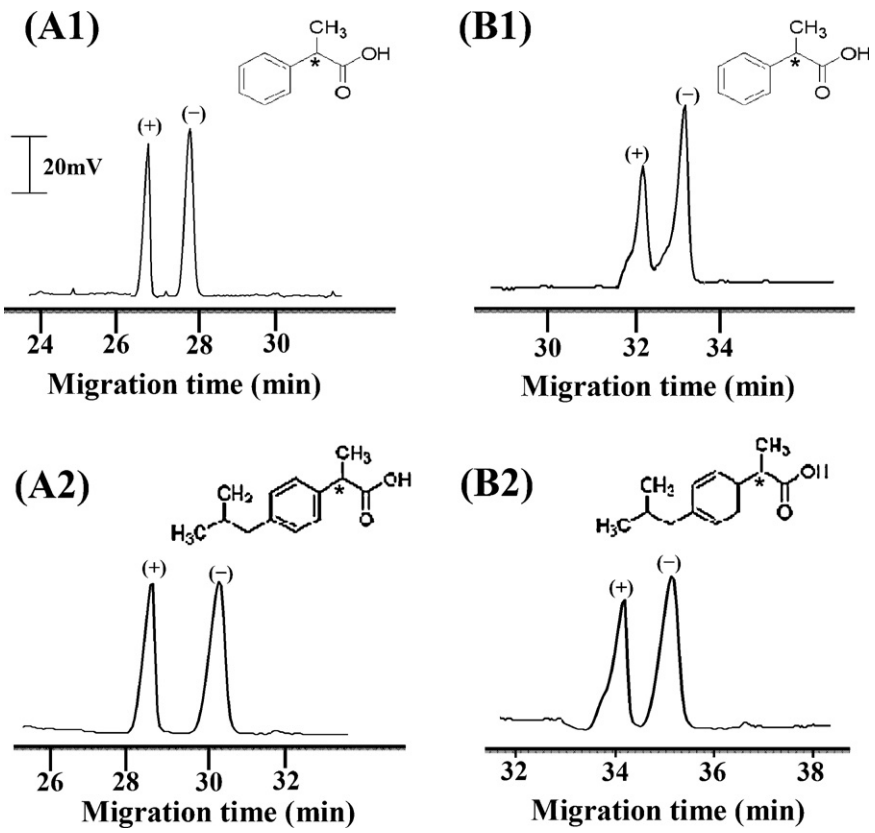

Fig. 6. Enantioseparations of α-phenylcarboxylic acids in the GTS–CS–s and SiH–CS–s capillaries. (A) BGE used in the GTS–CS–s capillary (60 cm (55 cm) × 75 μm I.D.): borate buffer, pH 7.5, 30 mM. (B) BGE used in the SiH–CS–s capillary (60 cm (55 cm) × 75 μm I.D.): borate buffer, pH 8.0, 10 mM. The applied voltage was 25 kV. Samples: hydrostatic injection of 10 cm for 5 s and detection at 214 nm. Samples: (1) 2-phenylpropionic acid; (2) ibuprofen.

having fewer attached CS units would exhibit poorer resolution than the GTS–CS–s capillary.

Ibuprofen, a derivative of PPA, is a nonsteroidal anti-inflammatory drug. Indeed, (*S*)-(+)-ibuprofen was found to be the active form both *in vitro* and *in vivo*. The electrochromatograms of racemic ibuprofen separated in the GTS–CS–s and SiH–CS–s capillaries using the same borate buffers are, respectively, shown in Fig. 6(A2) and (B2). In comparison with PPA, the resolution of ibuprofen was improved, but longer separation times were required. The isobutyl group in ibuprofen could contribute to the chromatographic retention in the CEC phases and increase the migration times, although the electrophoretic flow toward the injection (anode) end for the anionic ibuprofen ($pK_a = 4.4$) was slower than for PPA.

4. Conclusions

Based on the use of the different silanization agents, GTS and TES, two stepwise strategies to immobilize the CS chiral selectors on silanized capillary wall surfaces were provided in this study to compare their chiral separation abilities with that of the MAA–CS capillary, which copolymerized the CS moiety *in situ* with acrylamide monomers in a previous study. The crosslinking agent, succinic acid, was added with CS during the syntheses of the GTS–CS–s and SiH–CS–s capillaries to increase CS loading and the chiral resolution power; notably, no resolution of the tryptophan enantiomers was observed for the GTS–CS capillary without added succinic acid. Due to the higher CS loading, which was supported by the EOF measurements, the GTS–CS–s capillary exhibited better resolution than the SiH–CS–s capillary. Although the column efficiency and the resolution of the tryptophans separated in the two capillaries did not exceed those of the MAA–CS capillary, the performance of the GTS–CS–s capillary was close to that of the GTS–BSA capillary, in which the fixed chiral selector, BSA, demonstrated stronger chiral recognition of tryptophan ($\alpha = 4.0$) than CS ($\alpha = 1.4$–1.6). For the chiral separation of (±)-catechin, the electrostatic repulsions between the anionic catechins and the succinic acid-modified phases greatly affected the performance of the GTS–CS–s and SiH–CS–s capillaries while the performance of the MAA–CS capillary was hindered by the hydrogen-bonding between the five catechin hydroxyl groups and the polyacrylamide backbone. For the acidic racemates, 2-phenylpropionic acid and its derivative, ibuprofen, their chiral separations could achieve the satisfactory resolutions.

Acknowledgements

Support of this work by the National Science Council of Taiwan (NSC-98-2113-M-039-003-MY3) and by China Medical University (CMU 99-S-48) is gratefully acknowledged.

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