Enantioseparation with cationic β-cyclodextrin chiral stationary phases in supercritical fluid chromatography and high-performance liquid chromatography

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Chapter 1

General Introduction

1.1 Chiral drugs and compounds

The basic biological components such as amino acids, carbohydrates, proteins and polysaccharides in organisms are chiral and exist mainly in single enantiomeric form. Over the past three decades, stereoselectivity in drug action and disposition has become a well-recognized consideration in clinical pharmacology and development of chiral drugs. It is well established that chirality of drugs may influence significantly their pharmacological [1], toxicological [2], pharmacodynamic and pharmacokinetic [3, 4] properties. Therefore, it is important to obtain optically pure enantiomers of the racemic drugs. More than half of the marketed drugs are chiral [5]. Although not all the racemic drugs are marketed in optically pure form, the trend is towards marketing more single-enantiomer drugs [6].

The effects of chirality on drugs are now integrated into the process of drug development [7] and strict regulations have been demanded by US F.D.A. [8]. It is thus inevitable to investigate the necessity of developing optically pure enantiomer in new drugs' developments. This decision should be made based on sufficient scientific data. Only if the metabolic interconversion of enantiomers or the identity of the effects can be found, then the drug is allowed to be marketed as a mixture of enantiomers. Otherwise, it is necessary to obtain optically pure drugs. In those cases, the enantiomer with unwanted activity is considered as an impurity and efforts are devoted to detect and eliminate its presence.

In the past two decades, the development of asymmetric synthesis and

efficient techniques for analytical control of the enantiomeric composition of chiral compounds has made significant breakthroughs in the commercialization of single enantiomeric drugs [88-90]. However, asymmetric synthesis process usually requires involutedly protective and deprotective steps and tedious reaction conditions as well; the synthesis routes are not universally applicable. On the other hand, the enantioselective separation techniques are more practical and convenient to conduct. Thus, the enantioseparation techniques have become mature and popular in analysis and preparation of enantiomericly pure drugs [39, 91-93].

1.2 Enantiomeric separation technology preview

1.2.1 Development of chiral selectors

Enantioseparations have been studied extensively ever since Davankov's finding that ion exchange chromatography could be used for enantioseparation in the early 1970s [9]. Driven by the growth of asymmetric organic synthesis leading to chiral drugs, food additives, fragrances, agricultural chemicals and many other important chiral intermediates, the development of chiral selectors has grown rapidly. Many chiral selectors were developed and applied in various chiral resolution technologies. Firstly, Davankov *et al.* developed metal ion complexes for enantioseparations [9, 10]. After that, by linking small chiral molecules onto stationary phase, brush type chiral stationary phases were prepared [3, 5]. It is worthy to mention that Pirkle *et al.* developed the first commercial column with brush type chiral stationary phase for HPLC in 1981 [11]. Most recently, natural chiral macromolecules such as crown ethers [6-8], cyclodextrins [12, 13],

celluloses [14, 15], macrocyclic glypeptides [16], proteins [17, 18] as well as synthetic polymers [19] were modified for the application of enantioselective processes.

1.2.2 Development of chiral separation technologies

There are two emerging trends [20]: The first trend is the micro scaling of existing separation technologies, such as CE, CEC, microbore-LC, microbore-SFC, GC; the second trend is the development of chiral technology for large scale industrial applications wherein HPLC and SFC are most suitable to scale up. The following sections review the most commonly used chromatographic techniques for enantioseparations.

Gas chromatography (GC)

Gas chromatography has been widely employed for chemical analysis due to its high speed and efficiency. Volatile organic chemical samples are able to be evaporated at high temperature and the gaseous analytes are flushed through fused silica capillary with inert gas to achieve separations. GC analysis is the most efficient among chromatographic techniques. In many cases, the stationary phases in the capillary were modified to enhance the selectivity [15, 17-19]. The modifiers were either immobilized as a thin film on the capillary wall or deposited on the packing material. Amongst all the chemically modifiers, cyclodextrin derivatives were also applied as GC stationary phases by coating onto the packing material. Moreover, the cyclodextrin GC stationary phases are commercially available [21]. Efficient enantioseparations of chiral organic precursors were attained on

cyclodextrin GC phases [22-24]. Additionally, GC has also been used in preparative scale to separate enantiomers of anesthetic drugs and the separations were performed in the overloaded elution mode [25, 26]. However, the choice of applicable inert gas as mobile phase is limited and the solubility of most organic chemicals in the gaseous mobile phase is poor. GC analysis also requires good volatility as well as stability of the analyte at the operation temperatures. Therefore, analytes which can be analyzed in GC are quite limited. Although the GC analysis takes on most of the chromatographic merits such as high efficiency and analysis speed, a relatively narrow area is applicable [20].

Liquid chromatography (LC)

The separation modes in LC analysis can be varied among normal phase liquid chromatography (NPLC), reversed phase liquid chromatography (RPLC) and polar organic phase chromatography (POLC) determined by the relative polarities between the stationary phase and mobile phase. A comparable application of enantioseparations on a cyclodextrin (CD) based chiral stationary phase (CSP) showed that the sequence of the enantiomers eluted out could be reversed in different separation modes [27] which suggested that when CD based CSPs were used in HPLC, the enantioseparation mechanisms were altered in different separation modes. It is considered that in NPLC and POLC, the CD cavity is occupied by hydrophobic organic solvent. The enantioseparation is attained through the enantioselective interactions between substituents on the CD rim and functional groups on the analytes. In RPLC, on the contrary, the cavity is

vacant and ready to form hydrophobic inclusion with hydrophobic moiety of the analytes. Because the hydrophobic inclusion with the CD cavity is so efficient to enable enantioseparation for many racemates, CD based CSPs are usually applied in RPLC.

A comparison amongst all enantioseparation techniques showed LC was the most versatile method applied in chromatographic separations and the publications on LC in the area of enantioseparation were growing rapidly especially in recent years [28]. However, there are no universal CSPs or chromatographic conditions enabling all chiral molecules' enantioseparations. Small changes in the analytes' structures and/or chromatographic conditions would exert a strong impact on the enantioseparation results. Thus, the optimization on CSPs and chromatographic conditions in LC requires a lot of time [35, 36]. The application of multi-column parallel screening for HPLC condition optimization has successfully solved this problem [29]. As many parameters of chromatography in LC can be optimized, almost all racemates are able to be resolved at least in LC. Many reviews elucidated the applications of HPLC in evaluating new CSPs [30-33]; a broad range of pharmaceutical racemates such as amino acids [34], aromatic carboxylic acids [35], flavonoids [36] cardiovascular medicines (antihypertensives, antiarrhythmics, antianginals, diuretics), adrenergic drugs (vasopressors), anti-inflammatory and analgesic compounds, topical anesthetics, antihistanminics and antimalarial therapeutics [37] *etc.* were performed enantioseparations in HPLC. Excellent enantioseparation abilities are usually achievable in LC whilst separation

speed is relatively slower [20].

The conventional analytical scale HPLC is normally using 2.1 ~ 4.6 mm I.D. columns in enantioseparations. Because of the large column sizes, both the costs of columns and the consumptions of expensive and hazardous organic solvents are high. On the other hand, long analysis time, high cost and significant environmental issues of conventional HPLC incumber parallel and multidimensional analysis, which is nevertheless becoming increasingly important as the number and complexity of the samples being enlarged in recent years [29]. Those drawbacks could be remedied using miniaturized techniques recently developed in LC [38]. The nano-LC systems are usually applying capillary columns with I.D. between 10-100 μm and a flow rate of 50-700 nl/min. Compared to HPLC, nano-LC may offer a higher column efficiency, faster analysis, higher sample sensitivity and lower sample dilution [39]. The chiral selectors in nano-LC are attached onto stationary phases [40, 41] or added into the mobile phases [39, 42]. As capillary columns applied in nano-LC, consumptions of valuable chiral mobile phase additives could be reduced significantly [43]. Although nano-LC is widely employed in chiral analysis and enantioseparations, problems were noticed as the volume of sample should be strictly controlled in order to avoid sample volume overloading in the capillary columns [44]. Vissers et al brought forward a “column switching and large volume injection” scenario to solve the problem, wherein the sample was pre-concentrated in a column of higher I.D. using appropriate conditions, before it was transferred into the capillary

column [45]. An alternative method is “the on-column focusing” where the sample is directly concentrated in the analytical column [46]. Giovanni et al. employed the on-column focusing method and injected relatively high sample values (1500 nL) in the nano-LC which successfully increased the method sensitivity [47]. Because the nano-LC has shown the advantages of high sensitivities and low sample / chiral selector consumptions, this method is especially valuable in the field of food and beverages’ quality control [48] and in cases when expensive chiral selectors are employed [49].

The development of LC is currently heading onto nano-separation technology. Nanoparticles and nanostructured materials have been applied in the enantioselective LC. Higher efficiency as well as separation speed was attained; the two qualities are greatly demanded in the development of high throughput analytical methods [50]. Accordingly, ultra high performance liquid chromatography (UHPLC) was developed to overcome the high pressure drop generated by the submicro or nano particle packing. Wu *et al* have discussed the correlations amongst separation time, particle size and pressure drop as well as the practical problems in the pump system, the special injection system and the column packing material on UHPLC [51].

Supercritical Fluid Chromatography (SFC)

The application of supercritical fluid chromatography (SFC) as well as subcritical fluid chromatography to enantioseparations has been investigated in recent years [52-54]. CSPs which have been employed in the SFC conditions are

including Pirkle-type phases, cyclodextrin and cellulose derivatives [41-44]. Relatively less work has been done using chiral selector entrained in the mobile phase in SFC conditions because of the low solubility [55]. Since Carbon dioxide has relatively low critical temperature and pressure, it is the most commonly used mobile phase in SFC. In order to increase the mobile phases' eluting power, CO_2 is usually incorporated with polar organic solvents [56].

The polarity of CO_2 is often equated with *n*-hexane applied in LC condition. In this way, the conditions applied in NPLC and SFC are sometimes comparable. However, in comparative studies between HPLC and SFC, it was found enantioseparations attained in NPLC, POLC or RPLC could be entirely achievable in SFC, which suggested that the separation mode in SFC overlapped all the three separation modes in LC [27]. Additionally, polar modifiers such as methanol are able to be employed in SFC while they are immiscible with *n*-hexane in NPLC.

The mobile phase is mainly CO_2 in SFC and it has low viscosity. As a result, the solute in the mobile phase has higher diffusion coefficient in SFC than when it is in LC mobile phase. Accordingly, higher flow rate of the mobile phase can be applied to shorten the analysis time whilst column pressure is lower [27]. In supporting the development of large scale synthetic manufacture of chiral drugs, it is inevitable to highlight the efficiency of developing optimum chiral chromatographic modalities for specific products and the potential intermediates of increasing number. Higher flow rate and lower mobile phase viscosity conduce increased peak efficiency and higher resolution in SFC [57].

In contrast to LC where normally two or more solvents can be varied, mobile phase in SFC is CO_2 mixed with one organic solvent, normally only the organic modifier should be optimized. Thus, it would be much simpler for mobile phase optimizations in SFC. On the other side, in SFC, the system rapidly attains equilibrium status after changing chromatographic parameters. Consequently, the time needed for condition optimization in SFC is much shorter than in LC. As the efficiency of finding out optimal separation condition in the analytical grade analysis is highly demanded in the modern pharmaceutical industry, application of SFC instead of LC could effectively shave time off the schedule of a drug development program [58].

Moreover, SFC has higher sensitivity than LC. A comparative investigation between HPLC and SFC has shown that SFC enabled better separation and detection of impurities whereas the peak ascribable to small amount of impurity was invariably obscurred by the major ingredients' peaks in HPLC [56, 59]. SFC is widely employed in the pharmaceutical industry both in high throughput manufacturing and rapid analyses of drugs.

i) SFC is cost effective. The product is easily recoverable from the solvents after collection [60, 61];

ii) In the analytical chromatography, SFC readily attains equilibrium when changing mobile phase parameters, allowing for the optimization of the chromatography parameters in a shorter time frame [27, 62];

iii) Low viscosity of CO_2 diminishes the column pressure drop and thus a

higher flow rate can be applied. The solute in SFC mobile phase has a higher diffusion coefficient than in NPLC mobile phase, and the optimum linear velocity is shifted to a higher value. In many cases, the increase in flow rate can reduce analysis time without compromising efficiency [63];

iv) Previous comparisons between SFC and HPLC indicated the column efficiency in SFC is generally higher than in HPLC [57].

v) The work done by Wong *et. al* [61] demonstrated the difference between SFC and HPLC in method screening approaches on three different types of CSPs.

Although it was emphasized that the analytical SFC was not always better than HPLC in chiral selectivity for a certain kinds of racemates, the application of SFC led to direct scaling up to preparative chromatography

In recent years, 20-mm-diameter chiral columns are used by the pharmaceutical manufacturers. Pump systems can accommodate up to 100-mm-diameter columns. Accordingly, a facile scale up of the column dimensions would afford the advantage of lower cost in SFC compared with LC.

Capillary Electrophoresis (CE) & Capillary Electrochromatography (CEC)

In CE, the sample to be separated is placed in the electrolyte and an electric field is applied. The components carrying a negative charge migrate to the anode while those carrying a positive charge migrate to the cathode. The species in the interior of a capillary which is filled with electrolyte are separated based on their size to charge ratios. Many factors such as chiral selectors' concentration,

background electrolyte's pH and ionic strength [64], addition of organic solvents [65], injection mode [66] as well as temperature [67] could influence the enantioseparation results in CE.

In CEC, on the other hand, the capillary is filled, packed or coated with stationary phase. The movement of the mobile phase as well as analytes is usually driven by an applied electric field. Additional pressure is sometimes added for assisting analytes mobility. The retention of an analyte may be determined by electrophoretic migration and chromatographic retention [68]. Different from CE where mobility differences are the only factor determines separation, in CEC, modification on stationary phase improves chromatographic results as well.

Although CE and CEC are relatively new separation technologies, they have built up strong positions in enantioseparation analysis because of their high sensitivity and efficiency [69, 70]. The reproducibility of peptides analysis was amongst the first notable successes of CE. In addition, some acidic peptides which achieve enantioseparations in LC with difficulty are readily separated in CE using β-CD as chiral mobile phase additive [71]. It is reported that CE & CEC are especially efficient in charged analytes' enantioseparation [72-75].

1.3 Cyclodextrin's property and applications.

Cycodextrins are toroidal structural molecules. The *α-,β-,γ-* cyclodextrins consist of six, seven and eight *α*-(1,4)-linked D-(+)-gluco-pyranose units respectively. The images and dimensions of *α-, β-, γ*-cyclodextrins are illustrated in Fig. 1.1, while their physical and chemical properties are summarized in Table 1.1.

Table 1.1: Physical and Chemical Properties of Cyclodextrin Molecules [76]

cyclodextrin	No. of glucose units	Molecular mass(g/mol)	Cavity diameter(nm)	No. of stereogenic centers	Water solubility (g/100 ml)
α	6	972	0.49	30	14.5
β	7	1135	0.62	35	1.85
γ	8	1297	0.79	40	23.2

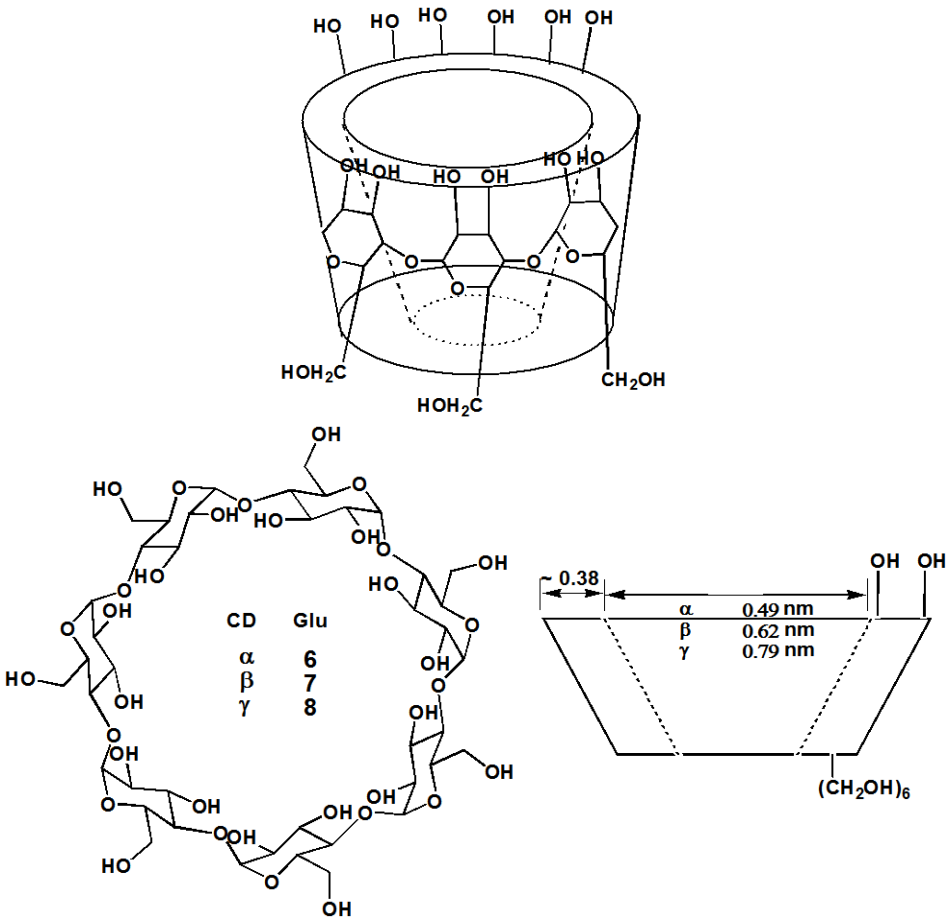


Figure 1.1: Three naturally occurring cyclodextrin molecules: α-, β-, γ-cyclodextrins

The special properties of cyclodextrins originate from their unique structures. Cyclodextrin contains a hydrophobic cavity and a hydrophilic surface. The hydrophobic cavity is able to entrap hydrophobic molecules. Entrapment and inclusion occurs without formation of chemical bonds. This peculiar property of cyclodextrins were useful in catalytic reactions and chromatographic separations [77, 78]. As illustrated in Fig. 1.1, three types of cyclodextrins (CD) have different

sizes. A general consideration is that small volume hydrophobic organic molecules form the most stable complexes with α-CD but the weakest with γ-CD. Secondly, neutral molecules generally bind more tightly with native CD than their charged equivalents. However, the stability of the complex formed in most cases, whether it is tight or loose, is not always straightforward and can be more complicated with functionalized CD [79]. A large number of reports on enantioseparations with CD derivatives in CE and LC have been published [31, 80] where the CDs are most frequently employed as chiral mobile phase additives (CMAs) because they have sufficient solubility in the mobile phase and limited UV absorbance. Nevertheless, in packed column SFC, the modifications of chiral selectors are usually on stationary phases and the CD-based CSPs are relatively less investigated. Most CSPs reported for SFC are polymer-multilayer polysaccharides [81]. In the analogical studies of immobilizing CD as CSPs, the CDs were bonded onto stationary phases with greater difficulty. For the simplification of preparation, polymerized CDs can be coated facilely onto silica particles and implemented as stationary phases, though the enantioseparations were attained only within a narrow range of conditions [82].

1.4 Mechanism of enantioseparation

The stereogenic difference between interactions of chiral compounds and a chiral receptor in the biological system was firstly explained by Easson and Stedman in 1933 [83]. As illustrated in Fig. 1.2, this is a statement on configuration-dependant three attractive contact point model. However, the

approach of chiral compound towards the receptor from interior condition is not allowed.

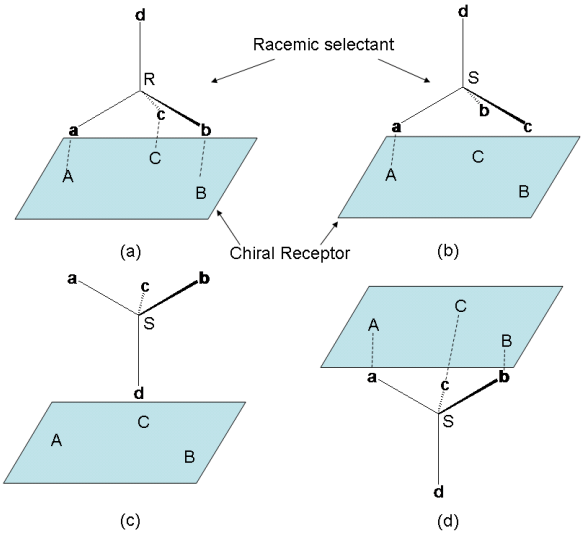


Figure 1.2: Easson-Stedman model (a, b, c) derived from an exterior approach of racemic selectant towards a chiral receptor. Each group on the chiral center of the selectant has a corresponding binding site on the chiral receptor. The interior approach of selectant (d) is not allowed.

To overcome the limitation of Easson-Stedman model, Topiol and Sabio brought forward a 4-contact point model in explaining the stereoselective binding of selectant enantiomers when the corresponding binding sites on the receptor were not in a plane (Fig. 1.3) [84]. Topiol also raised a more general criterion to explain that the stereogenic binding of chiral compounds and receptor relied on the inequality of the distance matrices of diastereometric complexes between enantiomers and a receptor [85]. Although all the models mentioned above have been used in explaining mechanisms in chromatographic enantioseparation, basically, “three-point interaction model” is the most prominent [86].

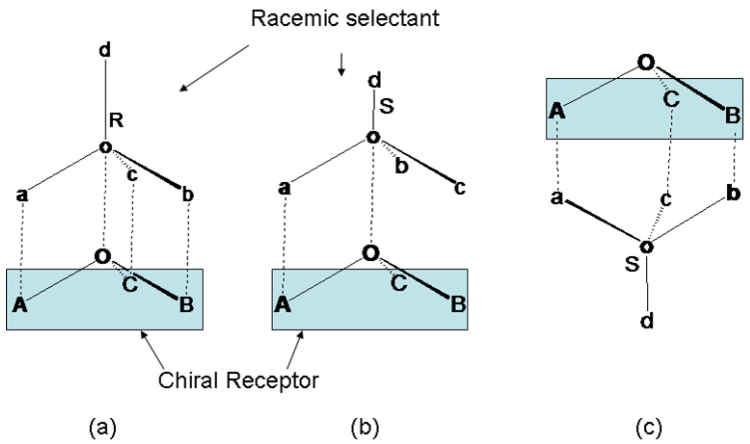


Figure 1.3: Topiol-Sabio 4-contact point model allows stereogenic bindings when selectants approach towards chiral receptors from both exterior (a,b) and interior (c) conditions.

Pirkle et al restated the "three-point interaction model" in discussing enantioseparations with chiral stationary phases (CSP) [87]. According to Pirkle's model, "chiral recognition requires a minimum of three simultaneous interactions between the CSP and at least one of the enantiomers, with at least one of these interactions being stereochemically dependant". Pirkle's three point interaction model can be illustrated by a representative enantioseparation in Fig. 1.4. Only one of the enantiomers affords three simultaneous interactions with the chiral selector. Although both the two enantiomers are able to form transient complex with the chiral selector, the formation energies of these two complexes are different. If the difference is great enough, the enantiomers can be resolved by this CSP. It was also suggested by Pirkle *et al* that when forming the complex, in many cases, not all the interactions are attractive. The interaction is sometimes steric hindrance or repulsive electrostatic force [87]. The repulsive force is usually combined with one or more attractive interactions and contributes to the ultimate chiral recognition.

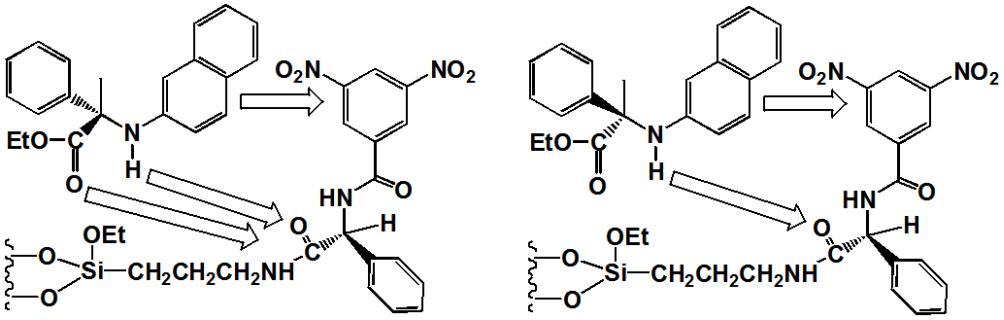


Figure 1.4: Examples of Pirkle's three point interaction model: only one of enantiomers affords three simultaneous interactions with the chiral selector. If energy difference between two diastereometric complexes is great enough, enantioseparation is able to achieve.

The interactions discussed in Pirkle's three point interaction model usually include:

i) Dipole dipole stacking

ii) Hydrogen bonding

iii) π-π stacking

iv) Steric hindrance

v) Hydrophobic inclusion

vi) Electrostatic force

The long range and nondirectional electrostatic force is much stronger than the hydrophobic interactions especially in apolar solvents [88]. On the contrary, dipole-dipole stacking, hydrogen bonding and π-π stacking are short range directional interactions. The electrostatic force is important as a strong binding interaction which is usually incorporated with secondary interactions such as hydrogen bonding, π-π stacking, dipole dipole stacking etc. to fit the three-point interaction model [86]. When polysaccharide and cyclodextrin are applied as chiral

selector, hydrophobic inclusion is usually accounted as an important enantioselective interaction. On the other hand, the charged analytes and chiral selectors generate electrostatic force mutually. "The interactions of electrostatic force and hydrophobic inclusion are important complementarities towards the enantioseparation mechanism on Pirkle's model" [89].

Transient complexes are formed between chiral selector and each of the enantiomers. According to the model, "only the difference between enthalpies of two transient complexes is directly related with enantioseparation whereas the enthalpy quantity of individual complex formed is unimportant in the consideration" [87]. The enantioseparation mechanism is usually investigated through computational method. The retention behavior and selectivity of selectand enantiomers on a chiral selector are computational results of free energy, enthalpy and entropy in forming diastereometric complexes (eq. 1.1-1.3). High values of enthalpy and entropy usually indicate that strong binding interactions between enantiomers and chiral selector are involved in the chiral recognition mechanism. Enthalpy-entropy compensation regression usually reveals that the solute retention mechanism is not related with the selectand molecular structures [90-93].

$$\Delta(\Delta G^{\circ}_{R,S}) = \Delta G^{\circ}_{S} - \Delta G^{\circ}_{R} \quad \text{.....eq. 1.1}$$

$$-\Delta(\Delta G^{\circ}) = RT \ln \frac{K_S}{K_R} = RT \ln \alpha \quad \text{.....eq. 1.2}$$

$$R \ln \alpha = -\Delta(\Delta H^{\circ})/T + \Delta(\Delta S^{\circ}) \quad \text{.....eq.}$$

1.3

When cyclodextrins are applied as chiral selectors, their hydrophobic cavities allow complexation or adsorption with the hydrophobic moieties on the selectand molecules. If the volume of the analytes' hydrophobic moiety matches up with the cavity size well, the complex formed is considered relatively inflexible. As the cavity itself is chiral, the inflexible hydrophobic complex usually contains stereoselective interactions between analytes and the CD cavity [87]. In many cases, the hydrophobic inclusion process effectively cooperates with other interactions, enhancing enantioseparation. The combined interactions between hydrophobic inclusion and electrostatic forces are illustrated with a representative example in Fig. 1.5 where three points' interactions are likely to afford successful enantioseparation.

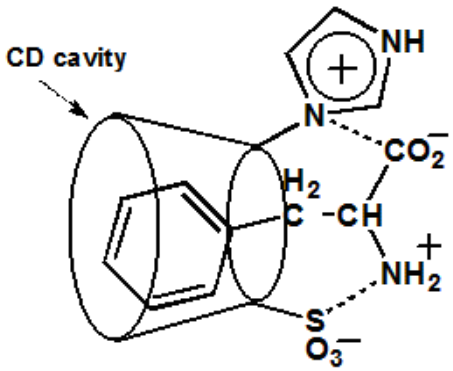


Figure 1.5: Example of hydrophobic complex combined with electrostatic force

1.5 Ionic liquids and ionic chiral selectors

It was found that Ionic liquids (ILs) are useful in efficient and environmentally benign chemical processing and chemical analysis. Amongst the applicable ILs, the imidazolium, pyridinium and ammonium ionic liquid had been

applied as solvents in catalytic reactions or liquid-liquid extractions [94-96]. The peculiar physicochemical properties of a series of ILs containing nitrile-functionalized imidazolium, pyridinium and quaternary ammonium cations were studied for the reference of relative investigations [97]. The alkylimidazolium IL had also been used as stationary phase in GC. Its dual nature to interact with the analytes through electrostatic and dispersive forces was shown [98]. In order to understand the dual nature of this kind of IL properly, a detailed study had shown the high charge region and low charge region on the IL molecule [99]. Moreover, the imidazolium, pyridinium and quaternary ammonium ILs have also been used as media in enantioselective reactions [100-102]. In those studies, aromatic and aliphatic cations were usually comparatively studied due to the distinct differences amongst their shapes and structures [94, 103]. However, the difference between aromatic cations was less investigated but more intriguing; for example, a comparison between influences of 1-butyl-3-methylimidazolium and butyl-pyridinium on the rates in phase transfer catalysis reactions had shown the imidazolium IL accelerated the reaction rate more than pyridinium IL [100].

It was reported that the ILs possessed dual capability of dissolving both polar and nonpolar species [104]. In the applications of chiral ionic liquids (CIL) as chiral selectors, the CILs also showed the “dual nature” properties, in that they separated nonpolar compounds as if they were nonpolar stationary phase and separated polar compounds as if they were polar stationary phases [105]. The CILs have been successfully applied as stationary phases for GC. A range of chiral

alcohols and diols, chiral sulfoxides, some chiral epoxides and acetamides was successfully resolved [105]. The CILs were also used as background electrolyte additives in CE [106] or pseudostationary phase in micellar electrokinetic chromatography [107]. In the study of Yuan *et. al* [108], CIL was applied as chiral selector in HPLC and CE for resolving compounds included racemic alcohol, acid, amino acid and amine *etc.* Therein, the interactions between chiral ionic liquid and analyte were experimentally investigated. The complicated interactions between the multifunctional CILs and the analytes could also be calculated theoretically with a linear free energy approach [109].

The charged moieties was first introduced into cyclodextrin and applied as charged chiral selector in CE by Terabe et al [110]. The application of charged chiral selectors such as quaternary ammonium-β-cyclodextrin, sulfobutyl ether-β-cyclodextrin, coboxylic-β-cyclodextrin successfully afforded enantioseparation towards a broad range of pharmaceutical racemates [111, 112]. The combination of CE and mass spectrometry provided high separation efficiency, sensitivity and on-line molecular structural elucidation [113]. A charged coboxylic-β-cyclodextrin which was employed as stationary phase in CE appeared to be pH responsible in the enantioseparation processes [114]. Tait *et al.* demonstrated that the use of anionic chiral mobile phase additives effectively increased the "separation window" as the maximum opportunity for separation might exist when the analyte and chiral selector migrated in opposite direction [115]. Stalcup *et al*. developed sulfated β-cyclodextrin derivatives and applied

them as chiral mobile phase additives in CE [116]. They also developed chemically bonded sulfated β-cyclodextrin derivatives as CSPs in HPLC [117]. This type of negatively charged β-cyclodextrin derivatives had shown versatility in enantioseparation of at least 16 racemates of pharmaceutical interests. The novel anionic stationary phases exhibited considerable potential for multi-modal retention properties and promised the development of more universal applicable separation medium [118]. On the contrary, there are few reports on positively charged β-cyclodextrin derivatives as CSPs in enantioseparations especially in HPLC & SFC.

Recently, our group [119-121] has reported the syntheses of cationic cyclodextrins containing imidazolium, pyridinium or ammonium moieties and applied them as chiral mobile phase additives in CE. These cationic chiral selectors are highly soluble in aqueous solutions or alcohol / aqueous medium. These cationic chiral additives have demonstrated efficient enantioseparations for phenyl hydroxyl acids, phenyl carboxylic acids and dansyl amino acids.

1.6 CSP immobilization methods

The most widely used CSPs can be classified into several categories: polysaccharide CSPs, CSP based on synthetic polymers, protein CSPs, cyclodextrin CSPs and macrocyclic antibiotic CSPs etc. [122].

CSPs based on polysaccharide and cyclodextrin derivatives are well developed and commercialized for enantioseparations [123]. These chiral selectors are immobilized onto silica gel either by physically coating or chemically bonding.

Since there is no chemical bond between silica gel and the functional material in coated CSPs, polar solvents such as chloroform and THF could swell or dissolve the chiral selectors and should be prohibited from usage [124]. In order to develop more robust CSPs, chemical immobilization of the chiral selector without the loss of its chiral recognition ability is highly desirable and it would help extending the range of applicable eluents. A more suitable eluent thus improves the chiral selectivities [125].

The methods for chemically bonding of polysaccharides onto silica were explored through variable reactions. Yamamoto *et al.* prepared immobilized CSP by chemically bonding of part of the hydroxyl groups of the polysaccharide derivatives to the amino group on the silica gel with diisocyanate [126]. These immobilizations might have caused a change in the higher order structure of the polysaccharide derivatives and the stronger bonding linker would make the polyglucose ring tortuous, therefore their chiral recognition abilities were lowered.

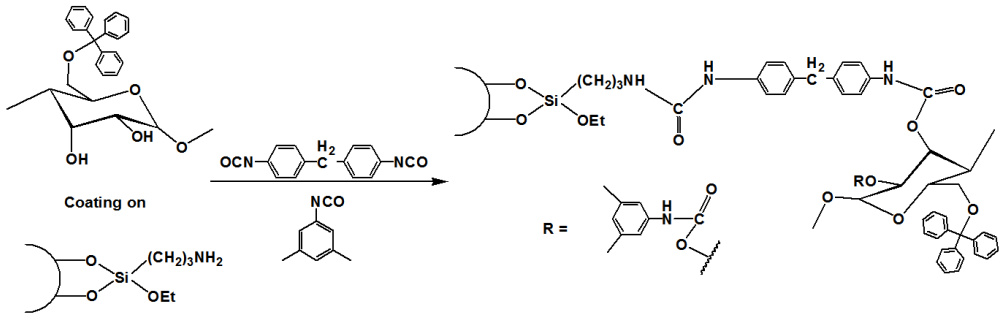


Figure 1.6 Immobilization of 3,5-dimethylphenylcarbamates of cellulose and amylose with diisocyanate [126]

The addition of vinyl monomers into the radical polymerization reaction enabled the efficient immobilization of the chiral selector without loss of chiral selectivities [127]. In another investigation, Staudinger reaction was used to connect azido

substituted polysaccharides with amino functionalized silica [128].

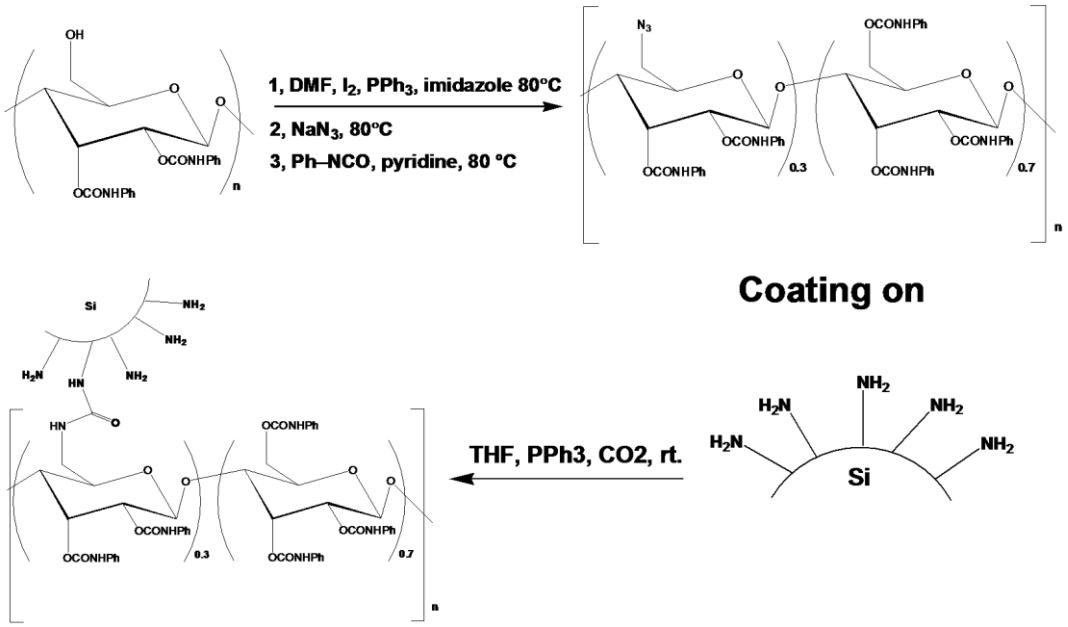


Figure 1.7 immobilization of azido cellulose phenylcarbamate onto silica gel via Staudinger reaction [128]

Our group facilitated direct immobilization of β-CD derivatives onto stationary phases through Staudinger reactions [129]. Another direct immobilization approach through catalytic hydrosilylation to bond vinylized β-CD with bare silica gel was also reported by our research group [130]. On the other hand, CD derivatives are usually immobilized onto the support indirectly. A polymer or other compounds are often employed as an intermediate arm [131-133]. Chu *et al.* made a thermoresponsive CSP by attaching β-CD moieties onto linear poly(N-isopropylacrylamide) chains. The chains acted as microenvironmental adjustors for β-CD molecules where high temperature would decrease the phase transition coefficient and the inclusion constant of CD as well [134]. Buchmesier *et al*. successfully applied ring-opening metathesis graft-polymerization in synthesizing a series of bonded CSPs based on β-CD derivatives. The resulted

CSPs were able to be used in a wide pH range from 2-10, however, a valuable ruthenium based catalyst was used in this synthesis route [135-137].

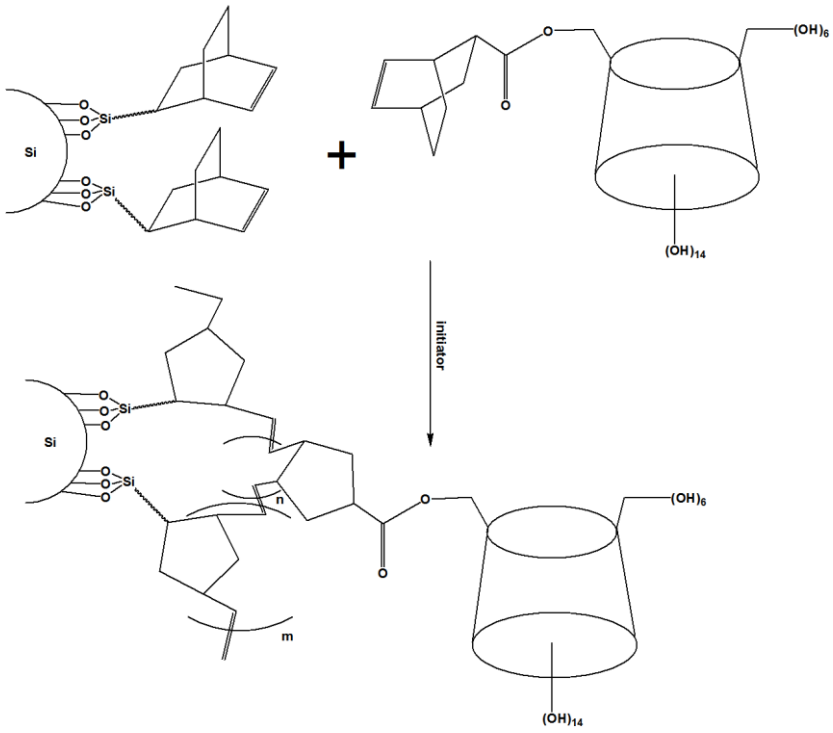


Figure 1.8 Immobilization of chiral β-CD based polymer via ring-opening metathesis graft-polymerization [135]

The comparison between the natures of coated and bonded β-CD CSP was made by Morcellet *et al*. The connection of β-CD onto silica surface through a short grafting linkage would result in a reduced CSP porosity. Furthermore, coated method was more preferable as non-toxic solvent and fewer reaction steps were required, comparing with bonded method [138].

Similarly, CSPs based on synthetic polymers are also prepared through physically coated [139, 140] or chemically bonded [141, 142] methods. Those CSPs are sustainable with a larger pH range and higher sample loading, but generally lower chiral recognition abilities were attainable than the polysaccharide type CSPs [143]. The protein based CSPs are usually immobilized through

crosslinking protocols. The most important ones are based on human serum albumin (HSA) [144]. The CSPs based on proteins require restricted pH and temperature operation conditions. Their loading capacities are insufficient for preparative applications too [145].

1.7 Loading study

Amongst the techniques extensively applied in chemical and biological analyses, electrophoretic methods such as CE and CEC provide high efficiency but very low throughput of the product. LC and SFC have shown the advantages of providing an appropriate output of optically pure intermediates for subsequent synthetic work or medical treatments. Their utmost productivities are able to be determined by the loading study performed on packed columns. Problems for loading study are usually divided into two parts [146]:

i) Sample volume overload (peak efficiency is reduced)

ii) Sample mass overload (non-linear isotherm causes peak distortion)

In the discussion of loading study, volume and concentration of the sample can be overloaded if baseline separation is achieved (Rs > 1.5). The utmost loading as well as output is able to be measured by increasing the amount of racemic analytes until the separated ingredients disperse in the column and start to merge at the outlet. This loading study is usually applied for evaluation of newly developed CSPs and acting as an intermediate investigation for scaling up [147-150]. Welch *et al*. had shown that the chromatographic schemes attained on microscale packed

column, conventional analytical column and preparative column were comparable [150]. In the loading study chromatography obtained on analytical column, it was found the utmost loading of sample with concentration of 90 mg/ml appeared when an injection volume of 150 μl was performed and its Rs was around 1.5. Loading study on the analytical column achieved enantioseparation of an amount up to tens of milligrams.

1.8 Scope of this thesis

The main objective of this project is to prepare a series of novel cationic β-CD CSPs. The CSPs were prepared either by coating or bonding synthesized cationic β-CD derivatives onto silica gel. Their chromatographic properties were evaluated in HPLC and SFC with racemic compounds and drugs.

Efforts were made to investigate the relationship between the CSPs' structures and enantioseparations. The novel CSPs synthesized herein comprise a cationic moiety which is different from conventional neutral CD based CSPs. Comparison between our cationic CSPs and neutral CSPs with similar structures would reveal the role of cationic moiety in enantioseparations processes. The influence of the cationic substituent on enantioseparations is further evaluated through a comparative study amongst imidazolium, pyridinium or ammonium moiety using a broad range of conditions and racemic analytes. Besides the cationic substituents, the cationic β-CD derivatives were fully derivatized with O-phenylcarbamate

groups. CSPs with the same cationic imidazolium substituent were either fully derivatized with O-phenylcarbamate or O-3,5-dimethylphenylcarbamate groups. Their functions were being compared in the same condition in SFC and HPLC to reveal these achiral and neutral substituents' influences on enantioseparation. Furthermore, in a detailed study on the CSPs structures, the substituent on the cationic imidazolium ring was varied between methyl and *n*-octyl substituent. The influence of the alkyl substituents' length on enantioseparation is discussed.

In order to improve our CSPs' performances in HPLC and SFC, optimal methodologies were explored for bonding cationic β-CDs onto stationary phases, wherein hydrosilylation and radical co-polymerization approaches were adopted and compared. As bonded cationic β-CD CSPs could tolerate a broad range of mobile phases, the chiral selectivities and capacity factors were optimized by varying mobile phase composition, pH and ionic strength. The discrepancies between SFC and HPLC enantioseparation results on our bonded cationic β-CD CSPs were observed. The possible interactions between analytes and cationic CSPs in different conditions are discussed. Attempts were made to rationalize using viable separation mechanisms. Furthermore, loading studies on our cationic CSPs in SFC were also investigated.

Chapter 2 Preparation and Application of Coated Cationic Chiral Stationary Phases in Normal Phase Liquid Chromatography and Supercritical Fluid Chromatography

2.1 Introduction

In the applications of charged chiral selectors, it was found that enantioseparations were strongly dependent on the presence of opposite charges as well as the structural compatibility between chiral selector and analyte [107]. François *et al.* [151] has found that applying the ionic liquids of ethyl- and phenylcholine bis (trifluoromethylsulfonyl) amide alone did not afford any enantioseparation on tested racemates. However, enantioseparations were attainable by applying those chiral ionic liquids together with cyclodextrin derivatives. A synergistic effect of the two selectors on enantioseparation was suggested. Investigation on chiral ionic liquid has also showed that applying the ionic liquid alone was unable to bring on enantioseparation. The cationic moiety in the ionic liquid could probably afford one or two interaction sites while at least 3 interaction sites were needed according to Pirkle's three-point model [152]. Accordingly, it would be interesting to investigate the chiral selector when the ionic liquid also incorporated with the β-CD moiety. In our CSPs, the positive charge was innovatively imposed onto CD rings through the imidazolium, pyridinium or ammonium moiety. Intensive investigations on those cationic moieties revealed their dual nature in their interactions with the analytes through electrostatic and dispersive forces. Their capability of supplying multiple interaction sites towards the analytes might be favorable to enantioseparations.

The procedure for coated CSPs' preparation is simple and the surface coverage of CD derivatives on CSPs can be easily adjusted. Coated CSPs are most

widely employed, for example, commercial column Chiralpak AD® is prepared by coating amylose tris(3,5-dimethylphenylcarbamte) onto underivatized silica; Chiralcel OD® is prepared by coating cellulose tris(3,5-dimethylphenylcarbamte). Both columns are widely applied for enantioseparations [153, 154]. In this chapter, the coated CSPs were prepared by coating functional cationic β-CDs onto underivatized 5 μm spherical silica gel. As the loading of CD derivatives on coated CSP can be facilely varied, CSPs of three different CD loadings were compared to determine the optimal one for enantioseparation. The coated cationic CSP was being compared with the corresponding chemical bonded neutral CSP, SINU-PC [155], to evaluate its capabilities in enantioseparation.

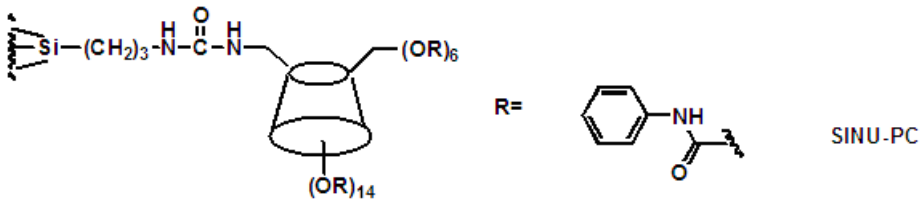
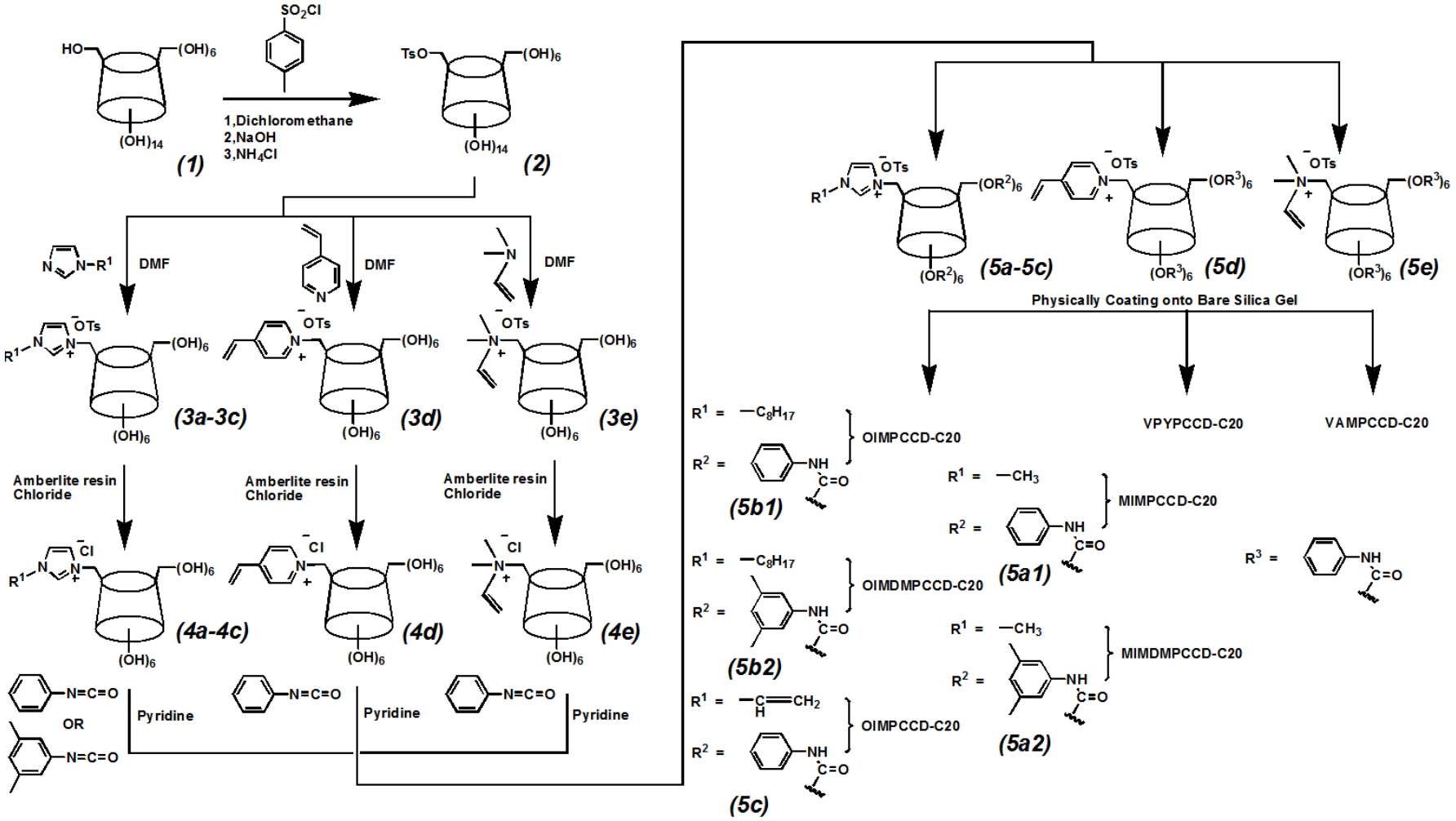


Figure 2.1: Structure of SINU-PC

2.2 Preparation of coated cationic CSPs

2.2.1 Synthesis of cationic β-cyclodextrins

As depicted in Scheme 2.1, the cationic β-CD derivatives (*3a*-*3e*) with cationic substituent of imidazolium, pyridinium and ammonium moiety at the 6-position were successfully prepared through reactions between 6^{A}-O-toluenesulfonyl-β-CD (*2*) with imidazole, pyridine or amines in anhydrous DMF with excellent yields using established procedures [156].



Scheme 2.1: The preparation route of cationic β-CD derivatives & coating onto stationary phases

The ESI-MS results of the cationic β-CD tosylate matched up with the calculated molecular mass (table 2.1). The NMR resonance peaks also corroborated the success of linking cationic moiety onto β-CD. In the NMR spectra of 6^A-(3-alkylimidazolium)-β-CD tosylate, three aromatic peaks presented hydrogens on the cationic imidazolium ring which appeared downfield between δ 7.6 to 9.4 ppm. In the NMR spectra of 6^A-(4-vinylpydinium)-β-CD tosylate, the characteristic peaks of hydrogens on the cationic pyridinium ring appeared downfield between δ 8.5 and 8.9 ppm. In the NMR spectra of 6^A-(N,N-allylmethylammonium)-β-CD tosylate, the hydrogen on the positively charged nitrogen depicted chemical shift at δ 7.96 ppm. Those hydrogens on the cationic substituent were more deshielded than the hydrogens on the neutral moiety of the molecule and therefore easily characterized.

Table 2.1: ESI-MS [M^+] Results of Cationic β-CD Tosylate

Code	3a	3b	3c	3d	3e
Calculated	1199.4	1297.5	1211.4	1222.4	1188.4
Found	1199.6	1297.7	1211.4	1222.2	1188.3

As it was tested that the cationic β-CD with chloride anion exhibited better enantioseparation abilities over those with tosylate anion when they were applied as chiral mobile phase additives in CE [121], anionic exchange were performed on the cationic β-CD tosylates using Amberlite resin chloride. The characteristic NMR peaks of tosylate group in the aromatic region between δ 7.0 and 7.5 ppm are no longer manifested, while the other resonance peaks ascribable to the remaining cationic β-CD moiety are unchanged.

On the other hand, prior investigative work on modified β-CD has shown that carbamoylated β-CD derivatives afforded higher chiral selectivities compared with those simply functionalized with ester, phenyl or carbonyl groups. It was shown that the O-phenylcarbamate groups on the carbamoylated CSPs were capable of providing additional sites for hydrogen bonding and / or dipole-dipole stacking interactions [157].

The prepared CSPs are β-CD fully derivatized by reactions with phenyl isocyanate or 3,5-dimethylphenyl isocyanate. Carbamoylation was conducted under dry N_2 atmosphere due to the sensitivity of the isocyanate moiety to moisture. During the reaction of CD carbamoylation, the by-reaction occurs when isocyanate forms a solid trimer: triphenyl isocyanurate or tri-(3,5-dimethylphenyl)-isocyanurate (Fig. 2.2). Both of the trimers were poorly soluble in pyridine, thus, the isocyanurate formed would precipitate, resulting reaction solutions cloudy. It was observed that tri-(3,5-dimethylphenyl)-isocyanurate was more easily generated than triphenyl isocyanurate as much more precipitates coming out during the reactions. If the isocyanate was added into the reaction in one portion, most of isocyanate added was transformed to isocyanurate, and the mixture turned cloudy quickly. However, dividing the addition of the same amount of isocyanate into quarter portions, the mixture kept clear throughout the reaction, and the yield of carbamoylated CD derivatives was found to be higher.

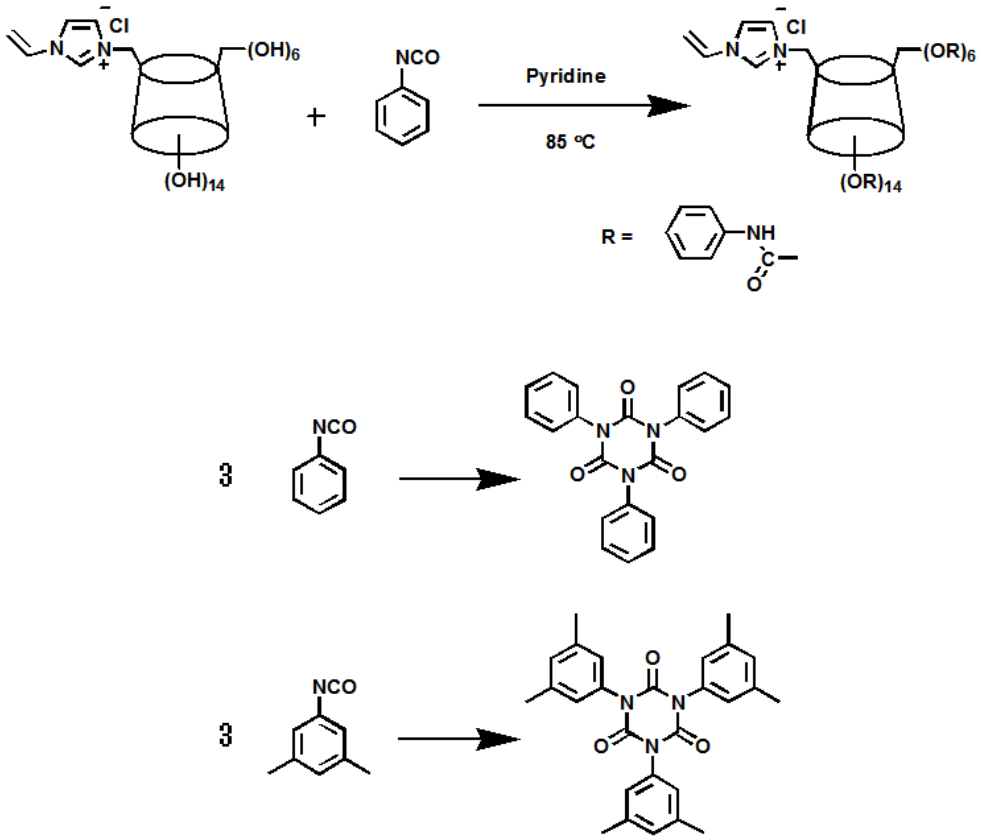


Figure 2.2: Carbamoylation of β-CD and by-reactions

The ESI-MS results of cationic perphenylcarbamoyl-β-CD and per(3,5-dimethylphenylcarbamoyl)-β-CD matched up with their respective calculated molecular masses.

Table 2.2: ESI-MS [M^+] Results of Carbamoylated Cationic β-CDs

-PCCD	MIM-	MIMDM-	OIM-	OIMDM-	VIM-	VPY-	VAM-
Calculated	3580.16	4143.56	3678.27	4241.74	3592.16	3603.17	3569.18
Found	3580.19	4144.03	3678.24	4242.68	3592.07	3603.67	3569.18

The NMR spectra exhibit aromatic peaks of phenylcarbamate substituents in δ 6.00 to 8.20 region and the peaks on β-CD framework are from δ 3.00 to 6.00. The ratios between the numbers of hydrogen in those two areas are consistent with the correct structural features of the prepared products. For

per(O-phenylcarbamoyl)- β-CD, the ratio between hydrogen atoms on phenyl groups in the phenylcarbamate moieties (100 hydrogen atoms) and those on the β-CD framework (49 hydrogen atoms) should be around 2. As 3,5-dimehylphenylcarbamoyl moiety only has 3 aromatic hydrogen atoms, per(3,5-dimethylphenylcarbamoyl)-β-CDs exhibit lower ratios of about 1.2 between hydrogen atoms in the two regions.

Table 2.3: ^{1}H NMR (300 MHz, $CDCl_3$) Characterization of Carbamoylated β-CD

Name	H [δ (ppm) 3.00-6.00]	H [δ (ppm) 6.00-8.20]	Ratio: (H [δ (ppm) 6.00-8.20] / H [δ (ppm) 3.00-6.00])
MIMPCCD	52	103	1.98
MIMDMPCCD	52	67	1.29
OIMPCCD	51	100	1.96
OIMDMPCCD	51	64	1.25
VIMPCCD	52	100	1.92
VPYPCCD	52	101	1.94
VAMPCCD	57	100	1.75

In addition, microanalyses experimental results are also consistent with the calculated results well.

Table 2.4: Elemental Analysis of Carbamoylated β-CD

Name	C% (Cal.)	C% (found)	H% (Cal.)	H% (found)	N% (Cal.)	N% (found)
MIMPCCD	61.75	62.44	4.87	5.79	8.51	8.80
MIMDMPCCD	64.95	62.68	6.15	6.33	7.37	7.25
OIMPCCD	62.38	61.04	5.13	5.78	8.29	7.61
OIMDMPCCD	65.43	63.30	6.34	6.56	7.20	7.00
VIMPCCD	61.87	60.25	4.86	5.13	8.49	9.11
VPYPCCD	62.35	62.50	4.87	5.39	8.08	8.47
VAMPCCD	61.94	61.11	4.97	5.35	8.15	8.92

2.2.2 Methodologies of coating cationic β-cyclodextrins onto silica gel

The percarbamoylated cationic CD derivatives prepared were dissolved in

chloroform (20 ml chloroform/1 g CD derivative) with sonication until clear. After that, it was filtrated through membrane filter (0.45 μm pore size) and coated evenly onto silica gel. The mixture was dried in vacuum at room temperature. The coated CSPs prepared in this way were packed into stainless steel column with slurry packing method [158, 159]. *n*-Hexane was used for packing coated CSP as polar solvent might dissolve the coated CD derivatives on the silica. On account of the relatively low viscosity of *n*-hexane, the flow rate of packing solvent was maximized to 24 ml/min. The pressure could reach as much as 8000 psi. A poor efficiency of the column was observed when a lower pressure of 4000 psi was applied for packing. Guansajonz *et al.* had also reported that a higher packing pressure would result in greater column efficiency [158]. Accordingly, it was better to apply high pressure for packing the coated CSPs.

The loading of CD derivatives on the silica gel was simply calculated by the amount of CD derivatives added into chloroform (as described above). By adding various amounts of CD derivatives into chloroform, coated CSPs with different CD loadings could be made. The coated CSPs were applied in normal phase liquid chromatography (NPLC) and SFC. The mobile phase used comprised mixtures of *n*-hexane and 2-propanol in NPLC and a mixture of CO_2 and 2-propanol in SFC. The ratio of 2-propanol applied in the mobile phase in both cases should not exceed 4% (v/v) and it was optimal to operate with 2-propanol below 3% in the mobile phase to avoid dissolution of the coated chiral selectors. The chromatographic results depicted good reproducibility after fifteen days'

continuous operations. The method for preparing coated CSPs is more facile than bonded CSPs since no additional reactions are required to immobilize the chiral selectors onto silica gel. The CD loading on coated CSPs can be simply determined and varied. However, the conditions which can be applied to coated CSPs in HPLC and SFC are severely limited, as higher content of polar organic solvent in the mobile phase would flush out the coated chiral selectors from the stationary phase.

The microanalysis results evaluated the amount of cationic CD derivatives loaded on the coated CSPs.

Table 2.5: Element Analysis of Coated CSPs

CSP	C% (found)	H% (found)	N% (found)	Surface coverage (μmol/m^2)
MIMPCCD-C15	**11.13**	**1.92**	**1.93**	**0.191**
MIMPCCD-C20	**12.31**	**1.96**	**2.00**	**0.216**
MIMPCCD-C35	**21.56**	**2.51**	**3.26**	**0.465**
MIMDMPCCD-C20	**11.38**	**1.60**	**1.94**	**0.159**
OIMPCCD-C20	**11.68**	**1.58**	**1.51**	**0.195**
OIMDMPCCD-C20	**13.34**	**1.86**	**1.46**	**0.188**
VIMPCCD-C20	**11.48**	**1.49**	**1.12**	**0.197**
VPYPCCD-C20	**11.78**	**1.32**	**1.06**	**0.201**
VAMPCCD-C20	**11.14**	**1.29**	**1.06**	**0.191**

2.3 Comparison on coated CSPs loadings

The loading concentration of CD derivatives has an influence on the enantioselectivity. Different amount of 6^A-(3-methylimidazolium)-6-deoxyper

-phenylcarbamoyl-β-CD chloride (MIMPCCD) (Fig. 2.3) was loaded onto 5 µm spherical silica gel.

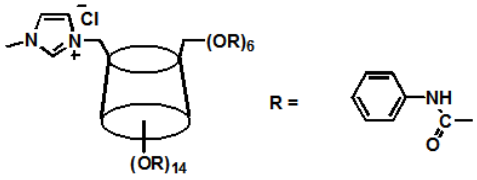


Figure 2.3: Structure of MIMPCCD

The ratio between CD and silica was set as 15:85 (w/w) (MIMPCCD-C15) 20:80 (MIMPCCD-C20) or 35:65 (MIMPCCD-C35) by weight. The coated CSPs were applied in HPLC. According to Fig. 2.4, MIMPCCD-C20 shows highest selectivity towards the racemates which means the best loading concentration of CSP should be close to 20 wt.%.

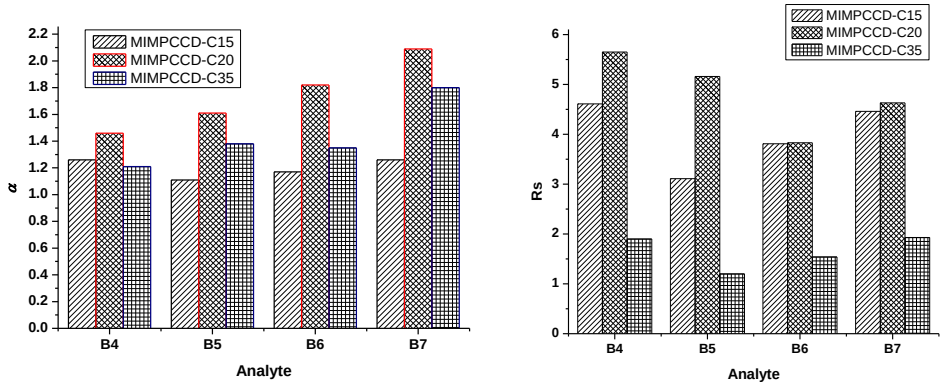


Figure 2.4: Influence of CD loading on enantioselectivities: (a) MIMPCCD-C20 has shown highest chiral selectivities; (b) Analytes attained highest resolutions on MIMPCCD-C20 (Condition: *n*-hexane : 2-propanol (97:3 v/v), flow rate 1.0 ml/min, oven temperature 25 °C)

Although sufficient loading concentration is necessary for enantioseparation, overloading chiral selectors on CSPs from 20 wt.% to 35 wt.% caused a decline of chiral selectivities. As the proportion of chiral selectors on CSPs increased, both enantiomers of analytes interacted with chiral selector more intensively. However, the difference between the interactions of each enantiomer and the chiral selector was not always increased.

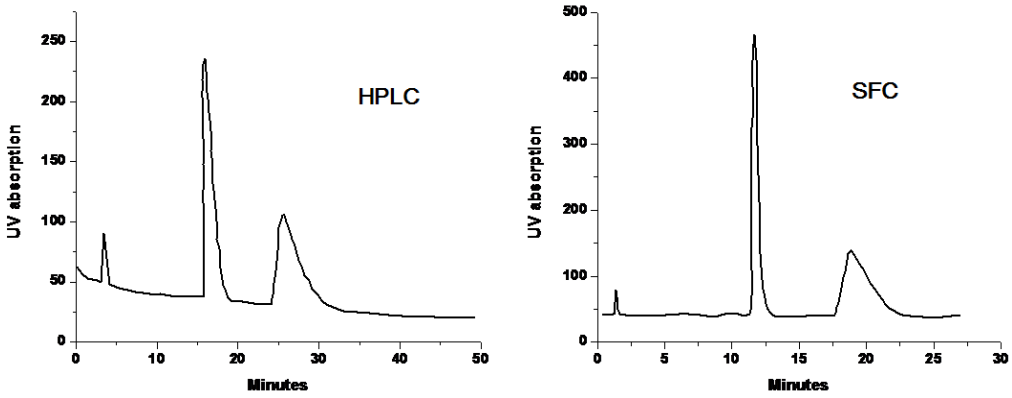


Figure 2.5: Typical charomatographic separation data for *p*-iodophenyl-1-ethanol using MIMPCCD-C20 under (a) HPLC conditions: *n*-hexane/2-propanol (97 : 3, v:v). flow rate 1 ml/min, column oven temperature 25 ℃ and (b) SFC conditions: CO_2/2-propanol (97:3. v:v), total flow rate 3 ml/min, back pressure 17 MPa and column oven temperature 40 ℃

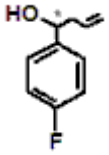
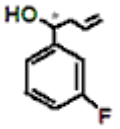
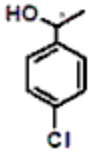
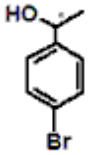
2.4 Comparison between MIMPCCD-C20 and SINU-PC

SINU-PC is a CSP derived from chemically bonding neutral β-CD trisphenylcarbamate derivatives onto silica [155]. The chiral selector in MIMPCCD-C20 is also a β-CD derivative fully O-functionalized with phenylcarbamoyl groups whilst only one hydroxyl group is replaced with a cationic imidazolium moiety. It is anticipated that the cationic CSP may display electrostatic interactions towards analytes, with additional available interaction

which may be favorable to enantioseparations.

The enantioseparations of the racemic α-phenyl alcohols had previously been demonstrated on CSP SINU-PC [160]. As shown in Table 2.5, MIMPCCD-C20 has shown higher enantioselectivities towards the racemates under the same HPLC conditions. The racemates attained high resolutions on coated CSP MIMPCCD-C20.

Table 2.6: Comparison between MIMPCCD-C20 and SINU-PC [160] in HPLC

Name	Structure	α		Rs	
		MIMPCCD-C20	SINU-PC	MIMPCCD-C20	SINU-PC
p-Fluorophenyl-3-buten-1-ol		1.76	1.34	2.64	1.35
m-Fluorophenyl-3-buten-1-ol		1.88	1.65	1.35	2.78
p-Chlorophenyl-1-ethanol		1.62	1.37	2.48	2.14
p-Bromophenyl-1-ethanol		1.83	1.72	2.32	3.07

Condition: *n*-hexane : 2-propanol (97:3 v/v), flow rate 1.0 ml/min, oven temperature 25 °C

2.5 Effect of different alkyl substituents on chiral resolution

OIMPCCD-C20 and MIMPCCD-C20 are similar to each other in structure, wherein the methyl group on the imidazolium moiety of MIMPCCD-C20 is changed to *n*-octyl group on the imidazolium moiety of OIMPCCD-C20.

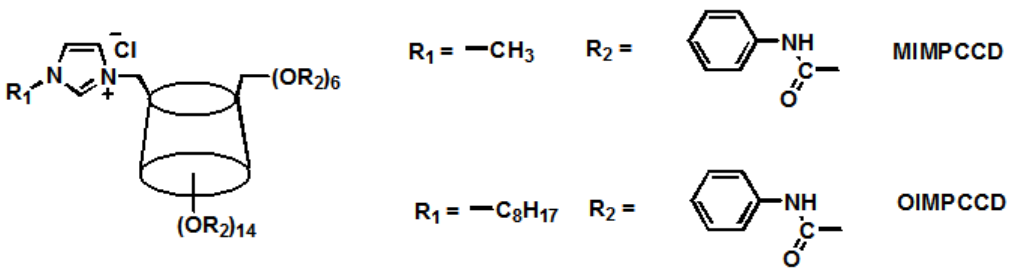


Figure 2.6: Structures of MIMPCCD & OIMPCCD

Their loading concentrations are both 20 wt.%, and the surface coverage of CD was calculated from C% of elemental analysis results [161]. The CD coverage of OIMPCCD-C20 was 0.194 μmol/m^2 and it was close to the one of MIMPCCD-C20 at 0.216 μmol/m^2.

The comparison between OIMPCCD-C20 and MIMPCCD-C20 was based on chromatographic results attained in both HPLC and SFC conditions with the analytes which were resolved on both CSPs.

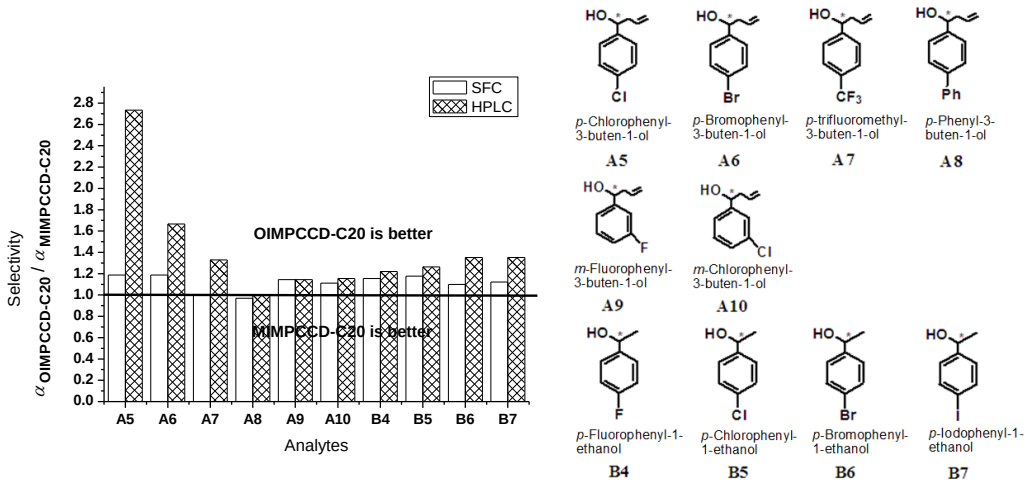


Figure 2.7: Comparison between OIMPCCD-C20 and MIMPCCD-C20 (Condition: HPLC: *n*-hexane : 2-propanol (97:3 v/v), flow rate 1.0 ml/min, oven temperature 25 °C; SFC CO_2 : 2-propanol (97:3 v/v), flow rate 2.0 ml/min, oven temperature 40 °C, BPR 15 MPa.)

In Fig 2.7, if the quotient between chiral selectivities obtained on two CSPs is more than one, the bar presents a higher chiral selectivity on OIMPCCD-C20. It is evident that almost all the analytes depicted enhanced enantioseparations on

OIMPCCD-C20 in both HPLC and SFC which would suggest that the interactions between racemates and alkyl substituent on the imidazolium moiety may influence enantioseparation. The longer alkyl chain stretching out would be more accessible and generate stronger hydrophobic interactions with the analytes. The longer alkyl substituent may also prevent two CD rings from being too close to each other so that they are able to work individually and afford a better selectivity [162]. This is in contrast to the former report that, when CD derivatives were employed as chiral mobile phase additives in CE, longer alkyl chain on the imidazolium ring might afford steric hindrance only, thus resulting in lower chiral selectivity [121]. The hydrophobic interactions were more profound and helped enantioseparations in normal phase liquid chromatography (NPLC) and SFC as applied here. Exceptionally, MIMPCCD-C20 has a higher chiral selectivity towards A8. This is likely due to A8 is the most bulky amongst all the analytes. The introduction of longer alkyl chain results in stronger steric hindrance which might be especially influential to bulky racemates. The advantage of OIMPCCD-C20 is more prominent in HPLC than in SFC. In SFC, the elution phase is mainly supercritical status CO_2. The viscosity is so small that all the analyte molecules move through the CSPs too quickly, making it difficult for the racemates to experience sufficient interactions with the alkyl substituent. Accordingly, the results obtained on both CSPs are similar in this instance.

2.6 Effect of different O-phenylcarbamoyl moieties on enantioseparations

The O-phenylcarbamoyl moiety on CD has proven to be the most useful functional group for enantioseparation [163]. It provides H-bonding, π-π stacking and dipole-dipole stacking sites. These interactions, when combined, are able to achieve enantioseparations [87]. Accordingly, the CSPs synthesized here are fully derivatized with O-phenylcarbamoyl groups. The analyte molecules may be in contact randomly with any O-phenylcarbamoyl group on the CD rims. We compare herein two CSPs, OIMPCCD-C20 where the free hydroxyl groups are derivatized fully as O-phenylcarbamoyl groups and OIMDMPCCD-C20, as the O-3,5-dimethylphenyl- carbamoyl groups.

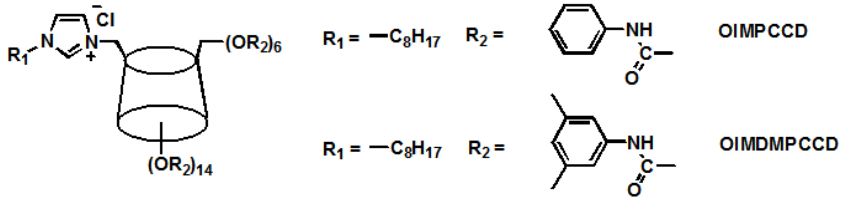


Figure 2.8: Structures of OIMPCCD & OIMDMPCCD

The CD surface coverage of OIMPCCD-C20 was 0.194 μmol/m^2, and it was close to the one on OIMDMPCCD-C20 at 0.188 μmol/m^2. OIMPCCD-C20 and OIMDMPCCD-C20 were compared under the same conditions in HPLC and SFC. According to Fig. 2.9, greater enantioseparation abilities were attained on OIMPCCD-C20.

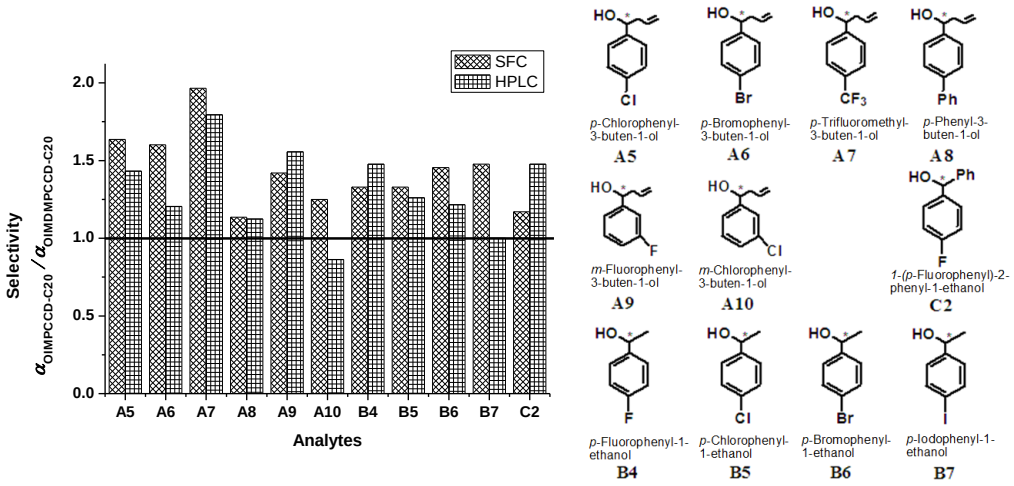


Figure 2.9: Comparison between OIMPCCD-C20 and OIMDMPCCD-C20, **(**Condition: HPLC: *n*-hexane : 2-propanol (97:3 v/v), flow rate 1.0 ml/min, oven temperature 25 ℃; SFC CO_2 : 2-propanol (97:3 v/v), flow rate 2.0 ml/min, oven temperature 40 ℃, BPR 15 MPa.)

2.7 Inspection of relationships between analytes' structures and enantioseparations

On the basis of chemical structures, the analytes could be divided into three series. In Fig. 2.10, Series ***A*** were derivatives of derivatives of 1-phenyl-3-butene-1-ol; series ***B*** were derivatives of 1-phenylethanol, whilst the remaning racemates relegated to series ***C***. The enantioseparations of analytes were compared on OIMPCCD-C20 under the same HPLC conditions. 15 out of 21 analytes were successfully resolved. Several conclusions can be drawn through careful analysis of Fig. 2.10.

i) An electron withdrawing group on the *para*-position of the aryl ring appears preferable for enantioseparation. Thus, the coated cationic β-CD CSP has shown good resolutions towards A5, A6, A7, A9, A10 and B4, B5, B6, B7. All of the analytes have an electron withdrawing group on the aryl ring

which is connected directly to the chiral center. A5, A6, A7 are *para*-substituted 1-phenyl-3-butene-1-ol. The substituents are "-Cl", "-Br", "-CF_3". The groups' size diminishes in the sequence: -CF_3 > -Br > -Cl and the selectivity is A7 > A6 > A5. Similarly, B4, B5, B6, B7 are *para*-substituted 1-phenylethanols. The substituents are "-F", "-Cl", "-Br", "-I" and selectivities: B7 > B6 > B5 > B4. The results are suggestive that a bulky electron withdrawing group on the *para*-position of the aryl ring afforded more favorable enantioseparations. The bulky group is easily polarized and more accessible to functional groups on CSP which may account for one interaction in the Pirkle's three-point model.

ii) The π-π stacking between analytes and CSP is likely to be enantioselective. The allylic group connected with the chiral center in A5, A6 is replaced with methyl group respectively in B5, B6. Selectivity is A5 > B5, A6 > B6. It is evident that allylic group on the chiral center affords improved the stereoselective interactions towards the chiral selector. The allylic group may form π-π stacking with CSP. As π-π stacking is determined by the distance between two pairs of π-electrons, one enantiomer of the racemates should afford more favorable π-π stacking than the other. However, in another case, in the compound C2 which has a phenyl group in place of the methyl group in B4, although the phenyl group can also form π-π stacking with CSP, the selectivity is in the sequence: B4 > C2. It is likely that the steric bulk of the phenyl group would diminish resolution of enantiomers.

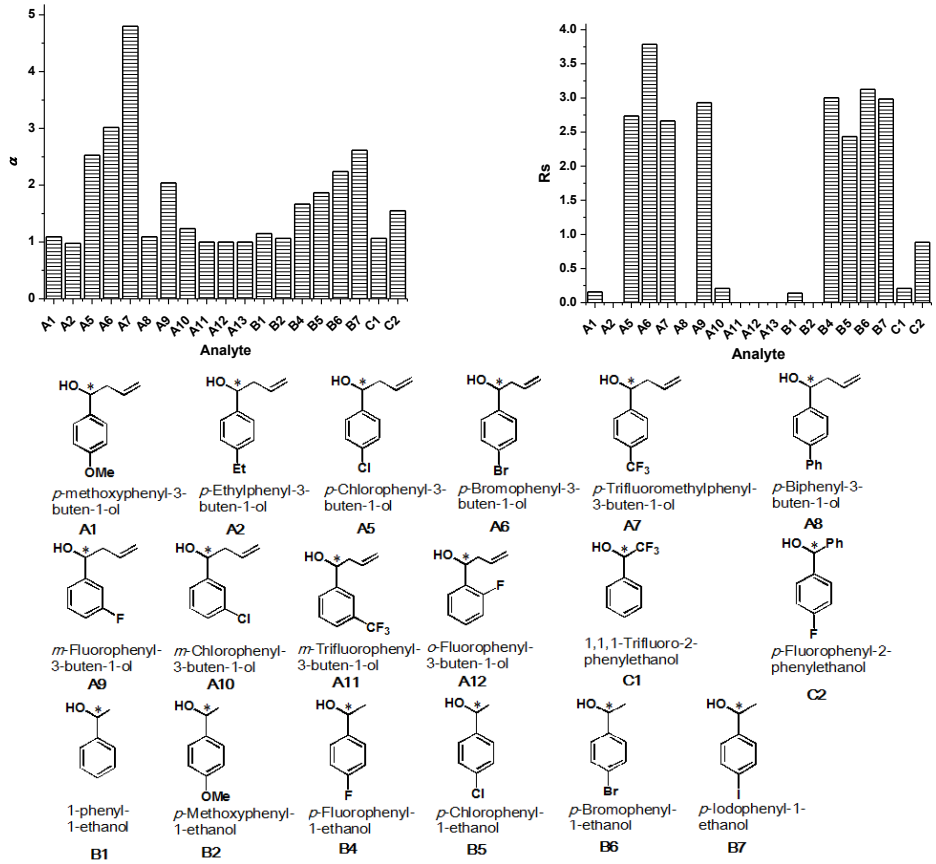


Figure 2.10: Comparison between different analytes on OIMPCCD-C20 in HPLC (Condition: *n*-hexane : 2-propanol (97:3 v/v), flow rate 1.0 ml/min, oven temperature 25 °C.)

iii) A small electron withdrawing group on the *meta*-position of the aryl ring is preferable; electron withdrawing groups on the *ortho*-position of the aryl ring are not helpful to enantioseparations.

A9, A10, A11 are substituted by an electron withdrawing group at *meta*-position with -F, -Cl and -CF_3 respectively. Selectivity and resolution of A9 are higher than A10 while A11 has no enantioseparation. It is inferred that if there is no electron withdrawing group on *para*-position of the aryl ring but a small electron withdrawing group on *meta*-position, it may still be possible to form H-bond with CSP and afford enantioseparation. However, if the

group on *meta*-position is large, such as $-CF_3$ in A11, it may afford strong steric hindrance unfavorable to enantioseparation. A12 and A13 have small halogen substituents on *ortho*-position of the aryl ring, but no separations are observed which would suggest that presence of an electron withdrawing group on *ortho*-position is not helpful to enantioseparation

iv) Electron donating groups on the *para*-position of the aryl ring is unfavorable to enantioseparation

Compounds A1, A2, B2 which have electron donating groups at *para*-position of the aryl ring have marginal or no enantioseparation on this CSP. A8, however, containing a phenyl group at the *para*-position is weakly resolved. Although phenyl group can form π-π stacking with CSP, its influence is not obvious. The presence of groups on the *para*-position of the aryl ring seems to be the dominating influence in enantioseparations. However, weak or no enantioseparations were attainable when they are electro donating groups.

The cationic β-CD CSP is capable of achieving good chiral resolution towards selected racemates. The structural features for the most favorable analytes are those where chiral center is connected onto a phenyl group and a strong electron withdrawing group is on the *para*-position of the aryl ring. It is better if the electron withdrawing group is soft and easily polarisable. The enantioseparation is also enhanced by an allylic group connected directly onto the chiral center to provide additional π-π stacking sites.

2.8 Effect of cationic moiety on enantioseparation

Coated CSPs with CD derivatives containing an imidazolium, pyridinium or ammonium cationic moiety were compared. Their structures are illustrated in Fig. 2.11.

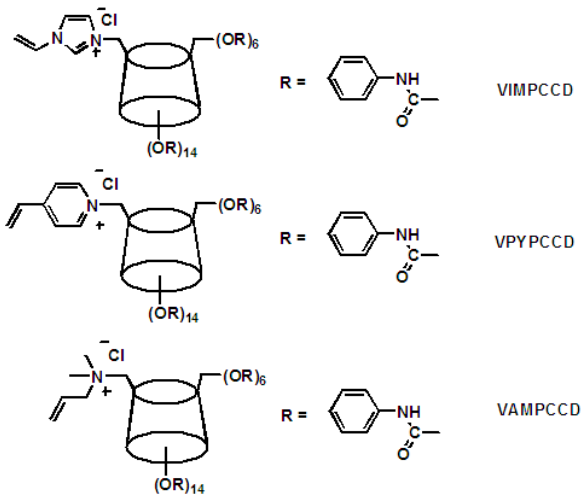


Figure 2.11: Structures of CD derivatives with different cationic moiety

According to the comparison in Fig. 2.12, the analyte which achieved good enantioseparation on one CSP was also resolved relatively well by the other cationic CSPs due to the similarity amongst the CSPs' structures. It is therefore inferred that the analyte undergoes similar enantioseparation mechanisms on the three cationic coated CSPs.

However, different cationic moieties such as imidazolium, pyridinium or ammonium substituent on the CSPs could have variable interaction strength toward the analytes. VAMPCCD-C20 has shown lower enantioselectivities towards analytes A7, A9, A10, B4, B6 and B7 than the other two CSPs. Those analytes which contain an electron withdrawing group on the *para*-position of aryl ring afforded favorable structural feature for stereoselective interactions towards our cationic CSP as discussed previously in *Chapter* 3.6. On the other hand,

VAMPCCD-C20 affords much better enantioseparation towards A1, A2, A8 and A14 while VIMPCCD-C20 and VPYPCCD-C20 depicted marginal or no enantioseparations towards those samples.

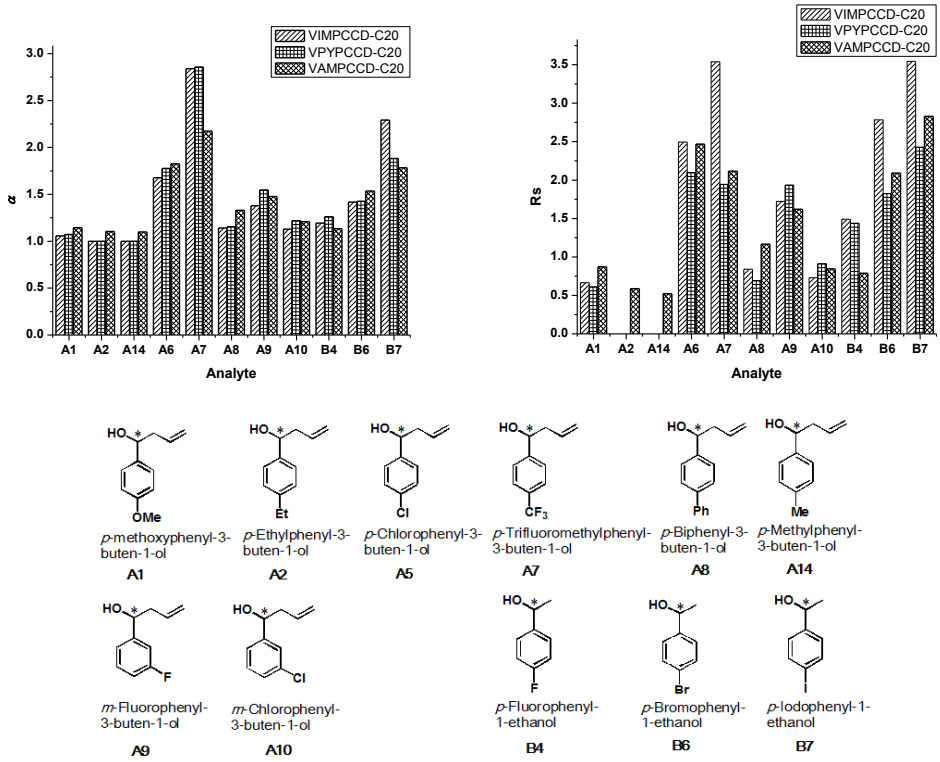


Figure 2.12: Comparison between cationic moieties on chiral selectivities of analytes in HPLC (Condition: *n*-hexane : 2-propanol (97:3 v/v), flow rate 1.0 ml/min, oven temperature 25 °C.)

2.9 Reproducibility of the coated CSP

The content of 2-propanol in the mobile phase should be limited to below 4% because higher proportion of 2-propanol may dissolve or swell the chiral selectors on the coated CSPs. The coated CSPs are found to be stable under the operation conditions applied in HPLC and SFC.

The reproducibility of MIMPCCD-C20 is representative of other coated CSPs due similarity amongst the cationic CDs' chemical structures. Its reproducibility was tested with the analyte, *p*-iodophenylethanol (B4) and the time interval

between the 2 sets of experiments was around fifteen days (Fig. 2.13). During the time, the coated CSP was applied continuously under the same condition on analysis of other samples. According to the results, it is obvious that the coated CSP was more stable in HPLC condition. The high pressure and flow rate in SFC might have shortened the applicable period of the coated CSP.

The decreased ratio of capacity factor (k_1') and selectivity (α) can be calculated:

HPLC:

$$\Delta k_1'\% = \frac{k_1'_{300} - k_1'_1}{k_1'_1} \times 100\% = \frac{3.78 - 3.81}{3.81} \times 100\% = -0.79\%$$

$$\Delta\alpha\% = \frac{\alpha_{300} - \alpha_1}{\alpha_1} \times 100\% = \frac{1.82 - 1.83}{1.83} \times 100\% = -0.55\%$$

SFC:

$$\Delta k_1'\% = \frac{k_1'_{300} - k_1'_1}{k_1'_1} \times 100\% = \frac{8.42 - 9.35}{9.35} \times 100\% = -9.94\%$$

$$\Delta\alpha\% = \frac{\alpha_{300} - \alpha_1}{\alpha_1} \times 100\% = \frac{1.70 - 1.74}{1.74} \times 100\% = -2.30\%$$

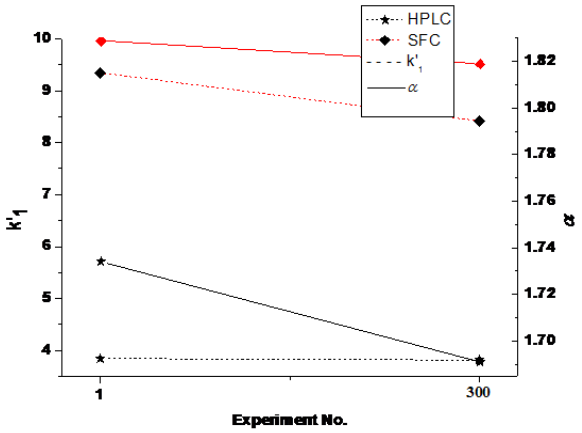


Figure 2.13: Reproducibility of sampling chromatographic results. (Condition: HPLC: *n*-hexane: 2-propanol (97%:3% v/v), flow rate 1.0 ml/min, column temperature 25 °C; SFC: CO_2: 2-propanol (97%:3% v/v) flow rate 2.0 ml/min, column temperature 40 °C, BPR 17 MPa.)

Conclusion

Cationic β-CD derivatives were coated onto silica gel and being investigated as CSPs in NPLC and SFC. To our best knowledge, these studies are the very first attempt towards applying cationic β-CD as CSPs for enantioseparation. Our cationic β-CD CSPs have shown even greater enantioseparation abilities than chemically bonded CSP (SINU-PC) which is based on neutral β-CD derivative. The cationic moieties as well as alkyl substituents attached to the cationic linker were being involved in the enantioseparation processes. Longer alkyl chain depicted more favorable enantioseparations as it affords easier to access and consequent stronger hydrophobic interactions towards analytes. Additionally, O-phenylcarbamate derivatives have depicted higher chiral selectivities than O-3,5-dimethylphenyl- carbamate derivates in the enantioseparations. The cationic β-CD derivatives have shown extraordinarily strong enantiomeric separations towards racemic *α*-phenyl alcohols with electron withdrawing groups on the *para*-position of the phenyl ring which is connected with the chiral center. Our coated CSPs afforded good reproducibilities in continuous operations in both NPLC and SFC under the recommended conditions where the proportion of 2-propanol in mobile phase was below 3%.

Chapter 3 Preparation and Application of Bonded Cationic Chiral Stationary Phases in Normal Phase Liquid Chromatography and Supercritical Fluid Chromatography

3.1 Introduction

In Chapter 3, it was demonstrated that the cationic β-CD CSPs had strong chiral separation abilities which was even better than related chemically bonded CSP based on neutral β-CD derivatives (SINU-PC). The investigation in this chapter aims at chemically bonding the cationic β-CD derivatives onto stationary phases.

The resulting bonded phase CSPs would be anticipated to tolerate wide range of solvents for use as mobile phases [164]. As the content of organic modifiers and acidic/basic additives in the mobile phases would influence the ultimate enantioseparations [107, 165, 166], the chromatographic conditions in both NPLC and SFC were generally optimized to attain maximum chiral selectivities. Acid / basic additives were also employed to minimize peak tailing and increase resolution [167, 168]. On account of the different separation mechanisms between NPLC and SFC [27, 169] performances of the cationic β-CD CSPs in NPLC and SFC were compared as well.

The β-CD CSPs prepared herein contain an aromatic or aliphatic cationic substituent. The differences between aromatic and aliphatic quaternary ammonium salts had been extensively investigated in the studies of ionic liquid (IL) where they were applied as solvents or reaction medium [95, 97, 103, 170-172]. The dissimilarities between cationic aromatic or aliphatic moiety on the stationary phases on enantioseparations are also discussed herein.

Furthermore, it is important to test the maximum loading of samples on

packed columns for evaluating the functions of new CSPs, so as to achieve higher throughput of optically pure enantiomers. Welch *et al* has demonstrated that by injecting a sample of proportional amounts into a micro scale packed column or a large scale preparative column which was scaled up to one million folds, the chromatographic results such as retention times and chiral selectivities obtained on two columns were quite similar [173]. Accordingly, output of big dimensional packed columns is predictable by the loading studies on small dimensional packed columns. Loading studies on the analytical columns had been reported by other researchers as well, where the samples were overloaded either by overloading volume or concentration [15, 174-176]. We had also investigated our cationic CSPs' capabilities in the loading studies. The injected samples were overloaded by volume or concentration, which would afford variable adsorption mechanisms in the chromatogram.

Hereby, the analytes chosen for analyses in NPLC and SFC should contain multi functional groups to afford stereoselective interactions towards the cationic CSPs. Firstly, analytes containing hydroxyl groups which has modest polarity were considered; secondly, it was observed in our trial experiments that phenyl group or phenoxy group were favorably interacting with CD cavities in CSPs; thirdly, polar analytes such as α-phenyl acid and primary amines which exhibited improperly long retentions were excluded. The main classes of the analytes discussed here are listed as follows:

i) α-Phenyl alcohol and its derivatives: A series of α-phenyl alcohols

which are substituted by four types of halogens (F, Cl, Br, I) and at different position on the benzene ring (*o*-, *m*-, *p*-) were used. Some highly electron withdrawing group such as -CF_3 substituted α-phenyl alcohol and electron donating group substituted α-phenyl alcohol were also discussed.

ii) Polyphenols: These are compounds such as flavanone and its derivatives which are chiral compounds extracted from plants. They belong to the larger group of natural flavonoids widely distributed in edible plants such as fruits, vegetables, nuts and tea. The numerous health related properties of flavonoids include "exhibition of antiviral activities and anti-inflammatory, inhibition of human platelets aggregation and anticancer activity".

iii) Dansyl amino acids: A series of racemic dansyl amino acids comprising both monocarboxylic acids and dicarboxylic acids have been tested on the cationic CSPs. These racemic acids have previously been successfully resolved into enantiomers by the cationic β-CD when it was applied as chiral mobile phase additive in CE [177].

iv) Diuretic drugs: Bendroflumethiazide, althiazide, indapamide, and trichlormethiazide are examples. The cationic β-CD derivative CSPs have shown strong enantioselectivities towards these racemates.

v) Other racemates: Fenoterol and trans-4-cotininecarboxylic acid have also depicted very good enantioselectivities and resolutions.

3.2 Preparation of bonded CSPs

3.2.1 Hydrosilylation

Our group has developed neutral bonded CSP using 6^{A}-N-allylamino -6-deoxyperphenylcarbamoyl-β-CD with hydrosilylation [130]. The CSP prepared with this method exhibited accentuated enantioseparation abilities in HPLC [178, 179]

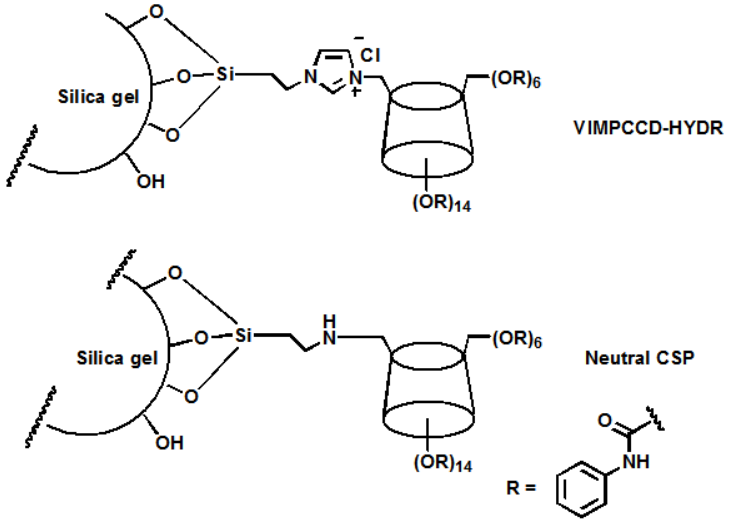
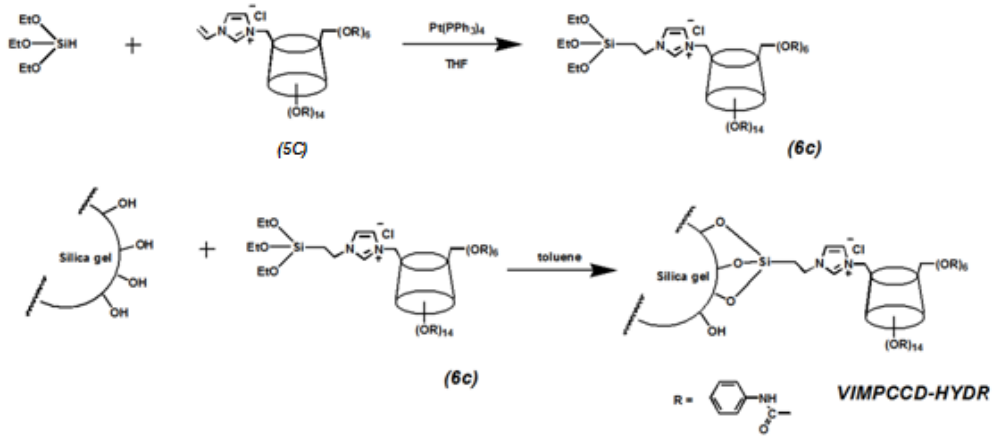


Figure 3.1: Structures of VIMPCCD-HYDR and neutral CSP [130] synthesized through hydrosilylation

As it has been illustrated in Scheme 3.1, the same process was also applied for the preparation of the cationic β-CD CSP. Firstly, 6^{A}-(3-vinylimidazolium)-6-deoxy perphenylcarbamoyl-β-CD chloride (*5c*) was reacted with triethoxy silane in the presence of tetrakis(triphenylphosphoine)platinum $\{Pt[P(C_6H_5)_3]_4\}$ as a catalyst. The crude product was filtered through silica gel column using dried ether. Afterwards, the solvent was evaporated. The residue (*6c*) was thereafter dispersed in dried toluene

and mixed with 5 µm spherical silica gel. The mixture was heated to 90 °C for 10 hours. After that, 1 ml water and 1 ml acetic acid was added to enhance the network formation of the silica gel with the hydrosilylated product. Microanalysis result: 7.242, C%; 1.375, H%; 0.767, N%. CD coverage on the silica gel was calculated as 0.078 µmol/m^2.



Scheme 3.1: Preparation of bonded CSP via hydrosilylation

In hydrosilylation, CD derivative was bonded onto bare silica directly. Other methods reported for bonding CD derivative and silica gel together required the pre-functionalization of the silica gel with reactive organic linkers [24, 180, 181]. However, the free linkers on the silica gel may not be completely bonded with CD derivative in the hydrosilylaion reactions. The remnant free linkers on the silica surface may be undesirable because they are able to interact with analytes non-stereoselectively [130].

On the other hand, hydrosilylation requires tedious condition to protect the reactive intermediate from being destroyed by moisture. It is especially difficult to handle the intermediate when a flash column separation process is required to

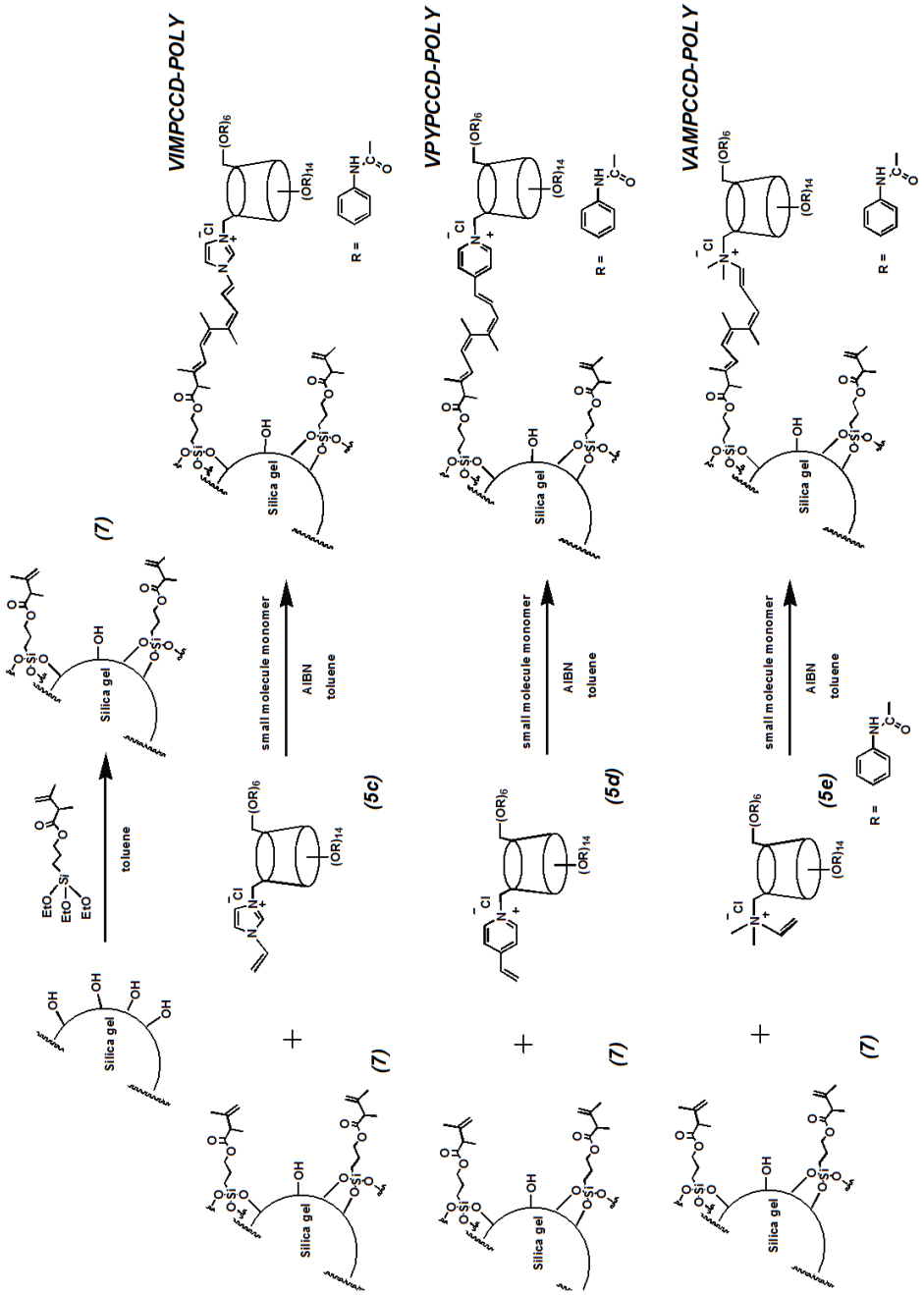
eliminate the catalyst from the mixture after the first step.

3.2.2 Co-polymerization

Polymerization methods are useful for immobilizing fully derivatized chiral selectors onto silica gel. Kimata *et al.* [182] immobilized cellulose *p*-vinylbenzoate onto silica gel through radical polymerization. Oliverous *et al.* [183] prepared bonded CSPs via radical polymerization of cellulose, amylose and chitosan derivatives with 10-undecenoylcarboxylate or 3,5-dimethylphenylcarbamate groups onto allyl silica gel. These immobilization reactions may cause change to the higher order structures of the polysaccharide derivatives. As a result, the chiral recognition abilities of these CSPs may be lowered [127]. To overcome this problem, Okamoto [127] and Chen X.M. *et al.* [184] have used small molecular monomers to help co-polymerizing the cellulose derivatives onto vinylized silica gel. In this way, the CD derivatives were successfully immobilized onto stationary phases whilst exhibiting strong enantioseparation abilities. Consequently, this method was adopted herein for immobilization of the vinylized cationic β-CD onto vinylized silica gel.

In the co-polymerization reactions (as depicted in scheme 3.2), the vinylized silica gel was firstly coated with vinylized cationic β-CD. The solid mixture formed was suspended in a dried organic solvent (toluene, DMF, THF or 1,4-dioxane). AIBN and small molecular monomer were added into the cloudy mixture thereafter. Heating or UV irradiation was used to initiate the reaction and optimal reaction conditions were explored. As the structures of cationic β-CD

derivatives were similar, the co-polymerization with 6^{A}-(3-vinylimidazolium)-6-deoxyperphenylcarbamoyl-β-CD chloride (VIMPCCD) was investigated as a representative example and discussed herein.



Scheme 3.2: The route of co-polymerization for preparation of bonded CSPs

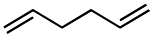
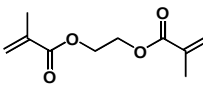
i) The small molecular monomers (SMMs) for co-polymerization

Among the available SMMs, there are many SMMs containing Nitrogen (N). In the elemental analysis (EA) result, if the nitrogen content comes exclusively

from the CD derivative, the amount of successfully bonded CD derivative onto the silica gel can conveniently be calculated. However, if the SMM also contains nitrogen atoms, as it would be bonded onto the silica gel simultaneously, it would be difficult to determine how much CD has been bonded based on the EA results. For the convenience of CD loading measurements on the bonded CSPs, only SMMs without nitrogen atoms such as 2,3-dimethylbutadiene (DMBD), 1,5-hexadiene (HD) and ethylene glycol dimethacrylate (EGD) were chosen in this work. VIMPCCD (scheme 2.1) was coated onto the surface of vinylized silica gel (33.3% w/w). Thereafter, AIBN was added and the solid mixture was suspended in anhydrous toluene. The SMM was then injected into the mixture and the reactions initiated by heating to 80 °C. The CD derivative's surface coverage on the resulted bonded CSPs were determined by the nitrogen content (N) (Eq. 3.1) [161]:

$$\frac{\mu mol}{m^2} = \frac{(\%N)(10^6)}{(S.A.)(n_N)(14.0067)[100 - \frac{\%N}{(n_N)(14.0067)}(M_r)]} \qquad \text{(Equation 3.1)}$$

Table 3.1: Elemental Analysis of CSPs Prepared with Different SMMs

Name	Structure	Code	C%	H%	N%	CD Surface coverage (μmol/m^2)
2,3-Dimethylbutadiene		DMBD	13.63	1.70	0.88	0.100
1,5-Hexadiene		HD	6.85	1.32	0.33	0.035
Ethylene glycol dimethacrylate		EGD	28.69	3.95	0.26	0.027

Conditions: initiated by heating to 80 °C; reacted in dried toluene

The EA results of CSPs prepared by using three different SMMs are summarized in Table 3.1. From the results, it was found that the maximum surface coverage of bonded CD derivative on silica surface was 0.1 μmol/m^2 which was achieved by using DMBD. EGD was highly reactive towards polymerization as the resulting CSP had the highest carbon content ("C%") of 28.69%, whereas its CD coverage on the silica surface was only 0.027 μmol/m^2. This would suggest that most of the EGD was self-polymerized in the reaction and it did not help in bonding the CD derivative. HD has a lower reactivity towards co-polymerization with CD derivative. Thus, the CSP prepared with HD had low C% (6.85%) and N% (0.33%) contents.

ii) The methods of initialization for co-polymerization

In our study, the vinylized silica gel coated with CD derivatives and AIBN were suspended in dried toluene. Two methods were used for initiating the co-polymerization reactions: UV irradiation or heating. Referring to the results from Table 3.1 and Table 3.2, the CD coverage of CSPs prepared by heating varied from 0.027 to 0.100 μmol/m^2, whilst by UV radiation initiation the CD coverage of CSPs was from 0.002 to 0.023 μmol/m^2. Heating was obviously the more suitable initiation method for use in the co-polymerization.

Table 3.2: Results of Reaction Initiated with UV

SMM	C%	H%	N%	CD Surface coverage (μmol/m^2)
DMBD	8.00	1.40	0.02	0.002
HD	7.25	1.20	0.09	0.009
EGD	9.17	1.40	0.22	0.023

Condition: reacted in dried toluene

iii) Solvents for co-polymerization

The effects of solvents used in co-polymerizations are compared in Table 3.3. In comparison between toluene (s/n 1) and DMF (s/n 2) are compared. It is found that self-polymerization of EGD was slower in DMF than in toluene. The carbon content of CSP obtained in DMF was 9.26% which was obviously lower than the one obtained in toluene (28.69%). However, the difference between the nitrogen contents is marginal (0.26% in toluene; 0.21% in DMF). As the nitrogen content represents the successfully bonded CD derivatives, the CD derivatives' surface coverage of CSP obtained in DMF (0.021 μmol/m^2) was comparable with the one attained in toluene (0.027 μmol/m^2). The CD derivative was more soluble in DMF which might afford a faster polymerization with the CD derivatives involved. However, it appeared that the radicals have a shorter life in DMF. Thus, although radical chain propagation time was longer in toluene than in DMF, it had lower activity to bond with the CD derivatives in toluene. Therefore, the bonding efficiencies of CD derivatives in toluene and in DMF were similar.

Table 3.3: The Effect of Solvents on Co-polymerizations

No.	SMM	Solvent	C%	H%	N%	CD Surface coverage (μmol/m^2)
1	EGD	Toluene	28.69	3.95	0.26	0.027
2	EGD	DMF	9.26	1.47	0.21	0.021
3	HD	THF	9.23	1.51	0.28	0.030
4	HD	1,4-Dioxane	9.27	1.59	0.14	0.014
5	HD	Toluene	6.85	1.32	0.33	0.035
6	HD	Paraffin oil	32.51	5.58	0.06	0.006

Amongst reactions from s/n 3 to 6 in Table 3.3, it is inferred that the

efficiency sequence for CD immobilization was: toluene > THF > 1,4-dioxane > paraffin oil. It is suggested the oxygen atom in the solvent molecules such as THF or 1,4-dioxane could terminate the chain growth [185]. Since paraffin oil was inert to annihilate the free radicals, polymerization was able to proceed for a longer time in paraffin oil. Longer polymer chains could be formed in paraffin oil which resulted in the highest C% (32.51%). However, in paraffin oil, the CD derivative was poorly soluble. Consequently, the CD derivative was polymerized with difficulty. The CSP obtained in paraffin oil had a poor surface coverage of CD at a value of 0.006 μmol/m^2. Toluene was relatively inert toward termination chain and thus allowing for better dispersion of CD derivatives. The combined effects for toluene afforded the highest CD loading of 0.035 μmol/m^2 at 80 ℃ in 18 hours.

iv) Sequences of small molecular monomer (SMM) additions

The SMM is competing with the CD derivative during the chain propagation. Three component mechanism of grafting was proposed [186]:

i) Approach *A*: addition of monomer to CD radicals followed by propagation of the grafted radical chains;

ii) Approach *B*: simultaneous reaction of radical homopolymer chains and CD radicals;

iii) Approach *C*: simultaneous reaction of radical homopolymer chains and grafted radical chains.

HD was chosen to evaluate the results of all the three reaction modes because

it has relatively moderate reactivity towards polymerization. A mixture of vinylized silica and coated CD (0.8 g : 0.2 g) and 1.4 mg AIBN was added to 5 ml dried toluene. The reaction was initiated by heating to 80 °C. HD was added in one of three ways. In the first, 1.04 ml HD was added to the reaction after it was heated to 80 °C for 1 hour (case A); in the second instance, 1.04 ml HD was added at the beginning before heating, thus, HD, CD derivative and vinyl groups on silica were initiated at the same time (case B); in the third instance, 0.52 ml HD was added at the beginning of the reaction, and after the reaction mixture was heated to 80 °C for 1 hour, another 0.52 ml HD was added (case C). The elemental analysis results of the three cases are summarized in Table 3.4.

Table 3.4: Different Grafting Mechanism with Varied SMM Addition Sequences

Approach	C%	H%	N%	CD Surface coverage ($\mu mol/m^2$)
A	5.88	1.22	0.30	0.032
B	7.02	1.27	0.51	0.056
C	6.85	1.32	0.33	0.035

It is obvious that in case “B”, CSP with highest CD surface coverage of 0.056 $\mu mol/m^2$ was obtained. It seems to be necessary to initiate both CD derivative and SMM at the same time in this reaction. The CD derivative had a low solubility in toluene while SMM were well dissolved. As long as SMMs’ radicals formed short chains, they could connect the radicals form from the vinyl groups on the silica gel and vinyl groups on CD. At the beginning of the reaction, if there was no SMM radical, the radicalized vinyl group on the silica gel or on CD substituent might be directly quenched by solvent molecules. As a result, the efficiency of

bonding CD derivatives would be lower. In case "C", the carbon content of the CSP is 6.85% which is close to 7.02% of carbon in case "B" but the nitrogen content is 0.33% which is close to 0.30% in case "A". This is suggestive that the second half of HD added did not help in the bonding of CD in the reaction. The radical on CD might already have lost its activity when the second half portion of HD was added and the SMM might have undergone self polymerization.

3.2.3 Comparison between co-polymerization and hydrosilylation

In the co-polymerization reaction, 6^A-(3-vinylimidazolium)-6-deoxyper -phenyl-carbamoyl-β-cyclodextrin chloride was pre-coated onto 5 μm vinylized silica gel and then swelled in anhydrous toluene. 2,3-Dimethylbutadiene was then added in proportion to 30 mol% the total vinyl groups. AIBN was added and the reaction proceeded at 80 °C for 48 hours. The product was washed with THF and then MeOH in sequence and dried at 100 °C overnight. Calculated based on nitrogen content in elemental analysis results (Table 3.5), the CD derivatives' grafting coverage is 0.088 μmol/m^2 on the surface of silica.

In hydrosilylation reaction, 6^A-(3-vinylimidazolium)-6-deoxy-perphenyl carbamoyl-β-cyclodextrin chloride was reacted with triethoxysilane, catalyzed by $Pt[P(C_6H_5)_3]_4$ in anhydrous THF at reflux temperature for 4 days. Solvent was removed in vacuum thereafter. The product was mixed with 5 μm vinylized silica gel at 1:1 (w/w) ratio and suspended in anhydrous toluene at 90 °C for 24 hours. The CD derivatives' grafting coverage was also calculated based on nitrogen content of elemental analysis results which is 0.078 μmol/m^2 on the surface of

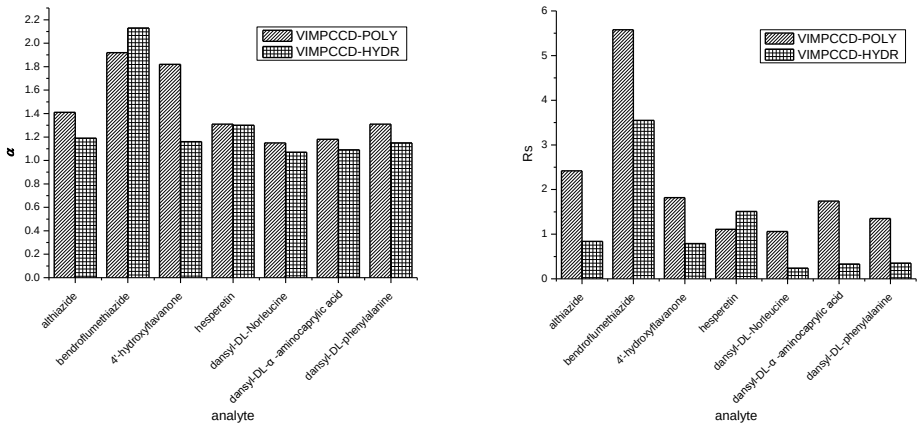
silica gel.

Table 3.5: Different Immobilization Methods

Approach	C%	H%	N%	CD Surface coverage ($\mu mol/m^2$)
Co-polymerization	12.66	1.54	0.78	0.088
Hydrosilylation	7.24	1.38	0.77	0.078

By comparing two CD immobilization approaches, it is found that the co-polymerization method is more facile while the hydrosilylation reaction requires more rigorous anhydrous reaction conditions. The co-polymerization approach is thus simpler, requiring less reaction time. However, the CSPs derived in the two reactions have similar surface coverage of bonded CD on silica.

The bonded CSPs prepared with hydrosilylation (VIMPCCD-HYDR) and co-polymerization (VIMPCCD-POLY) were packed into stainless steel columns of same dimensions (ϕ 2.1×150 mm). The performances of the two CSPs were compared under the same conditions in SFC.



(a) (b)

Figure 3.2: Comparison between VIMPCCD-POLY & VIMPCCD-HYDR in SFC: (a) CSP prepared through co-polymerization has shown higher chiral selectivities towards the analytes (b) Analytes attained higher resolutions on CSP prepared through co-polymerization

In the comparison, the CSP synthesized through co-polymerization has shown

better chiral selectivities towards almost all the samples (Fig. 3.2 (a)). Additionally, CSP synthesized through hydrolysis displayed poorer column efficiency in comparison with the one synthesized using the polymerization approach (Fig.3.2 (b)). The chiral selector bonded onto silica gel through hydrosylization through a short linker. The functional groups on the rim of the CD rim as well as the CD cavity are too close to the surface of silica gel. Thus, the interactions between chiral selector and analytes were prohibited by steric hindrance [187]. On the other hand, in co-polymerization, silanol groups are able to be covered by the polymeric chiral selectors [188]. The co-polymerization method was therefore adopted to prepare the bonded cationic CSPs incorporating the imidazolium, pyridinium or ammonium moieties throughout the project.

3.2.4 Characterization of bonded CSPs

The grafted CD derivatives are characterized with the typical vibration bands in FT-IR. The aromatic "C=C" vibration bands between 1400-1650 cm^{-1} characterized the phenyl groups in the phenylcarbamate moieties.

Table 3.6: Typical FT-IR Vibration Bands of Bonded CSPs

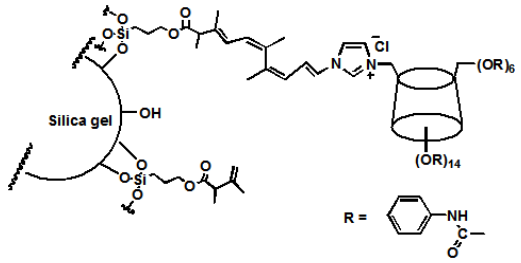
Bonded CSP	Aromatic "C=C" vibration bands (cm^{-1})
VIMPCCD-POLY	1647
VPYPCCD-POLY	1631
VAMPCCD-POLY	1634

Microanalyses results, on the other hand justified the success of bonding β-CD derivatives onto silica. The amounts of CD surface coverage on the bonded CSPs are calculated based on the nitrogen content in the microanalysis results.

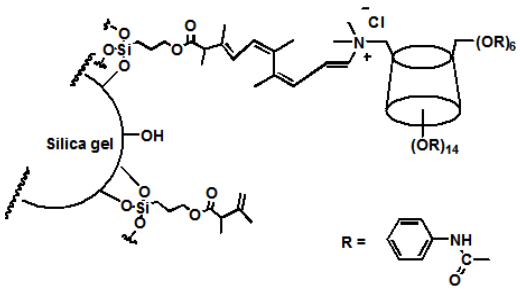
Table 3.7: Microanalyses of Bonded CSPs

Bonded CSP	C%	H%	N%	CD surface coverage ($\mu mol/m^2$)
VIMPCCD-POLY	12.66	1.544	0.785	0.088
VPYPCCD-POLY	9.495	1.397	0.797	0.094
VAMPCCD-POLY	9.432	1.394	0.775	0.091

3.3 Differences between aromatic and aliphatic cationic substituents on the bonded CSPs in enantioseparations



VIMPCCD-POLY



VAMPCCD-POLY

The bonded CSPs **VIMPCCD-POLY** and **VAMPCCD-POLY** have similar structures but different cationic linkers which is an aromatic cationic substituent in **VIMPCCD-POLY** but an aliphatic cationic substituent in **VAMPCCD-POLY**.

The enantioseparation results of flavanone derivatives and racemic thiazides on **VIMPCCD-POLY** and **VAMPCCD-POLY,** using similar conditions, are summarized in Fig. 3.3. In HPLC, the enantioseparation of most racemates on **VIMPCCD-POLY** was found to be accentuated in comparison with **VAMPCCD-POLY**. In SFC, however, all the racemates acquired higher chiral selectivities on **VIMPCCD-POLY**. It was evident that the cationic CSP with aromatic cationic substituent afforded more favorable enantioseparations than that with aliphatic cationic substituent.

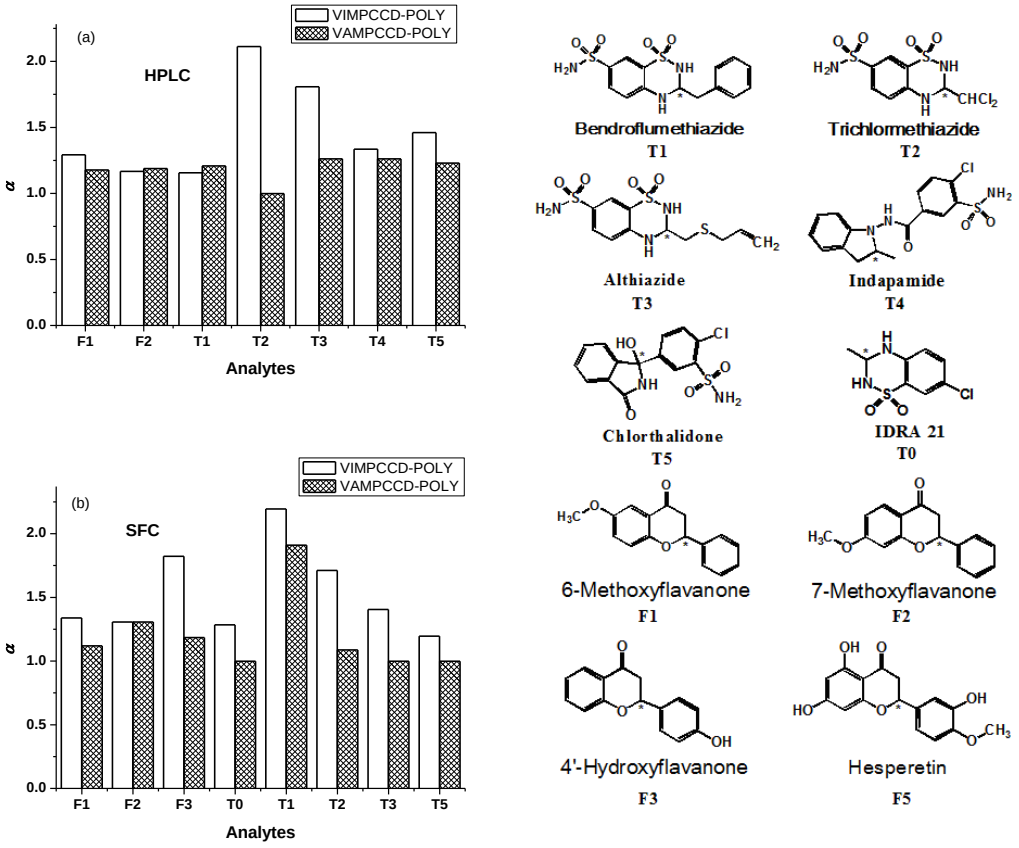


Figure 3.3: Comparison between enantioseparations attained on **VIMPCCD-POLY** and **VAMPCCD-POLY** (Condition: (a) NPLC: flow rate 0.4 ml/min, oven temperature 25 °C; T1-T5: *n*-hexane: 2-propanol 70:30 (v/v); F1, F2: *n*-hexane: 2-propanol 97:3 (v/v); (b) SFC: flow rate 1.0 ml/min, oven temperature 40 °C, BPR 15 MPa; T1-T4: CO_2: MeOH 70:30 (v/v); T0: CO_2: MeOH 90:10 (v/v); T5: CO_2: MeOH 80:20 (v/v); F1, F2:CO_2: 2-propanol 99:1 (v/v); F3: CO_2: 2-propanol 97:3 (v/v).)

In the aromatic cationic substituent, the planar shape of aromatic ring makes for easy interaction with the positive charged substituents and the analytes. In the CSP with aliphatic ammonium linker, however, the substituents on the nitrogen atom are tetrahedron in shape affording less favorable interactions between the analyte and the cationic substituent. Furthermore, the aromatic imidazolium moiety could also interact with analytes through π-π stacking, which is not

possible with the aliphatic ammonium group. The aromatic cationic moiety is therefore compatible with multiple interaction types, facilitating various enantioseparation processes.

3.4 Evaluation of cationic β-CD bonded CSPs with pharmaceutical drugs

3.4.1 Flavanone derivatives

The retentions of **F4** and **F5** were found to be higher than that of **F3**. **F1** and **F2** presented even lower retention than **F3**. Accordingly, the analysis in both NPLC and SFC required a lower content (1% v/v 2-propanol or methanol in the mobile phase) of polar organic modifier for analysis of **F1** & **F2**, modest amount of polar organic modifier (3%) for **F3** and a higher ratio (10%) for **F4** & **F5**. The enantioseparations of 5 flavanone derivatives on three bonded CSPs were studied and the results summarized in Fig 3.4. As flavanone derivatives with more phenolic groups depicted higher retention, it is suggestive that the attractive interactions between phenolic groups and chiral selectors would account for the retention of the flavanone derivatives. The polar phenolic groups in flavanone derivatives most probably interacted with the substituents on the CD rings, whilst not likely included into the hydrophobic CD cavity. Although the retention as well as the attractive interaction between phenolic groups and chiral selectors in **F3** was not the strongest amongst flavanone derivatives, its chiral selectivity was the best. It is obvious that the stronger interactions between phenolic groups and chiral selectors may not always result in higher stereoselectivity whereas the position of

phenolic group on flavanone is important for enantioseparation: cationic CSPs have shown enhanced enantioselectivity if the phenolic group is in 4'-position (**F3**); their enantioselectivities towards flavanone derivatives with multiple phenolic groups were reduced especially for flavanone derivatives with phenol groups on both 4'- and 6-positions (**F4**). The strong influence of 4'-phenolic group on enantioseparation was also mentioned in a comparative study where native β-CD stationary phase (**Cyclobond I**) was applied for flavanone derivatives' enantioseparations [189].

Previous works on enantioseparation of flavanone derivatives with β-CD derivatives were usually carried out in RPLC. Therein hydrophobic inclusion phenomenon was considered to be the main factor in the mechanism of enantioseparations. Analytes containing highly polar moieties were not likely separated on the neutral β-CD CSPs in RPLC, for example, the enantioseparation of 4'-hydroxyflavanone (**F3**) on neutral β-CD CSP **Cylcobond I**® using methanol / water failed due to the analyte's high polarity [189]. Nonetheless, on our cationic β-CD CSP (**VIMPCCD-POLY**), a resolution of 1.94 for 4'-hydroxyflavanone (**F3**) was attained. The enantioselectivities of naringenin (**F4**) and hesperetin (**F5**), which contain several phenolic groups, were 1.10 and 1.15 on neutral bonded methylated β-CD CSP using methanol / aqueous buffer as mobile phase [190]. On the cationic CSP, accentuated selectivities of 1.33 and 1.31 were attained for naringenin (**F4**) and hesperetin (**F5**) respectively. The bonded cationic β-CD CSPs provided additional electrostatic interaction sites compared with

neutral β-CD CSPs, affording enhanced enantioseparation towards polar analytes.

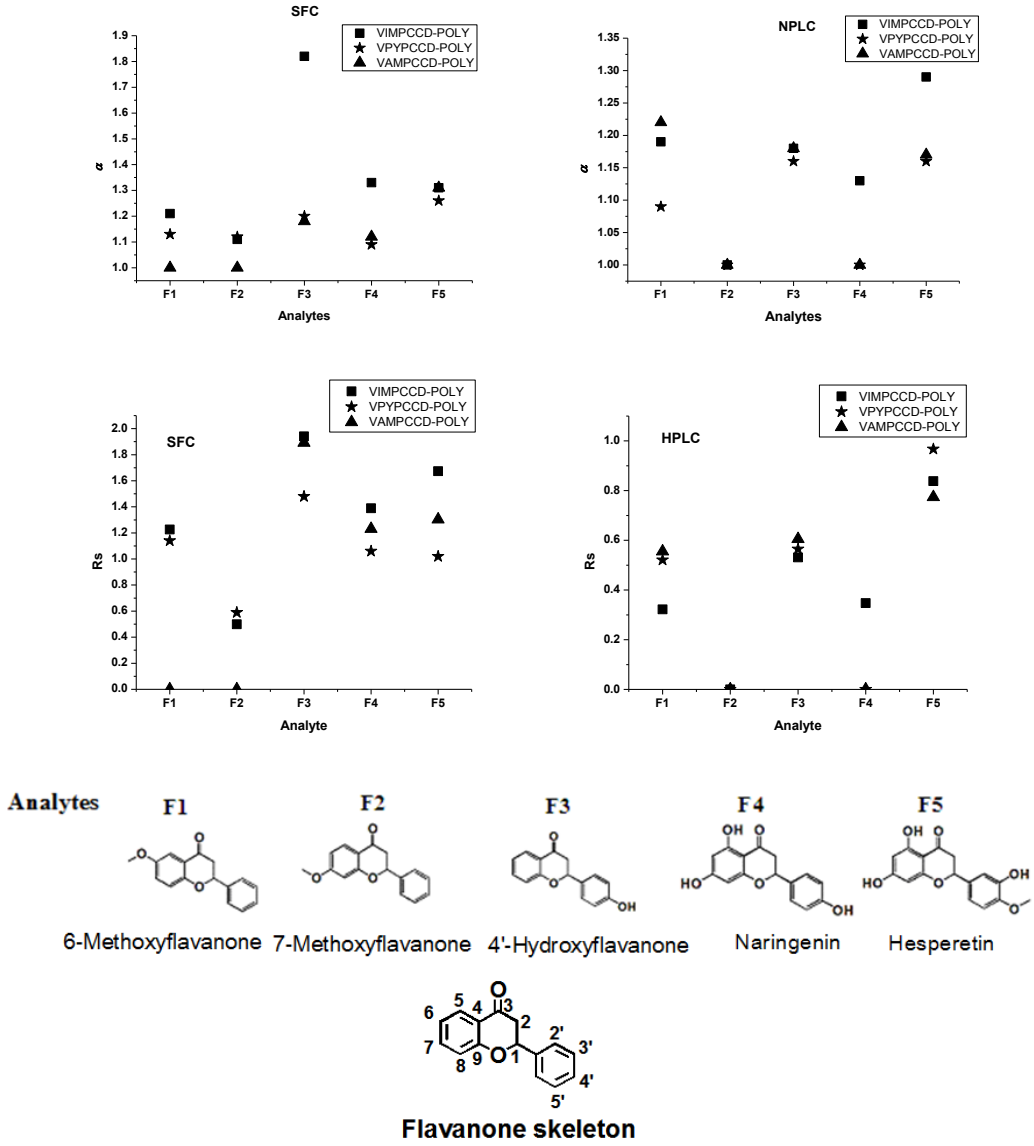


Figure 3.4: Enantioseparation results of flavanone derivatives. (Condition: SFC flow rate 1.0 ml/min, oven temperature 40 °C, BPR 15 MPa, **F1** & **F2**:CO_2: 2-propanol 99:1 (v/v), **F3**: CO_2: 2-propanol 97:3 (v/v), **F4** & **F5**: CO_2: 2-propanol 90:10 (v/v); NPLC: flow rate 0.4 ml/min, oven temperature 25 °C, **F1** & **F2**: *n*-hexane: 2-propanol 99:1 (v/v); **F3**: *n*-hexane: 2-propanol 97:3 (v/v); **F4** & **F5**: *n*-hexane: 2-propanol 90:10 (v/v))

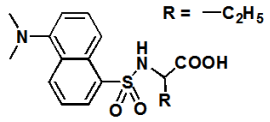
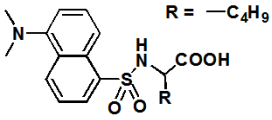
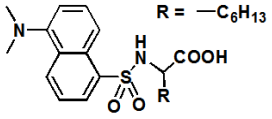
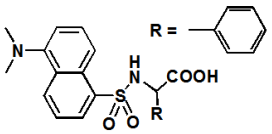
3.4.2 Dansyl amino acids

SFC enantioseparations of dansyl amino acids were acquired on bonded CSP **VIMPCCD-POLY** and **VPYPCCD-POLY** using mobile phase comprising CO_2 / 2-propanol (60:40/ v:v).

For dansyl amino acids (Table 3.8), enantioseparations were obtained using 2-propanol as modifier. However, enantioseparation was not attainable using methanol modifier. As methanol has a higher polarity than 2-propanol, it probably interacted tightly with the substituents on the CD rim, masking the interaction sites on the substituents and thus weakening its enantioseparation ability [27]. On the other hand, comparing the selectivities and resolutions amongst the dansyl amino acids, it was found that when the chain length of the alkyl group ("R") was prolonged from ethyl to *n*-hexyl substituent, **VIMPCCD-POLY** have shown increased chiral selectivity from 1.09 to 1.18 and **VPYPCCD-POLY** from 1.07 to 1.21; as a result, resolutions of the analytes increased. It can therefore be inferred that hydrophobic inclusion between the CD cavity and the "R" group was involved in the enantioseparation processes for dansyl amino acids. The "R" group with higher hydrophobicity afforded a better interaction with the CD cavity. Moreover, **VIMPCCD-POLY** afforded analyte dansyl-DL-phenylalanine an enantioselectivity of 1.29 which contains a phenyl group. The same CSP has shown a lower chiral selectivity towards dansyl-α-aminocaprylic acid (1.18) which has a *n*-hexyl group. The phenyl group's interaction with CD cavity in **VIMPCCD-POLY** was apparently more favorable to enantioseparation than an alkyl chain (n-hexyl group); but on **VPYPCCD-POLY**, chiral selectivity of dansyl-phenylalanine was 1.18, lower than a selectivity of 1.21 towards dansyl-α-aminocaprylic acid. It was considered the pyridinium ring was more bulky than imidazolium ring which would cause a stronger steric hindrance to the

phenyl group in dansyl-*α*-aminocaprylic acid, reducing the stability of the complex formed between the CD cavity and phenyl group.

Table 3.8: Enantioseparations of Dansyl amino acids in SFC.

Analytes		*α*	Rs	Column
Dansyl-DL-α-amino-butyric acid		1.09	0.72	A
		1.07	0.65	B
Dansyl-DL-norleucine		1.15	1.08	A
		1.20	0.78	B
Dansyl-DL-α-aminocaprylic acid		1.18	1.29	A
		1.21	0.85	B
Dansyl-DL-phenylalanine		1.29	1.74	A
		1.18	0.91	B

Conditions: CO_2 : 2-propanol (60 : 40 v:v) flow rate 1 ml/min, oven temperature 40 °C, BPR 15 MPa (Column A: **VIMPCCD-POLY**; column B: **VPYPCCD-POLY**)

3.4.3 Diuretic thiazides

In previous studies, applications of neutral CD derivatives in CE or RPLC reported weak enantioseparations of diuretic thiazides where the hydrophobic cavity of CD could form only unstable inclusion with the thiazides [191-194]. Comparative studies on neutral CD additives such as native β-CD, (2-hydroxylpropyl)-β-CD and methyl-β-CD had shown that native CD depicted the best enantioseparation towards the thiazide of chlorthalidone, albeit with a modest enantioselectivity of 1.12 [192]. Charged β-CD derivatives were also applied for the enantioseparation of thiazides. A dual additive comprising anionic β-CD (carboxymethyl-β-CD) and neutral β-CD in the mobile phases had shown enantioseparations towards chlorthalidone with an optimized resolution of 1.5.

However, the enantioseparation was the combined effect between neutral and charged β-CD additives. It was unclear whether the presence of charged β-CD additive alone would afford better enantioseparation [193]. Cationic β-CD additive, on the other hand, also afforded enantioseparation towards chlorthalidone (α 1.149). Meanwhile, derivatizing β-CD with charge was also desirous as it would enhance the solubility of CD additives. With higher concentration of cationic CD derivatives in the mobile phase, the enantioselectivity of chlorthalidone was optimized at 1.188 [194]. However, our bonded cationic β-CD CSP VIMPCCD-POLY, afforded a higher chiral selectivity of 1.29 towards chlorthalidone (T5).

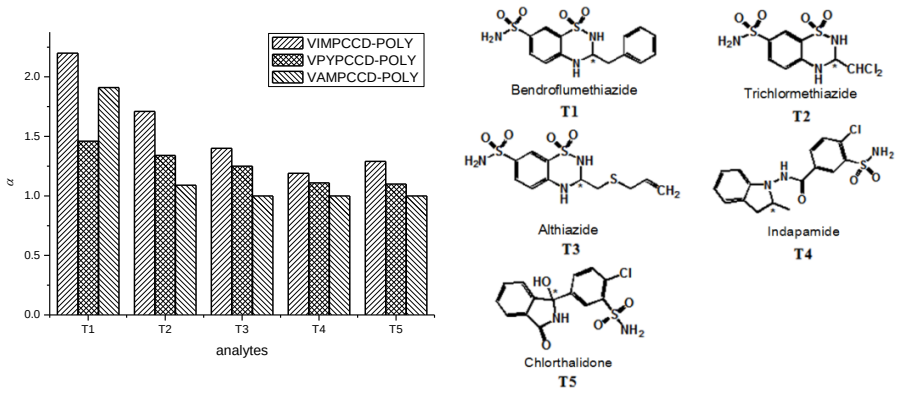


Figure 3.5: Enantioseparation of thiazides on bonded cationic β-CD CSPs in SFC (Condition: CO_2 : methanol (90 : 10 v:v) flow rate 1 ml/min, oven temperature 40 °C, BPR 15 MPa)

Enantioseparations of bendroflumethiazide were previously attainable on bonded native β-CD CSP with a selectivity of 1.11 [195, 196]. It was suggested in these reports that the enantioseparations of the thiazides might require π-π stacking while the substituent in their bonded CSP could only form hydrogen bonding with the analytes. The CSPs with O-naphthylethylcarbamoyl substituents on (R)- and

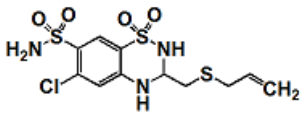
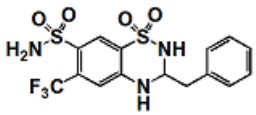
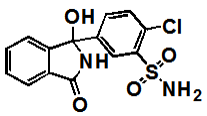
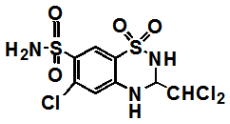
(S)-naphthylethylcarbamate- β-CD could provide π-π stacking sites and showed accentuated enantioseparations towards althiazide (α 1.02-1.03), bendroflumethiazide (α 1.10-1.22) and indapamide (α 1.04-1.18) in RPLC and SFC [27, 197]. However, our cationic β-CD derivatives contain rather appropriate function groups and therefore afford rather better enantioseparations towards althiazide (α 1.40), bendroflumethiazide (α 2.20) and indapamide (α 1.19) in SFC. Enantioseparation results on the bonded cationic β-CD CSPs are summarized in Fig. 3.5. It is apparent that bendroflumethiazide (**T1**), trichlormethiazide (**T2**), althiazide (**T3**) are well resolved. Meanwhile the cationic CSPs' selectivity diminishes along the sequence **VIMPCCD-POLY**, **VPYPCCD-POLY** and **VAMPCCD-POLY** for almost all racemates. On the basis of literature results, it was recognized the enantioseparations of such thiazides containing bicyclic or tricyclic structures required multiple interaction sites on the CD rim to form hydrogen bond, dipole-dipole stacking or π-π stacking [195]. The CSPs with an aromatic cationic substituent would offer more interaction sites and could therefore enhance enantioseparation in comparison to that with aliphatic cationic substituent. Moreover, partially hydrophobic inclusion between the bulky hydrophobic moiety of thiazide and CD cavity may also be involved in the enantioseparation mechanism [160]. It is considered that the somewhat bulkier pyridinium moiety might cause higher steric hindrance for the analytes' access to the CD cavity and resulted in a relatively lower chiral selectivity than the one with imidazolium moiety.

3.5 Influence of organic modifier on enantioseparation

Chemically bonded CSPs are amendable for use with highly polar organic mobile phases. The presence of organic modifier in NPLC and SFC serves to increase the elution ability of the mobile phase as well as to improve the solubility of the solute in the mobile phase [56, 197-199]. Moreover, different organic modifiers have variable hydrogen bonding ability. Accordingly, their interactions with chiral selectors are diverse [200-202]. In this study, the enantioseparations were performed on **VIMPCCD-POLY** in SFC and NPLC. The cationic CSP enabled enantioseparations of racemic samples of thiazides, flavanone derivatives, aromatic acids and dansyl amino acids. The influence of the type as well as the proportion of organic modifiers on enantioseparations was investigated.

3.5.1 Organic modifiers' influence on enantioseparation of thiazides in SFC

Table 3.9: Enantioseparations of Thiazide with Different Organic Modifiers

Analytes		Modifiers	α	Rs
Althiazide (**T3**)		a	1.49	2.68
		b	1.40	2.55
Bendroflumethiazide (**T1**)		a	1.43	1.56
		b	2.20	6.91
Chlorthalidone (**T5**)		a	-	-
		b	1.29	2.44
Trichlormethiazide (**T2**)		a	1.41	1.56
		b	1.71	3.94

Conditions: Flow rate 1.0 ml/min, oven temperature 40 ºC, BPR 15 MPa; modifier content in CO_2: a 30 vol% 2-propanol; b 10 vol% MeOH; "-" means no separation

2-Propanol and methanol were applied as organic modifiers. Use of methanol

was found to afford better enantioseparation for most thiazides than 2-propanol (Table 3.9). As 2-propanol is more hydrophobic than methanol, it could competitively block the racemic thiazide molecules' inclusion into hydrophobic cavity of β-CD, thus depicting diminishing enantioselectivities [203].

A representative enantioseparation chromatogram of a thiazide on bonded CSP **VIMPCCD-POLY** in SFC is presented in Fig 3.6. The two main peaks with equal area are recognized as enantiomers.

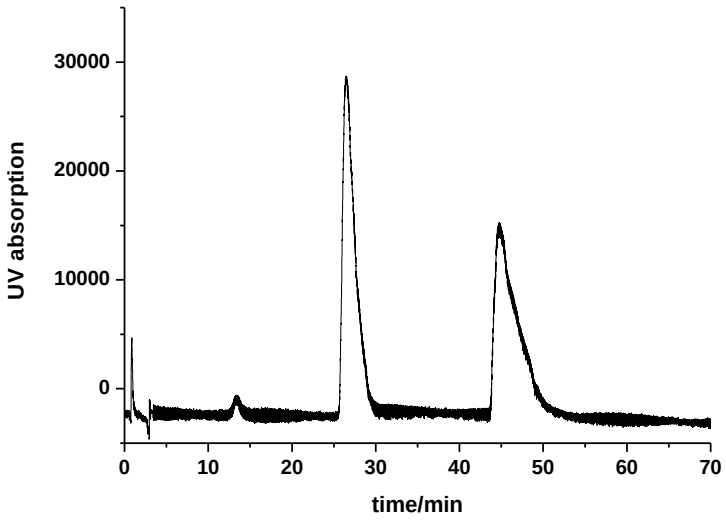


Figure 3.6: Representative SFC chromatogram for trichlormethiazide. (Condition: CO_2 : MeOH (90:10 v/v), flow rate 2 ml/min, oven temperature 40 ^oC, BPR 15 MPa.)

3.5.2 Effect of the proportion of 2-propanol on the retention & enantioseparation of racemic aromatic acids in NPLC

In NPLC, the CD cavity is occupied by *n*-hexane molecules [27]. In contrast to SFC where CO_2 is miscible with both highly polar and modestly polar organic solvents, in NPLC condition, *n*-hexane is only miscible with modifier of modest polarity. In this study, enantioseparations of two series of aromatic acids were

investigated using 2-propanol / *n*-hexane as mobile phases in NPLC. The enantioseparation results obtained are compared in Fig. 3.7. Amongst the acids of series I (**B1**, **B2**), it was found that polar mobile phases containing higher ratio of 2-propanol afforded shorter retention times for the acid analytes but better enantioselectivities. In this case, decreasing the attractive interactions between CSP and acids of series I might improve the enantioseparations. *α*,4-Dimethylphenylacetic acid (**B2**) has a better enantioselectivity than 2-phenylpropionic acid (**B1**) while having a lower retention, which is suggestive that the additional methyl substitute on the *para*-position to the chiral center might affect the enantioseparation progress.

The aromatic acids of series II were 2-phenoxylpropionic acid and its derivatives. It was found that by increasing the ratio of 2-propanol in the mobile phase, the retention times and enantioselectivity were lowered simultaneously. This would suggest that the attractive interactions between the acids and the chiral selector would enhance the enantioseparation process in this case. Increasing solvent polarity would weaken the attractive interactions which not only reduce retention but also lower chiral selectivity.

It has been reported that the substituents on the chiral center of the analyte could be used to predict the value of its enantioselectivity which is affordable on specified chiral selector [204]. Comparing the structures of acid analytes between series I and series II, it is found that the key difference lies in the presence of the phenyl or phenoxy moiety adjacent to the chiral center. Analytes with a phenoxy

group showed higher retention than the analytes with phenyl groups under the same conditions. By reducing the content of organic modifier, increased retention time was found favorable to enantioseparation of analytes with a phenyl group but unfavorable for the analytes with a phenoxy group, which would suggest that the substituent on this position was influential to the analytes' retention and enantioseparation processes.

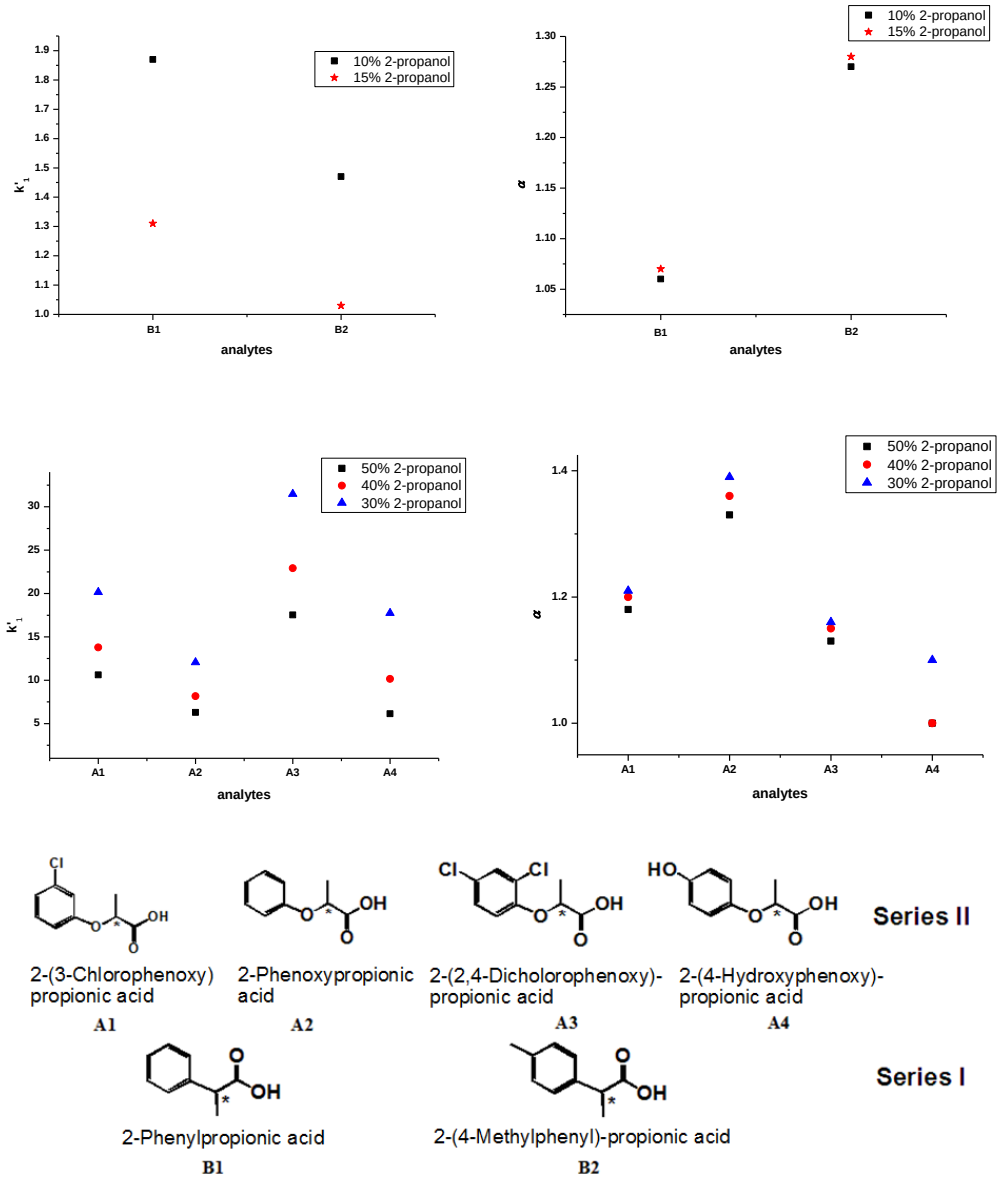


Figure 3.7: Selectivities & retention *vs.* modifier content for acid series II on **VIMPCCD-POLY** in NPLC (Condition: flow rate: 0.4 ml/min, oven temperature: 25 ºC)

3.5.3 Effect of the proportion of 2-propanol on the enantioseparation of flavanone derivatives and thiazides in NPLC

The cationic CSP **VPYPCCD-POLY** afforded enantioseparations towards thiazide racemates with high chiral selectivities but only modest enantioseparations towards flavanone derivatives in NPLC. The amount of 2-propanol added in the mobile phase also has a significant impact on the enantioseparations of thiazide and flavanone derivatives. Their enantioseparation results are illustrated in Fig. 3.8.

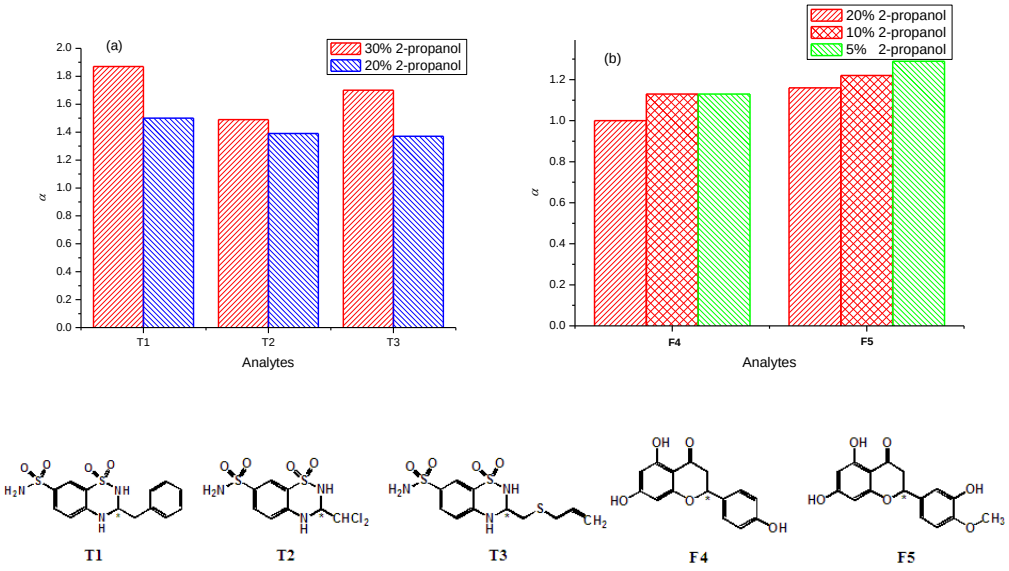


Figure 3.8: Selectivity vs. modifier content for thiazide (a) & flavanone (b) derivatives in NPLC on **VPYPCCD-POLY**

It was recognized previously that the alcohol content in the mobile phase of NPLC was one major parameter affecting the quality of enantioseparation [205]. In our investigations, it was found that enantioseparation of racemic thiazides was enhanced with higher content of 2-propanol in the mobile phase [Fig. 3.8 (a)]. On the other hand, the flavanone derivatives usually have higher enantioselectivity with less 2-propanol in the mobile phase [Fig. 3.8 (b)]. The results suggested that changing the content of 2-propanol would influence the interactions between chiral

selector and analytes enantioselectively. The enantioselective interactions are considered either in the hydrophobic inclusion of the CD cavity or between the analytes and the substituents on the CD rim. However, in this case, as the CD cavity was occupied by *n*-hexane in NPLC, it was not possible to afford hydrophobic inclusion. The stereoselective interactions being affected by 2-propanol must be between analytes and substituents on CD rim.

3.6 Influence of acid additive on enantioseparation

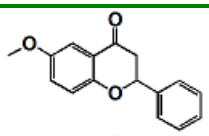
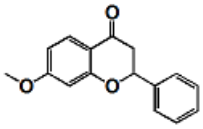
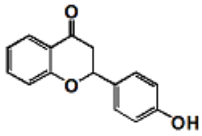
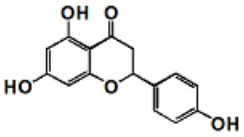
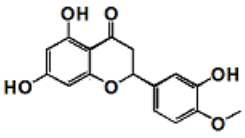
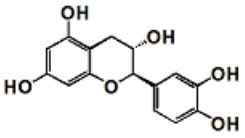
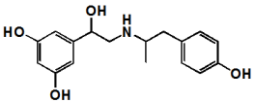
3.6.1 Acid additive's influence on the retention and enantioseparation of the racemates in SFC

In our investigation, acetic acid was added at 0.1% volume ratio to the organic modifier (2-propanol).

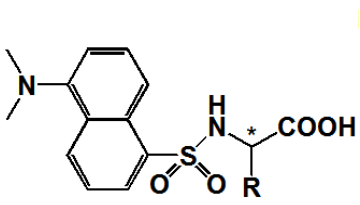
Each racemic flavanone derivative was subjected to different separation conditions in SFC as summarized in Table 3.10, together with enantioselectivities observed. The dansyl amino acids as listed in Fig. 3.9, performed enantioseparation using a mixture of CO_2 / 2-propanol (60/40 v/v) as the mobile phase.

The mobile phases with or without added acetic acid (0.1 vol%) containing same proportion of organic modifiers were being compared. The acid additive has affected the retention and enantioseparation of the racemates in SFC. The discrepancies amongst the results are illustrated in Fig. 3.10.

Table 3.10: SFC Conditions Applied on Enantioseparation of Weakly Acidic Racemates.

Analyte	Structure	k_1	α	Rs	Condition
6-Methoxyflavanone (**F1**)		4.11	1.11	1.23	a
7-Methoxyflavanone (**F2**)		5.84	1.10	0.83	a
4'-Hydroxyflavanone (**F3**)		12.66	1.86	1.94	b
Naringenin (**F4**)		16.48	1.33	1.39	c
Hesperetin (**F5**)		14.52	1.31	1.11	c
Catechin (**F6**)		16.51	1.23	0.85	d
Fenoterol (**F0**)		12.91	1.51	2.69	e

Condition: Flow rate 1.0 ml/min, oven temperature 40 ºC, BPR 15 MPa; modifier content in CO_2: **a** 1% 2-propanol; **b** 5% 2-propanol/0.1% v/v AcOH; **c** 10% 2-propanol/0.1% v/v AcOH; **d** 20% 2-propanol; **e** 20% MeOH/0.1% v/v AcOH



R= —C_2H_5 Dansyl-DL-α-aminobutyric acid (D1)

R= —C_3H_7 Dansyl-DL-α-novaline (D2)

R= —C_4H_9 Dansyl-DL-α-norleucine (D3)

R= —C_6H_{13} Dansyl-DL-α-aminocaprylic acid (D4)

R= —C_6H_5 Dansyl-DL-α-phenylalanine (D5)

Figure 3.9 Structures of dansyl amino acids in the study on acid additives' influence

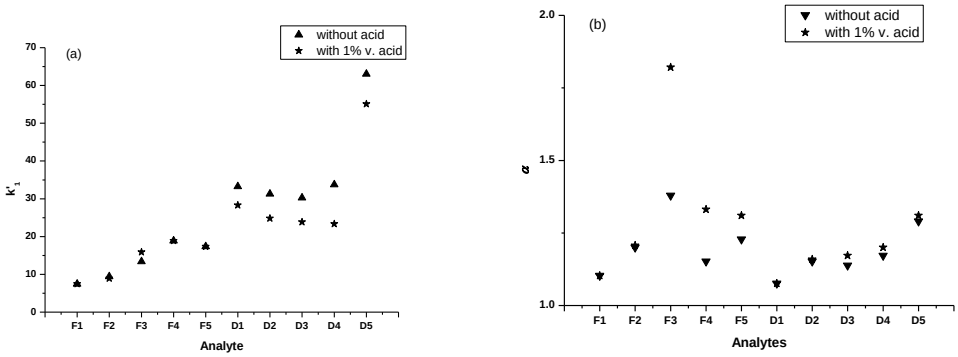


Figure 3.10: Change of k_1' and selectivities of analytes with addition of acetic acids

In perusal of the results in Fig. 3.10, two conclusions can be drawn:

i) Acid additive shortens strong acidic analytes' retention time obviously but has little or no influence on weakly acidic or neutral analytes.

In Fig. 3.10 (a), dansyl amino acids exhibited higher retention than weakly acidic (i.e. containing phenolic group) and neutral compounds. Acid additive reduced the retentions of dansyl amino acids significantly while the retention times of weakly acidic and neutral compounds were not influenced too much. The acidic groups in the analytes could generate attractive electrostatic force with cationic CSP. Acid additive competed to interact with the cationic moiety on the chiral selector masking the positive charge on the chiral selectors. As a result, the electrostatic force between analytes and chiral selectors was weakened. The influence of acid additive on the retentions of dansyl amino

acids was clearly evident，corresponding the importance of electrostatic forces in the analytes' retentions.

ii) Acid additive improves enantioselectivity of weakly acidic analytes while it does not help enantioseparations of the strongly acidic analytes and neutral analytes.

In Fig. 3.10 (b), it is interesting to find that the cationic CSP's selectivities towards weakly acidic compounds (**F3** to **F5**) were improved by acid additive, but enantioselectivities of both strongly acidic analytes (**D1** to **D5)** and neutral compounds (**F1** and **F2**) were not changed. Acid additive could suppress the dissociation of acidic compounds in mobile phase. The CD cavity may then form stable hydrophobic inclusion with undissociated analytes and acquire higher chiral selectivities. However, neutral compounds (**F1**, **F2** without phenolic groups), which could only interact with CSP through hydrophobic inclusion, attained lower chiral resolutions than weakly acidic compounds (**F3**, **F4**, **F5** with phenolic groups). It is suggested that the hydrophobic inclusion alone is not sufficient for enhanced enantioseparations whereas a combination of electrostatic force and hydrophobic inclusion would be better. For strong acids, although the electrostatic force could be weakened by acid additive, it was still excessively strong, thus overwhelming stereoselective interaction which was possibly derived from CD inclusion. Accordingly, the acid additive only shortened the retention times but did not improved upon the chiral selectivities, and in both cases, the selectivities of strong acids were lower

than weak acidic analytes (Fig. 3.10 (b)). In Stalcup's study on anionic β-CD [117], it was also showed that, excessively strong electrostatic force between protonated aminoglutethimide and anionic chiral was unfavorable to enantioseparation. On the other hand, weakly electrostatic force could be adjusted by the acidic additives to cooperate with other interactions better and resulted in a higher enantioselectivity.

3.6.2 Acid additive's influence on the retention and enantioseparation of the racemates in NPLC

The acid additive's influence in NPLC is much stronger than in SFC probably because of its higher solubility in the organic solvents. Polar organic modifier in NPLC should be coupled with acid additives in the analyses of acid racemates since mobile phase without acid additive was too weak to elute them out. According to our experience, decreasing the proportion of polar organic modifier by half would normally prolong the retentions of neutral / weak acid analytes significantly. It is interesting that when acid additive was applied on the cationic β-CD CSP, the proportion of 2-propanol did not show strong influence on the retentions of the acid analytes. It is considered that the decrease of the attractive force between the chiral selector and analytes was mainly caused by the acid additive in the mobile phase. Although a higher proportion of 2-propanol might also result in a decrease of the retention, the effect appears to be only marginal. However, when acid additive was present, 2-propanol still had an influence on the enantioselectivities. It is therefore concluded that the stereoselective interactions

between chiral selector and analytes were being affected by 2-propanol in this case.

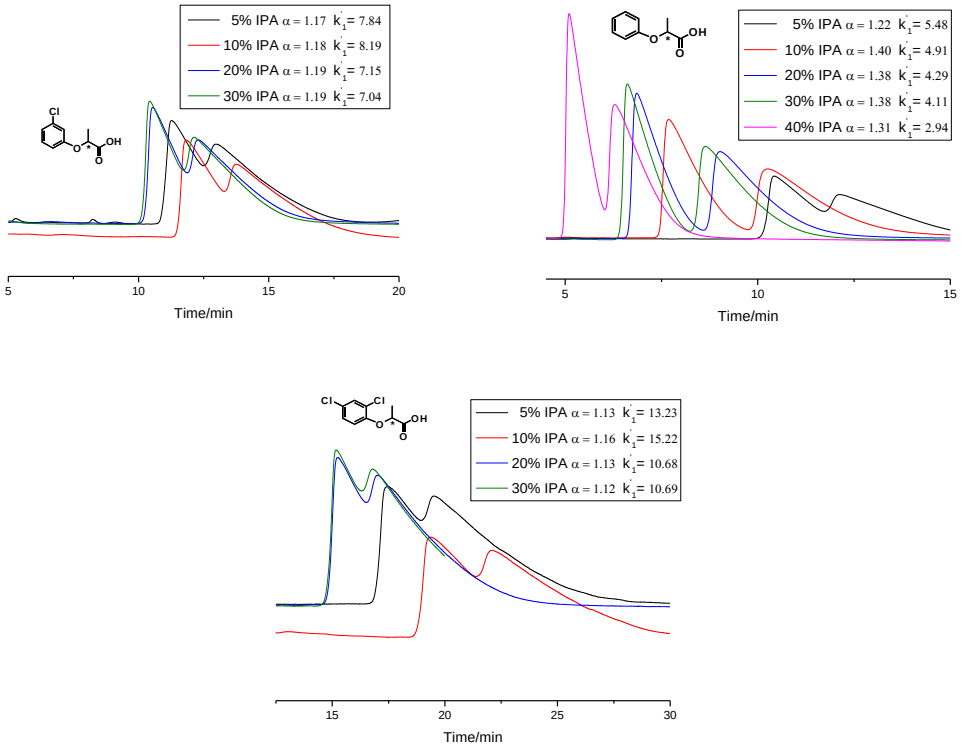


Figure 3.11: Influence of acid additive (1.0 vol%) for chiral separation of racemic acids in NPLC (**VIMPCCD-POLY**)

3.7 Cationic CSPs' performances in NPLC and SFC

Enantioseparations of a series of racemates have been attained under both SFC and NPLC conditions on CSP **VIMPCCD-POLY** and **VPYPCCD-POLY**. The cationic CSPs afforded baseline enantiomeric separations of most of the analytes.

The analytes tested in HPLC and SFC comparisons are summarized in Fig. 3.12. IDRA 21 [7-Chloro-3-methyl-3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxide (**T0**)] is an ampakine drug derived from aniracetam. **T0** is also a chiral

drug, with (+)-7-chloro-3-methyl-3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxide being the active form. Analytes from **F1** to **F5** are flavanone derivatives. The same with flavanone derivatives, Fenoterol (**F0**) also contains phenolic groups, and it contains a phenyl substituent free to rotate around a single C-C bond.

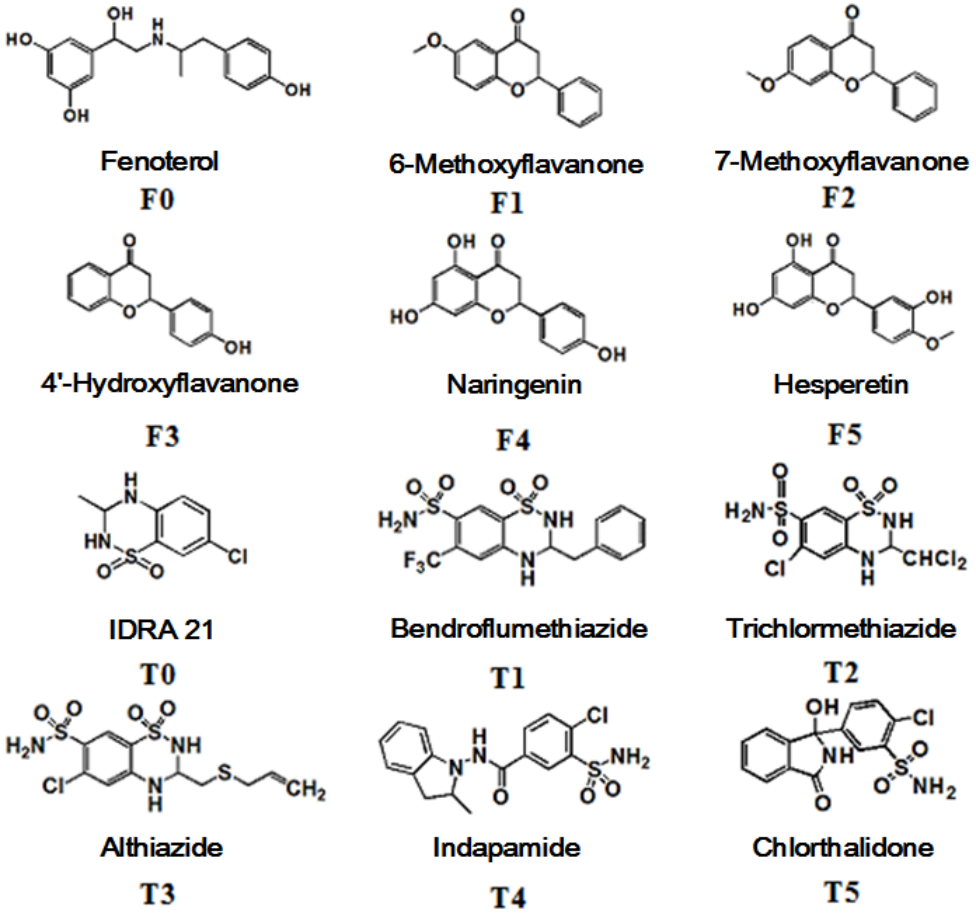


Fig 3.12: Structures of analytes for NPLC & SFC comparison

The enantioseparation results are summarized in Fig. 3.13. The cationic CSPs depicted higher chiral selectivities towards analytes with phenolic groups (**F0**-**F6**) in SFC than in NPLC. In contrast with NPLC, under SFC conditions, the CD cavity is occupied by CO_2 which is easy to be replaced by analyte [169, 206]. The formation of hydrophobic inclusion is favorable to enantioseparation. Enhanced enantioseparations of analytes of **F0**-**F6** in SFC confirmed the importance of the

inclusion phenomenon in the enantioseparation mechanism.

Analytes from **T0** to **T5** were thiazides. According to previous discussion, their interactions toward the chiral selectors were mainly with the substituents on the CD rim. The hydrophobic inclusion between analytes and CD cavity was not dominant. In this comparison, 4 out of 6 samples attained better enantioseparations on **VIMPCCD-POLY** in NPLC than in SFC; on **VPYPCCD-POLY**, 3 out of 5 were better resolved in NPLC. In this instance, a general trend cannot be drawn amongst the enantioselectivities of thiazides in both conditions. In comparison with the results achieved in NPLC, the lower enantioselectivities of some thiazides in SFC might be the result of the solvation of amine groups in the analytes by supercritical CO_2 [207]. Comparison between HPLC and SFC in previous reported CSPs also revealed that enantioseparations attained in SFC conditions were not always greater than LC [57, 61, 62].

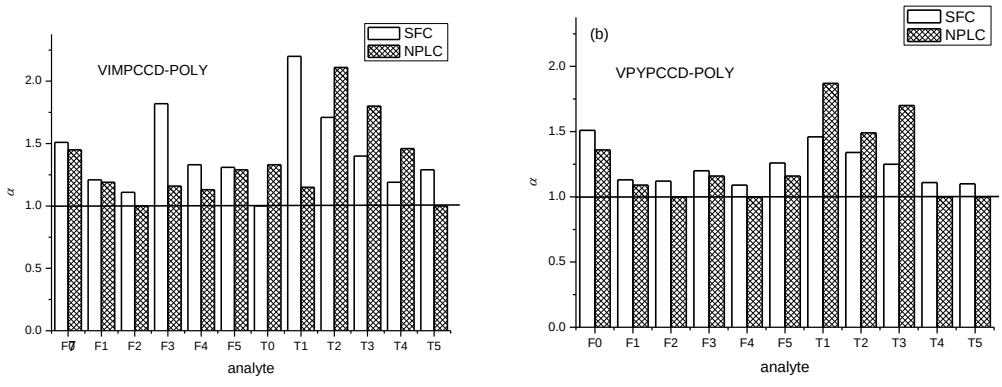


Figure 3.13: NPLC *vs.* SFC on bonded cationic β-CD CSPs

3.8 Loading study

3.8.1 Loading study on VIMPCCD-POLY

Bendroflumethiazide (**T1**) was chosen for loading studies because it had the highest chiral selectivities on the bonded cationic β-CD CSPs. On CSP **VIMPCCD-POLY**, solutions of bendroflumethiazide with three different concentrations (50 mg/ml, 100 mg/ml and 200 mg/ml) were prepared using HPLC grade methanol. The results in Fig. 3.14 showed the column was capable of achieving baseline separation when the sample concentration was 200 mg/ml. In our experiment, it was also determined that the maximum solubility of bendroflumethiazide in methanol at room temperature was around 200 mg/ml.

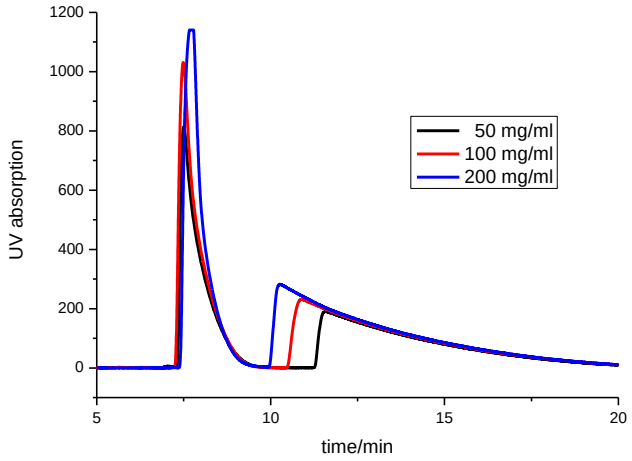


Figure 3.14: Loading study of bendroflumethiazide on **VIMPCCD-POLY** (CO_2 : MeOH (90:10 v:v); flow rate 1.0 ml/min; oven temperature 40 °C; BPR 15.0 MPa; injection volume 5.0 µl).

3.8.2 Loading study on VPYPCCD-POLY

The loading on **VPYPCCD-POLY** was lower than on **VIMPCCD-POLY**. The injection volume was reduced to 1.0 µl for the baseline separations of highly concentrated samples. The maximum sample loading on **VPYPCCD-POLY** was

1.0 µl of 200 mg/ml bendroflumethiazide which was about one fifth the amount on **VIMPCCD-POLY** (Fig. 3.15).

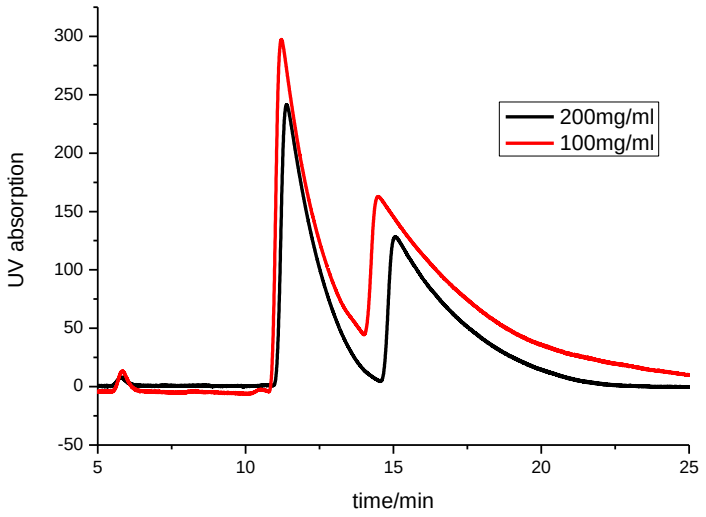


Figure 3.15: Loading study of bendroflumethiazide on VPYPCCD-POLY with different sample concentration (CO_2 : MeOH (90:10 v:v); flow rate 1.0 ml/min; oven temperature 40 °C; BPR 15.0 MPa; injection volume 1.0 µl)

The other technique of loading was by fixing the concentration of analytes while increasing the injection volume (Fig. 3.16). It was found the maximum loading was about 3 µl injection volume of 100 mg/ml sample.

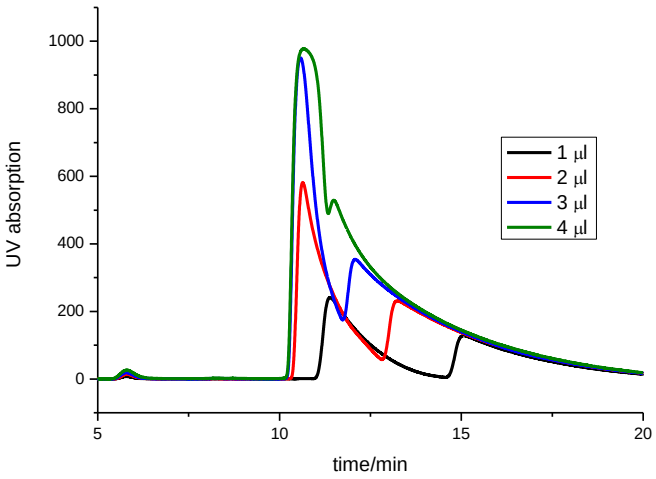


Figure 3.16: Loading study of bendroflumethiazide on VPYPCCD-POLY with different sample injection volumes (CO_2 : MeOH (90:10 v:v); flow rate 1.0 ml/min; oven temperature 40 °C; BPR 15.0 MPa; sample concentration 100 mg/ml)

It was found that overloading by increasing concentration or injection volume of sample afforded different chromatographic results. In Fig. 3.17, it showed although in both conditions, the loadings were 400 ng, using high concentration sample afforded a better separation but the peaks were broadened.

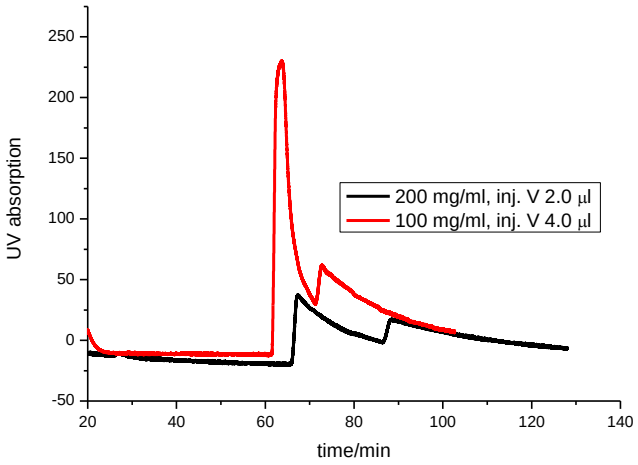


Figure 3.17: Loading study of bendroflumethiazide on VPYPCCD-POLY (CO_2 : MeOH (95: 5 v:v); 1.0 ml/min; oven temperature 40 °C.; BPR 15.0 MPa)

3.8.3 Mobile phase optimization in loading study

The chromatographic conditions were optimized in order to maximize the loading of analyte. Fig. 3.18 revealed that the best enantioseparation could be achieved with a lower content of methanol as modifier in the mobile phase. However, the downside for the improvement of the chiral resolution would be the longer time interval between two enantiomeric peaks.

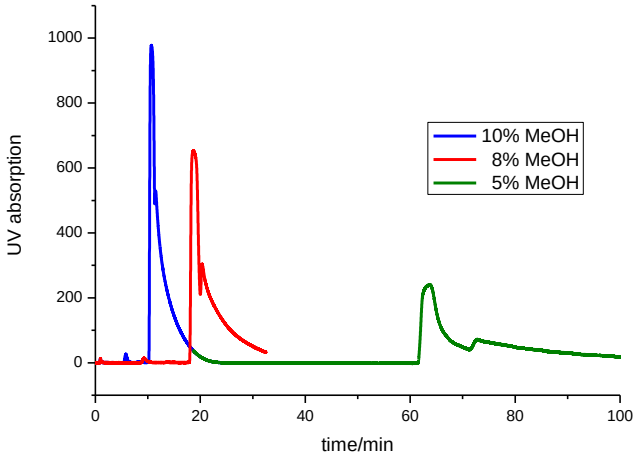


Figure 3.18: Loading study of bendroflumethiazide on VPYPCCD-POLY (flow rate 1.0 ml/min; oven temperature 40 °C.; BPR 15.0 MPa sample concentration 100 mg/ml; injection volume 4.0 µl)

Conclusion

Cationic β-CD derivatives containing an imidazolium, pyridinium or ammonium moiety were covalently bonded onto silica gel and applied as CSPs in NPLC and SFC. The bonded cationic CSPs afforded good enantioseparations toward thiazides, flavanone derivatives and dansyl amino acids. Organic modifier would compete with the analytes to occupy the CD cavity or form hydrogen bonding with the substituents on the CD rim. Therefore, it influences the retention as well as enantioseparation of racemates. Acid additive was found useful for

tuning of weak electrostatic force to act in concert with other interactions between analytes and CSPs and to enable improved enantioseparations. It was inferred that a combined effect of hydrophobic CD cavity comprising inclusion and electrostatic forces between analytes and chiral selector was involved in the enantioseparation process. In comparative studies made, it was revealed that the CSPs with aromatic cationic moiety depicted greater enantioseparation abilities over the CSPs with aliphatic cationic moiety. Additionally, the bonded CSPs with aromatic cationic moiety are also capable of separating greater quantity of racemates in loading studies. It has shown that overloading analytes with high concentration would give a better separation but the peaks were broadened. Meanwhile, overloading analytes with bigger injection volume resulted in higher column efficiencies but lower chiral selectivities.

Chapter 4 Investigation of Cationic Chiral Stationary Phases in Reversed Phase Liquid Chromatography

4.1 Introduction

Cyclodextrin derivatives have been universally employed as chiral selectors in liquid chromatographies (LC) [123]. Different elution modes in LC have been applied: normal phase, reversed phase and polar-organic modes with enantioselectivities in the three modes are mutually complementary [125]. In Chapters 3 and 4, cationic CSPs were applied in normal phase liquid chromatography (NPLC) and SFC. As discussed in Chapter 4, the cationic β-CD derivatives chemically bonded onto silica gel can be applied for use in polar solvents. The bonded CSPs were found to be stable using mobile phases containing additives of acetic acid or triethylamine. The bonded CSPs can also be able to be applied in reversed phase liquid chromatography (RPLC).

In RPLC, buffers and organic modifiers in which the ionic analytes dissociate and dissolve well are usually used as mobile phase. Accordingly, RPLC is widely applied for the separation of ionic analytes [208]. When CD based CSPs are applied in RPLC, the hydrophobic inclusion or adsorption between analytes and the CD cavity is a factor in both retention and the enantioseparation process. The molecular sizes as well as configuration of the analytes regulate the tightness of the hydrophobic inclusion and affect the chiral selectivity [209].

In recent years, ionic chiral selectors have been attracting more interests in the enantioseparations arena [210-212]. Stalcup *et al.* prepared sulfated CD CSP and applied it for enantioseparation of basic racemates in HPLC [213]. The anionic CD CSP interacted with the analytes through electrostatic forces and hydrophobic

inclusion. The extent of each interaction's influence on retention and enantioseparation of analytes could be adjusted by changing pH and ionic strength of buffer. Along with the application of anionic β-CD derivatives in HPLC, the anionic β-CDs had been extensively investigated as chiral selectors in CE. It is notable that the anionic β-CDs were potent in enantioseparation of a broad range of racemates with high resolutions [165, 214]. Accordingly, it would be interesting to investigate the cationic β-CD's performance in RPLC, since its interactions toward analytes would also be anticipated to be the combined effects of electrostatic forces and hydrophobic inclusion. However, compared with anionic chiral selector, the cationic β-CD derivatives have a higher isoelectric point and are easily protonated under acidic conditions. The chromatographic results with cationic β-CD may be complementary with those achieved on anionic β-CD under similar conditions. Since anionic β-CDs were generally effective for enantioseparation of basic compounds, it was envisaged that the acidic racemates might be properly interacting with the cationic β-CD CSPs.

For the mobile phase in RPLC, three factors affect the enantioseparation process: organic modifier, pH and ionic strength [201]. The organic solvent molecules interact competitively with the CD cavity as well as forming hydrogen bonds with the substituents on the chiral selector [215, 216]. Thus, an organic modifier enriched mobile phase would be able to elute out the analytes more quickly and the enantioselectivity would usually be decreased simultaneously. However, in some special cases the organic modifiers compete with the analytes

but only decrease their non-enantioselective interactions towards the chiral selector [217]. As the stereoselective interactions' influences become dominant, the enantioselectivity increases along with higher fraction of organic modifiers [214, 216, 218].

The acidic or basic analytes may generate undesirably strong interactions with the stationary phase which might lead to excessive retention. It is a common practice to employ acidic additives for acid analytes and basic additives for base analytes to improve separation efficiency [219]. Accordingly, acidic or basic additives are widely applied in NPLC [220, 221], RPLC [205, 222] and SFC [167, 168]. The additives are also useful for suppressing the interactions between analytes and free silanol group on silica gel. By adding acidic / basic additives, the problems of peak distortion and broadening can be resolved. In RPLC, both ionic analytes and ionic chiral selector could be dissociated in the buffer. The pK_a values of analytes and ionic groups on chiral selector are usually different. At a pH condition when both chiral selectors and analytes are dissociated, the electrostatic force and hydrogen bonding between analytes and CSPs would be strong. The resulting intensive interactions may result in strong retention of the analytes. However, at certain pH conditions when the chiral selector would be dissociated but the analytes are essentially undissociated, the interactions between the chiral selector and analytes are modest which would result in modest retention of the analytes but with somewhat higher resolution. As the pK_a of analytes is related with the "net charge" of the ionic molecules at a specified pH condition, the pK_a

values of chiral selector and analytes would be critical for selecting an optimal pH for enantioseparation [208, 223]. In this chapter, we present on our investigation on cationic β-CD CSP under various conditions in RPLC whereby the organic modifier, pH and ionic strength are varied.

4.2 Method development strategy

The cationic CSP **VIMPCCD-POLY** was chosen for investigations in RPLC since the best enantioseparation results were attained on this CSP in NPLC and SFC as discussed in Chapter 4. The preparation of this CSP has been described in *Chapter* 2. Its chemical structure is illustrated in Fig 4.1. The CSP was packed into ϕ 2.1 × 150 mm stainless steel column.

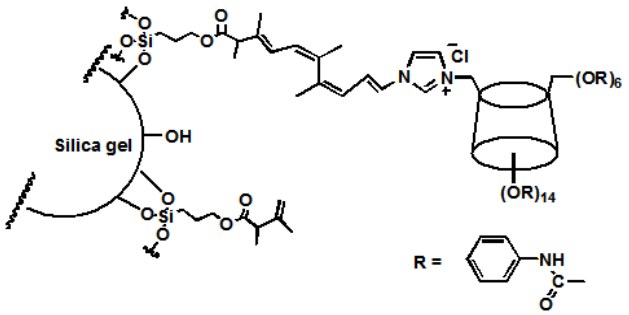


Figure 4.1 Structure of bonded CSP applied in RPLC

Since many parameters need to be varied for optimization of the mobile phase during our investigations, the standard gradient elution approach [224] was applied in RPLC as illustrated in Fig. 4.2. Initially, the mobile phase was set consistently as MeOH : water (30:70, v:v) at a flow rate of 0.2 ml/min to gain a quick overview of the samples' retentions and separations profile. It is well known that the retention time of analytes decreases with higher proportion of organic modifier in

mobile phase [225]. The samples are not able to interact with the chiral selector sufficiently with higher organic modifier so the samples are eluted out quickly. Lowering the proportion of organic modifier would increase the analytes' retention time and is likely to improve enantioseparations. On the other hand, it is sometimes necessary to increase the ratio of MeOH to elute out the analytes more quickly when the analytes are eluted out too slowly. In some cases, interactions between polar analytes and the silanol groups on the silica surface caused the sample peak broadening in the chromatography, whilst in extreme cases, the peaks coming out broad with low UV absorbance are obscured by the baseline noises. Acetic / basic additives are generally used to diminish the non-stereoselective interactions and enable sharpening of the sample peaks. After the samples' retention times were being adjusted to within an appropriate range, optimizations of chiral resolutions were effected by applying additives, varying pH and ionic strengths of the mobile phases.

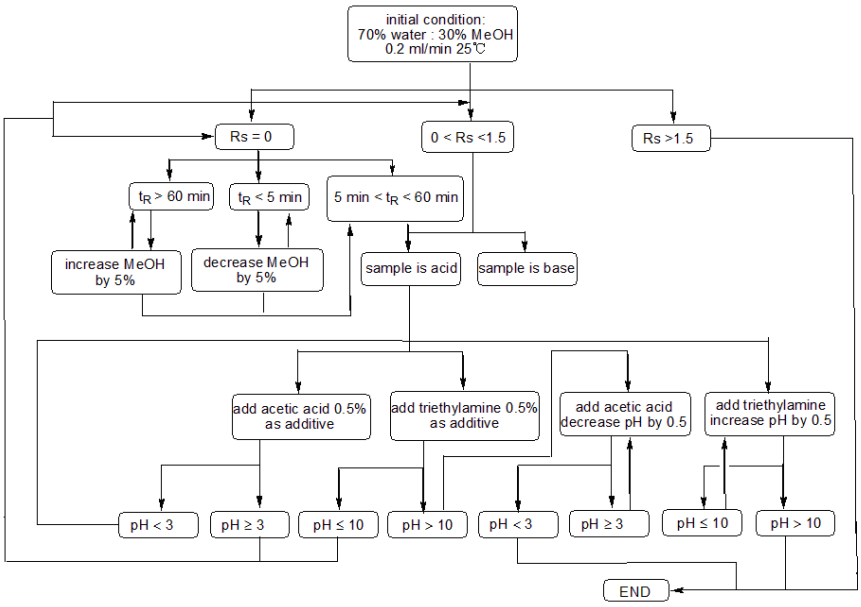


Figure 4.2: Scheme of designed gradient elution approach

4.3 The application of cationic CSP in RPLC

4.3.1 Influence of organic modifier on the chromatographic results

4.3.1.1 Influence of organic modifier on the retention of analytes

The organic modifier content is a determining factor for the analytes' retention time. The organic modifier applied in this work was MeOH. Its proportion in the mobile phase was varied from 10 to 50% (v/v). The chromatographic results with varying amounts of MeOH are summarized in Fig 4.3. Increasing proportions of MeOH afforded a decreased retention of "profen" compounds while this has little influence on smaller acid molecules such as mandelic acid and tropic acid. The organic modifier competed with the analytes for inclusion into the hydrophobic CD cavity [203, 226]. Consequently, if the analyte molecules rely mainly on inclusion in the hydrophobic CD cavity for retention on CSP, lowering the MeOH content in the mobile phase would prolong the retention time of the analyte significantly. The analytes in Fig 4.3 are organic acids with pK_a values lower than 5.0 [227]. Accordingly, the acid analytes would be significantly ionized in the mobile phase where the pH was 5.1. While the hydrophobic CD cavity would afford stable inclusion complexes with neutral molecules, polar ionized carboxylic group would be repelled from the cavity and stretching towards the outside of the CD rim [228]. Perusal of the analytes' structures revealed that each profen molecule has an apolar moiety situated away from the polar carboxylic group which can easily be included into the CD cavity. On the other hand, in mandelic acid and tropic acid, the carboxylic group is only one carbon removed

from the hydrophobic phenyl group. Inclusion of the phenyl groups into the CD cavity would consequently be anticipated to be less facile in these cases. As the inclusion process is no longer an important factor for the retention of the analytes, a change of organic modifier content in mobile phase would not afford a substantive influence on the retentions of mandelic and tropic acids.

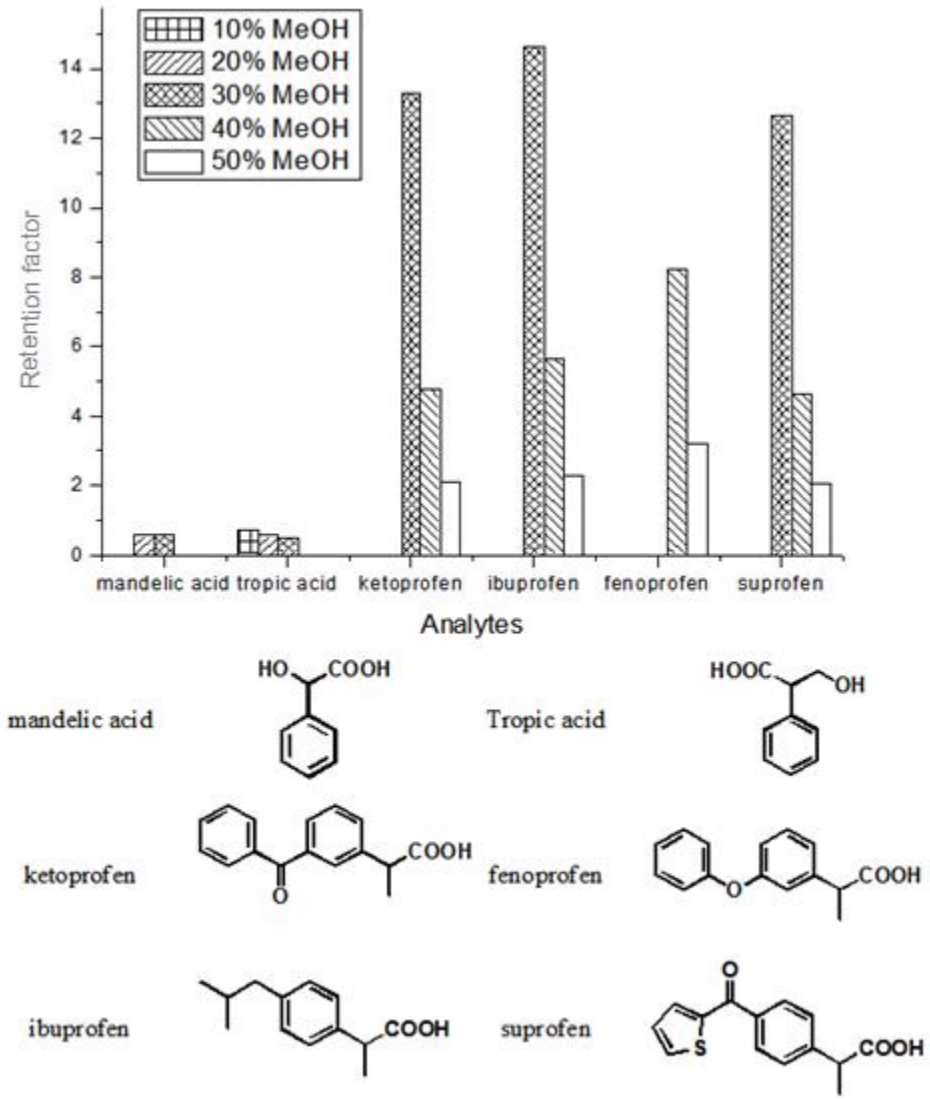


Figure 4.3: Influence of methanol composition on retention time of profens and small molecular acids. (Condition: MeOH/Buffer (0.16%w/v TEAA, pH 5.1), flow rate 0.2 ml/min, oven temperature 25 °C)

4.3.1.2 Influence of organic modifier on enantioseparation

Enantioseparations of the weakly acidic analytes are illustrated in Fig 4.4. The weakly acidic analytes, 4-hydroxylflavanone and hesperetin, are neutral or marginally ionized under the conditions applied (pH 5.94). As a result, the hydrophobic complexes formed between these analytes and CD cavity should be stable. It is indicated that higher content of MeOH decreased retention as well as chiral resolutions of the analytes. This would suggest that the inclusion process is important for both the retention and enantioseparation of the weakly acidic analytes under conditions applied herein.

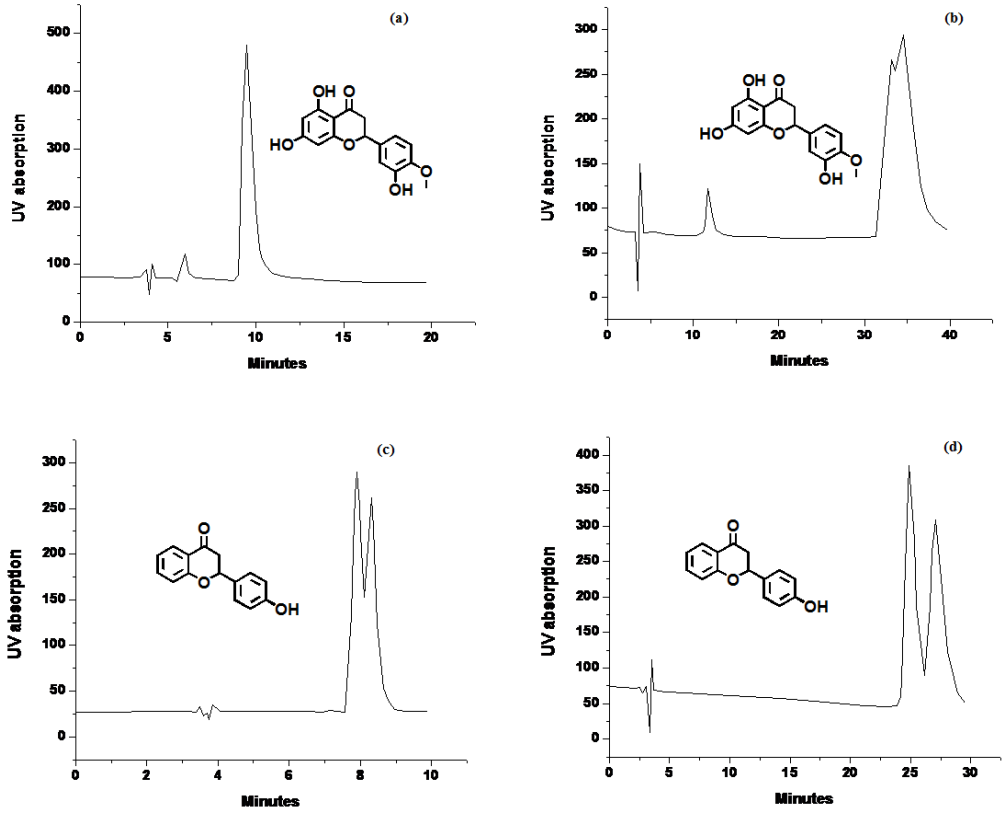


Figure 4.4: Chromatographs of hesperetin and 4-hydroxyflavanone at different methanol composition. (a) Hesperetin. MeOH/Buffer (1% w/v, pH 5.94) = 70/30 (b) Hesperetin. MeOH/Buffer (1% w/v, pH 5.94) = 50/50 (c) 4-Hydroxyflavanone. MeOH/Buffer (1% w/v, pH 5.94) = 70/30, (d) 4-Hydroxyflavanone. MeOH/Buffer (1% w/v, pH 5.94) = 50/50.

Basic compounds, such as alprenolol and atropine would be in their protonated forms under acidic conditions. Consequently, inclusion complexes in the hydrophobic CD cavity would be less favorable. As a result, any enantioseparations achieved on these basic compounds would have mainly resulted from their stereoselective interactions with the substituents on the CD rim. As shown in Fig. 4.5, the retentions of these basic compounds are noticeably reduced obviously with increasing MeOH in the mobile phases but their resolutions remained relatively unchanged. Thus, it is suggested that higher content of organic modifier would also weakened the interactions between substituents on chiral selectors and analytes but these changes are non-stereoselective.

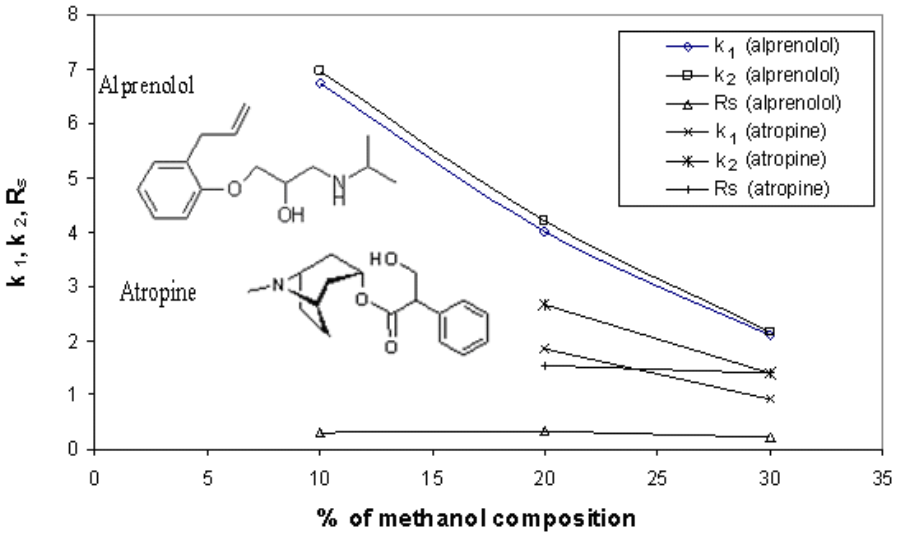


Figure 4.5: Influence of methanol composition on capacity factor and resolution of atropine and alprenolol. (Condition: MeOH/Buffer (0.16%w/v TEAA, pH 5.2))

4.3.2 Influence of pH on the chromatographic results

4.3.2.1 Influence of pH value of mobile phase on retention of analytes

Analytes of strong acid or alkali are elucidated in both acidic (pH = 5.2) and basic (pH = 8.13) conditions on the cationic β-CD CSP. It is apparent that basic analytes (**B1, B2**) showed higher retention time in basic condition and acidic analytes (**A1**-**A5**) had higher retention in acidic condition (Fig. 4.6).

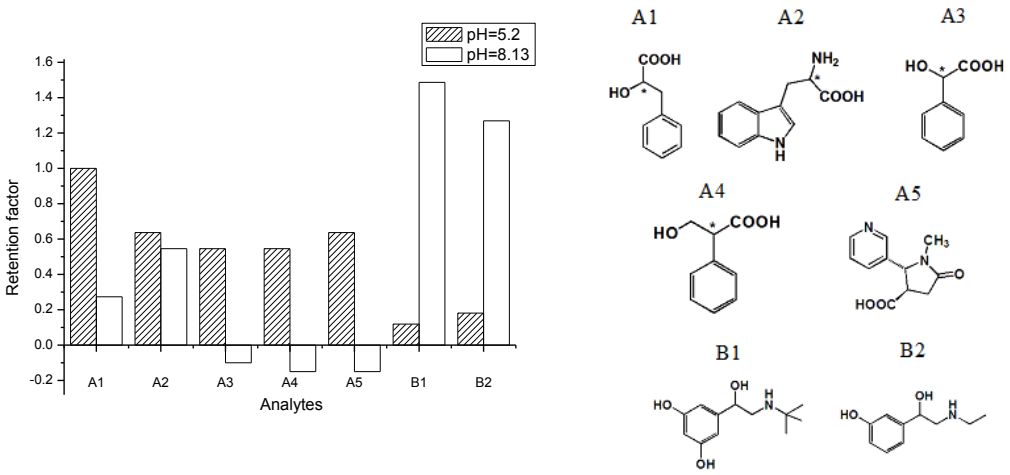


Figure 4.6: Influence of pH capacity factor of acidic & basic compounds (A1: 2-hydroxy-3-phenylpropionic acid; A2: tryptophan; A3: *α*-hydroxyphenylacetic acid; A4: 2-phenyl-3-hydroxypropionic acid; A5: *trans*-4-cotininecarboxylic acid B1: terbutaline B2: etilefrine) (Condition: MeOH/Buffer= 30/70, 0.5 ml/min.)

On the other hand, the flavanone derivatives summarized in Fig. 4.7 which are weakly acidic or neutral compounds are undissociated neutral molecules at pH 5.94. However, when pH was 4.27, the flavanone derivatives could be weakly protonated. The protonated analytes would afford a repulsive electrostatic force with the cationic CSP. At the same time, the inclusion formed between the protonated analytes and CD cavity would be less stable in comparison with the neutral analytes. Consequently, the flavanones' retentions were found to decrease

by reducing the pH from 5.94 to 4.27.

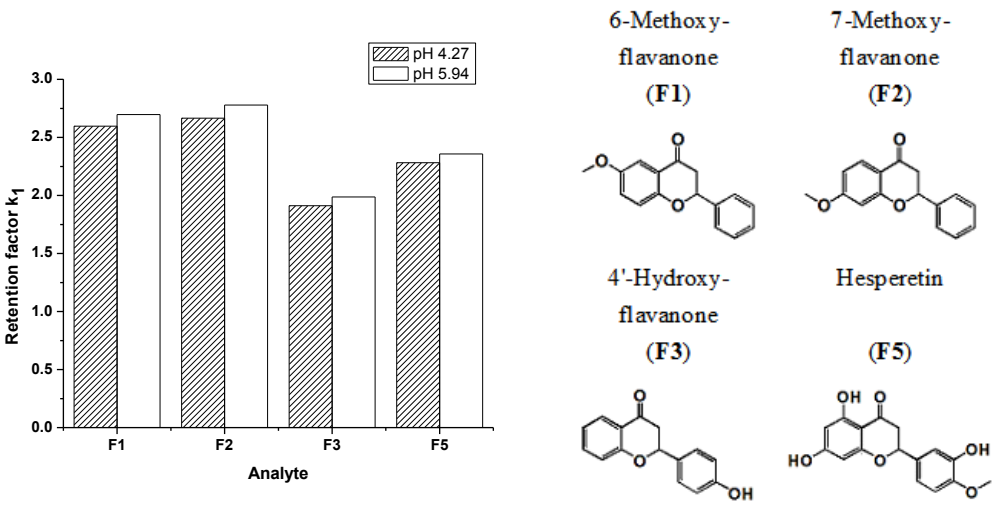


Figure 4.7: Influence of pH on capacity factor of flavanones (Condition: MeOH/Buffer (1% w/v) = 70/30, flow rate 0.5ml/min, oven temperature 25 °C)

In addition, the relationship between the retention of the profen compounds and pH of mobile phase was investigated under acidic conditions (Fig. 4.8). The profens are acidic compounds with pK_a values of 4.0 ~ 4.5 [227]. When pH was lowered from 7 to 4.33 (which was close to profens' pKa values), the retentions were increased. This could be ascribed to analytes turning from the dissociated anionic form (pH 6.98) to the predominantly neutral undissociated form (pH 4.33), with the attractive electrostatic forces between anionic analytes and cationic CSP becoming weaker but the hydrophobic inclusions becoming more favorable. As illustrated in Fig 4.8, higher retentions of the analytes are attained at pH 4.33, which would be suggestive that hydrophobic inclusion would be the dominant factor. However, when pH of mobile phases was lower to 3.55, below that of analytes' pK_a, the analytes remained largely at the neutral undissociated states and

the acidic additive interact competitively with the chiral selector [213]. This factor worked alone and resulted in the lower retentions.

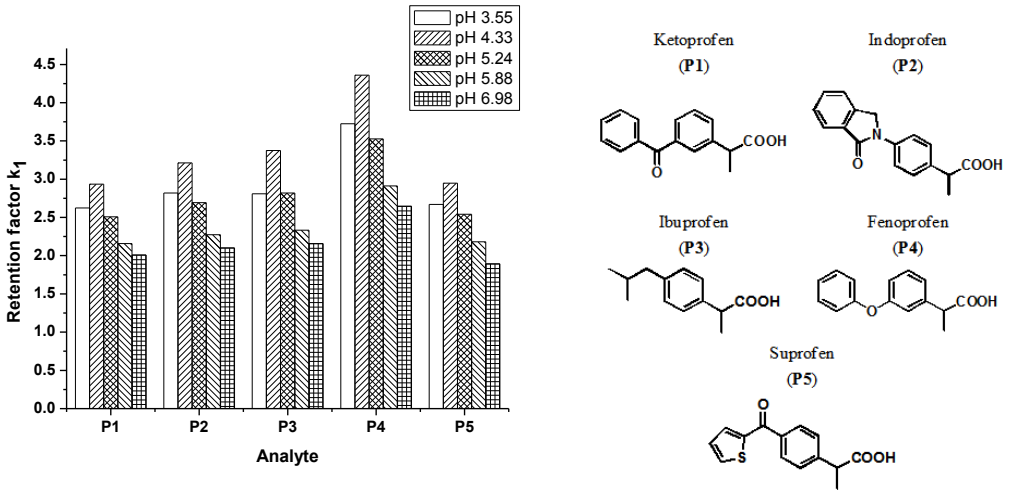


Figure 4.8: Influence of pH on capacity factor of profens (Condition: MeOH/ Buffer (1% w/v TEAA) = 50/50 oven temperature 25 °C, flow rate 0.5 ml/min,)

4.3.2.2 Influence of pH value of mobile phase on enantioseparation

Enantioseparations of weakly acidic flavanone derivatives have been attained on the bonded cationic CSPs under acidic conditions in RPLC. As indicated in Fig. 4.9, the influence of pH on their enantioselectivities is ambiguous.

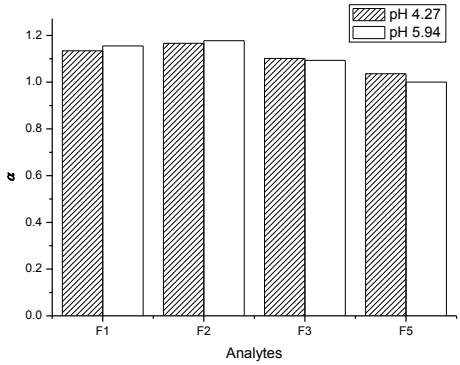


Figure 4.9: The pH of mobile phases' influence on enantioseparation of flavanone derivatives (Condition: MeOH/Buffer = 70/30; 0.5 ml/min)

Under lower pH 4.27, the electrostatic force between the analytes and CSP is

enhanced because of the protonation of analytes. However, hydrophobic inclusion between them is less marked. The two factors act competitively and afford opposing trends on specified analytes.

As depicted in Fig. 4.10, good enantioseparations of the basic compound fenoterol have been attained on the cationic CSP under acidic conditions.

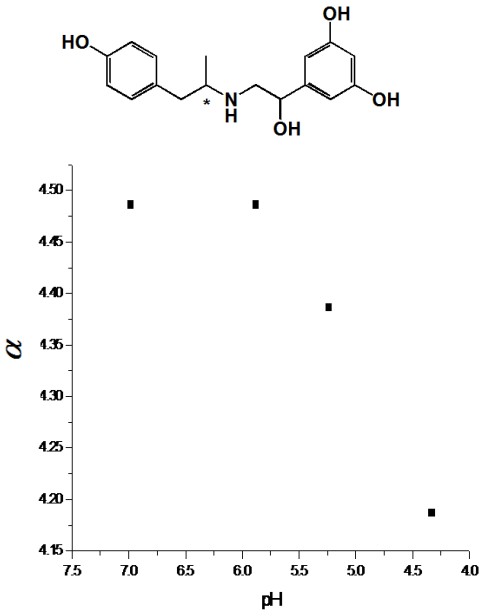


Figure 4.10: Chromatographs of fenoterol at different pH. (Condition: MeOH/ Buffer (1% w/v TEAA) = 50/50 0.5 ml/min)

In the acidic conditions, fenoterol should be predominantly protonated because its pK_a is around 9.0 [229]. The protonated ionic analyte then afforded repulsive electrostatic interaction with the cationic CSP. Since the analyte is protonated more extensively at the lower pH, the net charge of the analyte as well as the repulsive electrostatic force would be increased while the extent of hydrophobic inclusion into the CD cavity would diminish. As illustrated in Fig. 4.10, improved enantioseparation of this compound would be attainable near neutrality where the repulsive electrostatic force would be weak but hydrophobic

inclusion more prevalent. Both of these two factors act synergistically to afford good chiral recognition.

4.3.3 Influence of ionic strength on the chromatographic results

Fig. 4.11 shows that dobutamine's peaks are sharpen using mobile phase with higher ionic strength of additives, with consequent improvement in the resolution. In the CSP used in this work, our β-CD derivative was covalently linked onto the surface of silica gel through co-polymerization with small molecular monomers. These organic linkers between CD and silica gel might interact with the analytes in a non-enantioselective way which could result in unnecessarily long retention time and peak distortion. Acidic / basic additives with higher ionic strength could diminish the negative influence more significantly, affording sharper peaks.

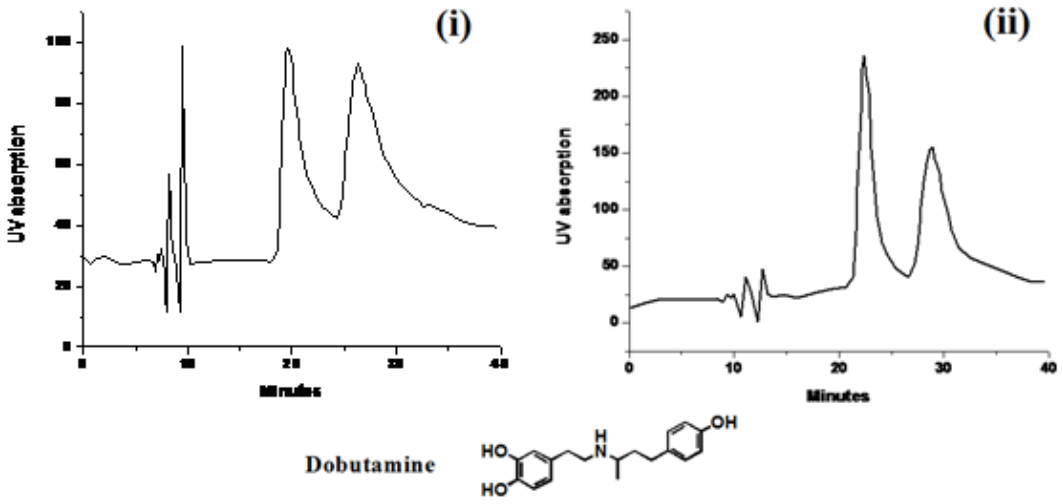


Figure 4.11: Chromatographs of dobutamine at different ionic strength of mobile phases (Condition: (i) MeOH/Buffer (0.65% w/v TEAA) = 40/60 (ii) MeOH/Buffer (1% w/v TEAA) = 40/60)

On the other hand, by increasing the ionic strength, the retentions of profens decreased (Fig. 4.12). The additives would compete with the analytes in occupying

the CD cavity [230]. Accordingly, the analytes would have a lesser capacity for inclusion into the CD cavity with consequently diminished retention.

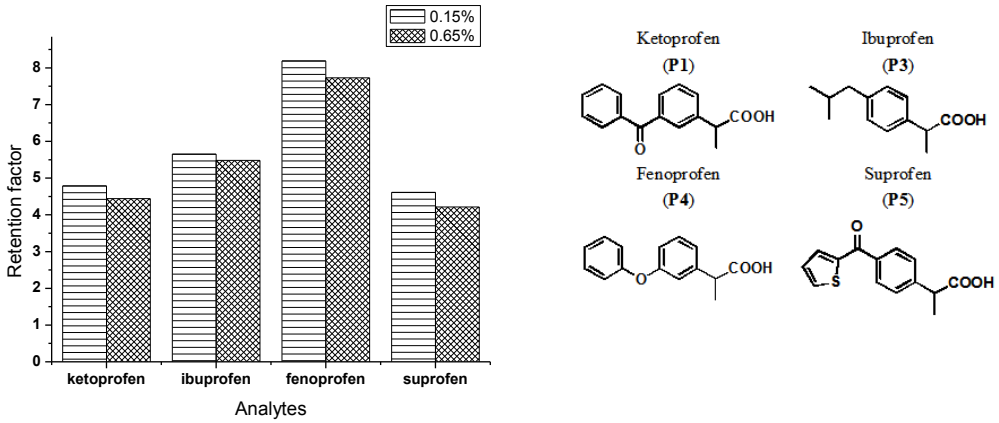


Figure 4.12: Influence of concentration of TEAA on capacity factor of arylpropionic acids (Condition: MeOH/Buffer (TEAA, pH 5.2) = 40/60)

Conclusion

In this chapter, the performance of cationic β-CD has been thoroughly investigated using RPLC. A screening strategy was applied to optimize the chromatographic condition by adjusting mobile phase parameters, such as the proportion of organic modifier, pH value and ionic strength. It was found that the MeOH in the mobile phase would compete with the analytes to interact with the CD cavity as well as hydrogen bonding with the substituents on the CD rims. In the cases when the hydrophobic inclusion between CD cavity and analytes was a key factor for the retention and enantioseparation, a higher proportion of organic modifier in the mobile phase would make the analytes elute out faster and affording lower chiral selectivities. The pH of mobile phase affects the charge status of acidic or basic analytes. The hydrophobic inclusions between CD cavity and charged analytes are less favorable. On the other hand, electrostatic forces

between analytes and cationic substituents on the chiral selector were also involved in the retention and enantioseparation process. Acid additives might competitively interact with the cationic substituent on the CSP and weaken electrostatic force between analytes and CSP. However, diminished electrostatic forces might result in a better enantioseparation when it was combined with the effects of other interactions between analytes and chiral selector. Additionally, increasing the ionic strength of the mobile phase would reduce non-stereoselective interactions between analytes and CSP, so that, the chromatographic peak became sharp. In summary, the cationic CSP prepared can be applied for RPLC mode in HPLC and affords efficient enantioseparations under optimized conditions.

Chapter 5

Experimental

5.1 Reagent

Anhydrous β-cyclodextrin, imidazole, 1-methylimidazole and phenyl isocyanate were purchased from Merck; *p*-toluenesulphonyl chloride was from Fluka; methylene chloride, ethyl acetate and hexane were from Fisher; 1-bromooctane and Amberlite resin IRA-900(Cl) were from Alfa Aesar; anhydrous 1,4-dioxane, 3,5-dimethylphenyl isocyanate and calcium hydride were from Sigma-Aldrich; sodium hydride was from Acros; anhydrous DMF was collected from solvent reflux system, pyridine was from Baker analyzed®; chloroform was from Infinity®; magnesium sulphate was from GCE.

5.2 General experimental

Structures of all compounds were assigned by NMR spectra which were operated on a Bruker ACF300 FT-NMR spectrometer supplied by Bruker Biospin (Fällanden, Switzerland). Mass spectra of all compounds were obtained using the QSTAR XL LC/MS/MS System, which comprises a high-performance hybrid quadrupole time-of-flight mass spectrometer by Applied Biosystems (Foster City, California, USA). The loading concentrations of CSPs were determined by TG-DTA supplied by PerkinElmer Thermogravimetric Analyzers Company (USA). Elemental analyses of all compounds were performed on Vario EL III universal CHNOS element analyzer supplied by Elementar Analysensysteme (Hanau, Germany). FT-IR results were detected by PerkinElmer FT-IR spectrometer (Waltham, Massachusetts, United States of America). Melting points were determined on Büchi Melting Point apparatus B-545 (USA).

Organic solvents methylene chloride and pyridine were dried over calcium hydride. The solvents were refluxed with calcium hydride for 15 hours before collecting for use.

5.3 Full HPLC & SFC Experiments

5.3.1 HPLC analysis instrumentation and conditions

The HPLC setup comprises of an Agilent 1100 series degasser, quaternary pump, auto sampler and a variable-wavelength detector. The newly packed column is flushed with 97:3 (v:v) hexane : 2-propanol (IPA) at a flow rate of 0.2 ml/min for 24 hours. Increase the flow rate to 0.5 ml/min and wait for getting baseline stable. The ratio of hexane and 2-propanol was then adjusted to reach the most appropriate condition. All analyses were performed at ambient temperature at 25 °C. UV absorbance was detected at wavelength of 220 nm. The analytes were prepared at a concentration of 500 ppm by dissolving them in mobile phases. Typically, a sample volume of 5 μl was injected. Typical analysis flow rates were 1.0 ml/min for the coated CSP columns, 0.5 ml/min for the bonded CSP columns in NPLC; 0.2 ml/min in RPLC. The analysis time was adjusted according to each sample's capacity factor. In RPLC, buffer was prepared by adding triethylamine (TEA) into D.I. water with acetic acid added thereafter to adjust pH. The amount of additives in the buffer was recorded as the total weight of both acetic acid and TEA in buffer (w/v).

5.3.2 SFC analysis instrumentation and conditions

The SFC setup comprises of a Jasco BP-2080 *plus a*utomatic back pressure

regulator, UV/Vis Detector, column thermostat, rheodyne 6-way valve manual injector 20 µl, HPLC pump, solvent selection unit and a CO_2 delivery pump. Liquid CO_2 is supplied by Singapore Oxygen Air Liquide (SOXAL). In SFC operations, back pressure regulator (BPR) was set beyond 10 MPa, oven temperature 40 °C, total flow rate was set in a range of 1.0-3.0 ml/min and variable content of IPA or MeOH were mixed in mobile phase as organic modifier, UV absorbance was detected at 220 nm wavelength. The samples were prepared at a concentration of 500 ppm by dissolving them in pure 2-propanol and the sample injection volume was typically 5µl.

5.4 Column packing approach

There are two types of CSPs prepared in the experiment: coated CSP and bonded CSP. The coated CSP can only be packed with non-polar solvent while the bonded CSP can be packed with both polar and non-polar solvent. Because the functionalized silica gel can be dispersed well in methanol, methanol is usually chosen for packing bonded CSP.

The coated CSP (4 g) was dried in vacuum at 40 °C overnight, suspended in approximately 15 ml HPLC grade hexane and then added into slurry reservoir. The CSPs were packed into a stainless steel column (250 mm × 4.6 mm I.D.) with a pump (SSI Scientific Systems, Inc LabAlliance, PA US). Firstly, applying flow rate 24.00 ml/min and pressure 4000 Psi, checking the joints to make sure there is no leak in the packing line. After that, increase pressure stepwise until packing pressure reaches 8000 Psi. Keep this pressure and flow rate for 1 hour to make sure

the column is packed tightly. The CD derivatives' loading on the silica gel were detected with element analysis.

Bonded CSP is suspended in 15 ml HPLC grade methanol and packed into smaller I.D., shorter length stainless steel columns (150 mm × 2.1 mm I.D.) using the same procedure as coated CSPs.

5.5 Synthesis of 6^{A}-O-toluenesulphonyl-β-cyclodextrin …(2)

(1) Synthesis of 1-(*p*-Toluenesulfonyl)imidazole

Imidazole (65 g, 0.95 mol) was added into 1 L three necked round bottom flask (RBF), followed by adding 250 ml anhydrous methylene chloride; Sirring until imidazole was completely dissolved. The solution of imidazole was cooled down to 0 °C. *p*-toluenesulfonyl chloride (80 g, 0.42 mol) was dissolved in 250 ml anhydrous methylene chloride, and transferred into pressure-equalizing-funnel. Then it was added into the solution of imidazole dropwise under N_2 protection within 1.5 hours.

After the solution of *p*-toluenesulfonyl chloride has been added, the resulting mixture was allowed to react at room temperature for 2 hours. The resulting mixture was filtered through a pad of silica gel (approx. 100 g). After that, the pad was washed with 500 ml of 1:1 ethyl acetate-hexane; the solvent in filtrate was removed on a rotary evaporator to afford white solid product. The white residue was purified through recrystallization from D.I. water with hexane (approximately 100 ml/ 10 ml). 1-(*p*-toluenesulfonyl)imidazole crystallized from the solution. Yield: 83.44% [77.8 g (0.35mol)]. ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 2.28 (s

3H, CH_3) 7.12 (s, 1H, 4-imiH) (“imi” represents “imidazolium”), 7.50 (s, 2H, 3,5-C_6H_4, *J* = 6.3Hz), 7.74 (s, 1H, 5-imiH), 7.99 (d, 2H, 2,6- C_6H_4, *J* = 6.3Hz) 8.36 (s, 1H, 2-imiH). ^{13}C NMR (300 MHz, DMSO-d6, δ ppm) 21.23, 119.76, 125.93, 128.70, 134.76, 135.88, 138.63, 145.41. The NMR analysis data are matched with the reference [231].

(2) Synthesis of 6^A-O-toluenesulphonyl-β-cyclodextrin …*(2)*

Anhydrous β-cyclodextrin (40 g, 35.2 mmol) was added into 2 L three necked round bottom flask then dissolved in 900 ml D.I water at 60 °C. The solution was cooled down to room temperature with continuous stirring. β-cyclodextrin precipitated out of the solution but suspended well in water. 1-(*p*-Toluenesulfonyl)-imidazole (31.3 g 141 mmol) was added in one portion. The mixture was stirred for 2 hours to afford a uniform suspension. A solution of NaOH (18 g 0.45 mol) in 50 ml D.I. water was added over 20 min and continued reacting for 10 min. Unreacted solid was filtrated with sintered-glass-funnel. NH_4Cl (48.2 g 0.90 mol) was added into the filtrate to quench the reaction. The solids dissolved within a short time but white solid precipitates from the solution thereafter. The precipitate was filtrated out, and dried in oven. It was washed with 750 ml water at 60 °C and dried in vacuum. White product of 6^A-O-toluenesulphonyl-β-cyclodextrin was obtained. Yield: 26.18% [12.08g (9.22mmol)]. ESI-MS spectra: (expected) 1311.4; (found) 1311.3. ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 2.41 (s, 3H, -CH_{3tosyl}) (“tosyl” represents “*p*-toluenesulphonyl- substituent”) 3.33-3.82 (overlapped with solvent peak, 40H, H-2,3,4,5,6) 4.25-4.60 (m, 2H, H-6) 4.61-4.76 (m, 5H, OH-6)

4.76-4.83 (m, 7H, H-1) 5.73-5.83 (m, 14H, OH-2,3) 7.43(d, 2H, 3,5-C_6H_4, *J* = 6.0Hz) 7.74 (d, 2H, 2,6- C_6H_4, *J* = 6.0Hz). ^{13}C NMR (300 MHz, DMSO-d6, δ ppm) 21.2, 60.39, 72.49-73.51 (m), 81.99, 102.41, 119.77, 125.96, 128.57. The NMR analysis data are matched with the reference [231].

5.6 Synthesis of cationic β-cyclodextrin tosylate …(3)

5.6.1 Synthesis of 6^{A}-(3-methylimidazolium)-β-cyclodextrin tosylate …*(3a)*

To a solution of 6^{A}-O-toluenesulphonyl-β-cyclodextrin (12.91 g 0.01 mol) in 20 ml anhydrous DMF in 100 ml three-necked-RBF, 3 ml 1-methylimidazole was added. The mixture was stirred under N_2 protection at 90 °C for 48 hours. The resultant solution was poured in 100 ml acetone to precipitate product out. The product was filtrated through sintered-glass-funnel and washed with 100 ml acetone. Repeated the washing for several times until the solid turned white. The solid was dried in vacuum. Yield: 86.6% [11.87 g (8.66 mmol)]. ESI-MS spectra [M^+]: (expected) 1199.4; (found) 1199.6. ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 2.28 (s, 3H, =CH_{3tosyl}) (tosyl represents tosylate anion) 2.72 (t, 1H, H-2, *J* = 3.6Hz), 2.88 (t, 1H, H-4, *J* = 3.6Hz), 3.00-3.15 (t,1H, H-5, *J* = 5.5Hz), 3.34 (m, 12H, H-2,4), 3.40-3.75 (overlap with solvent peak, 27H,H-3,5,6) 3.82 (overlap with solvent peak ,3H CH_{3imi}), 4.60-4.70 (t, 1H, OH-6, *J* = 5.5Hz), 4.73 (m, 4H, OH-6, *J* = 3.4Hz) 4.82 (m, 6H, H-1, *J* = 2.0Hz) 4.95-5.10 (d, 1H, H-1, *J* = 2.2Hz) 5.74-5.87 (m, 13H, OH-2,3) 5.95-6.10 (d, 1H, OH-2, *J* = 3.4Hz), 7.12-7.14 (d, 2H, 3,5-C_6H_4, *J* = 4.8Hz), 7.47 (d, 2H, 2,6-C_6H_4, *J* = 5.0Hz) 7.62 (d, 2H, =CH-5_{imi},

CH-4_{imi}, *J* = 3.0Hz) 8.94 (s, 1H, =CH-2_{imi})

The NMR analysis data are in consistency with reference [232]

5.6.2 Synthesis of 6^A-(3-octylimidazolium)-β-cyclodextrin tosylate ...*(3b)*

This compound was prepared by reaction of 6^A-O-toluenesulphonyl-β-cyclodextrin and 1-octylimidazole followed the same procedure as described in Chapter 6.6.1. Yield: 89.54% [13.15 g (8.95 mmol)]. ESI-MS spectra [M^+]: (expected) 1297.5; (found) 1297.7. ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 0.80-0.95 (t, 3H, -CH_3, *J* = 6.6Hz) 1.10-1.40 (s, 10H, 5 -CH_2-) 1.70-1.90 (t, 2H, -CH_2-) 2.29 (s, 3H, =CH_{3tosyl}) (tosyl represents tosylate anion) 2.72 (m, 1H H-2), 2.88 (m, 1H, H-4), 3.00-3.15 (m,1H, H-5), 3.39 (m, 12H, H-2,4), 3.20-3.75 (overlap with solvent peak, 27H,H-3,5,6) 3.73 (overlap with solvent peak ,2H -CH_2), 4.50-4.60 (m, 1H, OH-6), 4.60-4.70 (m, 4H, OH-6) 4.82-4.85 (m, 6H, H-1) 4.95-5.10 (d, 1H, H-1) 5.76-5.82 (m, 13H, OH-2,3) 5.95-6.10 (d, 1H, OH-2), 7.14 (d, 2H, 3,5-C_6H_4, *J* = 6.0Hz), 7.46-7.49 (d, 2H, 2,6-C_6H_4, *J* = 6.0Hz) 7.71 (s, 1H, CH-4_{imi}) 7.77 (s, 1H, =CH-5_{imi}) 9.06 (s, 1H, =CH-2_{imi}) (imi represents imidazolium)

5.6.3 Synthesis of 6^A-(3-vinylimidazolium)-β-cyclodextrin tosylate ...*(3c)*

This compound was prepared by reaction of 6^A-O-toluenesulphonyl-β-cyclodextrin and 1-vinylimidazole followed the same procedure as described in Chapter 6.6.1. Yield: 87.5% [12.10 g (8.75 mmol)]. ESI-MS [$\mathbf{M}^+$]: (expected) 1211.4; (found) 1211.4. ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 2.74 (m, 1H H-2), 2.80 (m, 1H, H-4), 3.00-3.15 (m,1H, H-5), 3.32-3.45 (overlap with solvent peak,

12H, H-2,4), 3.20-3.80 (overlap with solvent peak, 27H, H-3,5,6)， 4.30-4.40 (m, 1H, OH-6), 4.48-4.59 (m, 6H, OH-6， $=CH_{2vinyl}$) 4.84-4.86 (m, 6H, H-1) 5.00 (d, 1H, H-1) 5.40-5.50 (m, 1H, $-CH_{vinyl}$) 5.64-5.84 (m, 13H, OH-2,3) 5.95-6.10 (d, 1H, OH-2), 7.54(d, 2H, 3,5-C_6H_4, J = 6.0Hz) 7.76 (d, 2H, 2,6- C_6H_4, J = 6.0Hz) 7.87 (s, 1H, =CH-4_{imi}) 8.18 (s, 1H, CH-5_{imi}) 9.42 (s, 1H, =CH-2_{imi})

5.6.4 Synthesis of 6^A-(p-vinylpyridinium)-β-cyclodextrin tosylate …*(3d)*

This compound was prepared by reaction of 6^A-O-toluenesulphonyl-β-cyclodextrin and *p*-vinylpyridine followed the same procedure as described in Chapter 6.6.1. Yield: 91.3% [12.72 g (9.13 mmol)]. ESI-MS spectra [$\mathbf{M}^+$]: (expected) 1222.4; (found) 1222.2. ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 2.29 (s, 3H H-CH_{3tos}), 2.73 (m, 1H H-2), 2.83 (m, 1H, H-4), 3.10 (m,1H, H-5), 3.29-3.48 (overlap with solvent peak, 12H, H-2,4), 3.49-3.64 (overlap with solvent peak, 27H, H-3,5,6)， 4.30 (m, 1H, OH-6), 4.47 (m, 6H, OH-6) 4.83 (m, 6H, H-1) 5.00-5.10 (m, 1H, H-1) 5.40-5.60 (m, 1H, $=CH_{vinyla}$) 5.73 (m, 13H, OH-2,3) 5.95-6.10 (d, 2H, OH-2 $=CH_{vinylb}$), 7.10-7.13 (d, 2H, 3,5-C_6H_4, J = 4.8Hz), 7.35 (m, 1H, $-CH_{vinyl}$), 7.47-7.49 (d, 2H, 2,6-C_6H_4, J = 5.0Hz), 8.53 (d, 2H, 3,5-H_{pyr} J = 6.0 Hz) 8.90 (d, 2H, 4,6-H_{pyr} J = 7.5 Hz)

5.6.5 Synthesis of 6^A-(N,N-allylmethylammonium)-β-cyclodextrin tosylate …*(3e)*

This compound was prepared by reaction of 6^A-O-toluenesulphonyl-β-cyclodextrin and N,N-methylallylamine followed the same procedure as described in Chapter 6.6.1. Yield: 82.6% [11.23g (8.26 mmol)]. ESI-MS spectra [$\mathbf{M}^+$]: (expected) 1188.4; (found) 1188.3. ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 2.29 (s,

3H H-CH_{3tos}), 2.73 (t, 1H H-2), 2.89 (m, 1H, H-4), 3.19 (m,1H, H-5), 3.20-3.46 (overlap with solvent peak, 12H, H-2,4), 3.55-3.80 (overlap with solvent peak, 32H, H-3,5,6 -CH_{3N}^{+} -CH_{2allyl}), 4.36 (m, 1H, OH-6), 4.45 (m, 6H, OH-6), 4.84 (m, 6H, H-1), 4.89 (d, 2H, =CH_{2allyl}), 5.00-5.10 (d, 1H, H-1) 5.67-6.00 (m, 14H, OH-2,3 -CH_{allyl}), 7.10-7.12 (d, 2H, 3,5-C_6H_4, *J* = 4.8Hz), 7.46-7.49 (d, 2H, 2,6-C_6H_4, *J* = 5.0Hz), 7.96 (s, 1H, -NH^{+})

5.7 Anion exchange …(4)

5.7.1 Anion exchange from 6^A-(3-methylimidazolium)-β-cyclodextrin tosylate to 6^A-(3-methylimidazolium)-β-cyclodextrin chloride …*(4a)*

6^A-(3-methylimidazolium)-β-cyclodextrin tosylate exchanged anion with Amberlite resin chloride. The Amberlite resin was suspended in D.I. water then poured into column. The resin was flushed with D.I water several times until PH of fluent was equal to 7. A solution of cationic β-cyclodextrin tosylate (2 g) in 20 ml D.I. water was then poured into column. The liquid was controlled to flow out of the column slowly. Then 80 ml D.I. water was added to flush the sample out. After about 4 hours, 100 ml solution was collected. Then, refilled the solution into the column, repeated the anionic exchange process to ensure completely anion exchange with chloride. Totally 200 ml aqueous solution is collected. The solvent was removed on a rotary evaporator to afford 6^A-(3-methylimidazolium)-β-cyclodextrin chloride. Yield: 88.67% (1.60 g). m.p. 248-255 °C. ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 2.72 (t, 1H, H-2, *J* = 3.6Hz), 2.88 (t, 1H, H-4, *J* = 3.6Hz), 3.00-3.15 (t,1H, H-5, *J* = 5.5Hz), 3.34 (m, 12H,

H-2,4), 3.15-3.75 (overlap with solvent peak, 27H,H-3,5,6) 3.84 (overlap with solvent peak ,3H CH_{3imi}), 4.31 (t, 1H, OH-6, *J* = 5.5Hz), 4.55-4.62 (m, 5H, OH-6, *J* = 3.4Hz) 4.75-4.85 (m, 6H, H-1, *J* = 2.0Hz) 4.95-5.10 (d, 1H, H-1, *J* = 2.2Hz) 5.66-5.82 (m, 13H, OH-2,3) 6.01 (d, 1H, OH-2, *J* = 3.4Hz), 7.68 (d, 2H, =CH-5_{imi}, CH-4_{imi}, *J* = 3.0Hz) 9.01 (s, 1H, =CH-2_{imi}) (imi represents imidazolium)

By comparison to the ^{1}H NMR data of 3-methylimidazolium-β-cyclodextrin tosylate the peaks at 2.29 (s, 3H, =CH_{3Tosyl}) (tosyl represents tosylate) 7.12-7.14 (d, 2H, 3,5-C_6H_4), 7.47 (d, 2H, 2,6-C_6H_4) disappear in ^{1}H NMR data of 3-methylimidazolium-β-cyclodextrin chloride while the other parts are not changed which means the tosylate has been converted to chloride completely while the other parts of the molecule is not changed. The NMR analysis results are in consistency with reference [233].

5.7.2 Anion exchange from 6^A-(3-octylimidazolium)-β-cyclodextrin tosylate to 6^A-(3-octylimidazolium)-β-cyclodextrin chloride ...*(4b)*

6^A-(3-methylimidazolium)-β-cyclodextrin tosylate proceeded anion exchanged with Amberlite resin chloride following the same procedure as described in Chapter 6.7.1. Yield: 61.30% (1.11g). m.p. 276-286 °C. FT-IR (KBr) 3300 (O-H) 2922 (C-H), 1639 cm^{-1} (C=N), 1158 (C-N), 1032 cm^{-1} (C-O) 706 cm-1 (C-H). ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 0.80-0.95 (t, 3H, -CH_3, *J* = 6.6Hz) 1.10-1.40 (s, 10H, 5 -CH_2-) 1.70-1.90 (t, 2H, -CH_2-) 2.84 (m, 1H, H-4), 3.00-3.15 (t,1H, H-5), 3.39 (m, 12H, H-2,4), 3.15-3.75 (overlap with solvent peak, 27H,H-3,5,6) 3.75-4.00 (2H -CH_2), 4.63 (m, 5H, OH-6) 4.81-4.84 (m, 6H, H-1) 4.95-5.10 (d, 1H, H-1) 5.76-5.84 (m, 13H, OH-2,3) 6.01 (d, 1H, OH-2), 7.70 (s,

1H, CH-4_{imi}) 7.77 (s, 1H, =CH-5_{imi}) 9.07 (s, 1H, =CH-2_{imi}) (imi represents imidazolium). By comparison to the ^{1}H NMR data, only the peaks at 2.29 (s, 3H, =CH_{3Tosyl}) (tosyl represents tosylate) 7.14 (d, 2H, 3,5-C_6H_4), 7.46-7.49 (d, 2H, 2,6-C_6H_4) disappear while the other parts are not changed which means the tosylate has been converted to chloride completely while the other parts of the molecule is not changed.

5.7.3 Anion exchange from 6^A-(3-vinylimidazolium)-β-cyclodextrin tosylate to 6^A-(3-vinylimidazolium)-β-cyclodextrin chloride …*(4c)*

6^A-(3-vinylimidazolium)-β-cyclodextrin proceeded anion exchanged with Amberlite resin chloride following the same procedure as described in Chapter 6.7.1. Yield: 65.54% (1.18 g). m.p. 254-268 °C. FT-IR (KBr) 3300 (O-H) 2930 (C-H), 1638 cm^{-1} (C=N), 1156 (C-N), 1030 cm^{-1} (C-O) 708 cm-1 (C-H). ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 2.73 (m, 1H H-2), 2.88 (m, 1H, H-4), 3.00-3.15 (m,1H, H-5), 3.32-3.45 (overlap with solvent peak, 12H, H-2,4), 3.20-3.80 (overlap with solvent peak, 27H, H-3,5,6)， 4.30-4.40 (m, 1H, OH-6), 4.48-4.59 (m, 6H, OH-6， =CH_{2vinyl}) 4.84-4.86 (m, 6H, H-1) 5.00 (d, 1H, H-1) 5.40-5.50 (m, 1H, -CHvinyl) 5.64-5.84 (m, 13H, OH-2,3) 5.95-6.10 (d, 1H, OH-2), 7.87 (s, 1H, =CH-4imi) 8.18 (s, 1H, CH-5imi) 9.42 (s, 1H, =CH-2imi)

5.7.4 Anion exchange from 6^A-(4-vinylpyridinium)-β-cyclodextrin tosylate to 6^A-(4-vinylpyridinium)-β-cyclodextrin chloride …*(4d)*

6^A-(4-vinylpyridinium)-β-cyclodextrin tosylate proceeded anion exchanged with Amberlite resin chloride following the same procedure as described in Chapter 6.7.1. Yield 72.23% (1.30 g). m.p. 262-286 °C; FT-IR (KBr) 3300 (O-H)

2930 (C-H), 1639 cm^{-1} (C=N), 1157 (C-N), 1031 cm^{-1} (C-O) 708 cm-1 (C-H). ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 2.74 (m, 1H H-2), 2.80 (m, 1H, H-4), 3.08 (m,1H, H-5), 3.35 (overlap with solvent peak, 12H, H-2,4), 3.51-3.67 (overlap with solvent peak, 27H, H-3,5,6)， 4.24 (m, 1H, OH-6), 4.45 (m, 6H, OH-6) 4.84 (m, 6H, H-1) 5.00-5.10 (m, 1H, H-1) 5.28 (m, 1H, =CHvinyl$_a$) 5.70 (m, 13H, OH-2,3) 5.98-6.10 (d, 2H, OH-2 =CHvinyl$_b$), 7.35 (m, 1H, -CHvinyl), 8.53 (d, 2H, 3,5-Hpyr J = 6.0 Hz) 8.94 (d, 2H, 4,6-Hpyr J = 7.5 Hz)

5.7.5 Anion exchange from 6^A-(N,N-allylmethylammonium)-β-cyclodextrin tosylate to 6^A-(N,N-allylmethylammonium)-β-cyclodextrin chloride …*(4e)*

6^A-(N,N-allylmethylammonium)-β-cyclodextrin tosylate proceeded anion exchanged with Amberlite resin chloride following the same procedure as described in Chapter 6.7.1. Yiled 66.34% (1.19 g). m.p. 290-296 °C. FT-IR (KBr) 3300 (O-H) 2930 (C-H), 1641 cm^{-1} (C=C), 1157 (C-N), 1031 cm^{-1} (C-O) 708 cm-1 (C-H). ^{1}H NMR (300 MHz, DMSO-d6, δ ppm) 2.73 (t, 1H H-2), 2.82 (m, 1H, H-4), 3.19 (m,1H, H-5), 3.34 (overlap with solvent peak, 12H, H-2,4), 3.53-3.91 (overlap with solvent peak, 32H, H-3,5,6 -CH_{3N}^{+} -CH_{2allyl}), 4.19 (m, 1H, OH-6), 4.65 (m, 6H, OH-6), 4.82 (m, 6H, H-1), 5.01 (m, 3H, =CH_{2allyl}, H-1) 5.73 (m, 14H, OH-2,3 -CH_{allyl}), 8.13 (s, 1H, -NH_{imi})

5.8 Carbamoylation …(5)

5.8.1 Synthesis of 6^A-(3-methylimidazolium)-6-deoxyperphenylcarbamoyl-β-cyclodextrin chloride (MIMPCCD) …*(5a1)*

6^A-(3-methylimidazolium)-β-cyclodextrin chloride (1.80 g, 1.46 mmol) was

dissolved in 30 ml dry pyridine at 85 °C. Phenyl isocyanate (10.94 g, 0.09 mol) was added and the reaction was processing at 85 °C under nitrogen atmosphere for 19 hours. The excess pyridine was distilled off under vacuum. After that, the product **MIMPCCD** was purified by flash column chromatography [hexane : ethyl acetate 60:40(v:v)].

Due to the high polarity of the macromolecular product (**MIMPCCD**), its R_f value is small. The impurities contain neutral non-polar organics or unreacted smaller molecular reagents. When hexane : ethyl acetate 40:60 (v:v) was used as elution phase, the impurities were flushed out (R_f = 0.85, 0.44), but the point remaining on the starting point dragged obviously (R_f = 0.12) which was suggestive that there must be a big loss of the final product using this eluent ratio. When hexane : ethyl acetate 50:50 (v:v) was applied, the impurities can also be flushed out (R_f = 0.88, 0.78, 0.51, 0.20) and the point remaining on the starting point was not dragged obviously (R_f = 0.05). However, the impurities can also be separated when hexane : ethyl acetate 60:40(v:v) is applied (R_f = 0.78, 0.51, 0.36, 0.20, 0.12). The target product showed almost zero R_f in this condition. Therefore, hexane : ethyl acetate 60:40(v:v) was used for flushing out the impurities, then the product (**MIMPCCD**) was eluted out with methanol. Yield: 62% [3.27 g (0.90 mmol)]. m.p. 192-195 °C. ESI-MS [$\mathbf{M}^+$]: (expected) 3580.16; (found) 3580.19. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 3.24-5.53 (m, 52H, H-cyclodextrin, H-CH_{3im}), 6.46-7.39 (m, 103H, H-Phenyl); Microanalysis for $C_{186}H_{175}N_{22}O_{54}Cl$ (expected) C: 61.75 %, H: 4.87 %, N: 8.51 %, (found) C: 62.44 %, H: 5.79 %, N: 8.80 %.

The phenyl isocyanate, pyridine and chloroform used in this step are high toxic and should be handled in fumehood all the time.

5.8.2 Synthesis of 6^A-(3-methylimidazolium)-6-deoxyper-(3,5-dimethyl phenylcarbamoyl-β-cyclodextrin chloride (MIMDMPCCD) ...*(5a2)*

6^A-(3-methylimidazolium)-β-cyclodextrin chloride (1.72 g, 1.39 mmol) was suspended in 20 ml pyridine to form a milky white colour mixture at 85 °C. 3,5-dimethylphenyl isocyanate (12 ml, 0.085 mol) was added into the mixture in 4 portions, every portion added in an interval of half a hour. The reaction processed at 85 °C under N_2 protection for 17 hours. The muddy mixture turned clear and brown solution, which showed the product, which was easier to dissolve in pyridine, was generated. Excess pyridine was vacuum distilled off and the residue was dissolved in 250 ml chloroform. The solution was washed with 250 ml water by 3 times. The organic phase was collected and dried over magnesium sulphate overnight. The solution was filtered and condensed to approx. 10 ml. Flash column separation was applied to purify the product [hexane: ethyl acetate 70:30 (v: v) (R_f = 0.95, 0.57, 0.45, 0.34, 0.07)]. Yield: 44.8% [2.60 g (0.62 mmol)]. m.p. 216-222 °C. ESI-MS [$\mathbf{M}^+$]: (expected) 4143.56; (found) 4144.03. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 1.90-2.25 (m, 124H, H-3,5-CH_3), 3.24-6.00 (m, 52H, H-cyclodextrin, H-CH_{3im}), 6.20-7.39 (m, 67H, H-Phenyl) Microanalysis for $C_{226}H_{255}N_{22}O_{54}Cl$ (expected) C: 64.95%, H: 6.15%, N: 7.37%, (found) C: 62.68%, H: 6.33%, N: 7.25%.

5.8.3 Synthesis of 6^A-(3-octylimidazolium)-6-deoxyperphenylcarbamoyl-β-cyclodextrin chloride (OIMPCCD) …*(5b1)*

6^A-(3-Octylimidazolium)-β-cyclodextrin chloride (1.802 g, 1.352 mmol) was suspended in 20 ml anhydrous pyridine reacted with phenyl isocyanate (10 ml, 0.092 mol) at 85 °C for 18 hours. Excess pyridine was distilled off. The residue was dissolved in 200 ml chloroform and washed with 250 ml water by 3 times. The organic phase was separated and dried with anhydrous magnesium sulfate overnight. Filtered solution was distilled on rotary evaporator to precipitate the solute. In the TLC analysis, the point stayed at the starting point was the product **OIMPCCD**. Similar to the considerations as discussed in Chapter 6.8.1, hexane: ethyl acetate, 60:40 (v: v) was used to flush out the impurities (R_f = 0.67, 0.62, 0.33, 0.23). The product of **OIMPCCD** was flushed out with methanol thereafter. Yield: 51.85% [2.60 g (0.70 mmol)]. m.p. 202-205 °C. ESI-MS [$\mathbf{M}^+$]: (expected) 3678.27; (found) 3678.24. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 0.80-1.35(m, 13H, H-Octyl) 3.00-6.00 (m, 51H, H-Cyclodextrin, H-CH_{2im}) 6.00-8.20 (m, 100H, H-Phenyl) Microanalysis for $C_{193}H_{189}N_{22}O_{54}Cl$ (expected) C: 62.38%, H: 5.13%, N: 8.29%, (found) C: 61.04%, H: 5.78%, N: 7.61%.

5.8.4 Synthesis of 6^A-(3-octylimidazolium)-6-deoxyper-(3,5-dimethylphenylcarbamoyl-β-cyclodextrin chloride (OIMDMPCCD) …*(5b2)*

6^A-(3-Octylimidazolium)-β-cyclodextrin chloride (3.78 g 2.83, mmol) was dissolved in 50 ml anhydrous pyridine in a 250ml three-necked-round bottom flask. 3,5-dimethylphenyl isocyanate (25 g, 0.17 mol) was added. Reaction condition was the same as mentioned in Chapter 6.8.1, but reaction time was set as 16 hours.

In the TLC analysis, the point stayed at the starting point was the product

OIMDMPCCD. Similar to the considerations as discussed in Chapter 6.8.1, hexane: ethyl acetate 50:50 (v: v) was used to flush out the impurities (R_f = 0.89，0.68，0.60，0.32，0.23，0.12). The product of **OIMDMPCCD** was flushed out with methanol thereafter. Yield: 44.67% [5.41 g (1.26 mmol)]; m.p. 213-225 °C; ESI-MS [$\mathbf{M}^+$]: (expected) 4241.74; (found) 4242.68. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 1.70-2.40 (s, 123H, H-CH_{2oct}, H-3,5-CH_3) 0.75-0.95 (m, 3H, H-CH_{3Oct}) 0.95-1.35 (m, 12H, H-6×CH_{2Oct}) 3.00-6.10 (m, 51H, H-Cyclodextrin, H-CH_{2imi}) 6.35-7.25 (m, 64H, H-Phenyl) (imi represents imidazolium) Microanalysis for $C_{233}H_{269}N_{22}O_{54}Cl$ (expected) C: 65.43%, H: 6.34%, N: 7.20%, (found) C: 63.30%, H: 6.56%, N: 7.00%.

5.8.5 Synthesis of 6^A-(3-vinylimidazolium)-6-deoxyperphenylcarbamoyl-β-cyclodextrin chloride (VIMPCCD) ...*(5c)*

2.15 g 6^A-(p-vinylimidazolium)-β-cyclodextrin chloride (1.72 mmol) was suspended in 20 ml dried pyridine. The mixture was heated towards 85 °C under N_2 atmosphere. Phenyl isocyanate (12.0 ml, 110.32 mmol) was added quarterly within 2 hour. Then the mixture continues reaction for 20 hours. The reacted solution turns from green color to dark yellow at reaction termination. The excess pyridine was removed in vacuum at reaction temperature. The resulted solid was dissolved in 250 ml chloroform by three times. The organic phase was collected and dried with anhydrous magnesium sulfate. The filtered solution was condensed to approximately 10 ml. In the TLC analysis, the point stayed at the starting point was thought as the product **VIMPCCD**. Similar to the considerations as discussed in Chapter 6.8.1, hexane: ethyl acetate 70:30 (v: v) was used to flush out the

impurities (R_f = 0.80, 0.70, 0.52, 0.38, 0.25). The product of **VIMPCCD** was flushed out with methanol thereafter. Yield: 66.16% [4.13 g (1.14 mmol)]. m.p. 197-199 °C. ESI-MS [$\mathbf{M}^+$]: (expected) 3592.16; (found) 3592.07. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 3.00-6.00 (m, 52H, H-Cyclodextrin, H-Vinyl) 6.00-7.80 (m, 100H, H-Phenyl) Microanalysis for $C_{187}H_{175}ClN_{22}O_{54}$ (expected) C: 61.87%, H: 4.86%, N: 8.49%, (found) C: 60.25%, H: 5.13%, N: 9.11%.

5.8.6 Synthesis of 6^A-(p-vinylpyridinium)-6-deoxyperphenylcarbamoyl-β-cyclodextrin chloride (VPYPCCD) ...*(5d)*

6^A-(*p*-Vinylpyridinium)-β-cyclodextrin chloride (1.8 g, 1.43 mmol) was suspended in 20 ml dried pyridine. The mixture was heated at 85 °C under N_2 atmosphere. Phenyl isocyanate (9 ml 79.0 mmol) was added with two equal portions within 1 hour. Then the mixture was stirred for 20 hours. The mixture turned from green color to dark yellow when reaction completed. The excess pyridine was removed in vacuum. The resulted solid was dissolved in 250 ml chloroform and the solution was washed with 250 ml D.I. water by three times. The chloroform phase was collected, dried with anhydrous magnesium sulfate, and purified with flash column separations. In the TLC analysis, the point stayed at the starting point was the product **VPYPCCD**. Similar to the considerations as discussed in Chapter 6.8.1, hexane: ethyl acetate 75:25 (v: v) was used to flush out the impurities (R_f = 0.84, 0.74, 0.59, 0.31, 0.24). The product of **VPYPCCD** was flushed out with methanol thereafter. Yield: 68.75% [3.58 g (0.98 mmol)]. m.p. 200-204 °C. ESI-MS [$\mathbf{M}^+$]: (expected) 3603.17; (found) 3603.67. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 3.00-6.00 (m, 52H, H-Cyclodextrin, $=CH_{2vinyl}$) 6.00-7.70 (m,

101H, H-Phenyl, -CH_{vinyl}) Microanalysis for $C_{189}H_{176}ClN_{21}O_{54}$ (expected) C: 62.35%, H: 4.87%, N: 8.08%, (found) C: 62.50%, H: 5.39%, N: 8.47%.

5.8.7 Synthesis of 6^A-(N,N-allylmethylammonium)-6-deoxyperphenylcarbamoyl-β-cyclodextrin chloride (VAMPCCD) ...*(5e)*

6^A-(N,N-Allylmethylammonium)-β-cyclodextrin chloride (1.7 g, 1.43 mmol) was reacted with phenyl isocyanate (9 ml 79.0 mmol) followed the procedure as described in Chapter 6.8.6. In the TLC analysis, the point stayed at the starting point was the product **VAMPCCD**. Similar to the considerations as discussed in Chapter 6.8.1, hexane: ethyl acetate 80:20 (v: v) was used to flush out the impurities (R_f = 0.86, 0.78, 0.70, 0.33). The product of **VAMPCCD** was flushed out with methanol thereafter. Yield: 89.98% [4.64 g (1.29 mmol)]. m.p. 208-212 °C. ESI-MS [$\mathbf{M}^+$]: (expected) 3569.18; (found) 3569.18. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 3.00-6.00 (m, 57H, H-Cyclodextrin, H-N-CH_3, H-N-allyl) 6.00-7.80 (m, 100H, H-Phenyl) Microanalysis for $C_{186}H_{178}ClN_{21}O_{54}$ (expected) C: 61.94%, H: 4.97%, N: 8.15%, (found) C: 61.11%, H: 5.35%, N: 8.92%.

5.9 Immobilization reactions

5.9.1 Polymerization

5.9.1.1 Preparation of vinylized silica gel …***(7)***

Kromasil silica gel (5 μm 5 g) was pre-dried in vacuum at 150 ºC. In a 250 ml dried three-necked RBF, 100 ml anhydrous toluene and 3-methacryloyloxypropyl-trimethoxysilane (MPS) (2.3 ml) were added. The mixture was stirred vigorously in N_2 atmosphere for 30 min. Then the dried silica was added to the mixture and

stirred for 45 min. After that, the mixture was heated at 90 ºC for 18 hours. Product was collected by filtration through 0.45 μm pore size membrane and washed with MeOH in soxlet apparatus overnight. The solid was dried in oven at 100 ºC to afford the final product.

The surface coverage of organic functional material on the surface of silica gel was determined by elemental analysis: C, 5.81%; H, 1.28%. Accordingly, a surface coverage of MPS on silica gel was calculated as 2.16 μmol/m^2 based on the carbon content [161, 234]. FT-IR (KBr) 2964, 2855 cm^{-1} (C-H) 1705 cm^{-1} (C=O) 1635 cm^{-1} (C=C) 1130 cm^{-1} (C-O and Si-O). The characteristic peaks show the successful bonding of MPS onto silica surface.

5.9.1.2 Preparation of co-polymerized 6^A-(3-vinylimidazolium)-6-deoxy-perphenylcarbamoyl-β-cyclodextrin chloride CSP: **VIMPCCD-POLY**

VIMPCCD was physically coated onto the surface of vinylized silica with a loading of 33.3% (w/w). The CD coated silica (2.2 g) was suspended in 20 ml of anhydrous toluene. 2,3-Dimethyl-1,4-butadiene (DMBD, 2.6 ml) was then added dropwise and the mixture was stirred vigorously for 30 min under N_2. AIBN (0.1 mol% of the total amount of vinyl groups of the reactant) was added and the mixture was heated at 80 °C for 18 h. After this period, the product was filtered, washed with THF followed by EtOH and then finally washed with MeOH in a soxlet apparatus for 16 hr. The product was then dried in vacuo at 60 °C for 16h. The loading of CD derivative on the surface of silica gel was determined by

elemental analysis: 12.66, C%; 1.544, H%; 0.785, N%. The cyclodextrin derivatives' grafting coverage was calculated based on the nitrogen content, to be 0.088 μmol/m^2. FT-IR (KBr) 1720 cm^{-1} (C=O) and 1647, 1558, 1458 cm^{-1} (C=C phenyl group) 1130 cm^{-1} (C-O and Si-O). The characteristic peaks show the CD derivative has been successfully bonded onto silica surface.

5.9.1.3 Preparation of co-polymerized 6^A-(3-vinylpyridinium)-6-deoxy-perphenylcarbamoyl-β-cyclodextrin chloride CSP: **VPYPCCD-POLY**

VPYPCCD-POLY was synthesized following the same procedure as described in Chapter 6.10.1.2. The loading of CD derivative on the surface of silica gel can be determined by elemental analysis: 9.495, C%; 1.397, H%; 0.797, N%. The cyclodextrin derivatives' grafting coverage was calculated based on the nitrogen content, to be 0.094 μmol/m^2. FT-IR (KBr) 2982, 2873 cm^{-1} (C-H), 1712 cm^{-1} (C=O) and 1631, 1546, 1448 cm^{-1} (C=C phenyl group) 1130 cm-1 (C-O and Si-O).

5.9.1.4 Preparation of co-polymerized 6^A-(N,N-allylmethylammonium)-6-deoxyperphenylcarbamoyl-β-cyclodextrin chloride CSP: **VAMPCCD-POLY**

VAMPCCD-POLY was synthesized following the same procedure as described in Chapter 6.10.1.2. The loading of CD derivative on the surface of silica gel can be determined by elemental analysis: 9.432, C%; 1.394, H%; 0.775, N%. The cyclodextrin derivatives' grafting coverage was calculated to be 0.091 μmol/m^2. FT-IR (KBr) 2987, 2872 cm^{-1} (C-H), 1708 cm^{-1} (C=O) and 1634, 1541,

1447 cm^{-1} (C=C phenyl group) 1130 cm^{-1} (C-O and Si-O).

5.9.2 Hydrosilylation (VIMPCCD-HYDR)

VIMPCCD (1.41g), triethoxysilane (2 ml) and Pt(PPh3)4 (3.3 mg) were added in RBF. Dried THF (10 ml) was added thereafter. The solution was reacted at 75 °C for 3 days. The mixture was eluted through silica column with dried ether. Then the solvent was evaporated. Thereafter, dried spherical silica gel (5 μm, 1g) and a mixture of 8 ml dried toluene and 2 ml dried THF were added. The mixture was heated to 90 °C for 10 hours. The mixture was filtered and washed with toluene, MeOH and THF sequentially. The product **VIMPCCD-HYDR** was dried in oven at 60 °C overnight. Elemental analysis result: 7.242, C%; 1.375, H%; 0.767, N%. Based on the content of nitrogen, the CD coverage on the silica gel was calculated as: 0.078 $\mu mol/m^2$.

Chapter 6

Conclusion & Suggestions for Future Studies

6.1 Conclusions

A series of cationic β-CD derivatives have been synthesized and applied as CSPs in both SFC and HPLC. This would be the first attempt of applying cationic β-CD CSPs in HPLC and SFC to the best of our knowledge. The β-CD derivatives depicted good enantioseparations capability towards pharmaceutical compounds as well as other racemic compounds such as *α*-phenyl alcohols, aromatic acids *etc.* Although neutral β-CD CSPs have been reported as versatile and efficient for enantioseparations, the cationic β-CD CSPs herein have shown even greater chiral resolution capabilities. It is suggested that the cationic moieties as well as the alkyl substituent on the cationic moieties are involved in the enantioseparation processes. The substituents on cationic CSPs which are more accessible and able to provide more interaction sites are considered pivotal in the enantioseparation processes. In contrast to the neutral β-CD CSPs, the cationic β-CD CSPs present a dual interaction of electrostatic force and hydrophobic inclusion in the CD cavity towards the analytes.

The cationic CSPs have been prepared either by physically coating or chemically bonding approaches. Reaction steps required are less for the preparation of coated CSPs, and the loading of β-CD derivatives on the silica gel support can be easily tuned in the method of preparation. It is found chiral selectivities do not always increase with a higher CD loading while the best chiral selectivity was achieved on CSPs with 20% (w/w) CD loading. The cationic β-CD derivatives have also been chemically bonded onto stationary phases in order to

widen the applicability in broader range of mobile phases. By a co-polymerization approach, cationic β-CD derivatives were successfully bonded onto silica gel in the presence of small molecular monomers. The co-polymerization conditions have been optimized to achieve the highest loading of bonded β-CD.

When the cationic CSPs were applied in HPLC and SFC, the organic modifier would competitively interact with the hydrophobic CD cavity or the cationic substituent on the CD rim. The acidic / basic additives in the mobile phase would affect the enantioseparation results by occupying the CD cavity or masking cationic substituent. In RPLC conditions, the presence of acid / basic additives in buffer is capable of suppressing the dissociation of the ionic analytes as well. The enantioseparations attained on NPLC and SFC have been compared. It was found that partial insertion of hydrophobic analytes into CD cavity in SFC affected the chiral resolution processes. A combined effect of electrostatic force and hydrophobic inclusion resulted in an improved chiral selectivity.

6.2 Suggestions for future studies

In this study, the cationic β-CD CSPs have been applied in NPLC, RPLC and SFC. Chromatographic conditions have been optimized. Possible chiral recognition mechanisms have been proposed. However, the influences of electrostatic force and hydrophobic inclusion complexation on chiral separation are not quantitively calculated. Molecular modeling may be useful to achieve a better theoretical understanding and prediction of the chiral separation mechanism.

Only chloride ion was chosen as the counterion in our cationic CSPs. Investigations on chiral ionic liquid had revealed that anions may also affect enantioseparation processes. It will be interesting to change the counterions in the CSPs to investigate their influence on chiral resolution as well.

The success of applying cationic β-CD CSPs for chiral separation in SFC will make the scaling up process easier. The cationic β-CD CSPs can be further prepared and investigated in preparative chromatography.

The cationic CSPs showed stronger enantioselectivities towards racemates than commercialized neutral CSP (our cationic CSP has shown an average of 17% higher chiral selectivities than neutral CSP SINU-PC towards compared analytes). It is envisaged that by properly modifying the manufacture process, the coated or bonded cationic CSPs can be commercialized.

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