การแยกอิแนนทิโอเมอร์ของสารประกอบฟลาโวนอยด์โดยใช้คะพิลลารีอิเล็กโทรไคเนทิก โครมาโทรกราฟี

นางสาวเพ็ญพร ขจรกลิ่น

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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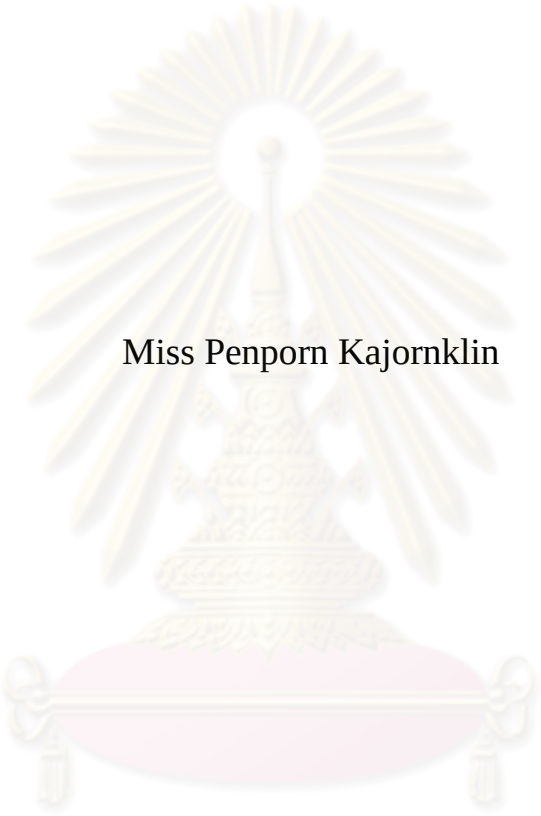
ปีการศึกษา 2553

ENANTIOMERIC SEPARATION OF FLAVONOID COMPOUNDS USING CAPILLARY ELECTROKINETIC CHROMATOGRAPHY

Miss Penporn Kajornklin



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เพ็ญพร ขจรกลิ่น : การแยกอิแนนทิโอเมอร์ของสารประกอบฟลาโวนอยด์โดยใช้คะพิลลารีอิเล็กโทรไคเนทิกโครมาโทกราฟี (ENANTIOMERIC SEPARATION OF FLAVONOID COMPOUNDS USING CAPILLARY ELECTROKINETIC CHROMATOGRAPHY) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.ธรรมนูญ หนูจักร, 43 หน้า.

ได้ศึกษาการแยกอิแนนทิโอเมอร์ของ 2'-, 4'- และ 7-ไฮดรอกซีฟลาวาโนนในคราวเดียวกันโดยใช้ไซโคลเดกซ์ทริน-อิเล็กโทรไคเนทิกโครมาโทกราฟี (CD-EKC) ที่มีไฮลีซัลเฟตไซโคลเดกซ์ทรินหนึ่งหรือสองชนิด (HS-β-CD และ/หรือ HS-γ-CD) ในฟอสเฟตบัฟเฟอร์ที่พีเอช 2.5 ค่าจำเพาะของอิแนนทิโอเมอร์ (κ) ซึ่งนิยามเป็นอัตราส่วนของค่าคงที่ของการจับกันสำหรับ อิแนนทิโอเมอร์กับไซโคลเดกซ์ทรินนั้น มีลำดับดังนี้ 2'- > 4'- ≈ 7- ด้วย HS-β-CD (3.16, 1.04 และ 1.02 ตามลำดับ) ในขณะที่ 4'- > 2'- > 7- ด้วย HS-γ-CD (1.40, 1.17 และ 1.09 ตามลำดับ) พบว่าค่าการแยกของอิแนนทิโอเมอร์ที่เหมาะสมสอดคล้องกับค่า κ: ได้แก่ ค่าการแยกสูงมากถึง 19 สำหรับ 2'- ด้วย HS-β-CD, ค่าการแยกเล็กน้อยสำหรับ 4'- และ 7- ด้วย HS-β-CD และค่าการแยกสมบูรณ์ที่ฐานพีก (R_s ≥ 1.5) สำหรับการแยกอิแนนทิโอเมอร์ด้วย HS-γ-CD ในคราวเดียวกัน

ในระบบไซโคลเดกซ์ทรินสองชนิดที่ใช้ CD1 ความเข้มข้นคงที่ (4 หรือ 8 mM HS-β-CD) และที่ใช้ CD2 ความเข้มข้นต่างๆ (2 ถึง 47 mM HS-γ-CD) นั้น พบว่าเมื่อเพิ่มความเข้มข้นของ CD2 การเปลี่ยนแปลงค่าจำเพาะของการแยกสาร (α) ซึ่งนิยามเป็นอัตราส่วนของค่าความสามารถในการเคลื่อนที่ทางไฟฟ้าของอิแนนทิโอเมอร์นั้น มีความสอดคล้องกับหลักการของค่า κ สำหรับไซโคลเดกซ์ทรินสองชนิด: ได้แก่ การเพิ่มขึ้นของค่า α สำหรับ 7- เนื่องจากการเคลื่อนที่ของอิแนนทิโอเมอร์มีลำดับเหมือนกันใน CD2 และ CD1 ซึ่งมี $\kappa_2 > \kappa_1$, การลดลงของค่า α สำหรับ 2'- เนื่องจากการเคลื่อนที่ของอิแนนทิโอเมอร์มีลำดับเหมือนกันใน CD2 และ CD1 ซึ่งมี $\kappa_2 < \kappa_1$ ในขณะที่ค่า α ของ 4'- ลดลงจนถึง 1 แล้วเพิ่มขึ้นเมื่อความเข้มข้นของ CD2 มากขึ้น เนื่องจากการสลับลำดับการเคลื่อนที่ของอิแนนทิโอเมอร์ใน CD2 และ CD1 ซึ่งมี $\kappa_2 > \kappa_1$

ภาควิชา เคมี ลายมือชื่อนิสิต เพ็ญพร ขจรกลิ่น

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PENPORN KAJORNKLIN: ENANTIOMERIC SEPARATION OF FLAVONOID COMPOUNDS USING CAPILLARY ELECTROKINETIC CHROMATOGRAPHY. ADVISOR: ASSOC.PROF. THUMNOON NHUJAK, Ph.D., 43 pp.

Simultaneous separation of 2'- 4'- and 7-hydroxyflavanone enantiomers was investigated using cyclodextrin-electrokinetic chromatography (CD-EKC) with single or dual highly sulfated-CD (HS-β-CD and/or HS-γ-CD in a pH 2.5 phosphate buffer. Enantioselectivity (κ) defined as the ratio of the binding constant K for enantiomers to CD, was obtained in order 2'- > 4'- ≈ 7- with HS-β-CD (3.16, 1.04 and 1.02, respectively) while 4'- > 2'- > 7- with HS-γ-CD (1.40, 1.17, and 1.09 respectively). The optimum enantiomeric resolution (R_s) was found to be consistent with the κ scale: very high R_s, up to 19 for 2'- with HS-β-CD, partial R_s for 4'- and 7- with HS-β-CD, and achieved baseline $R_s \geq 1.5$ for simultaneous separation of these three enantiomers with HS-γ-CD.

In dual CDs using the fixed concentration of CD1 (4 or 8 mM HS-β-CD) and varying the concentration of CD2 (2 to 47 mM HS-γ-CD), a change in separation selectivity (α), defined as the ratio of the electrophoretic mobilities for enantiomers, with an increase in the CD2 concentration was found to be consistent with the concept of dual enantioselectivities for dual CDs: an increase in α for 7- due to the same order of enantiomers in CD2 and CD2 with $\kappa_2 > \kappa_1$, a decrease in α for 2'- due to the same order of enantiomers in CD2 and CD1 with $\kappa_2 < \kappa_1$, while a decrease in α to 1.0 and then an increase in α at higher CD2 for 4'- due to the reversed migration order of enantiomers in CD2 and CD1 with $\kappa_2 > \kappa_1$.

Department : Chemistry Student's Signature Penporn Kajornklin
Field of Study : Chemistry Advisor's Signature Thumnoon Nhujak
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LIST OF ABBREVIATIONS

η	viscosity
μ	electrophoretic mobility
μ_{eo}	electroosmotic mobility
μ_{net}	total mobility
μ_0	electrophoretic mobility of free analyte
μ_∞	electrophoretic mobility of CD:analyte complex
BGE	background electrolyte
CD	cyclodextrin
CD1	cyclodextrin at fixed concentration
CD2	cyclodextrin at vary concentration
CD-EKC	cyclodextrin-electrokinetic chromatography
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
CMC	critical micelle concentration
CZE	capillary zone electrophoresis
DAD	diode array detector
EOF	electroosmotic flow
EKC	electrokinetic chromatography
HS-β-CD	highly sulfated-β-cyclodextrin
HS-γ-CD	highly sulfated-γ-cyclodextrin
i.d.	internal diameter
MEKC	micellar electrokinetic chromatography
MEEKC	microemulsion electrokinetic chromatography

SDS	sodium dodecyl sulfate
κ	enantioselectivity
ΔK	difference in binding constants for enantiomers to a selector ($\Delta K = K_2 - K_1$)
$\Delta\mu$	difference in electrophoretic mobility for enantiomers ($\Delta\mu = \mu_2 - \mu_1$)
C	free cyclodextrin concentration
$C_{\Delta\mu,\,max}$	concentration of cyclodextrin at give maximum mobility difference
E	electric field strength
I	ionic strength
K	binding constant
$\overline{K}$	average binding constant
L	total capillary length
l	length of capillary to detector
r_h	hydrodynamic radius
R_s	resolution
t_{eo}	migration time of EOF
t_m	migration time
t_R	retention time
V	applied voltage
v_{eo}	electroosmotic velocity
v_{ep}	electrophoretic velocity
v_{net}	total electrophoretic velocity
x_i	mole fraction of analyte
z	charge of an ion

CHAPTER I
INTRODUCTION

1.1 Chirality [Beesley and Scott 1998]

Chirality is the structural property of an object or molecule which lacks of the three elements (a plan, a center and axis) of symmetry exists in more than one form. A chiral compound consists of two or more configurations called "enantiomers" which differ from each other as nonsuperposable mirror images. Enantiomers have identical and chemical properties in an achiral environment, except for the direction of rotation of polarized light. However, one isomer may exhibit different pharmacological and toxicological effects from the another isomer when they are subjected to a chiral environment. A mixture of the enantiomers in equal amounts is called a racemic mixture or racemates which are optical inactivity because the rotation caused by one enantiomer is canceled by its complementary enantiomer. An example of a chiral compound is 2'-hydroxyflavanone (Fig. 1.1).

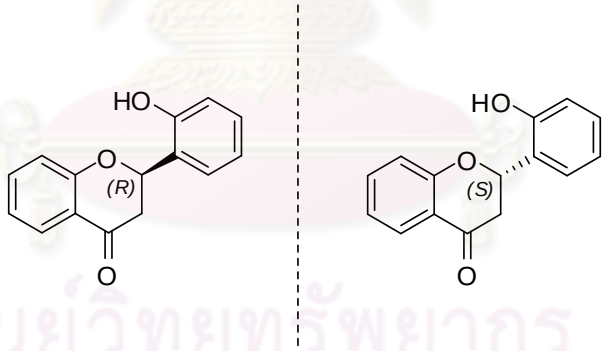


Figure 1.1 Structure of *R*-and *S*-2' hydroxyflavanone.

1.2 Importance of Chiral Separation

The chiral separation of enantiomers has been widely studied in analytical chemistry because chirality plays an important role in a wide variety of areas such as agrochemical, food and beverage as well as petrochemical industries. Especially, in the pharmaceutical and biological fields, approximately 50% of marketed drugs in currently use are chiral and only about 25% of these are single isomer [Gübitz and

Schmid 2008]. Additionally, about half of these chiral drugs may exist in the form of racemate, and each enantiomer may have a different pharmacological effects. For instance, thalidomide as a racemic mixture was used to prevent morning sickness during pregnancy in the 1960s. The (*S*)-enantiomer was proved to be as the therapeutic agent whereas the (*R*)-form was identified as the toxic agent. Furthermore, only the (*S*)-enantiomer of ibuprofen is effective as an anti-inflammatory agent, while the (*R*)-form has no physiological activity. Similarly, the anesthetic ketamine is administered as racemate, not only the (*R*)-enantiomer is less potent than the (*S*)-form but the (*R*)-form may cause post-operative effects. Because of this fact, the Food and Drug Administration (FDA) in America, Europe and Japan establish guidelines the requirement of chiral drugs as single isomers, in order to check the enantiomer purity, pharmacological and physiological testing before being placed on the market [Hutt and Valentová 2003, Shimaza *et al.* 2008].

Analytical methods which have been developed for the enantiomeric separation based on chromatographic techniques include direct and indirect separation methods. Indirect separation method involves using the chiral derivatization reagents to generate diastereomeric derivatives. Diasteriomers of the same compound have different physical and chemical properties therefore they can be separated by several separation techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) [Olsson 2008, Fanali 2009]. On the other hand, separation of diasteromers has many disadvantages. For example, it is time consuming in diastereomeric derivatives step, the analyte must contain active functional groups (hydroxyl, amino) to derivatize, it requires the use of very pure derivatizing agent, and the kinetics of the reaction can lead to different peak areas for the two diastereomers formed. Presumably due to these and other disadvantages of indirect separation method, the direct separation method is becoming very popular for enantiomeric separation. The direct separation method, called “chiral separation”, involves the use of chiral selectors which can interact with the enantiomers, leading to distinguish retention or migration behavior and the separation of enantiomers. There are two possible approaches to chiral separation: (1) The chiral selector as the stationary phase is employed for GC, HPLC and capillary electrochromatography (CEC), and (2) chiral selector is added to the mobile phase in HPLC or the buffer in capillary zone electrophoresis (CZE) or capillary electrokinetic

chromatography (CEKC) such as micellar electrokinetic chromatography (MEKC) and cyclodextrin electrokinetic chromatography (CD-EKC).

The chiral separation in CE is easier to perform than the indirect separation [Fanali 2009]. Because it is less time consuming since derivatization and purification are not required, a wide number of chiral selectors are commercially available, and small amounts of chiral selectors can be used. Furthermore, CE technique presents additional advantages over other techniques such as HPLC [Olsson 2008, Fanali 2009], including, (1) A relatively small volume of sample and buffer (nl or μl, respectively) is required (2) Expensive chiral columns can be avoided because the chiral selector can be easily added to the background electrolyte (BGE). (3) The separation is highly reproducible because the buffer with the chiral selector is replenished after each run. In CE, addition of chiral compounds to the electrolyte buffer is the most convenient approach to the separation of the enantiomers.

1.3 Chiral Selectors in CE [Gübitz and Schmid 2004, Olsson 2008]

To separate enantiomers, a chiral environment has to be created. The two enantiomers can only possess different properties if present in such a surrounding. The chiral selectors widely used as additives in the buffer can be divided into three main categories: inclusion systems (e.g. cyclodextrins (CDs) or crown ethers), enantioselective metal-ion complexes (e.g. copper (II)-L-histidine or copper (II)-aspartame), and optically active surfactants (e.g. chiral mixed micelles or bile acids). On the other hand, CDs and derivatized CDs are the most of widely reported in CE because they are many suitable properties that make them good chiral selectors for enantiomer separations. For example, they are stable in a widely pH (2-12) range used and also UV transparence that does not interfere with UV detection of UV absorbly enantiomers.

1.4 Literature Review

Chiral separation has received great attention for decades. This work involves chiral separation of hydroxyflavanones, in a class of flavonoid compounds. Flavonoids are of pharmaceutical importance due to their antioxidance capacities and antimicrobial activities. They are used as anti-inflammatory, antiviral, antiallergic, antibiotic, and anti-carcinogenic compounds [Heller and Forkmann 1988, Wollenweber *et al.* 1988]. For example, 2'-hydroxyflavanone can be used to inhibit proliferation and tumor vascularization in renal cell carcinoma [Nagaprashantha *et al.* 2011]. In some cases, only single isomer of flavaniod enantiomers has pharmaceutical activity [Lin *et al.* 2008]. Therefore, the development of analytical methods for enantioseparation of flavonoids is needed. Some previous work on chiral separation of flavonoids, including hydroxyflavanones, will be reviewed in this section.

Some flavonoids are non-ionizable, but hydroxyflavanones are weak acids that can carry negatively charge(s) at a basic pH of the buffer. Therefore, CD-EKC separation of negatively charged enantiomers can be performed using neutral and/or charged CDs with/without any chiral/achiral additive, while that of uncharged enantiomers can be performed using neutral CDs with addition of charged chiral/achiral additive or charged CD with/without addition of charged/uncharged chiral/achiral additive.

In the CD-EKC enantioseparation of hydroxyflavanone (eriodictyol) with single neutral CD in the borate buffer in a pH range of 9.3-10.1, 1.2% HP-γ-CD showed the optimal enantioseparation with the highest R_s (2.23), while β-CD and HP-β-CD gave the partial R_s for eriodictyol enantiomers [Pan *et al.* 2008].

Using single neutral CD (hydroxylpropyl (HP)-γ-CD, methyl (M)-γ-CD, M-β-CD) for separation of hydroxyflavanones (isosakuranetin, hesperetin, eriodictyol, homoeriodictyol, naringenin, 4'-hydroxyflavanone and pinostrobin) in the phosphate/borate buffer (pH 7-11) [Wistuba *et al.* 2006], M-γ-CD gave better enantiomeric R_s than did M-β-CD and HP-γ-CD, except for higher R_s of eriodictyol and naringenin with HP-γ-CD. In comparison with M-γ-CD or HP-γ-CD, negatively charged sulfato-β-CD was found to improve R_s for isosakuranetin and 4'-

hydroxyflavanone, but gave the achieved R_s of almost enantiomers except for eriodictyol and pinostrobin. Negatively charged carboxymethyl (CM)-β-CD gave the achieved R_s only for eriodictyol, while CM-γ-CD only for naringenin. In addition, no enantiomeric separation was achieved with sulfato-γ-CD.

Achiral MEKC separation of flavanone racemates was achieved for non-enantiomers using achiral SDS surfactant in the pH 9.2 borate buffer [Wang *et al.* 2007], but no enantiomeric separation was obtained due to no chiral selector in the buffer. Using CD-EKC separation of these flavanones with α-CD or β-CD, no enantiomeric separation was reported.

In comparison between chiral MEKC, using 100 mM sodium cholate surfactant, and CD-MEKC, using 20 mM γ-CD and 100 mM SDS, for enantiomeric separation of hydroxyflavanone glycosides and hydroxyflavanone aglycones in the pH 8.3 tris-borate buffer [Asztemborska *et al.* 2003], the chiral MEKC was reported to enable enantiomeric separation of the former hydroxyflavanones, but not applicable for the latter hydroxyflavanones, while the CD-MEKC was suitable for the latter but not for the former hydroxyflavanones.

Using the pH 3.0 phosphate buffer in CD-EKC separation of uncharged-hydroxyflavanones with negatively charged CD [Lin *et al.* 2008], sulfated (S)-β-CD was shown to achieve simultaneous enantiomeric separations for 2'-, 3'- and 4'-hydroxyflavanones, but poor peak shape was observed for 4'-. A dual CD system consisting of S-β-CD and neutral β- or γ-CD plus SDS improved peak shape and simultaneous separation of 2'- and 3'-hydroxyflavanones, but slightly worse enantiomeric separation for 4'-, in comparison with single S-β-CD.

Highly-sulfated CD (HS-CD) is another negatively charged CD that composes of a larger number of sulfate functional groups in comparison with the typical sulfated CD, such as 11, 12 and 13 sulfate groups for HS-α-, β- and γ-CD, respectively, while 7-11 sulfate groups for S-β-CD [Boer *et al.* 2000]. Single HS-CD has been reported for separation of the following enantiomers: HS-α-CD for propranolol; HS-γ-CD for praziquantel; HS-β-CD for warfarin [Perrin *et al.* 2003], using HS-α-, β- or γ-CD for

aromatic amino acids [Vaccher *et al*. 2006]. In some cases, simultaneous separation of enantiomers may not be obtained using only single CD [Nhujak et al 2005]. One approach to improve resolution of simultaneous separation of all analytes is the use of combination of two types of CD called dual CDs. The following dual neutral and anionic CDs have shown to enhance simultaneous separation of several pairs of enantiomers: HS-β-CD/TM-β-CD for enantiomers of fenoprofen, flurbiprofen, ibuprofen and ketoprofen [Abushoffa *et al.* 2002], and α-, β- or γ-HSCD/DM-β-CD, TM-β-CD or HP-β-CD for pharmaceutical compounds (16 basics, 8 acids and 1 neutral) [Matthijs *et al.* 2004], where HS, DM and TM stand for highly-sulfated, heptakis (2,6-di- *O-methyl*) and heptakis (2,3,6-tri-*O-methyl*), respectively.

In comparison with a single chiral selector as charged CD, dual chiral selectors as neutral and charged CDs have been reported to provide benefit of better resolution and peak shape [Lurie *et al.*1994]. A review, covering the literature up to 2008, of enantiomeric separation of drugs and pharmaceutical preparations using dual charged and neutral CDs has been reported by Scriba [2008].

1.5 Aim and Scope

In previous works, enantiomeric separations of 2'-, 3'- and 4'-hydroxyflavanones, in uncharged form at low pH of buffer (pH = 3), were carried out using the following chiral selectors: single S-β-CD with the achieved simultaneous separation of all enantiomers, dual S-β-CD/β-CD plus SDS or dual S-β-CD/γ-CD plus SDS both systems with the achieved baseline resolution for 2'-, 3'- but partial resolution for 4'-enantiomers. In our initial study, enantiomeric separation of 7-hydroxyflavanone was not achieved using a single S-β-CD. As previously mentioned, negatively charged HS-CDs have been shown to have high resolving ability and their possibility to separate neutral or ionic racemates using either single or dual CDs. However, up to date, enantiomeric separation of hydroxyflavanones using single or dual HS-CDs has not been previously reported.

Therefore, the aims of this work are to investigate simultaneous enantiomeric separation of three pairs of flavanone enantiomers, 2'-, 4'- and 7-hydroxyflavanones as

shown in Figure 1.2, using CD-EKC with single and dual charged HS-β-CD and/or HS-γ-CD, and to use the concept of dual enantioselectivities [Nhujak *et al.* 2005] to explain the migration order and a change in separation selectivity for these three enantiomers in dual CDs.

In initial work, the single CD, HS-β-CD or HS-γ-CD, will be used as a chiral selector for enantiomeric separation of 2'-, 4'- and 7-hydroxyflavanones in a phosphate buffer pH 2.5. The binding constant (*K*) of each isomer to each CD, and enantioselectivity of each CD for enantiomers will be determined, and used to explain and compare enantioseparation parameters, such as electrophoretc mobility difference, separation selectivity and resolution. Then, both HS-β-CD and HS-γ-CD will be used as dual chiral selectors for enantiomeric separation of 2'-, 4'- and 7-hydroxyflavanones, by using a fixed concentration of a particular CD and varying the concentration of another CD. The simultaneous separation of these enantiomers will be compared using single and dual CDs. The migration order and a change in separation selectivity for enantiomers in dual CDs will be discussed using the data of enantioselectivity determined from the single CD.

It is expected that this work will be useful for explanation of a change in separation selectivity (α) and resolution of enantiomers in CD-EKC using single and dual CDs and for optimization of simultaneous separation of several pairs of hydroxyflavanones. Especially, this work may be applied for drug quality control in order to determine enantiomeric purity check and toxicological investigations in pharmaceutical field. In addition, in the case that a single isomer is not available, dual CDs can be used to explain the same or reversed migration order of enantiomers in single CD.

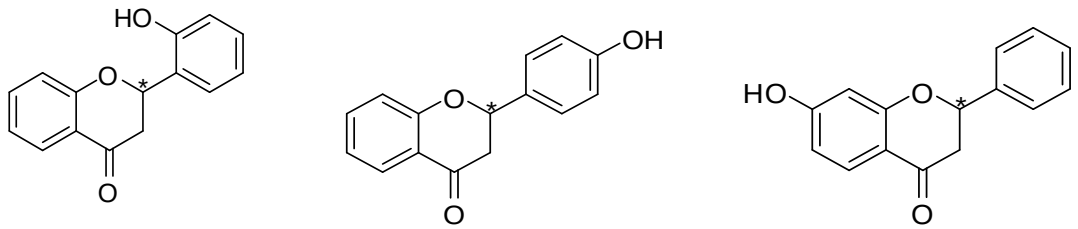


Figure 1.2 Chemical structures of 2'-, 4'- and 7-hydroxyflavan

CHAPTER II
THEORY

2.1 Introduction to CE

CE is an electrophoretic technique that use capillary containing a background electrolyte (BGE) solution under the influence of applied electric field. Separation mechanism is based on the differences in the electrophoretic mobility of analytes depending on the charge-to-size ratio. CE has become an increasingly popular separation technique, which can be used to analyze a wide variety of charged and neutral analytes covering both hydrophobic and hydrophilic compounds.

CE comprises of six basic modes which can be classified on the basis of the different separation mechanism, namely capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary electrochromatography (CEC), capillary gel electrophoresis (CGE), isotacchophoresis (ITP) and capillary isoelectric focusing (CIEF). More recently microemulsion electrokinetic chromatography (MEEKC) has been introduced as an alternative mode in CE. Each mode of CE has been appropriately used to separation of wide variety application. For instance, CZE is a technique that was developed for very efficient separations of charged solutes. CGE, the technique is useful for analysis of macromolecules and especially for DNA sequencing. Furthermore, MEKC and MEEKC were developed to help resolve neutral solutes that were not separable by CZE. At the same time, both of them were also used to separate charged analytes. In addition, many reports have been confirmed that MEEKC show obvious advantages over MEKC for the separation of hydrophobic compounds.

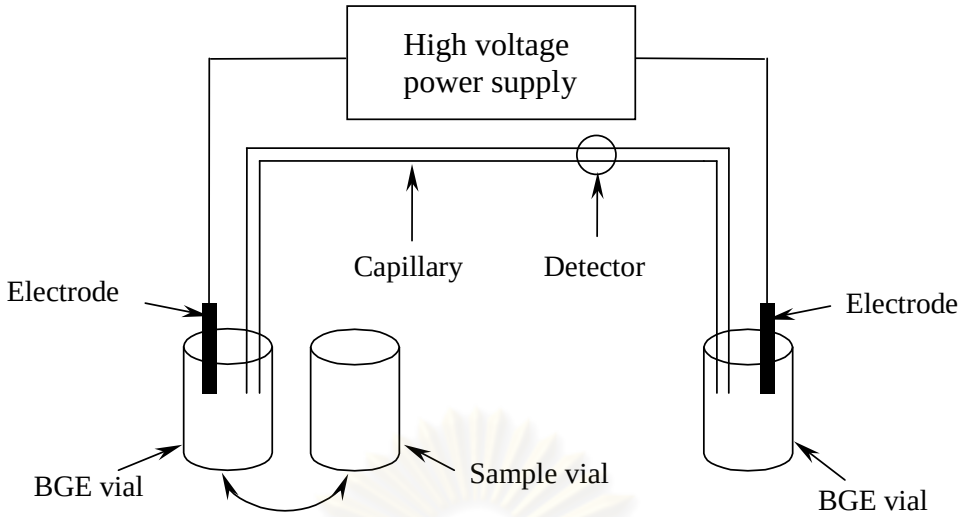


Figure 2.1 Schematic diagram of a basic CE instrument. Adapted from Weinberger [1993]

The schematic diagram of a typical CE instrument is displayed in Figure. 2.1. The components include a capillary, high voltage power supply, electrodes, buffer vials and detector. The capillaries are commonly narrow bore bare fused silica, ranging in diameter from 10 to 200 μm and length from 20 to 100 cm. The capillaries externally coated with polyimide to add flexibility to the normally brittle glass. The high voltage power supply is required to drive the separation, allowing voltages up between -30 and +30 kV in either normal or reversed polarity mode. Under normal polarity, the anode is located at the point of sample injection and the cathode is located towards the capillary outlet, near the detector. Two platinum electrodes commonly used to establish the potential. The most common detector format is UV-Vis that provides wavelength selection from 190 to 700 nm. Fluorescence detector is also currently employed due to its high sensitivity. However not all compounds possess high fluorescence so the derivatization must be required. Furthermore, a number of CE interfacing systems which couple with fast atom bombardment (FAB) or electrospray ionization (ESI) mass spectrometry (MS) have been reported.

2.2 Electrophoretic Mobility [Grossman et al. 1992, Camilleri 1993]

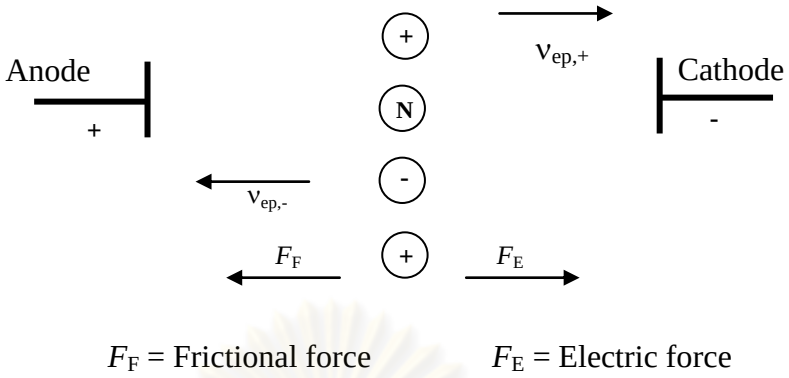


Figure 2.2 Migration behavior of each species. Adapted from Foret *et al.* [1993].

To understand the mechanism of separation in CE, it is important to appreciate which variables affect the electrophoretic migration of the analyte. The two primary factors that affect electrophoretic migration include the applied electric force (F_E) and the frictional force (F_F). When a charged analyte is placed in an applied electric field, it is migrate toward the electrode in the direction opposite polarity to its direction of the electric force. The electric force depends on the charge of the ion (q), which is proportional to the charge of an ion (z) and the electronic charge (e), and the electric field strength (E; $E = V/L$, where V is the applied voltage across L the total length of capillary).

$$F_E = zeE \tag{2.1}$$

According to the migration of the analyte is affected by the applied electric force. Meanwhile this migration is resisted by the frictional force which depends on a number of parameters, including the viscosity of the BGE (η) and the electrophoretic velocity (ν_{ep}) of an ion in BGE and size of the ion (in term the hydrodynamic radius of an ion, r_h)

$$F_F = 6\pi r_h \eta \nu_{ep} \tag{2.2}$$

Then, the acceleration of the ion will proceed until F_E is balanced by F_F, giving the equation:

$$zeE = 6\pi r_h \eta \nu_{ep} \quad (2.3)$$

The electrophoretic mobility, μ ($m^2\ V^{-1}\ s^{-1}$) is defined as the electrophoretic velocity of an ion migrating in BGE under the influence of an applied electric field and relates to parameters as the equation.

$$\mu = \frac{\nu_{ep}}{E} = \frac{ze}{6\pi\eta r_h} \quad (2.4)$$

With respect to Equation, mobility is directly proportional to the charge of the ion and inversely proportional to the radius of particle. Furthermore, it also depends on charge density of the analytes, ionic strength, viscosity of electrolyte, and temperature.

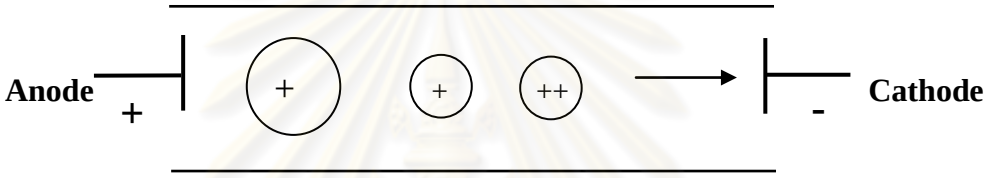
The another driving force in electrophoresis is electroosmosis generating electroosmotic flow (EOF) of an electrolyte solution through the capillary. Without EOF, the neutral analytes ($q = 0$) would simply not migrate at all because they do not possess an electrophoretic mobility, while anions and cations would migrate to the detection end of the capillary under the influence of their electrophoretic mobility.

Since a low pH of a phosphate buffer (pH 2.5) is used in this work in order to obtain analytes carrying a fully uncharged and suppress electroosmotic flow (EOF). Thus, details of EOF are not mentioned in this section. However, it can be demonstrated by Grossman *et al.* [1992] and Camilleri [1993].

2.3 Migration Behavior of Analytes in CE Without EOF

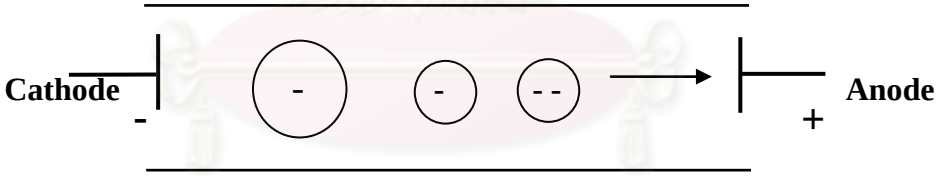
Without EOF in a CE buffer and capillary, the analytes migrate only by electrophoretic mobility. In the case of cationic analytes, the voltage is applied at the detector end as the cathode. Conversely, in the case of anionic analytes, the voltage is applied at the detector end as the anode**.** The electrophoretic mobility of the analytes depends on charge-to-size ratio. The higher ion charge and the smaller hydrodynamic radius migrate first.

a) Cationic analytes



Normal polarity of applied voltage

b) Anionic analytes



Reversal polarity of applied voltage

Figure 2.3 Migration behavior of analytes without EOF (a) cation and (b) anion. Adapted from Li [1992].

2.4 Cyclodextrin-modified Electrokinetic Chromatography (CD-EKC)

Electrokinetic chromatography (EKC), established by Terabe and coworkers in 1985, involves electrophoretic and chromatographic separation. Separation mechanism is based on different partitioning of the analytes between pseudostationary phase and aqueous phase with different effective mobilities.

In EKC, the chiral selector can also be an additive facilely to the electrolyte. CDs have been utilized the most common chiral selector in EKC. One of advantage of CDs is available in neutral, cationic or anionic forms, depending on the type and extent of derivation. Furthermore, the functionality of the derivation often plays a key role in defining the stereoselective association between the analyte and CD. The addition of CD in the CZE buffer, typically used in chiral separations, often refers to modified EKC or CD-EKC. In chiral separations, the most common CD-EKC buffer consists of CD or CD and surfactant such as sodium dodecyl sulfate (SDS) system, where concentration value less than critical micelle concentration (CMC). This system can be operated as the distribution process using only the host-guest interaction between CD and the solute. The differential migration between the host and guest molecules is permitted by electroosmosis and electrophoresis in capillary filled with CD solution.

2.5 CDs and Derivatized CDs

CDs are cyclic oligosaccharides bonded by cyclic α-(1,4)-linked oligomers consisting of six, seven or eight units for α-, β- and γ-CD, respectively. The chemical structure of the CD molecule is shown in Figure 2.4. The shape of CDs is similar to that of a truncated cone with cavity of different dimensions depending on the CD type. Their cavity is relatively hydrophobic and able to accept guest compounds of different types, particularly those with non-polar groups. The outside is relatively hydrophilic due to the presence of hydroxyl groups (primary and secondary). The main intermolecular forces are hydrogen bonding, hydrophobic, dipole-dipole and van der Waals interactions.

With respect to Figure 2.7, the hydroxyl groups on the CD rims can be easily derivatized. This offers additional possibilities for synthesize different CDs with properties desirable for different applications. Derivatized CDs provide additional properties compared to the native CDs. Depending on the substituents that are used, additional intermolecular forces can be introduced. Moreover, the increased solubility of derivatized CDs is a very important property. For instance, a number of derivatized neutral CDs containing methyl, hydroxyethyl and hydroxypropyl groups are commercially available and have been used successfully as chiral selectors in CE.

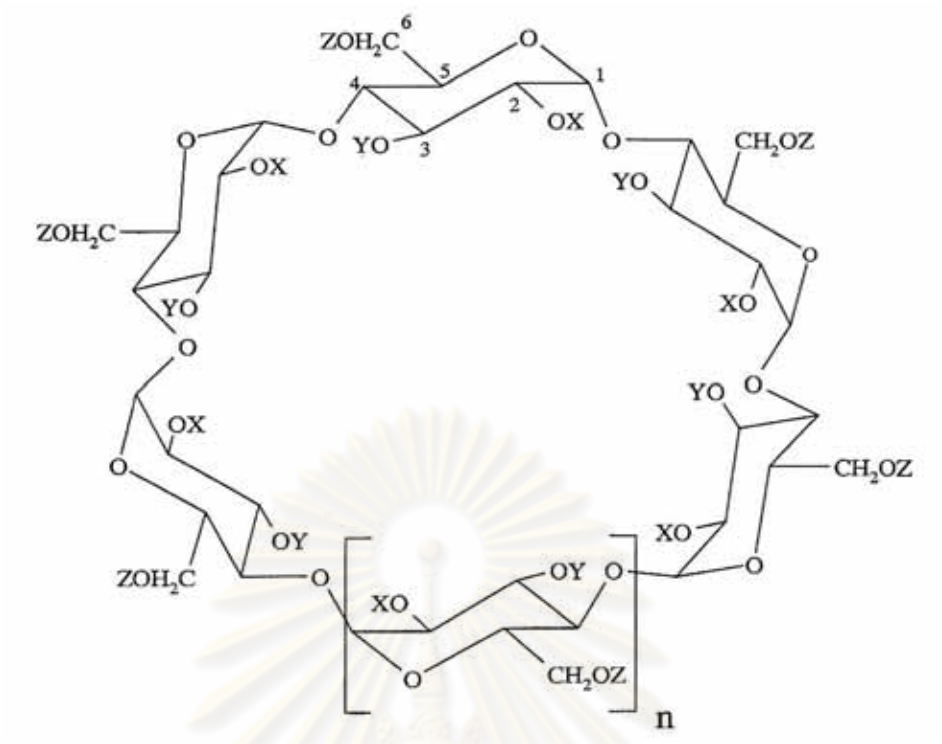


Figure 2.4 Structure of cyclodextrins (CDs), when n = 1, 2, 3 for α-, β- and γ-CD, respectively, and X, Y and Z = H or CH_3 for native or methyl ether derivatives.

In addition, charged CDs are also used for chiral separations. Three different types are used; anionic, cationic and amphoteric. Anionic CDs are much more frequently used than cationic CDs, and more varieties are commercially available than for cationic or amphoteric CDs. From chiral separation report, baseline separation can be achieved at lower concentration with charged CDs compared to neutral CDs. Moreover, chiral separation of neutral racemates is possible, with fast separations achieved due to the self-electrophoretic mobility.

Several anionic CD derivatives are available, such as carboxyalkylated, sulfoalkylated, sulfated, highly sulfated and phosphorylated CDs. They can be used for enantiomeric separation of cationic, anionic and neutral compounds

In this work, highly sulfated CD (HS-CD) was used. The HS-CDs are a proprietary distribution of cyclodextrins with an average number of 11 sulfates for the α-CD, 12 for the β-CD and 13 for the γ-CD. This distribution was "designed in" to provide increased resolution under defined separation conditions. The structure of HS- β-CD is shown in Figure 2.5

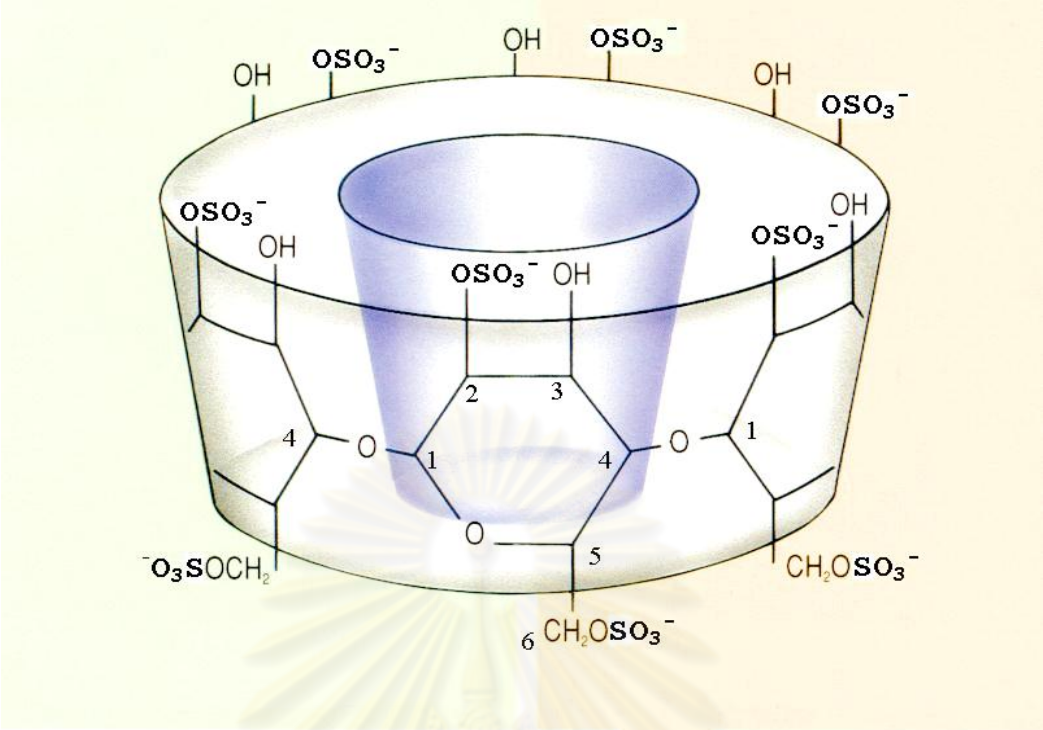


Figure 2.5 The structure of HS-β-CD. Reproduced from [Beckman Coulter]

2.6 Three Point Model Interaction

A simple model can be used to describe the mechanism of chiral recognition was proposed by Dalgliesh in 1952. A model is based on the "three-point interaction" theory. Figure 2.6 shows the interaction of each enantiomer with chiral selector. Differences in diastereomeric association lead to an apparent mobility difference in CE. Therefore, the resolution of enantiomers can occur. The main processes in chiral interactions include co-ordination to transition metals, charge-transfer interaction, hydrogen bonding and inclusion phenomena.

For charge-transfer interaction, both enantiomeric analytes and a chiral selector have π-electron sites, such as aromatic groups. The interaction points occur between the chiral selector (M, N and O) and the racemic analyte (X, Y and Z). When M and N are π-electron and acidic sites, respectively, and C is a basic or steric interaction site, whilst X and Y are π-electron and basic sites, respectively, and W and Z are acidic, basic, small or large sites. Enantioselectivity is based on an aromatic π-π interaction, together with additional polar interactions (hydrogen bonding, dipole interaction) or steric interaction.

Inclusion phenomena can occur when chiral selectors such as crown ethers and cyclodextrins have a cavity and act as hosts in host-guest equilibria. Selectively entails differential inclusion of the enantiomers as guest molecules. The stability of inclusion complexes depends on the size and shape of the analyte, hydrophobic and hydrophilic interaction, hydrogen bonding and solvent effects.

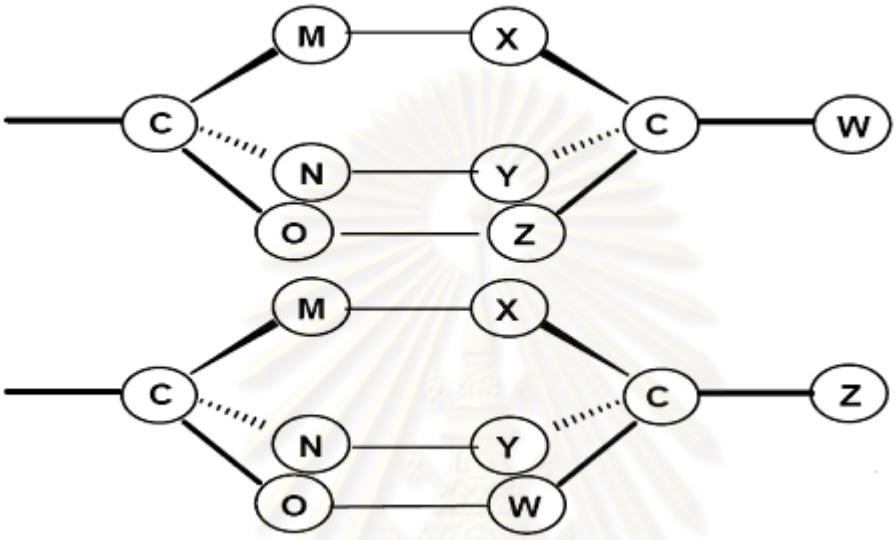
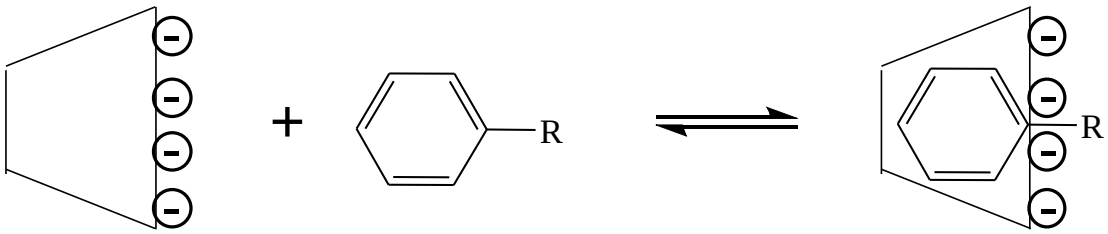


Figure 2.6 The three point interaction between a chiral selector and two enantiomers. Adapted from [Allenmark: 1991 and Nhujak: 2001]

2.7 Inclusion Complexation of CD and Enantiomers

In the presence of CD in the BGE, for charged CD and neutral enantiomers A, the formation of a complex with 1:1 stiochiometry can be expressed as follows;



$$\begin{aligned} &\overset{K_1}{\rightleftharpoons} \\ \text{A1} + \text{CD}^- &\rightleftharpoons \text{CDA1}^- \\ &\underset{K_2}{\rightleftharpoons} \\ \text{A2} + \text{CD}^- &\rightleftharpoons \text{CDA2}^- \end{aligned} \qquad (2.5)$$

Where K is binding constant, which can be expressed as

$$K = \frac{[\text{CDA}^-]}{[\text{CD}^-][\text{A}]} \qquad (2.6)$$

In presence of selector in BGE, the effective electrophoretic mobility, μ, of A is given by

$$\mu = x_{\text{A}}\mu_0 + x_{\text{CDA}}\mu_\infty \qquad (2.7)$$

where μ_o and μ_∞ are μ at zero and infinite concentration of CD, respectively. x_i is the mole fraction. It follows that [Wren and Rowe 1992a]

$$\mu = \frac{\mu_o + KC\mu_\infty}{1 + KC} \qquad (2.8)$$

Where C is the free concentration of CD, [CD], and may be assumed to be the initial concentration of CD ([S]) when [S] >> [A]. With known μ_o and C, the binding constant may be calculated by fitting the electrophoretic mobilities of the analyte as a function of CD concentration.

In the case of uncharged analytes and charged CD, μ_o is a equal to zero. Thus, Equation 2.8 may be rearranged to

$$\mu = \frac{KC\mu_{\infty}}{1+KC} \tag{2.9}$$

2.8 Enantioseparation Parameters

The enantioseparation parameters in CD-EKC can be expressed by electrophoretic mobility difference ($\Delta\mu$), separation selectivity (α) and resolution (R_s).

It follows from Equation 2.9 that the relationship between $\Delta\mu$ of enantiomers and C can be expressed by [Wren and Rowe 1992a, Penn *et al.*1993, 1994]

$$\Delta\mu = \frac{\Delta KC\mu_{\infty}}{\left(1+\overline{K}C\right)^2} \tag{2.10}$$

Where ΔK is the binding constant difference, $\overline{K}$ is the average binding constant ($\overline{K}$ = ($K_1 + K_2$)/2 or $\overline{K} = \sqrt{K_1 K_2}$). Therefore, it can be seen from Equation 2.10 that $\Delta\mu$ for enantiomers depends on ΔK for each enantiomer to CD, leading to enantiomeric resolution term.

The separation selectivity (α) is defined as the ratio of the electrophoretic mobilities for enantiomers [Chankvetadze 1997].

$$\alpha = \mu_2/\mu_1 \tag{2.11}$$

where μ_1 and μ_2 are the electrophoretic mobility of the analyte 1 and analyte 2, respectively, and $\mu_2 > \mu_1$.

A resolution (R_s) of two analytes is defined as the ratio of the difference in their migration times to their peak width at base as the equation

$$R_s = \frac{t_{m2} - t_{m1}}{0.5\left(w_{b1} - w_{b2}\right)} \tag{2.12}$$

R_s can be related to the mobility and the average number of theoretical plates, $\overline{N}$, as the equation

$$R_s = \frac{1}{4}\left(\frac{\Delta\mu}{\bar{\mu} + \mu_{eo}}\right)\sqrt{\bar{N}} \qquad (2.13)$$

where $\bar{\mu}$ is the average electrophoretic mobility of the analytes, and μ_{eo} the electroosmotic mobility. In CD-EKC without EOF, it follows from Equation 2.13 that the resolution of two analytes is given by

$$R_s = \frac{1}{4}\left(\frac{\Delta\mu}{\bar{\mu}}\right)\sqrt{\bar{N}} \qquad (2.14)$$

CHAPTER III
EXPERIMENTAL

3.1 Chemicals

Standard solutions of 20% (w/v) HS-β-CD (average MW = 2380.95) and HS-γ-CD (average MW = 2538.07), corresponding to 84.0 and 78.8 mM, respectively, were purchased from Beckman Coulter (CA, USA). 2'-, 4'- and 7-Hydroxyflavanone racemates were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphoric acid was purchased from Merck (Damstadt, Germany). MilliQ water was used for preparation of all solutions.

3.2 Preparation of Buffer and Analytes

A pH 2.5 phosphate buffer was prepared by titrating a 50 mM sodium dihydrogenphosphate solution with a 500 mM phosphoric acid solution to obtain a desired pH of 2.5. All buffer solutions were weekly prepared, stored in a refrigerator before use, and filtered through 0.45 µm syringe filters prior to CE analysis.

A phosphate buffer (pH 2.5) containing 0 to 50 mM HS-β-CD or 0 to 47 mM HS-γ-CD were prepared by diluting the appropriate amounts of 20% w/v HS-β-CD or HS-γ-CD with the phosphate buffer.

Stock solutions of each analyte, 2'-, 4'- and 7-hydroxyflavanones, were separately prepared at a concentration of 10,000 ppm in acetonitrile. The mixed standard solution of analytes at a concentration of 50 ppm each was obtained by pipeting each stock solution and then diluting this with milliQ water. All final solutions of test analytes were filterd through 0.45 µm PTFE syringe filters prior to CE analysis.

3.3 CE Conditions

A new capillary was conditioned with the following solutions for 15 min each; methanol, 1 M HCl, 1 M NaOH, 0.1 M NaOH, water and a pH 2.5 phosphate buffer. Prior to CE analysis each day, the capillary was rinsed with methanol, 0.1 M NaOH, water, and the phosphate buffer each for 10 min. Between consecutive run, the capillary was rinsed with methanol, 0.1 M NaOH for 2 min each and then with the buffer for 1 min. After analysis each day, the capillary was rinsed with water and methanol for 5 min each, and then 0.1 M NaOH and water for 10 min each.

All CE separations were performed using a Beckman Coulter MDQ-CE system equipped with a photo-DAD scanning from 190 to 300 nm and monitoring at 214 nm. An uncoated fused-silica capillary used was 60.2 cm in length (50 cm to detector) × 50 μm id, thermostated at 25 °C. Voltage was set at -20 kV. A sample solution was introduced by 0.5 psi pressure injection for 3 s. Each experiment was carried out in duplicate.

3.4 Enantiomeric Separation Using Single CD

Simultaneous separation of enantiomers was carried out using various concentrations of separately single CD (HS-β-CD or HS-γ-CD) and other CE conditions as in Sections 3.3. In the case of negligible or no EOF, observed electrophoretic mobility, $\mu_{obs,}$ of each enantiomer can be calculated from electropherograms using the equation 3.1

$$\mu_{obs} = \frac{lL}{Vt_m} \qquad (3.1)$$

where L and l are the total length of a capillary and the length of a capillary to detector, respectively, V is the applied voltage and t_m is the migration time of the analyte.

Since observed electrophoretic mobility also depends on the viscosity of the buffer containing CD [Penn *et al.*1993], corrected electrophoretic mobility of analytes, μ, was calculated using Equation 3.2,

$$\mu = \mu_{obs} \frac{\eta_c}{\eta_o} \tag{3.2}$$

where η_c / η_o is the relative viscosity of the buffer at a given to that at zero CD concentration. In this work, the values of relative viscosity of HS-β-CD and HS-γ-CD were assumed to be equal to those of β-CD and γ-CD, respectively, which were obtained from Nhujak [2001]

$$\eta_c / \eta_o = 1 + 2.638\times10^{-3}[\beta\text{-CD}] + 6.80\times10^{-6}[\beta\text{-CD}]^2 \quad ; r^2 = 0.9943 \tag{3.3}$$

$$\eta_c / \eta_o = 1 + 2.993\times10^{-3}[\gamma\text{-CD}] + 1.53\times10^{-5}[\gamma\text{-CD}]^2 \quad ; r^2 = 0.9983 \tag{3.4}$$

As can be seen in Equation 2.9, μ and $C_.$ have non-linear relationship. Therefore, in this work, the values of K and μ_∞ are obtained using the MATLAB program that is able to fit data points of μ as a function of C. It should be noted that μ_∞ is assumed to be equal for complexes of CD:enantiomers. The values of K and μ_∞ were obtained from the average μ from two separate runs. Results are shown in Section 4.2.

3.5 Enantiomeric Separation Using Dual CDs

In this work, dual CDs were carried out using the fixed concentration of CD1 (4 mM HS-β-CD) and varying the concentration of CD2 (2 to 47 mM HS-γ-CD change in separation selectivity (α) and the migration order was compared as sh in Section 4.4

CHAPTER IV
RESULTS AND DISCUSSION

4.1 pH and Concentration of Buffer

2'-, 4'- and 7-Hydroxyflavanones are weakly acidic compounds with pK_a values of 7.54, 8.00 and 7.35, respectively [Lin *et al.* 2008, Wang *et al.* 2007]. The degree of dissociation of the weak acid, α_{dis}, depends on the buffer pH as the equation [Khaledi, 1998]

$$\alpha_{dis} = \frac{1}{1+10^{(pK_a - pH)}} \qquad (4.1)$$

It follows from Equation 4.1 that the α_{dis} value is less than 0.01, indicating 99% of uncharged species, when pH is less than pK_a at least 2 units. Therefore, the pH 2.5 of a phosphate buffer was selected in order to allow the separation of uncharged enantiomers with charged CD and to eliminate EOF.

At pH of 2.5, the phosphate buffer contains the weak acid (H_3PO_4 or HA) and its conjugated base ($H_2PO_4^-$ or A^-) which can be expressed by a dissociation equilibrium as the equation

$$H_3PO_4\ (aq) + H_2O\ (aq) \rightleftharpoons H_2PO_4^-\ (aq) + H_3O^+\ (aq) \quad : pK_{a1} = 2.12 \qquad (4.2)$$

The pH of a solution containing the weak acid (HA) and its conjugated base (A^-) is given by the Henderson-Hasselbalch equation [Chang 2002]

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \qquad (4.3)$$

Since pK_{a1} of H_3PO_4 is equal to 2.12, the phosphate buffer at pH 2.5 in a range of pK_{a1} ± 1.0 was chosen in order to provide high buffering capacity, resulting in high precision in the suppressed EOF and migration times of analytes.

At low ionic strength (5-10 mM) phosphate buffers generately low currents and a reasonably fast EOF. However, the too low concentrations of the buffer result in poor precision of migration times due to electrolysis effect [Kelly *et al.* 1997] and poor peak shape due to high electromigration dispersion. However, the too high concentrations of the buffer generate high current and Joule heating which may limit the use of high voltage and temperature. Therefore, a concentration of the phosphate buffer at 50 mM was chosen to use in this work for suppressd EOF.

4.2 Simultaneous Separation and Binding Constant for Enantiomers to Single CD

Simultaneous CD-EKC separation of 2'-, 4'- and 7-hydroxyflavanones using single CD was carried out as a procedure described in Section 3.3. Electropherograms for separation of these enantiomers with HS-β-CD and HS-γ-CD are shown in Figures 4.1 and 4.2, respectively. From Figure 4.1a-d, five peaks are observed due to no enantiomeric separation of 7-hydroxyflavanone. In this work, a single enantiomer of each hydroxyflavanone is not available. Therefore, the letters of "a" and "b" are used to refer to the first and second migration order, respectively. Peak identification of enantiomers was conducted by spiking with the standard racemates. At pH 2.5 of the phosphate buffer, EOF is suppressed and negligible in comparison with the electrophoresis. Therefore, it may be assumed that the analyte, binding with negatively charged CD, migrates only due to effective electrophoretic mobility of its CD complex. Strickly speaking, the electrophoretic mobility (μ) of a negatively charged compound is assigned as the negative value. However, in this work, μ refers to $|\mu|$. From electropherograms in Figures 4.1 and 4.2, the higher μ contributes to the faster migration time. Owing to t_m in the order 2'a- < 2'b- < 4'a- < 4'b- < 7a- < 7b-hydroxyflavanones with HS-β-CD (Figure 4.1) or HS-γ-CD (Figure 4.2), the values of μ were obtained in order 2'a- > 2'b- > 4'a- > 4'b- > 7a- > 7b-.

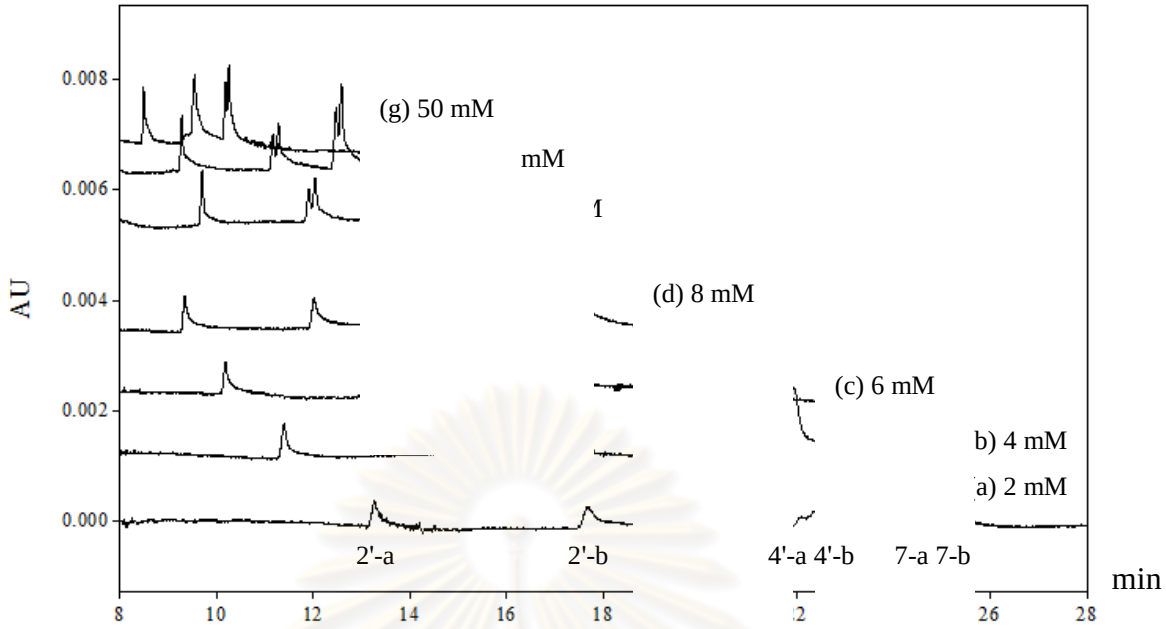


Figure 4.1 Electropherograms of the enantiomeric separatio l'- and 7-hydroxyflavanones using HS-β-CD as chiral selector. CE conditions: uncoated fused silica 50 µm i.d. × 60.2 cm (50 cm to detector), temperature 25 °C, BGE as 50 mM phosphate buffer pH 2.5, voltage -20 kV, 0.5 psi pressure injection for 3 s and UVdetection at 214 nm.

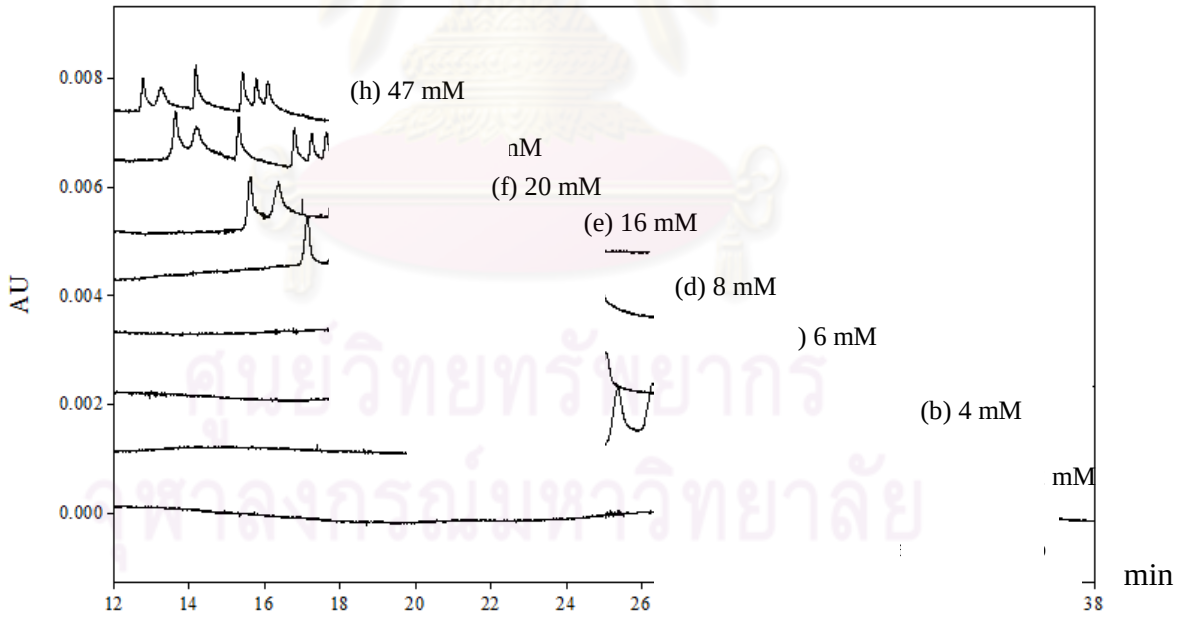


Figure 4.2 Electropherograms of the enantiomeric se 7-hydroxyflavanones using HS-γ-CD as chiral selector. CE con .

Figures 4.3 and 4.4 show binding curves of non-linear relationship between μ and *C* using a chiral selector HS-β-CD and HS-γ-CD, respectively. It should be noted that, at a given CD concentration, the corrected electrophoretic mobility (μ), due to a change in the buffer viscosity, was obtained from the observed value (μ_{obs}) as previously mentioned in Section 3.4

With an increase in the CD concentration, the higher μ (Figures 4.3 and 4.4) or the faster migration (Figures 4.1 and 4.2) of the enantiomers is seen due to an increase in the mole fraction of the charged complexes of uncharged enantiomers binding with negatively charged CD, according to Equations 2.7 and 2.9.

From experimental, μ data in Figures 4.3 and 4.4, *K* and μ_{∞} were obtained as shown in Table 4.1, using the MATLAB program of non-linear least-squares fit to data points of μ and *C* according to Equation 2.9.

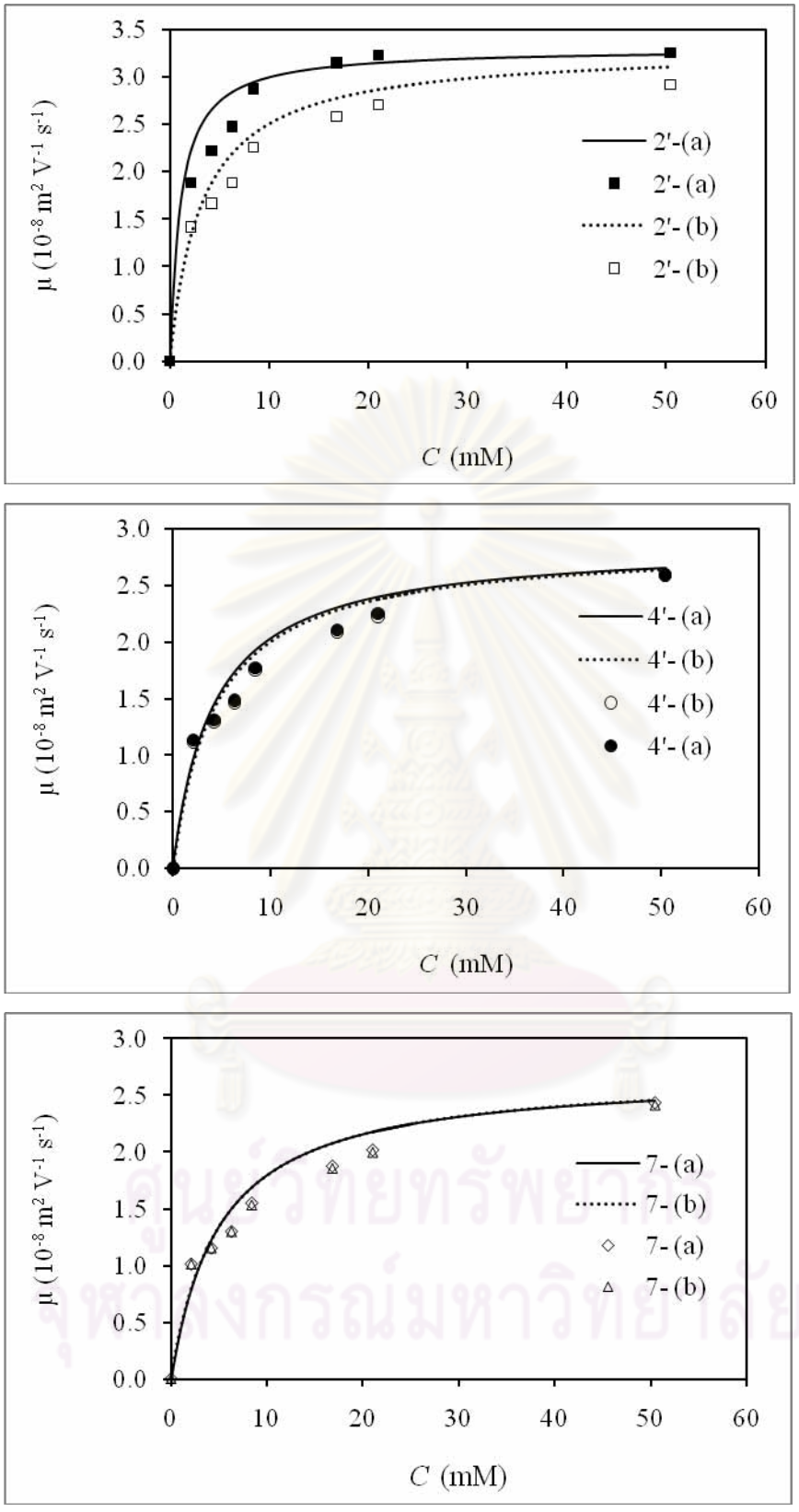


Figure 4.3 Binding curves for 2'-, 4'- and 7-hydroxyflavanone enantiomers to HS-β-CD. Experimental (symbols) μ is the average of two runs, and predicted (lines) μ is obtained using Equation 2.9 with data in Table 4.1.

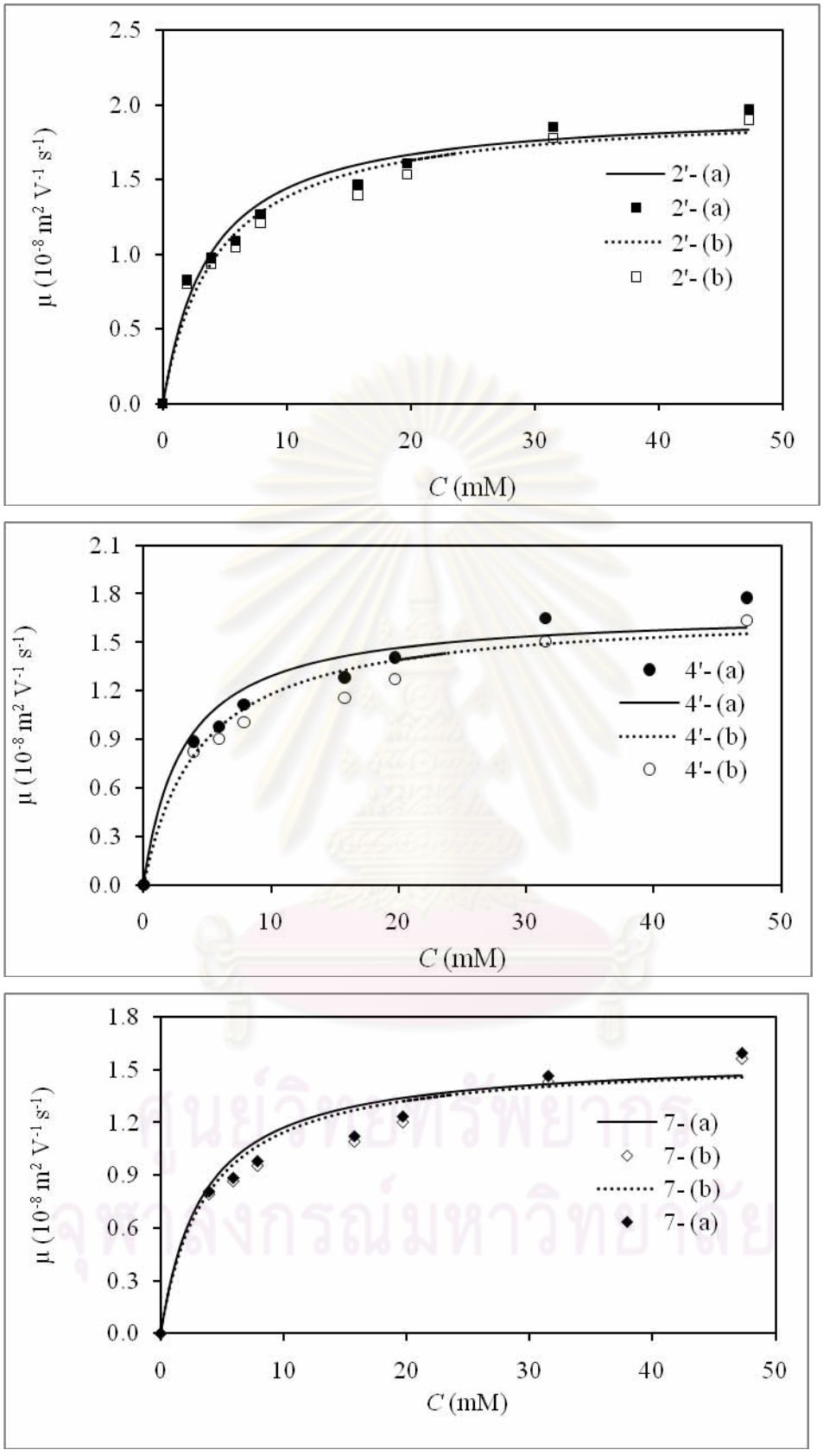


Figure 4.4 Binding curves for 2'-, 4'- and 7-hydroxyflavanone enantiomers to HS-γ-CD. Experimental (symbols) μ is the average of two runs, and predicted (solid lines) μ is using Equation 2.9 with data in Table 4.1.

Table 4.1 Binding constants**,** electrophoretic mobilities for 2'-, 4'- and 7-hydroxyflavanones

CD	enantiomers		Non-linear fitting			
			K/M^{-1}	μ (10^{-8}m^2.V^{-1}.s^{-1})		R^2
				μ_0	μ_∞	
HS-β-CD	2'-	a	991	0	3.30	0.88
		b	314			0.91
	4'-	a	242	0	2.87	0.95
		b	233			0.94
	7-	a	201	0	2.70	0.93
		b	197			0.92
HS-γ-CD	2'-	a	270	0	1.98	0.95
		b	231			0.93
	4'-	a	318	0	1.70	0.91
		b	228			0.92
	7-	a	291	0	1.57	0.91
		b	266			0.92

a and b refer to the first and second migration, respectively
K and μ_∞ are obtained from a non-linear fit of data points of μ as a function of C using Equation 2.12 and a non-linear fitting program (MATLAB)

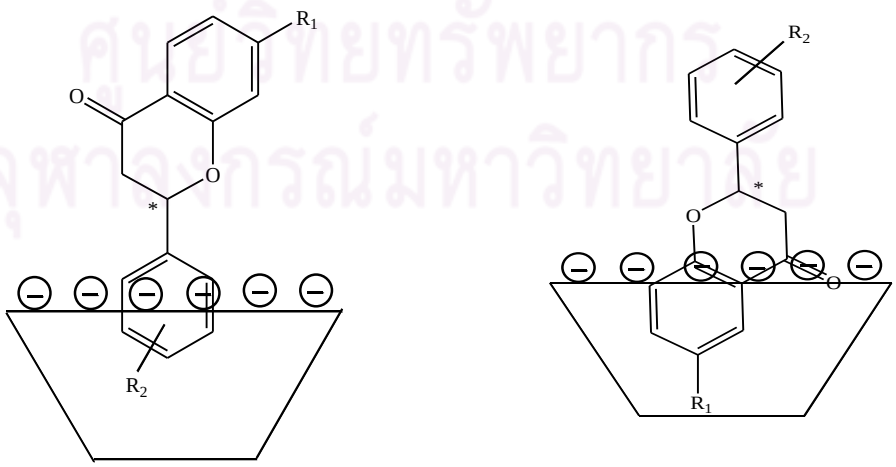
From Table 4.1 and Figures 4.3 and 4.4, the poor non-linearity was obtained with the R^2 values in a range of 0.88-0.95. It should be noted that an increase in the concentration of charged CD in the buffer also leads to higher Joule heating and higher ionic strength, resulting an increase in μ, for the former but a decrease in μ, for the latter. A change in μ due to these dual effects is difficult to correct and not taken into account in this work. Therefore, the deviation of experimental μ from the actual values can give poor fitting of non-linear relationship between μ and C.

In the case of uncharged enantiomers in CD-EKC with charged CD, μ_o of the uncharged enantiomers is equal to zero, and μ_∞ is assumed to be equal. According to Equation 2.9, the isomer with higher K gives the higher μ and the faster migration time. At a given CD concentration for the enantiomers, the higher μ for a than b (Figures 4.3 and 4.4) and the faster t_m for a than b (Figures 4.1and 4.2) are found to be consistent with the higher K for a than b (Table 4.1).

From Equation 2.9 at a given *C*, the effective electrophoretic mobility (μ) order of non-enantiomers A and B, such as 2'- and 4'-hydroxyflavanones depends on both *K* and μ_{∞}. For example, the non-enantiomers with the same order of *K* and μ_{∞} ($K_A > K_B$ and $\mu_{\infty A} > \mu_{\infty B}$) result the same order of μ and *K* ($\mu_A > \mu_B$). However, the reversed order of μ and *K* is possible ($K_A > K_B$ but $\mu_A < \mu_B$) when *K* and μ_{∞} have a reversed order ($K_A > K_B$ but $\mu_{\infty A} < \mu_{\infty B}$).

From Figure 4.1 and Table 4.1, the same order of μ and *K* was founded for 2'- > 4'- > 7-hydroxyflavanones with HS-β-CD (the t_m order 2'- < 4'- < 7- in Figure 4.1) due to the same order of *K* and μ_{∞} with 2'- > 4'- > 7-. However, HS-γ-CD with comparable *K* for 2'-, 4'- and 7- (Table 4.1) gave the μ order 2'- > 4'- > 7- (the t_m order 2'- < 4'- < 7- in Figure 4.2) due to the μ_{∞} order 2'- > 4'- > 7-.

It should be noted that inclusion complexation of CD and hydroxyflavanone may be formed below. The stability of the CD inclusion complex may be due to the hydrophobic interaction between the aromatic ring of analyte and the CD cavity, the hydrogen bonding or dipole-dipole interaction between a hydroxyl group of analyte and hydroxyl or sulfonated groups of CD, the suitable size of CD with analyte, steric effect of analyte, and position of substituent. It is difficult to compare the stability of these CD:enantiomers complexes without knowing their exact structure that may be comfirmed using the molecular modeling computation and/or X-ray crystallography.



4.3 Enantioselectivity and Enantioseparation for single CD

In this work, enantioselectivity (κ) is defined as the ratio of the binding constant, K_a / K_b where $K_a > K_b$ [Penn et al. 1994]. In this case, letters "a" and "b" refer to the first and the second migration order, respectively. κ = 1 indicates no enantiomeric resolution over a wide range of CD concentration. As previously mentioned in Section 2.7, the parameters used for describing separation can be expressed by electrophoretic mobility difference (Δμ), separation selectivity (α) and resolution (R_s). It follows from Equations 2.9 and 2.11, that Δμ and α of enantiomers may be rearranged to relate to κ by Equations 4.4 and 4.5, respectively.

$$\Delta\mu = 2\left(\frac{\kappa - 1}{\kappa + 1}\right)\frac{\bar{K}C\mu_{\infty}}{\left(1 + \bar{K}C\right)^2} \tag{4.4}$$

$$\alpha = \kappa\frac{1 + K_1C}{1 + \kappa K_1C} \tag{4.5}$$

From Equations 2.11 and 2.14, R_s may be rearranged to relate to α as the equation

$$R_S = \frac{1}{2}\left(\frac{\alpha - 1}{\alpha + 1}\right)\sqrt{\bar{N}} \tag{4.6}$$

As can be seen from Equations 4.4 to 4.6, enantiomeric separation occurs when enantiomers have different K or enantioselectivity greater than 1. The higher the value of enantioselectivity, the greater the enantioseparation parameter. The achieved baseline resolution is said when the resolution is at least 1.5 ($R_s \geq 1.5$).

Figure 4.5 shows graphs of enantioseparation parameters over a wide range of the CD concentration, and Table 4.2 shows the enantioselectivity and an example of enantioseparation parameters using ≈ 16 mM CD in the buffer. From Figure 4.5, the enantioseparation parameters (Δμ, α and R_s) increase to the maximum value with an increase in the CD concentration, and then fall down at the higher CD concentration.

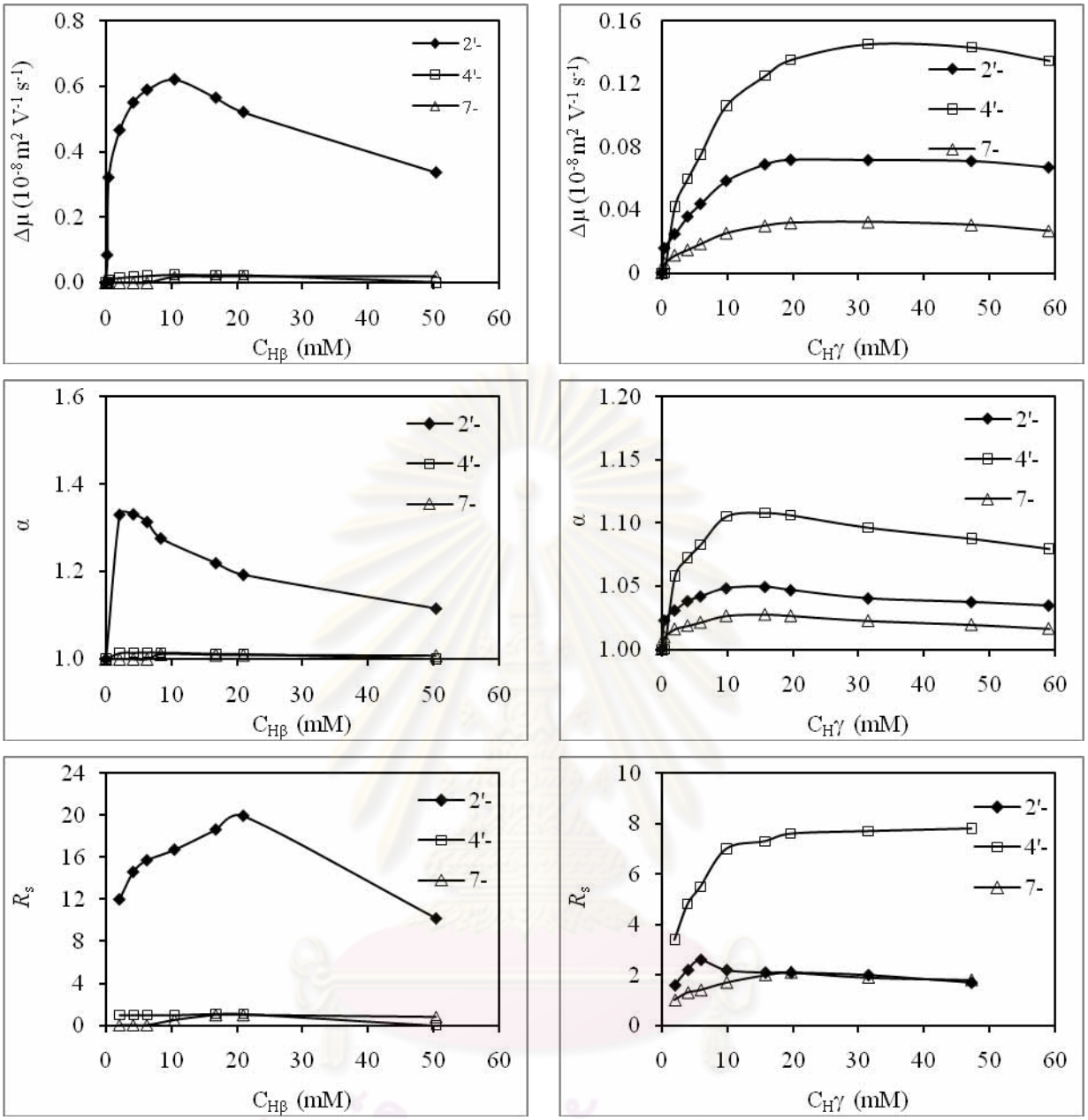


Figure 4.5 Enantioseparation parameters for 2'-, 4'- and 7-hydroxyflavanone enantiomers with HS-β-CD and HS-γ-CD in the concentration range used. Symbols are the average of experimental data from two runs and solid lines show the trend of data points.

Table 4.2 Enantioseparation parameters using ≈ 16 mM CD in the buffer

CD	analytes	κ	Enantioseparation parameters at ≈ 16 mM CD		
			Δμ (10^{-8} m^2 V^{-1} s^{-1})	α	R_s
HS-β-CD	2'-	3.16	0.565	1.219	18.6
	4'-	1.04	0.023	1.011	1.1
	7-	1.02	0.017	1.009	1.0
HS-γ-CD	2'-	1.17	0.070	1.049	2.1
	4'-	1.40	0.125	1.108	7.3
	7-	1.09	0.030	1.026	1.9

Over a wide range of the CD concentration used, HS-β-CD (2-50 mM) did not give the achieved enantiomeric resolution for 4'- and 7-hydroxyflavanones (R_s < 1.15 and < 1.01, respectively) due to the very small value of κ (1.04 and 1.02, respectively), while very high R_s up to 19 was observed for 2'- with HS-β-CD due to the very large value of κ (3.16). Therefore, HS-β-CD cannot be used for simultaneous separation of these three enantiomers. Using 2-47 mM HS-γ-CD, the achieved R_s of 2'- and 4'-enantiomers was obtained, while the achieved R_s of 7-hydroxyflavanone was found using 6-47 mM HS-γ-CD. Therefore, simultaneous separation of these three enantiomers can be achieved using CD-EKC with 6-47 mM HS-γ-CD. Generally, at a given CD concentration such as 16 mM as shown in Table 4.2, the enantioseparation parameters were obtained in order 2'- > 4'- ≈ 7- with HS-β-CD and 4'- > 2'- > 7- with HS-γ-CD, which is consistent with the enantioselectivity order (2'- > 4'- ≈ 7- with HS-β-CD and 4'- > 2'- > 7- with HS-γ-CD). In addition, in comparison with HS-β-CD, HS-γ-CD gave the better enantiomeric separation for 4'- and 7-, but worse enantiomeric separation for 2'-, which is consistent with the enantioselectivity order HS-γ–CD > HS-β-CD for 4'- and 7-, but HS-γ-CD < HS-β-CD for 2'-.

In previous work on enantioseparation of 2'-, 3'- and 4'-hydroxyflavanones with S-β-CD [Lin *et al*. 2008], the much higher R_s for 2'- than 3'- and 4'-enantiomers is probably due to the spatial configuration of the 2'-hydroxyl substituent which may form an intramolecular hydrogen bonding around the stereogenic center and/or possess a steric repulsive interaction between the 2'-hydroxyl substituent and the hydrophobic cavity of S-β-CD. Since HS-β-CD and S-β-CD have the similar structure

and cavity, the much higher enantioseparation for 2'- than 4'- and 7- with HS-β-CD in our work may be explained using a similar way with previous work [Lin *et al*. 2008].

4.4 Enantiomeric Separation Using Dual CDs

As previously mentioned in Section 1.4, dual CDs may improve simultaneous separation of all enantiomers in the case that a single CD cannot give the baseline resolution of these enantiomers. From Sections 4.2 and 4.3, the simultaneous enantiomric separation of 2'-, 4'- and 7-hydroxyflavanones was obtained using HS-γ-CD, therefore, the dual CDs may not be needed. However, the use of dual charged CDs was investigated in this work in order to examine if the concept of theoretical α models of charged enantiomers with dual neutral CDs [Nhujak *et al.* 2005] can be used to explain the migration order and a change in α of uncharged enantiomers using dual charged CDs. For example, in comparion with single CD1, dual CDs, with varying the concentration of CD2 in the buffer containing CD1 at the fixed concentration, can enhance α up to the maximum value when enantiomers migrate with the same order in CD1 and CD2 and have the values of $\kappa_2 > \kappa_1$, where κ_1 and κ_2 refer to enantioselectivity for enantiomers with CD1 and CD2, respectively. However, worse α is obtained for enantiomers with the same migration order in CD1 and CD2 and the values of $\kappa_2 < \kappa_1$. In the case of enantiomers with the reversed migration order in CD1 and CD2, the value of α decreases to 1.0 and then increases at higher CD2.

Figures 4.6 and 4.7 show the electropherograms of enantiomeric separation of 2'-, 4'- and 7-hydroxyflavanones with dual CDs by varying 2 to 16 mM HS-γ-CD (CD2) in the buffer containing 4 or 8 mM HS-β-CD (CD1). Figure 4.8 shows separation selectivity using dual charged CDs.

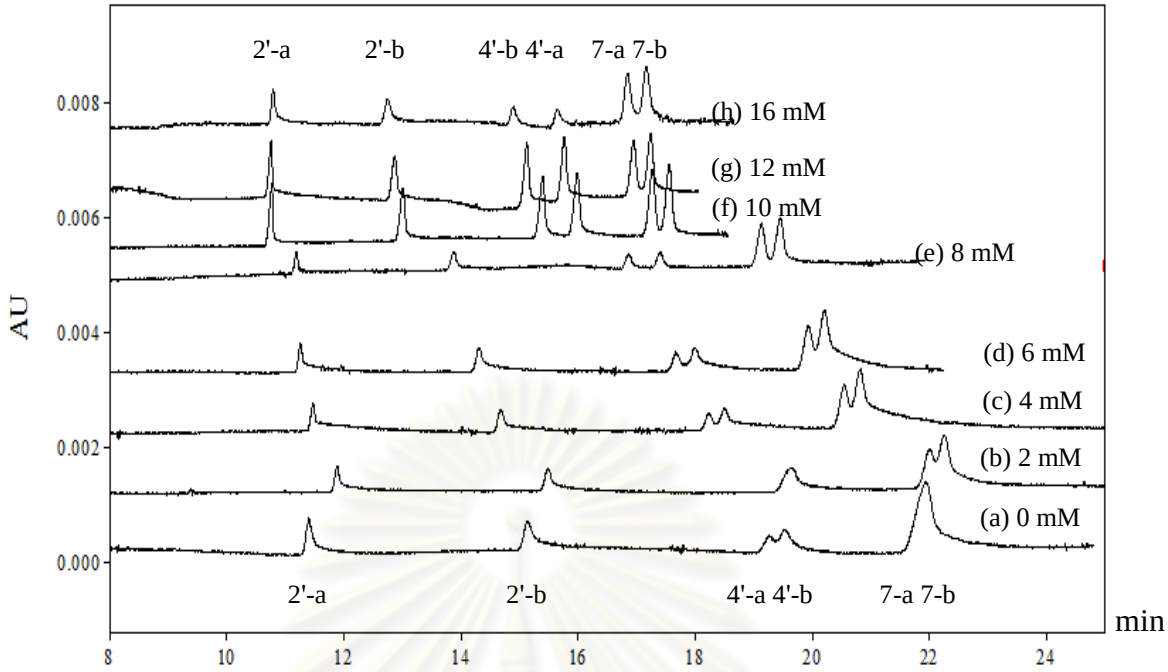


Figure 4.6 Electropherograms of the enantiomeric separation of 2'-, 4'- and 7-hydroxyflavanones using dual CDs as chiral selectors at fixed concentration of 4 mM HS-β-CD and varied concentration of HS-γ-CD. CE conditions as shown in Figure 4.1.

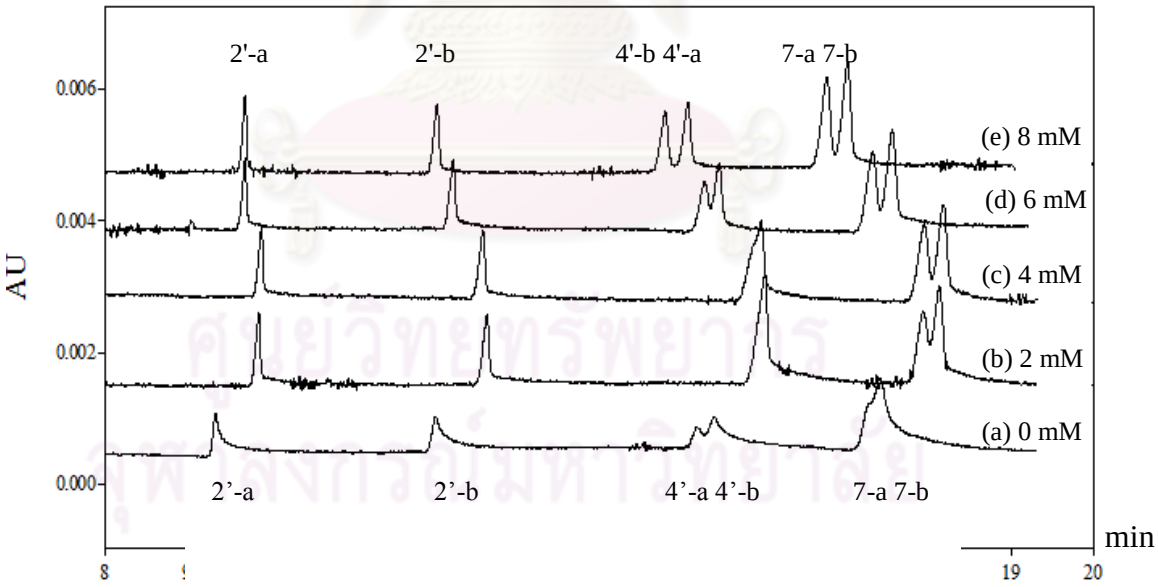


Figure 4.7 E
hydroxyflavanc
CD and varied

4'- and 7-
8 mM HS-β-

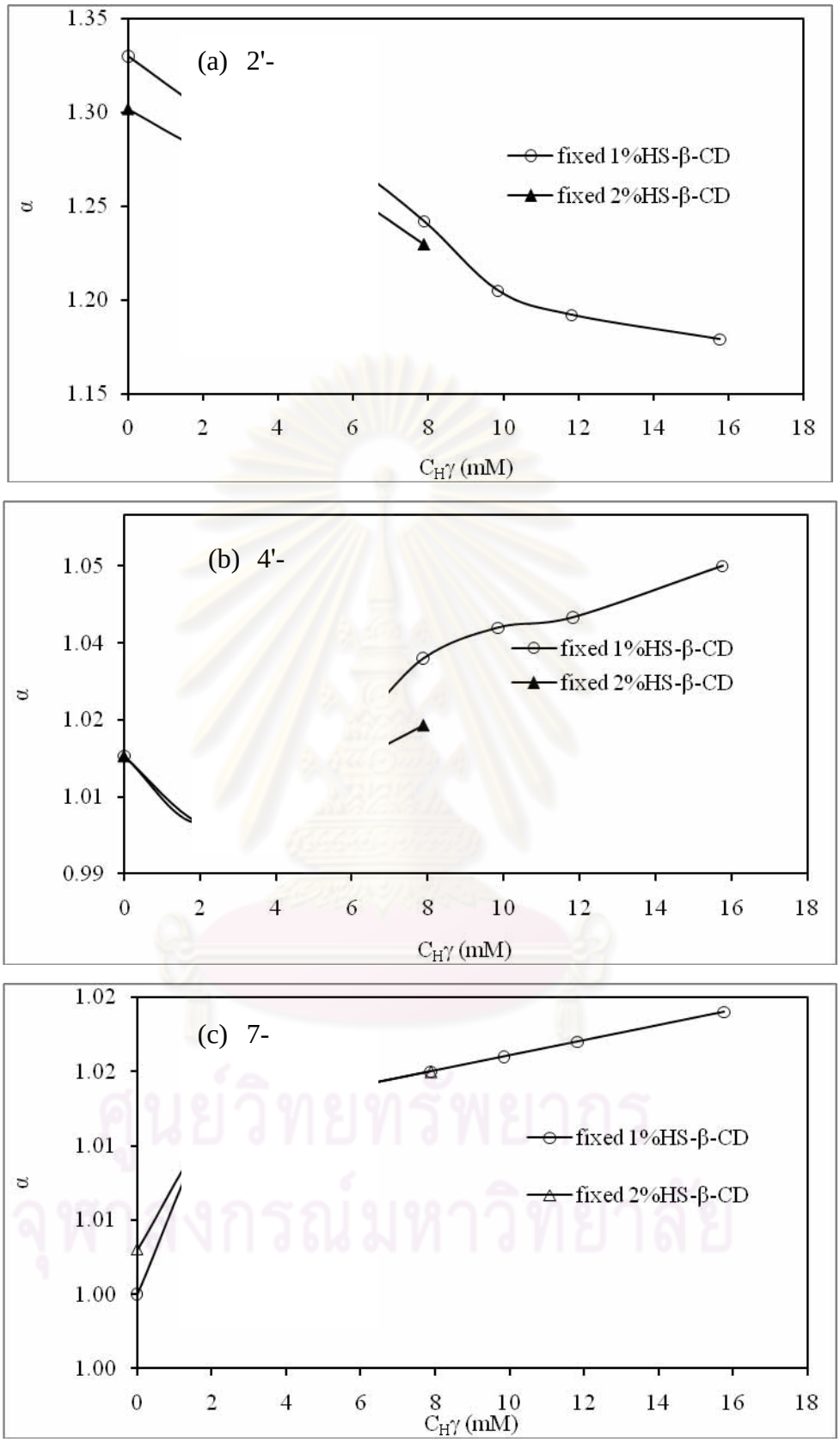


Figure 4.8 Separation selectivities for (a) 2'-, 4'- and 7-hydroxyflavanone enantiomers with fixed HS-β-CD and varied HS-γ-CD in the concentration range used. Symbols are the average of experimental data from two runs and solid lines show the trend of data points.

In comparison with single HS-β-CD at 4 mM (Figure 4.6a) or 8 mM (Figure 4.7a), the better enantiomeric separation for 7-hydroxyflavanone using dual CDs (Figures 4.6b-h, 4.7 b-e and 4.8) can be explained that HS-γ-CD gives higher enantioselectivity than does HS-β-CD ($\kappa_2 > \kappa_1$ with 1.09 and 1.02, respectively), implying the same migration order of enantiomers in HS-β-CD and HS-γ-CD. A slightly decrease in enantiomeric separation for 2'-hydroxyflavanone with dual CDs is due to $\kappa_2 < \kappa_1$ with 1.17 and 3.16 for HS-γ-CD and HS-β-CD, respectively, also implying the same migration order of enantiomers in HS-β-CD and HS-γ-CD. For 4'-hydroxyflavanone, the partial separation ($\alpha > 1$) was observed with the single HS-β-CD, while comigration ($\alpha = 1$) and then better separation ($\alpha > 1$) was obtained with dual CDs at low and high concentration of HS-γ-CD, respectively, implying the reversed migration order of enantiomers in HS-β-CD and HS-γ-CD. Strickly speaking, if the peak "a" in Figure 4.1 is assumed to *R*-isomer, that in Figure 4.2 would be *S*-isomer. In order to represent the reversed order of 4'-hydroxyflavanone enantiomers in single and dual CDs in Figures 4.6 and 4.8, the letter "b", instead of "a", is assigned to belong the first order for the electropherograms that the dual CDs result in the reversed migration order with the single CD giving the first order of "a".

CHAPTER V
CONCLUSION

The aims of this work are to investigate simultaneous enantiomeric separation of three pairs of flavanone enantiomers, 2'-, 4'- and 7-hydroxyflavanones, using CD-EKC with single and dual charged HS-β-CD and/or HS-γ-CD, and to use the concept of dual enantioselectivity to explain the migration order and a change in separation selectivity for these three enantiomers in dual CDs.

In the first part, the simultaneous CD-EKC separation of these uncharged enantiomers was carried out using single HS-β-CD or HS-γ-CD in a concentration range of 0-50 mM in a pH 2.5 phosphate buffer (50 mM NaH_2PO_4 adjusted with H_3PO_4). The binding constant (K) of each isomer to each CD was determined by using a non-linear fitting of the electrophoretic mobility and the CD concentration. Using K data, the values of enantioselectivity (κ) were obtained as the followings: 3.16, 1.04 and 1.02 for 2'-, 4'- and 7- with HS-β-CD, and 1.17, 1.40 and 1.09 with HS-γ–CD, respectively, where κ is defined as the ratio of K (K_a/K_b) and where the subscripts "a" and "b" refer to the first and the second migration order of enantiomers, respectively.

At the optimum concentration of CD, the enantioseparation parameters, such as electrophoretic mobility difference (Δμ), separation selectivity (α) and resolution (R_s) were found in order 2'- > 4'- ≈ 7- with HS-β-CD and 4'- > 2'- > 7- with HS-γ-CD, which is consistent with their κ order from a large to small scale. In addition, very high R_s up to 19 for 2'- with HS-β-CD was found due to a very large scale of their κ (3.16), while partial R_s for 4'- and 7- with HS-β-CD was observed due to a very small scale of their κ (1.04 and 1.02, respectively). In comparison with HS-β-CD, HS-γ-CD gave the better enantioseparation parameters for 4'- and 7- due to the higher κ with HS-γ-CD than HS-β-CD, while poorer ones for 2'- due to the smaller κ with HS-γ-CD than HS-β-CD. In conclusion, HS-γ-CD in a range of 8 to 47 mM, particularly at 16 mM, provided the achieved baseline resolution (R_s > 1.5) for simultaneous separation enantiomers of these hydroxyflavanones.

In the second part, both HS-β-CD and HS-γ-CD were used as dual chiral selectors for enantiomeric separation of these hydroxyflavanones by using a fixed concentration of HS-β-CD at 4 or 8 mM and varying the concentration of HS-γ-CD in a range of 2-16 mM. The migration order and a change in α of uncharged enantiomers with dual charged CDs in this work was explained using the concept of theoretical α models of charged enantiomers with dual neutral CDs [Nhujak *et al.* 2005]. A change in α with an increase in the CD2 concentration was found to be consistent with the concept of dual enantioselectivities for dual CDs: an increase in α for 7- due to the same order of enantiomers in CD1 and CD2 with $\kappa_2 > \kappa_1$, a decrease in α for 2'- due to the same order of enantiomers in CD1 and CD2 with $\kappa_2 < \kappa_1$, while a decrease in α to 1 and then an increase in α at higher CD2 for 4'- due to the reversed order of enantiomers in CD1 and CD2 with $\kappa_2 > \kappa_1$.

REFERENCES

Abushoffa, A. M., Fillet, M., Hubert, P., and Crommen. Prediction of selectivity for enantiomeric separations of uncharged compounds by capillary electrophoresis involving dual cyclodextrin systems. Journal of Chromatography A 948 (2002): 321-329.

Andrea, W., and Brown, P. R. HPLC and CE: Principles and Practice. San Diego: Academic Press, 1997, pp. 154-164.

Asztemborska, M., Miskiewicz, M., and Sybilska, D. Separation of some chiral flavanones by micellar electrokinetic chromatography. Electrophoresis 24 (2003): 2527-2531.

Beesley, T. E., and Scott, R. P. W. Chiral Chromatography. Chichester: John Wiley and Sons, 1998, pp. 1-25.

Boer, T., Zeeuw, R. A., Jong, G. J., and Ensing, K. The use of charged cyclodextrins in capillary electrophoresis for chiral separations in pharmaceutical analysis. Electrophoresis 21 (2000): 3220-3239.

Chang, R. Chemistry. 7th ed. New York: Mcgraw-Hill, 2002, p. 611.

Chankvetadze, B. Capillary Electrophoresis in Chiral Analysis. Chichester: John Wiley and Sons, 1997, pp. 5-72.

Fillet, M., Chankvettadze B., Crommen, J., and Blaschke, G. Enantiomeric separations of drugs using mixtures of charged and neutral cyclodextrins. Electrophoresis 20 (1999): 2691-2697.

Grossman, P. D., and Colburn, J.C. Capillary Electrophoresis: Theory and Practice. San Diego: Academic Press, 1992, pp. 14-22, 31, 99, 112-114.

Gübitz, G, and Schmid, M. G. Review chiral separation by capillary electromigration techniques. Journal of Chromatography A 1204 (2008): 140-156.

Kelly M. A., Altria, K. D., and Clark, B. J. Approach used in the reduction of the buffer electrolysis effects for routine capillary electrophoresis procedures in pharmaceutical analysis. Journal of Chromatography A 768 (1997): 73-80.

La, S., Kim, J., Goto, J., and Kim, K. Simultaneous chiral discrimination of multiple phofens by cylodextrin-modified capillary electrophoresis in normal and reversed polarity modes. Electrophoresis 24 (2003): 2642-2649.

Li, S. F. Y. Capillary Electrophoresis: Principles, Practice and Applications. Asterdam: Elsevier Science Publishers B. V, 11-12, 40.

Lin, C., Fang, W., Kuo, C., Chang, W., Liu, Y., Lin, W., Wu, J., and Lin, C. Chiral separation of hydroxyflavanones in cyclodextrin-modified capillary zone electrophoresis using sulfated cyclodextrins as chiral selectors. . Journal of Chromatography A 1188 (2008): 301-307.

Lurie, I. S., and Klein, R. F. X. Chiral resolution od cationic drugs of forensic interest by capillary electrophoresis with migtures of neutral and anionic cyclodextrins. Analytical Chemistry 66 (1994): 4019-4026.

Matthijs, N., Hemelryck, S. V., Maftouh, M., Massart, D. L., and Heyden, Y. V. Electrophoretic separation strategy for chiral pharmaceuticals using highly-sulfated and neutral cyclodextrins based dual selector systems. Analytica Chimica Acta 525 (2004): 247-263.

Nhujak, T. Quantitative Aspects of Capillary Electrophoresis and Chiral Analysis. Thesis, University of York (2001).

Nhujak, T., Saatravaha, C., Palanuvej, C. and Petsom, A. Chiral separation in capillary electrophoresis using dual neutral cyclodextrins: Theoretical models of electrophoretic mobility difference and separation selectivity. Electrophoresis 26 (2005): 3814-3823.

Pan, J., Zhang, S., Yan, L., Tai, J., Xiao, Q., Zhou, Y., and Wu, J. Separation of flavanone enantiomers and flavanone glucoside diastereomers from *Balanophora involucrata* Hook. f. by capillary electrophoresis and reversed-phase high-performance liquid chromatography on a C_{18} column. Journal of Chromatography A 1185 (2008): 117-129.

Penn, S. G., Goodall, D. M. and Loran, J. S. Differential binding of tioconazole enantiomers to hydroxyproply-β-cyclodextrin studied by capillary electrophoresis. Journal of Chromatography A 636 (1993): 149-152.

Penn, S. G., Goodall, D. M., and Loran, J. S. Capillary electrophoresis with chiral selectors: optimization of separation and determination of thermodynamic parameters for binding of tioconazole enantiomers to cyclodextrins. Analytical Chemistry 66 (1994): 2866-2837.

Penn, S. G., Bergstom, E. T., Knights, L., Lui, G., Ruddick, A and Goodall, D. M. Capillary electrophoresis as a method for determining binding constants: application to the binding of cyclodextrins and nitrophenolate. Journal Physical Chemistry 99 (1995): 3875-3880.

Perrin, C., Fabre, H., Maftouh, M. and Massart, D. Robustness testing of chiral separations by capillary electrophoresis using highly-sulfated cyclodextrins. Journal of Chromatography A 1007 (2003): 165-177.

Scriba, G. Review cyclodextrins in capillary electrophoresis enantioseparations-recent developments and applications. Journal Separation Science 31 (2008): 1991-2011.

Sepaniak, M. J., Copper, C. L., Whitaker, K. W., and Anigbogu, V. C. Evaluation of a dual-cyclodextrin phase variant of capillary electrokinetic chromatography for separations of nonionizable solutes. Analytical Chemistry 67 (1995): 2037-2041.

Vaccher, M.P., Bonte, J.P. and Vaccher, C. Capillary electrophoresis resolution of enantiomers of aromatic amino-acids with highly sulfated α-, β- and γ-cyclodextrins. Chromatographia 64 (2006): 51-55.

Wang, S., Fu, M., and Wang, M. Seeparation mechanism and determination of flavanones with capillary electrophoresis and high-performance liquid chromatography. Journal of Chromatography A 1164 (2007): 306-312.

Weinberger, R. Practical Capillary Electrophoresis. San Diego: Academic Press, 1993, pp. 56, 141-142, 366.

Wistuba, D., Trapp, O., Gel-Moreto, N., Galensa, R., and Schurig, V. Stereoisomeric separation of flavanones and Flavanone-7-*O*-glycosides by capillary electrophoresis and determination of interconversion barriers. Analytical Chemistry 78 (2006): 3424-3433.

Zhu, X., Ding, Y., Lin, B., Jakob, A., and Koppenhoefer, B. Transient state of chiral recognition in a binary mixture of cyclodextrins in capillary electrophoresis. Journal of Chromatography A 888 (2000): 241-250.

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