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COMPLEX FORMATION AND ENANTIOSELECTIVITY STUDIES OF TRIAZOLE FUNGICIDE ENANTIOMERS USING CAPILLARY ELECTROPHORESIS

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TAJUK PROJEK : COMPLEX FORMATION AND ENANTIOSELECTIVITY STUDIES OF TRIAZOLE FUNGICIDE ENANTIOMERS USING CAPILLARY ELECTROPHORESIS

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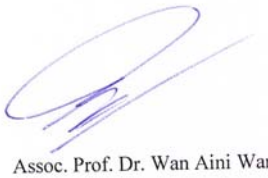
Assoc. Prof. Dr. Wan Aini Wan Ibrahim

A report submitted in fulfillment of the final progress research for VOTE number 78074

Faculty of Science
Universiti Teknologi Malaysia
FEBRUARY 2010

We declare that this report entitled "Complex formation and enantioselectivity studies of triazole fungicide enantiomers using capillary electrophoresis" is the result of our own research except as cited in the references.

Signature : 

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ABSTRACT

(Keywords: Micellar electrokinetic chromatography, cyclodextrin, triazole fungicides)

Several cyclodextrin modified-micellar electrokinetic chromatography (CD-MEKC) methods were developed for the successful triazole fungicides separation. In the first part, an efficient method was developed for the simultaneous enantioseparation of cyproconazole (4 stereoisomer), bromuconazole (4 stereoisomer) and diniconazole (2 stereisomer) enantiomers using CD-MEKC with a dual mixture of neutral cyclodextrins as chiral selector. The best simultaneous separation of cyproconazole, bromuconazole, and diniconazole enantiomers was achieved with a mixture of 27 mM HP-β-CD and 3 mM HP-γ-CD in 25 mM phosphate buffer (pH 3.0) containing 40 mM sodium dodecyl sulfate (SDS) and 15% iso-propanol as organic modifier. Complete separation of 10 stereoisomer of triazole fungicides were obtained in a single run with good resolution (R_s 1.74–26.31) and high peak efficiency (N > 400 000). In the second part of the study, enantioseparation of hexaconazole, penconazole, myclobutanil, and triadimefon was investigated. Simultaneous enantioseparation of penconazole, myclobutanil, and triadimefon was achieved under acidic condition (pH 3.0) using 25 mM phosphate buffer, 50 mM SDS, and 30 mM HP-γ-CD, with R_s greater than 0.9 whereas, simultaneous enantioseparation of hexaconazole, penconazole, and myclobutanil was successfully achieved under neutral condition (pH 7.0) using 25 mM phosphate buffer, 40 mM SDS, and 40 mM HP-γ-CD, with R_s greater than1.6. In order to improve detection sensitivity, on-line preconcentration technique was investigated. It was found that sweeping technique as an on-line preconcentration technique improved the detection sensitivity of the enantioseparation of cyproconazole, bromuconazole, and diniconazole by 30 to 60-fold, with good repeatabilities in the migration time, peak area and peak height were obtained with RSDs in the range of 0.08–0.32%, 0.03–2.44%, and 2.13–8.44% respectively. Furthermore, sweeping technique improved the detection sensitivity of the enantioseparation of hexaconazole, penconazole and myclobutanil by 62- to 67-fold. Good repeatabilities in the migration time, peak area and peak height were obtained with RSDs in the range of 2.39–3.90%, 1.96–6.15%, and 2.80–6.64% respectively. Finally, the formation constant of diniconazole enantiomers with HP-γ-CD under neutral and acidic condition was investigated using CD-MEKC.

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ABSTRAK

(Kata kekunci: Kromatografi elektrokinetik misel, siklodekstrin, fungisid triazol)

Beberapa kaedah kromatografi elektrokinetik misel-siklodekstrin terubahsuai (CD-MEKC) dibangunkan untuk pemisahan beberapa fungisid terpilih. Dalam bahagian pertama, satu kaedah cekap pemisahan serentak enantiomer siprokonazol (4 stereoisomer), bromukonazol (4 sterosiomer), dan dinikonazol (2 stereoisomer) telah dibangunkan menggunakan kaedah CD-MEKC dengan campuran dwi siklodekstrin neutral sebagai pemilih kiral. Pemisahan terbaik enantiomer siprokonazol, bromukonazol, dan dinikonazol secara serentak diperoleh dengan menggunakan, campuran dwi pemilih kiral 27 mM HP-β-CD dan 3 mM HP-γ-CD dalam 25 mM larutan penimbal fosfat (pH 3.0), yang mengandungi 40 mM natrium dodekil sulfat (SDS) dan 15% (v/v) iso-propanol sebagai pengubahsuai organik. Pemisahan lengkap 10 stereosiomer fungisid triazol ini telah diperoleh dalam satu larian dengan resolusi yang baik (R_s 1.74 - 26.31) dan kecekapan puncak yang tinggi (N > 400 000). Dalam bahagian kedua kajian, pemisahan enantiomer heksakonazol, penkonazole, miklobutanil, dan triadimefon telah dikaji. Pemisahan serentak enantiomer penkonazol, miklobutanil, dan triadimefon dicapai di bawah keadaan berasid (pH 3.0) menggunakan larutan penimbal fosfat 25 mM, SDS 50 mM, dan HP-γ-CD 30 mM, dengan R_s > 0.9. Pemisahan serentak enantiomer heksakonazol, penkonazol, dan miklobutanil dicapai di bawah keaddan neutral (pH 7.0) menggunakan larutan penimbal fosfat 25 mM, SDS 40 mM dan HP-γ-CD 40 mM, dengan R_s >1.6. Teknik pra-pemekatan dalam talian telah digunakan untuk meningkatkan kepekaan pengesanan. Teknik sapuan sebagai teknik pra-pemekatan dalam talian didapati boleh meningkatkan kepekaan pengesanan pemisahan enantiomer siprokonazol, bromukonazol, dan dinikonazol sehingga 30-60 kali ganda, dengan kebolehulangan masa migrasi, luas puncak,dan ketinggian puncak yang baik diperoleh dengan sisihan piawai relatif (SPR) masing-masing antara 0.08-0.32%, 0.03-2.44%, dan 2.13-8.44%. Tambahan lagi teknik sapuan didapati boleh meningkatkan kepekaan pengesanan pemisahan enantiomer heksakonazol, penkonazol, dan miklobutanil sehingga 62-67 kali ganda, dengan kebolehulangan masa migrasi, luas puncak,dan ketinggian puncak yang baik diperoleh dengan sisihan piawai relatif (SPR) masing-masing antara 2.39–3.90%, 1.96–6.15%, dan 2.80–6.64%. Akhir sekali pemalar pembentukan komplek enantiomer diniconazole dengan HP-γ-CD pada keadaan neutral dan asid telah dikaji.

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LIST OF ABREVIATIONS

BGE	-	Background electrolyte
CD	-	Cyclodextrin
CD-EKC	-	Cyclodextrin-modified electrokinetic chromatography
CD-MEKC	-	Cyclodextrin-modified micellar electrokinetic chromatography
CE	-	Capillary electrophoresis
CEC	-	Capillary electrochromatography
CMC	-	Critical micelle concentration
CZE	-	Capillary zone electrophoresis
DAD	-	Diode-array detection
EKC	-	Electrokinetic chromatography
EOF	-	Electroosmotic flow
GC	-	Gas chromatography
HPLC	-	High-performance liquid chromatography
LOD	-	Limit of detection
MEKC	-	Micellar electrokinetic chromatography
MRL	-	Maximum residue limits
SDS	-	Sodium dodecyl sulphate
SEF	-	Sensitivity enhancement factor
SFC	-	Supercritical fluid chromatography
SPE	-	Solid-phase extraction
TLC	-	Thin-layer chromatography
UV	-	Ultraviolet

LIST OF SYMBOLS

cm	-	Centi meter
µA	-	Micro ampere
μ_i	-	Electrophoretic mobility
μ_0	-	Electroosmotic mobility
µg	-	Micro gram
µL	-	Micro liter
µm	-	Micro meter
nL	-	Nano liter
i.d.	-	Inner diameter
l	-	Effective capillary length
L	-	Total capillary length
N	-	Efficiency
R^2	-	Correlation coefficient
R_s	-	Peak resolution
T	-	Temperature (˚C)
t_m	-	Migration time
V	-	Volt
γ	-	Gamma

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CHAPTER 1

SUMMARY OF REPORT

1.1 Background

The use of pesticide in agriculture and in insect control can not be avoided i.e. herbicides, fungicides, and insecticides. However, has well known many chemical products used in agrochemical are used as racemates. Racemate is a mixture of a pair of enantiomers because the compound has a chiral structure. Thus the use of pesticides in agriculture is also often used as racemates. The members of enantiomeric pairs frequently show rather different biological effects (Juvancz, Kendrovics, Ivanyi, and Szente, 2008). Moreover it has recently that one of the two enantiomers pairs may be more toxic than the other (Ali and Aboul-Enein, 2004).

Pesticides in agriculture and in insect control have grown as pollutant in the environment and have created a potential danger to both aquatic life and human health. Pesticides are also representing a risk of toxicity for humans, some animals, birds as well as useful insects. Moreover, pesticides have contaminated the food. Fungicides reported powerfully potential for causing adverse effects in humans. The most fungicides currently in use are from systemic fungicides category and triazole fungicide is the most important in this category. Chirality of several triazole fungicides can lead to important consequences regarding their bioactivity. Therefore, the chiral selective synthesis, pharmacological studies and analysis for chiral triazole fungicides are necessary. For chiral analysis, capillary electrophoresis (CE) is becoming a powerful analytical tool, which is more advantageous in terms of resolution, cost performance and simplicity has many advantages compared with other chromatographic methods, in terms of resolution, cost performance and simplicity.

1.2 Summary

This study was carried out to investigate chiral separation of sevral triazole fungicides namely cyproconazole, bromuconazole, diniconazole, hexaconazole, penconazole, myclobutanil, and triadimefon. Micellar elektrokinetic chromatography was used to separation selected triazole fungicides enantiomers. Micellar electrokinetic chromatography (MEKC) is a mode of electrokinetic chromatography using CE instrument in which surfactants (micelles) are added to the buffer solution (Terabe, 1993). This study was divided to three major studies. First is enantioseparation of cyproconazole, bromuconazole, and diniconazole. Secondly, enatioseparation of hexaconazole, penconazole, myclobutanil, and triadimefon. Lastly, is determination of determination of formation complex constant, K_f, between diniconazole enantiomers with HP-γ-CD. Especially for enantioseparation of cyproconazole, bromuconazole, and diniconazole was divided into three steps, are: screening cyclodextrin as chiral selectors, optimization of several parameters MEKC, and the last is the use of on-line sample preconcentration technique to improve detection sensitivity in MEKC. This chapter summarizes every chapter covered in this work.

Chapter 2 presents the introduction toseveral fungicides and explores triazole fungicides as one of the most important fungicides in detail. This chapter compiles the enantioseparation pesticide (including triazole fungicides) by several analytical methods. Micellar elektrokinetic chromatography (MEKC), especially for chiral analysis is covered in this chapter. However, MEKC using cyclodextrin as selected method for this study is shown in special part of this chapter. Furthermore, this chapter goes into detail for cyclodextrin as chiral selector and the most used chiral selector. The objectives of this study and the scope of this work are also covered in this chapter.

Chapter 3 explores the enantioseparation of cyproconazole, bromuconazole, and diniconazole using CD-MEKC. Inthis chapter discussed screening cyclodextrin

for suitable chiral selector and optimization of MEKC paramteres to improve separation performance. In this chapter is discussed the separation by single CD system, i.e use native CDs namely γ-CD, HP-γ-CD, and HP-β-CD. Since the separation was not successfully achieved, screening cyclodextrin is continued using dual CD system. Based on the results in single CD system, it was decided to use HP-γ-CD and HP-β-CD as chiral selector in Dual CD system.This chapter reports the optimization using dual CD system and the performance separation of selected triazole fungicide. Morover, the optimization of the several parameters in micellar electrokinetic chromatography (MEKC) for chiral separation of selected triazole fungicides are discussed. The pH effect, buffer phosphate concentration, surfactant (SDS) concentration, effect of separation temperature, effect applied voltage, and effect of addition organic modifiers were explored. Effect of several organic solvent was used in this study, namely methanol, ethanol, *n*-propanol, *i*-propanol, acetonitril, on enantioseparation of selected triazole fungicides are described in detail. Effect the addition of mixture of organic solvent, i.e methanol and acetonitril was also reports in this chapter.

Chapter 4 reports the enantioseparation of several triazole fungicides namely penconazole, myclobutanil, triadimefon, and hexaconazole using CD-MEKC with HP-γ-CD as chiral selector. Simultaneous enantioseparation of penconazole, myclobutanil and triadimefon was successfully achieved under acidic condition, whereas simultaneous of hexaconazole, penconazole, and diniconazle was achieved under neutral conditions.

Chapter 5 discusses the on-line preconcentration technique, i.e. sweeping used to improve the detection limit of separation of selected triazole fungicide. In this chapter is reported te optimization of sweeping on-line preconcentration for enantioseparation of selected triazole fungicides. The sweeping-CD-MEKC (pH 3.0) optimized combined with solid-phase extraction (SPE) pretreatment.

Chapter 6 explores the determination of formation complex constant, K_f, between diniconazole enantiomers with HP-γ-CD using MEKC. Determination of K_f

of diniconazole- HP- γ-CD was investigated under neutral and acidic condition at severaltemperature. Furthermore, K_f, of triadimefon-HP- γ-CD was also investigated at acidic condition and reported in this chapter.

Lastly, chapter 7 presents the overall conclusions and sugestion for further studies. This chapter summarizes the result obtained throughout the study such as the optimized conditions and the analytical performance of the enantioseparation of selected triazole fungicides. Suggestions are presented and discussed for further improvement of the study for future usage.

CHAPTER 2

INTRODUCTION

2.1 Fungicide

Organomercurials, the first synthetic organic chemicals used as agricultural fungicides, were introduced in the early twentieth century. Then in the 1930s and thereafter several organic fungicides was introduced such as dithiocarbamates, quinines, *N*-haloalkylthiodiarbiximides, and dinitrophenol derivatives. Most organic fungicides developed in the early stages have chemical reactivity which is another reason for their higher activity, and later known they are reliable to act on organism other than their targets (Uesugi, 1998).

Old types of fungicides action are often non-selective when their site of action is common among a variety of organism. Thus, non-selective action of the conventional fungicides sometimes caused undesirable effects on crop plants, mammals and other beneficial organisms. Then was developed the novel fungicides which are specifically active to plant diseases but not toxic to crop plants, mammals and other beneficial organisms. Most novel fungicides are a systemic fungicide that has to be absorbed by the plant, while most conventional fungicides are contact fungicide that kills fungi when sprayed on its surface. Sites of action of the novel fungicides are generally specific to the pathogenic fungi. There are several classes of the novel fungicides: benzimidazoles, *N*-arylcarbamtes, sterol biosynthesis inhibitors (SBI), acylalanines/ phenylamides, anilinopyrimidines, organophosphorus, melanin biosynthesi inhibitors (MBI), probenazole, dicarboximides, phenylpyrroles, arylcarboxyanilides, and methoxyacrylate (Uesugi, 1998).

2.2 Triazole Fungicides

Triazole fungicides are the most important group among the ergosterol biosynthesis inhibitors (EBI), groups as Figure 2.1 (Spindler and Fruh, 1998; Uesugi, 1998). EBI is the original name for sterol biosynthesis inhibitors (SBI) fungicides group, because ergosterol was believed to be the main sterol in most fungi, but now agricultural fungicides called sterol biosynthesis fungicides (SBI) rather than EBI fungicides (Uesugi, 1998). Triazole fungicides represent the most important systemic fungicides. Table 2.1 shows that sales most fungicides marketed decreased from 1990 to 1995, except triazole fungicides, substituted anilides and other newer systemics increased in absolute sales.

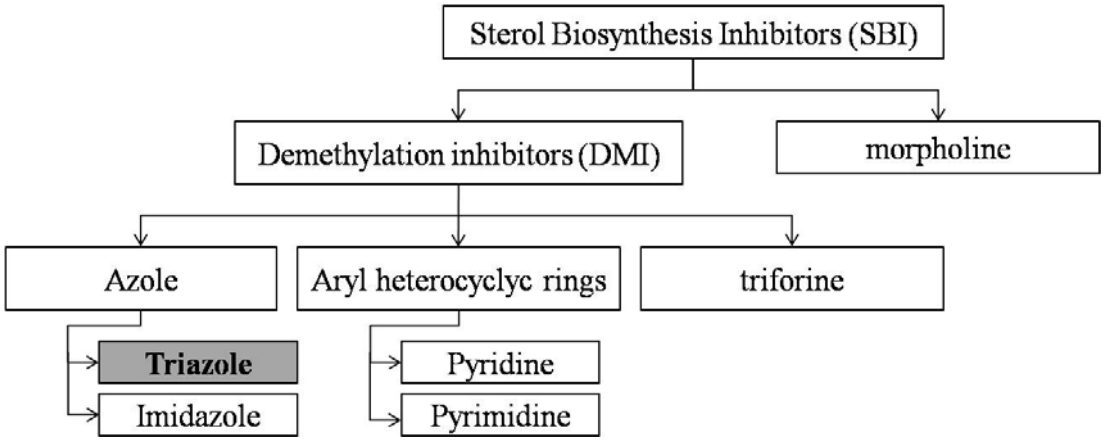

Figure 2.1 Groups of sterol biosynthesis inhibitors (SBI) fungicide.

Triazole fungicides have a common structural moiety, the 1, 2, 4-triazole ring (circled in Figure 2.2) that is connected to a hydrophobic backbone through position 1, which the hydrocarbon backbone has a substituted phenyl group at one end, and alkyl group or a different substituted phenyl group at the other end. This is the reason that most of the triazole fungicides have asymmetrical carbons, and makes most of triazole fungicides exist as chiral compound. Furthermore, chirality can lead to important consequences regarding their bioactivity (Wu *et al.*, 2001).

Table 2.1 Trends in the total sales of systemic and non-systemic fungicides in the period 1990-1995 (Uesugi, 1998).

	Sales ($ million)			Sales ($ million)	
Group	1990	1995	Group	1990	1995
Systemic compounds			**Non-systemic compounds**		
Benzimidazoles	673	430	Dithiocarbamates	801	680
Triazoles	1172	1385	Inorganics	702	520
Subtituted anilides	638	680	Other non-systemic	1033	780
Organophosphorus	232	205			
Morpholines	366	295			
Other systemic	818	880			
Total	3899	3875	**Total**	2536	1980

Triazole fungicides used in this study are cyproconazole, bromuconazole, diniconazole, penconazole, myclobutanil, triadimefon, and hexaconazole (Figure 2.2). Cyproconazole and bromuconazole have two chiral centers, while diniconazole has one chiral center. Thus, cyproconazole and bromuconazole have four enantiomers respectively, whereas diniconazole has two enantiomers. The biological activity of each enantiomer is different, for example the (-)-(R)-isomer of diniconazole showed strong antifungal activity in a liquid medium at more than 10^{-5} M, whereas the (+)-(S)-isomer exhibited no effect on fungal growth even at more than 10^{-4} M (Takano *et al.,* 1992). Enzymatic reactions in rat microsomal material, shows that under physiological conditions of a rat, trans isomer of bromuconazole reacts faster than the cis. Moreover, *trans*-bromuconazole was found to undergo stereoselective metabolism as evidenced by a change in the enantiomeric ratio (*trans*(-)/ *trans*(+)) with respect to time (Mazur *et al.,* 2007).

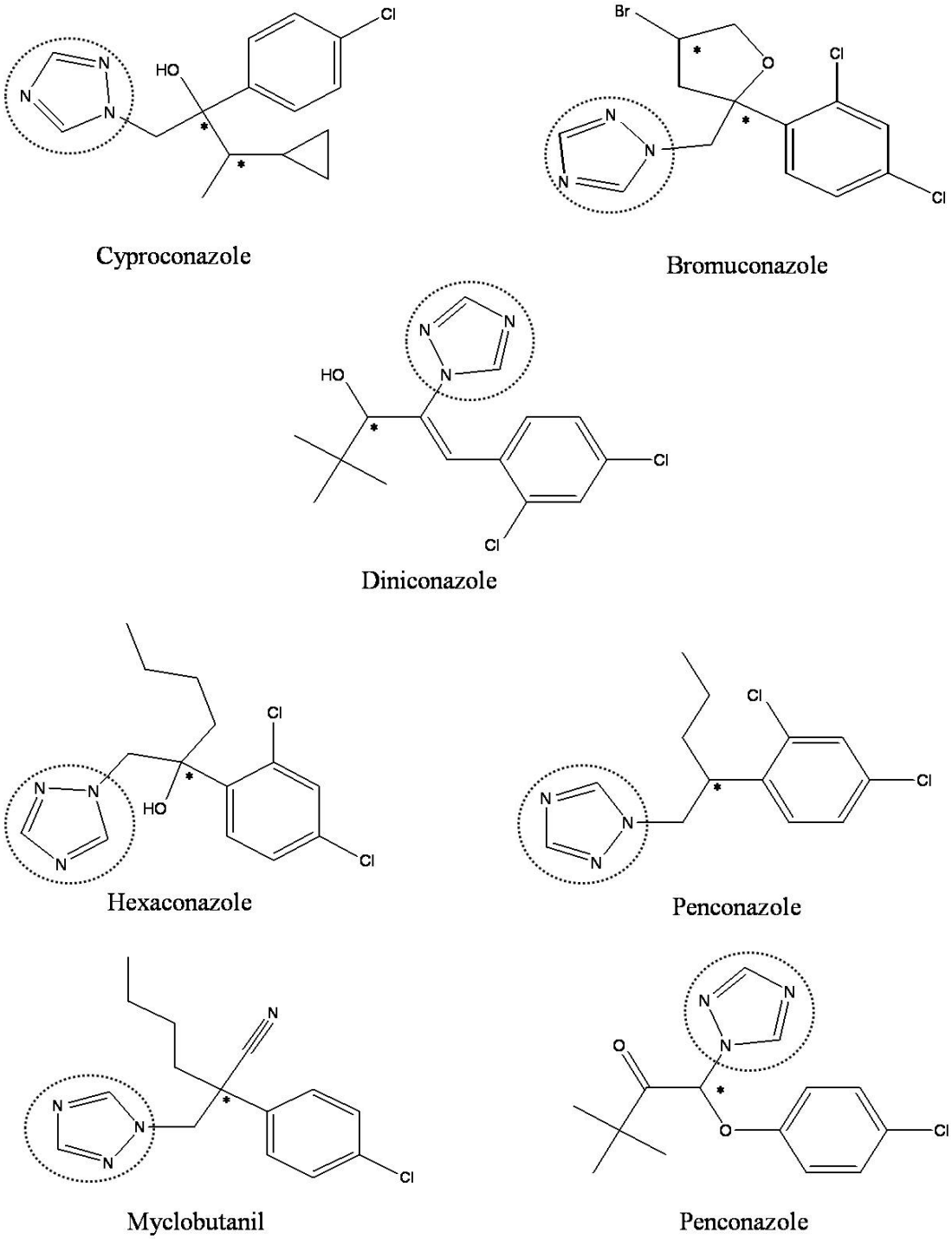

Figure 2.2 Structure of selected triazole fungicides (asterisk indicates the position of the asymmetric center) (http://syres.com/esc/kowwin.htm).

2.3 Enantioseparation of pesticides

Since the understanding of enantiomeric discrimination in environmental compartments is becoming important, the analytical techniques development for the separation of environmental chiral compounds is thus increasing. Numerous chromatographic methods have been reported for enantiomeric separations including thin layer chromatography (Sajewicz *et al.,* 2005), gas chromatography (Konig *et al.,* 2005, Fidalgo-Used, *et al.*, 2006a; Fidalgo-Used *et al.,* 2006b;), high-performance liquid chromatography (Wang *et al.,* 2005; Liu, *et al.*, 2006; Wang *et al.,* 2007; Wang *et al.,* 2008), supercritical fluid chromatography (Nishikawa, 1993, Del Nozal, *et al.,* 2003; Toribio, *et al.,* 2004), and capillary electrochromatography (Altria, 1999; Karcher and El Rassi, 1999; Zang and El Rassi, 2000). Primarily enantiomeric separation of pesticides are performed by gas chromatography (GC) (Table 2.2) and high-performance liquid chromatography (HPLC), and the majority of the published procedures for separating enantiomers of organophosphorus compounds have involved the use of chiral HPLC columns (Table 2.3) (Heiger, 2000; Meier *et al.*, 2001). However, HPLC methods for chiral analysis often suffer from low separation efficiencies, resulting in a lack of baseline resolution, which makes quantitation of traces levels of enantiomers difficult, and special chiral columns are expensive (Zang and El Rassi, 2000; Meier, *et al.*, 2001; Ali and Aboul-Enein, 2004).

Capillary electrophoresis (CE) has been found to be a powerful alternative to chromatographic techniques and several chiral separation principles has been applied in HPLC have been transferred to CE successfully. The advantages of CE are the small amounts of chiral selector and solvents required. This permits the use of expensive reagents and makes it easy to change the selector and the electrolyte when screening for a suitable selector and conditions. Furthermore, only small sample volumes are required and efficiency is very high (Gubitz, Schmid, and Martin, 1997; Pico, Rodrigues, Manes, 2003). Therefore, studies reported on the use of capillary electrophoresis in chiral separation are fast growing (Chankvetadze, 1997; Nevado, 2005; Kodama, 2006). However, CE also has limitations compared with

chromatographical technique. The comparative study of different analytical techniques to determine pesticide is summarized in Table 2.4 (Pico, *et al.*, 2003).

Table 2.2 Recent enantioseparation of some pesticide enantiomers using GC

Compounds	**Enantioseparation Remark**	**Ref.**
OP insecticide: naled, metamidophos, trichlorfon, malaoxon, malathion, isofenphos, phenthoate, phenamiphos, leptophos, chloretoxyphos, ruelene and pyraclophos	GC with flame ionisation detection (FID) using two different columns, Chirasil-Val (l-valine-*tert* butylamide) and CP-Chirasil-Dex CB (heptakis (2,3,6-tri-*O*-metil)-β-cyclodextrin)	Fidalgo-Used, Blanco-Gonza´lez, *et al.*, 2006
OP insecticide: ruelene	CP-Chirasil-Dex CB	Fidalgo-Used, *et al*., 2006
phenoxypropionic herbicides: mecoprop, dichlorprop	Column containing 1:1 mixture of per-*O*-pentylated and per-*O*-methylated ϒ-CD	Konig, 2005

Table 2.3 Recent enantioseparation of pesticide enantiomers using HPLC method

Compounds	Enantioseparation Remark	Ref.
Triazole fungicides: hexaconazole, triadimefon, tebuconazole, diniconazole, flutirafol, propiconazole, difenconazole	Normal phase HPLC using chiralcel OD columns and chiralcel OJ columns	Zhou *et al.,* 2008
Pyrethroid insecticide: λ-cyhalothin	Compared between different chiral columns: Chiralpak AD, Chiralpak AS, Chiralcel OD, and Chiralcel OJ column. Best separation was achieved using Chiralcel OD column	Xu *et al.*, 2008
OP insecticide: isocarbophos	Achieved using Chiralcel OD column	Lin *et al.*, 2008
Imidazolin herbicides imazethapyr, imazapyr, imazaquin	Compared between different chiral columns: chiralpak AD, chiralcel OD, and chiralcel OJ column. Best separation was achieved using chiralcel OJ column	Lin *et al.*, 2007
Phosphonate herbicide: 1-(substituted phenoxyacetoxy) alkylphosphoate herbicide	Compared between different chiral columns namely: chiralpak AD, chiralpak AS, chiralcel OD, and chiralcel OJ column. Best separation was achieved using chiralpak AD column	Li *et al.*, 2007
OP insecticide: Chloramidophos	Achieved using chiralpak AD column	Zhou *et al.*, 2007
Triazole fungicides: Tebuconazole, hexaconazole, myclobutanil, diniconazole, uniconazole, paclobutrazol, triadimenol	Compared between a Pirkle type (S,S)-whelk 01 chiral column of four different cellulose derivative columns: cellulose tribenzoate (CTB), cellulose tris(4-methylbenzoate) (CTMB), cellulose triphenylcarbamate (CTPC), and cellulose tris(3,5-dimethylphenyl carbamate) (CDMPC), in normal phase mode. Best separation was achieved using CDMPC	Pan *et al.*, 2006
OP insecticide: trichloronate	Achieved using chiralcel OJ column	Liu, *et al.*, 2006
Pyrethroid insecticides: bifenthrin, permethin, cypermethin, cyfluthin	Bifenthrin and permethin was achieved using Sumichiral OA-2500-1 column, while cypermethin and cyfluthin was achieved using Chirex 006-3019-DO column	Liu, *et al.*, 2005
OP insecticide: fenamiphos	Achieved using Chiralcel OD column	Wang, *et al.*, 2004
OP insecticide: penthoate ***Triazole fungicides:*** uniconazole, diniconazole, propiconazole ***Pyrethroids:*** β-cypermethin, β-cyfluthrin, α-renvalerate	Achieved using Chiralcel OD column and Pirkle type Chirex 3020 column	Li *et al.*, 2003
OP insecticide: lepthophos	Achieved using whelk-01 column	Yen, *et al.*, 2003

Table 2.4 Comparative study of different analytical techniques to determine pesticides (Pico, *et al.*, 2003).

Technique	Advantages	Disadvantages	Solutions
GC	• High resolving power and ability to resolve individual analytes. • High sensitivity and selectivity. • Existence of mass-spectrum libraries for screening unknown samples.	• Inadequate for polar, thermo-labile and low-volality compounds. • High consumption of expensive, high-purity gases.	• Derivatisation
LC	• Application to virtually any organic solute regardless of its volality or thermal stability. • Both mobile and stationary phase compositions are variables. • Capable of automation and miniaturization (microchip technology).	• Insufficient separation efficiency and selectivity. • Large amounts of expensive and toxic organic solvent used as mobile phase.	• Development of more efficient and selective columns material (immunoadsorbents, MIPs and restricted acces materials).
CE	• High separation efficiency. • Small consumption of expensive reagents and toxic solvents. • Capable of automation and miniaturization (microchip technology).	• Inadequate limits of detection. • Lack of selective detectors.	• Sample enrichment (SPE, stacking). • Increase detection path length. • Development of coupling methods to combine CE with highly selective detectors.

Applied CE techniques for chiral pesticides separations are capillary zone electrophoresis (Desiderio *et al.*, 1997; Mechref and El Rassi, 1997; Penmesta *et al.*, 1997; Aqa *et al.*, 1999; Wu *et al.*, 2000; Shi *et al.*, 2004; Klein *et al.*, 2006) with the addition of a chiral selector to the background electrolyte (BGE), and electrokinetic

chromatography (Martin-Biosca *et al.,* 2000; Garc'ya-Ruiz *et al*., 2005) using a chiral pseudostationary chiral selector. Several enantioseparation of chiral pesticides using CE is summarized in Table 2.5.

Chiral separation by capillary zone electrophoresis (CZE) is a rapidly developing area owing to the high efficiency and resolution that can be achieved and the option of adding molecular agents to the electrolyte (Harvey, 2000). However CZE is not able to separate neutral species. Micellar electrokinetic chromatography (MEKC) overcomes this limitation by adding a surfactant, such as sodium dodecyl sulfate (SDS) to the buffer solution (Terabe, 1993; Harvey, 2000). Adding an ionic surfactant to the buffer system provides the possibility of separation to both, neutral and charged analytes simultaneously. In the literature, MEKC is also often referred to as MECC (micellar electrokinetic capillary chromatography) since the separation are most often performed in capillary tube (Terabe, 1993). In MEKC method, the uncharged compounds are separated by different distributions between the aqueous and micellar phase (Otsuka and Terabe, 1996; Schmitt *et al.,* 1997; Garc´ýa-Ruiz *et al*., 2005).

Table 2.5 Enantioseparation of some pesticides using CE technique

Compounds	Enantioseparation	Ref.
Chloroacetanilide herbicide: metolachlor	γ-CD was used as chiral selector and borate buffer at pH 9.0	Klein *et al.*, 2006
OP insecticides: isomalathion, naled malathion, fenamiphos, penthoate	using CM-β-CD and tris buffer (pH 7), except for naled were used CM-β-CD, borate buffer (pH 9)	Garc'ya-Ruiz *et al.*, 2005
Herbicides: 2-phenoxypropionic acid (PPA), cis-3-(2-Chloro-3,3,3- trifluoropropenyl)-2, 2-dimethylcyclopropanecarboxylic acid (cis-PA), 1-phenyl-2-(p-tolyl) ethylamine (PTE) and 1-phenyl-2-(p-methoxyphenyl) ethylamine (PME).	Î²-cyclodextrin polymer (CD polymer) as chiral selector. The optimal operate conditions were obtained with 12 kV as separation voltage, 14 g/L CD polymer in 30 mmol/L tris-HCl as background electrolyte buffer.	Shi, *et al.*, 2004
Triazole fungicides: uniconazole and diniconazole	CM- β-CD as chiral selector and phosphate buffer at pH 6.5	Martin-Biosca, *et al.*, 2000
Triazole fungicides: bitertanol, cyproconazole, difenconazole, diniconazole, flutriafol, hexaconzole, myclobutanil, paclobutrazol, penconazole, propiconazole, tebuconazole, tetraconazole, triadimefon, triadimenol	Sulfated- β-CD as chiral selector and phosphate buffer (pH 3.0)	Wu, Lee, Li, 2000
Herbicides: (5S)-metolachlor OXA, acetochlor ESA, acetochlor OXA, and racemic metolachlor OXA	Î³- cyclodextrin as chiral selector in CZE, complete separation of all four isomers of enantiomerically enriched (5S)-metolachlor OXA, while the enantiomers of acetochlor ESA, acetochlor OXA, and racemic metolachlor OXA were partially separated.	Aqa, et al., 1999
Herbicides: Mecoprop,fenoprop, dichlorprop, haloxyfop, fluazitop, diclofop, fenoxaprop, flamprop, 2-phenoxy propionic acid	Vancomycin as chiral selector, and buffer Britton-Robinson at pH 5	Desiderio *et al.*, 1997
Phenoxyacid herbicides: silvex, dichlorprop, mecoprop, 2,4-CPPA, 2,2-CPPA, 2-PPA	Alkylglucoside as chiral surfactant	Mechref and El Rassi, 1997
Herbicides: imazaquin, Diclofop, imazamethabenz.	DM-β-CD, TM-β-CD, and HP-γ-CD were used as chiral selector. The best separation was obtained using mixture of DM-β-CD, TM-β-CD	Penmesta, *et al.*, 1997

2.4 Micellar Electrokinetic Chromatography

Pesticides are analyzed by micellar electrokinetic chromatography (MEKC) by adding micelles to the running buffer and the separation of the pesticides is based on their relative partitioning between the water and the micellar phase (Schmitt *et al.*, 1997). When an anionic surfactant such as sodium dodecyl sulphate (SDS) is employed, the micelles migrate toward the positive electrode by electrophoresis. The electroosmotic flow transports bulk solution towards the negative electrode. The electroosmotic flow (EOF) is usually stronger than the electrophoretic migration of the micelle under neutral or alkaline conditions and, therefore, the anionic micelle also migrate towards the negative electrode at a retarded velocity. When a neutral analyte is injected to the micellar solution, a fraction of it is incorporated into micelle and it migrates at the velocity of the micelle. The remaining fraction of the analyte remains free from the micelle and migrates at the electroosmotic velocity and depending on the distribution coefficient between the micellar and the non-micellar (aqueous) phase. The greater the percentage of analyte that is distributed into the micelle, the slower it migrates (Terabe, 1993).

The addition of chiral selectors to the running buffer that selectively bind the different enantiomers allows the electrophoretic separation of enantiomers of charged removal processes of the chiral compounds (Schmitt *et al*., 1997). Chiral separation is based upon the formation of diastereomeric complex between the enantiomer and a chiral selector and separation can be obtained only if these complexes have different equilibrium constants (also called binding constants or formation constants) of complex formation.

There are five factors wich can be control led to increase the selectivity in MEKC: temperature, choice of surfactant, modification of the micelle, choice of the aqueous phase, and modification of the aqueous phase. The temperature effect on selectivity is not dramatic but temperature does, however, affect the migration time. Surfactant choice affects the selectivity because a surfactant molecule has a hydrophobic part and hydrophilic part. Since most analytes interact with the micelles

at their surfaces, the hydrophilic group or ionic group is generally more important in terms of selectivity. The micellar phase can be modified by adding a second surfactant to form a mixed micelle or by selecting a different counter ion, since a mixed micelle of an ionic and a nonionic surfactant has lower surface charge and large size, its electrophoretic mobility will be lower than a single ionic micelle. The aqueous phase effect on surfactant ionization, the pH of the buffer is a critical parameter for the separation of ionizable analytes. The last is the modification of the aqueous phase which is very effective in manipulating selectivity, is by the use of additives. The additives applicable in MEKC are cyclodextrins (CDs), ion-pair reagents, urea, organic solvents and metal salts (Terabe, 1993; Otsuka and Terabe, 1996). In chiral compounds separation, the CDs addition functions as a chiral selector. Different mixtures between neutral CDs, organic modifiers and other chiral selectors like bile salts were assayed to reach the enantiomeric resolution (Nevado *et al.*, 2005).

2.5 Micellar Electrokinetic Chromatography Separation Employing Cyclodextrin

Cyclodextrins (CDs) or their derivatives are the most successful and useful chiral selectors for enantiomeric separation, especially for the separation of enantiomeric drugs (Wang *et al*., 1998; Blanco and Valverde, 2003; Juvancz *et al.*, 2008). MEKC with the addition of CDs for anionic surfactants such as SDS, CDs mix with SDS micelles, effectively lining the interior of the micelle with enantioselective agents. This gives the CD an effective net negative charge as it migrates against the EOF. Figure 2.3 shows a schematic illustrating the partitioning of a neutral analyte into a micelle and into a cyclodextrin. When enantiomers are solubilized by the micelle, the stereoisomer forming the more stable complex with the CD is solubilized to a greater extent and elutes later (Rogan *et al.*, 1995).

Cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) for the separations of pesticides was introduced by Schmitt *et al.* (1997), who used SDS as surfactant, and the experimental was limited to the non-ionizable pesticides

(neutral pesticides). Schmitt *et al*. reported enantioseparation for three organophosphorus pesticides (OPPs), namely malathion, ruelen, and dialifos. HP-β-CD was used as the chiral selector for enantioseparation of malathion and ruelen, while the enantioseparation of dialifos was achieved using γ-CD as chiral selector.

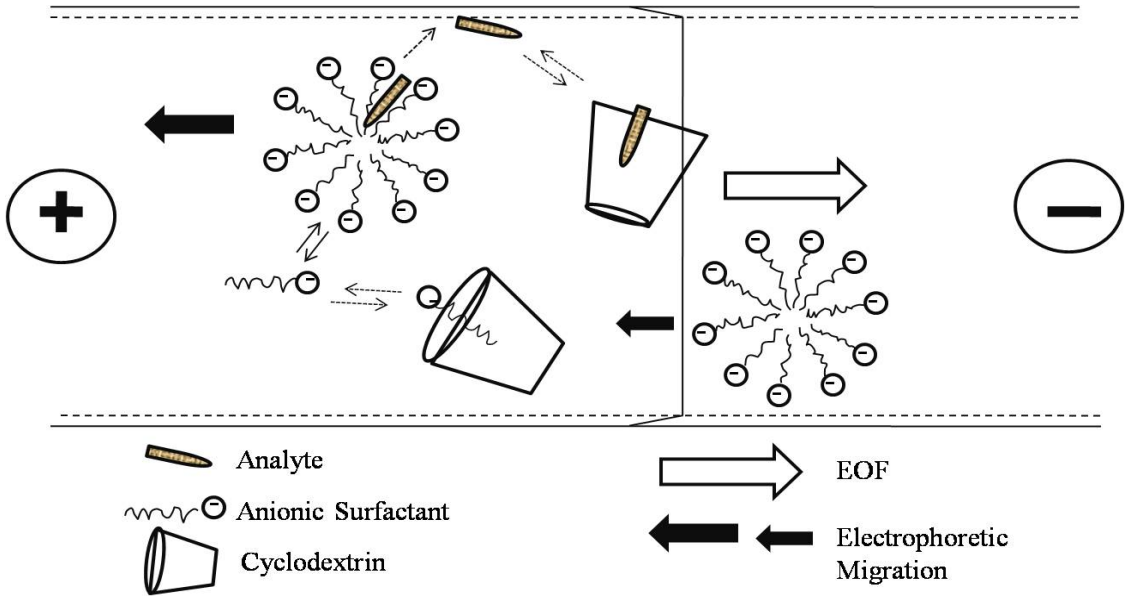

Figure 2.3 Schematic illustrating the partitioning of a neutral analyte into a micelle and into a cyclodextrin (Terabe, 1993)

Huang *et al.* (2007) reported enantiomeric separation of four OPPs, i.e. profenofos, prothiofos, sulprofos, and pyraclofos using nonaqueous media or aqueous-organic media as the running solution. The separation of four pesticides was achieved using γ-CD as chiral selector. Furthermore, CD-MEKC was also successful for enantioseparation of fungicides. Enantiomer of triadimenol, can be separated using HP-γ-CD and also using heptakis-6-sulfato-β-CD as chiral selector (Otsuka and Terabe, 2000). Enantiomer of propiconazole was separated using HP-γ-CD as chiral selector in appropriate conditions (Wan Ibrahim *et al.*, 2007). Recently CD-MEKC was succesfully achieved for chiral separation of mixture of hexaconazole, penconazole, and myclobutanil using HP-γ-CD as chiral selector (Wan Ibrahim *et al.*, 2009). Other enantioseparation of pesticides have been reported, for vinclozolin using γ-CD (Kodama *et al.*, 2002a), thiobencarb using HP-γ-CD (Kodama *et al.*,

2002b), and enantioseparation of malathion, crufomate, and fensulfotion using CM-β-CD (Anigbogu *et al.,* 2003).

Bile salts were also found to exhibit a synergistic effect in chiral recognition in combination with CDs (Gubitz and Schmid, 1997). Bile salts are able to form micelles and have been employed as an alternative to SDS. Bile salts such as sodium cholate, sodium deoxycholate, and their taurine conjugates are natural and chiral anionic surfactants, which form helical micelles of reversed micelle conformation (Terabe, 1993; Otsuka and Terabe, 2000). MEKC with the addition of CDs for chiral surfactant such as bile salts, were assayed to reach the enantiomeric resolution of citalopram (Nevado *et al.,* 2005).

2.6 Chiral Selector

Cyclodextrins (CDs) are non reducing oligosacahrides cyclic consisting of D-glucopyranose units bonded through α-1,4 linkages. CDs have been effectively employed for chiral separation and for improvement of a large number of separation processes in many chromatographic techniques, either as mobile-phase additives or as stationary phase or stationary phase additives (Cserhati and Forgacs, 2003). CDs or their derivatives are the most successful and useful chiral selectors for enantiomeric separation, especially for the separation of enantiomeric drugs. The three natives CDs are α-CD, β-CD, and γ-CD (Figure 2.4). CDs forms cavity dimension, which is lined by the hydrogen atoms and the glycosidic oxygen bridges. The outside surface of cavity is relatively hydrophilic, while the inside of caity is relatively hydrophobic (Terabe, 1993; Cserhati and Forgacs, 2003). The C-2 hydroxyl group of one glucopyranose unit can form a hydrogen bond with the C-3 hydroxyl group of the neighbouring glucopyranose unit, often refered as secondary hydroxyl rim or secondary hydroxyl belt (Cserhati and Forgacs, 2003). Table 2.6 shows the most important characteristics of native CDs; γ-CD has the biggest cavity, and α-CD the smallest.

The solubility of β-CD is relative low, it is caused in β-CD molecule secondary rim is formed completely, so β-CD is a rather rigid structure compared to

other native CDs (Cserhati and Forgacs, 2003). Addition of a high concentration of urea such as 2 M increases the solubility. For many compounds, γ-CD is a more effective additive than β-CD in MEKC. This is even the case for the compounds such as eleven trichlorobiphenol isomers, successfully separated by HPLC with the β-CD bonded phase. Conceivably, co-inclusions, of the surfactant molecule into the cavity of γ-CD would result in less available space for the analyte molecule (Terabe, 1993). Others characteristics of CDs are thermostable to a reasonable temperature which is important in GC, stable over very wide pH range (pH 2-12), and do not adsorb radiation in the region normaly association with UV detection (200-300 nm) (Cserhati and Forgacs, 2003).

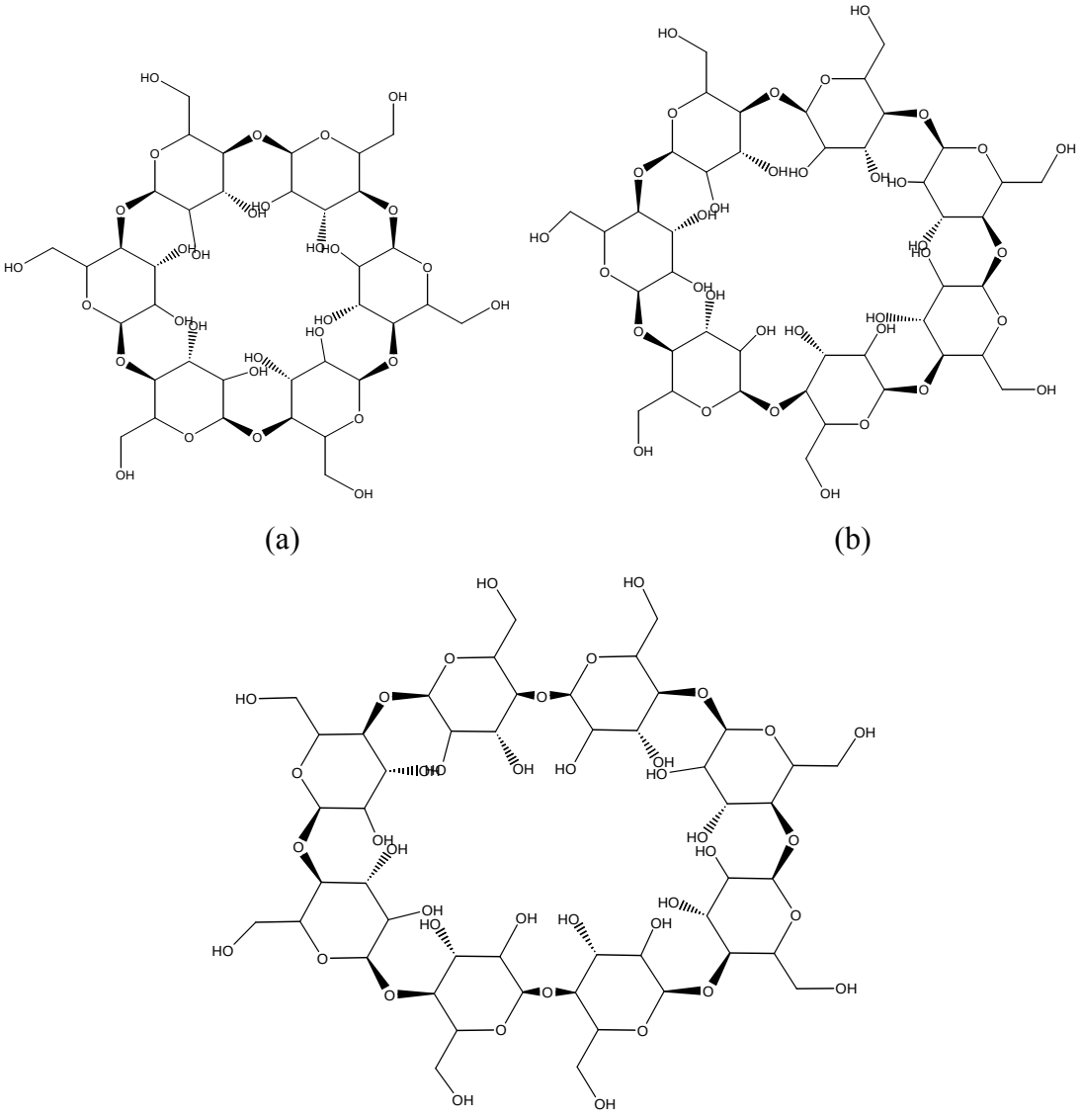

Figure 2.4 Schematic structures of native (a) α-, (b) β-, and (c) γ-CDs. (Chemexper Inc., 2008)

Table 2.6 Characteristics of Native CDs.

Cyclodextrin	α-CD	β-CD	γ-CD
Number of Glucose units	6	7	8
Molecular weight	972.9	1135.0	1297.2
Internal diameter of the cavity (nm)	0.47-0.52	0.62-0.64	0.75-0.83
Outside diameter (nm)	1.46	1.54	1.75
Height of the cavity (nm)	0.79-0.80	0.79-0.80	0.79-0.80
Solubility in water at 25˚C (mg mL^{-1})	14.50	1.85	23.2
pKa Value	12.3	12.2	12.1

Selectivity of CD is based on their cavity (Huang *et al.,* 2007). Thus CDs are capable of forming inclusion complexes with compounds having a size compatible with the dimensions of the cavity. Inclusion complexes are entities comprising two or more molecule which is the "guest" consisting "host". CDs are typical host molecules, while the guest molecules included several molecules having the size of one or two benzene rings, or even larger compounds, which have a side chain of comparable size to form inclusion complexes (Cserhati and Forgacs, 2003). The effect of cyclodextrin types are (Huang *et al.,* 2007):

- α-CD → perfect for monocyclic aromatic hydrocarbon (i.e. benzena and phenol)
- β-CD → matched within the size of the naphthalene ring
- γ-CD → Steady with compounds with 3 aromatic rings (antracene and phenantrene)

Table 2.7 Some native and derivatized CDs that have been used for enantioseparation of pesticides.

No	CDs	Pesticides	Reference
1	α-CD	ruelene	Terabe, 1993
2	β-CD	ruelene	Terabe, 1993
3	γ-CD	profenofos, porthiofos, sulprofos	Huang *et al.* 2007
		metolachlor	Klein *et al.*, 2006
		vinclozolin	Kodama *et al.,* 2002a
		malathion, ruelen, dialifos	Schmitt *et al.*, 1997
		malathion, dialifos	Terabe, 1993
4	HP-β-CD	malathion, crufomate, and fensulfotion	Anigbogu *et al.,* 2003
5	HP- γ-CD	hexaconazole, penconazole, and myclobutanyl	Wan Ibrahim *et al.*, 2009
		propiconazole, tebuconazole, and fenbuconazole	Wan Ibrahim *et al.,* 2007
		thiobenzacarb	Kodama *et al.,* 2002b
		triadimenol	Otsuka and terabe, 2000
		imazaquin, diclofop, imazamethabenz	Penmesta *et al.*, 1997
6	CM-β-CD	isomalathion, naled, malathion, fenamiphos, penthoate	Garc'ya-Ruiz *et al.*, 2005
		malathion, crufomate, and fensulfotion	Anigbogu *et al.,* 2003
		uniconazole and diniconazole	Martin-Biosca *et al.*, 2000
		ruelene	Terabe, 1993
7	DM-β-CD	imazaquin, diclofop, imazamethabenz.	Penmesta *et al.*, 1997
		malathion, ruelene	Terabe, 1993
8	TM-β-CD	imazaquin, diclofop, imazamethabenz.	Penmesta *et al.*, 1997
9	Sulfated-β-CD	bitertanol, cyproconazole, difenconazole, diniconazole, flutriafol, hexaconzole, myclobutanil, paclobutrazol, penconazole, propiconazole, tebuconazole, tetraconazole, triadimefon, and triadimenol	Wu *et al.*, 2000
10	Heptakis-6-sulfato-β-CD	triadimenol	Otsuka and Terabe, 2000

The use of mixtures of CDs has been reported for chiral separation. Although the use of mixture of CD can obviously improve separation and sensitivity (Wei *et al.,* 2006; Penmesta *et al.*, 2007), it is not always suitable for chiral separation (Abusofa

et al., 2003). Thus, enantioseparation of pesticides namely imazaquin, diclofop, and imazamethabenz using of mixture of DM-β-CD and TM-β-CD as chiral selector can obviously improve separation (Penmesta *et al.*, 2007). Several CDs have been used for enantioseparation of pesticides and is summarized in Table 2.7. According to the structure and hydrophobicities of the selected triazole fungicides, CDs that are suitable for separation of their enantiomers are native or derivatized β-CD and γ-CD.

2.7 Problem Statement

Some possibilities of the use of various native cyclodextrins CDs and derivatives CDs as chiral selector on the enantiomeric separation of pesticides have been shown previously (Wan Ibrahim, *et al.*, 2007; Schmitt et al., 1997, Terabe 1993). CD discriminates between enantiomers via inclusion into their hydrophobic cavity (Wren, 1993). Therefore it would be expected that compounds with greater hydrophobicity values will have greater affinity for CD (migrated slower) and optimum chiral selector concentration is lower for the more hydrophobic compounds. To the knowledge of the researchers, currently there are no systematic studies on selected triazole fungicides using capillary electrophoresis investigating these hypotheses. Such studies can add new body of knowledge confirming the proposed hypothesis to a broader range of compounds apart from enantiomeric pharmaceutical compounds since agrochemicals are widely used throughout the world. A more systematic work is needed to confirm this hypothesis, since currently there are no systematic studies on slected triazole fungicide enantiomers using capillary electrophoresis especially CD-modified MEKC.

2.8 Objectives of the Study

The first objective of this study was to carry out enantioseparation of selected triazole fungicides namely cyproconazole, bromuconazole, diniconazole, penconazole, myclobutanil, triadimefon, and hexaconazole using CD-MEKC. The effects of single and dual native cyclodextrin (CD) in the separation of selected triazole fungicides was studied and also to recognize the chiral separation mechanism

using CD-MEKC. The second objective was to study the effect of several separation parameters using CD-MEKC, such as effect of buffer pH, buffer concentration, micelle (SDS) concentration, separation temperature, applied voltage, and organic modifiers addition. The last objective was to study the on-line sample preconcentration technique i.e. sweeping-CD-MEKC for enhancing the detection sensitivity of enantioseparation of the selected triazole fungicides.

2.9 Scope of Study

This research is limited to the study of three selected triazole fungicides namely cyproconazole, bromuconazole, and diniconazole using MEKC with SDS as micellar phase, and cyclodextrin (CD) as chiral selector. CDs used are limited to neutral CD, selected based on structures predicted suitable to separate selected triazole fungicide enantiomers. Separation parameters investigated is limited to effect of buffer pH, buffer concentration, micelle (SDS) concentration, separation temperature, applied voltage, and organic modifiers addition. According to previous study, Sweeping-CD-MEKC showed a good enhancement factor of up to 100 fold compared to other techniques for triazole fungicides separation (Wan Ibrahim *et al*., 2007). Because of that Sweeping-CD-MEKC was used to enhancing the separation of selected triazole fungicides in this study. The good performance of separation is expected to give resolutions of greater than 1.5, with acceptable RSD of migration time, peak height, and peak area. The acceptable RSD refers to EC document SANCO/3030/99 rev.4 11/07/00 (79 (Biocides and Pesticides Unit, NY).

CHAPTER 3

SIMULTANEOUS ENANTIOSEPARATION OF CYPROCONAZOLE, BROMUCONAZOLE, AND DINICONAZOLE USING CYCLODEXTRIN-MODIFIED MICELLAR ELEKTROKINETIC CHROMATOGRAPHY

3.1 Introduction

Since the stereochemistry has a significant influence on the biological activity, the development of analytical methods for enantiomeric separation at low cost and short analysis time is increasing. As mentioned in chapter 2, CE has been found to be a powerful alternative to chromatographic techniques for enantiomeric separation. CE can provide rapid analysis with high efficiency and high resolution (Gubitz and Schmid, 1997; Pico *et al.,* 2003). Therefore, studies reported on the use of CE in chiral separations are fast growing (Chankvetadze, 1997; Nevado *et al.*, 2005; and Kodama *et al.*, 2006). Several review papers have recently been published on the chiral separation of enantiomers (Gubitz and Schmid, 1997; Vespalec and Bocek, 2000; Lamerhofer, 2005; and Wang *et al.*, 2008).

Micellar electrokinetic chromatography (MEKC) is a mode of electrokinetic chromatography using CE instrument in which surfactants (micelles) are added to the buffer solution. MEKC has been shown to be a powerful separation technique for separation of enantiomers (Schmitt *et al.*, 1997; Otsuka and Terabe, 2000). There are many advantages of MEKC compared with conventional chromatographic method for enantiomers separation, such as the minimal use of expensive chiral reagents and solvents required. Furthermore, only small sample volumes are required and efficiency is very high. There are two MEKC modes for enantiomers separation. The first is MEKC using chiral surfactants and the second is MEKC using cyclodextrin (CD) as chiral selector (Nishi and Terabe, 1996). In this study, cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) was used for the simultaneous

separation of two pair of cyproconazole enantiomers, two pair of bromuconazole enantiomers, and a pair of diniconazole enantiomers. CDs are currently by far the most common chiral additives (Wang *et al.*, 1998; Blaschke and Chankvetadze, 2000; Blanco and Valverde, 2003; Juvanc *et al.*, 2008). Several CDs and derivatized CDs were successfully used for the enantioseparation of triazole fungicides compounds (Biosca *et al*., 2000; Wu *et al*., 2001; Wan Ibrahim *et al.*, 2007; Wan Ibrahim *et al.,* 2009). In this study, native CD and derivatized CDs namely γ-CD, HP-γ-CD, and HP-β-CD were used as chiral selector. The effects of single and dual selected CDs on the enantioseparation of the selected triazole fungicides were investigated.

3.2 Experimental

3.2.1 Chemicals and Reagents

Selected triazole fungicides namely Cyproconazole, bromuconazole, and diniconazole and selected cyclodextrin namely 2-Hydroxypropyl-γ-cyclodextrin (HP-γ-CD), γ-cyclodextrin (γ-CD), and hydroxypropyl-β-cyclodextrin (HP-β-CD) were obtained from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) was obtained from Fisher Scientific (Loughbrough, UK). Disodium hydrogen phosphate 12-hydrate and sodium hydroxide pellets were obtained from Riedel-de Haen (Seelze, Germany). HPLC grade methanol was obtained from J.T. Baker (California, USA). Organic solvent used i.e methanol (MeOH), ethanol (EtOH), n-propanol (n-PrOH), and i-propanol (i-PrOH) were obtained from were obtained from Sigma Aldrich (St. Louis, MO, USA). Water was collected from a Millipore Water Purification System (Molsheim, France). Stock solutions (2000 mg L^{-1}) of individual triazole fungicide were prepared in methanol. Final dilutions were prepared by diluting the stock solution with water to 200 mg L^{-1}. The separation solutions for CD-MEKC were prepared by dissolving appropriate amounts of SDS, cyclodextrin, methanol and acetonitrile in phosphate buffers and adjusting the pH of the buffer with phosphoric acid solution. All running buffers were filtered through a 0.45 μm nylon syringe filter from Whatman (Clifton, NJ, USA).

3.2.2 Instrumentations

All experiments were carried out on Agilent capillary electrophoresi system (Agilent Technologies, Waldbronn, Germany), equipped with a diode array detector. Data acquisition and system control was carried out by the 3D-ChemStation Software by Agilent Technologies. Separations were performed using an untreated fused silica capillary of 64.5 cm total length, 56 cm effective length and 50 μm i.d. Sample injections were performed hydrodynamically at a constant pressure of 50 mbar for 1s. The separation runs were performed at a constant temperature of 20˚C and an applied voltage of - 25 kV.

3.2.3 Conditioning of the Capillary

At the beginning of each day, the capillary was flushed for 5 min with deionized water, followed by 5 min of 1 M NaOH to activate the silanol groups of the capillary, then followed by 10 min of deionized water, and 5 min of running buffer. Before each sample injection, the capillary was rinsed for 3 min with 0.1 M NaOH, followed by 3 min of deionized water, and 3 min of running buffer.

3.2.4 Preparation Standard Selected Triazole Fungicides

The stock solutions of the individual selected triazole fungicides werw prepared in methanol in the concentration range 2000 – 4000 mg L^{-1}. Final dilutions for the analysis were prepared by diluting the stock solution with water and concentrated at 200 mg L^{-1}, either for single CD system or dual CD system.

3.2.5 Method for Screening CDs as Chiral Selctor in Separation of Selected Triazole Fungicide enantiomers

Separation using MEKC was done by adding micelles to the running buffer. In this study, running buffer containing 25 mM phosphate buffer (pH 3), was based on previous research (Wan Ibrahim, Hermawan, and Sanagi, 2007), the addition of 40 mM SDS to form micellar phase, and methanol (MeOH) : acetonitrile (AcN) 10%:5% v/v was added as organic modifier. In the first step, the effects of single and dual native cyclodextrins namely γ-CD, HP- γ-CD, and HP-β-CD were investigated. The selection of chiral selector is based on the cavity of cyclodextrin matching with the structure of the selected triazole fungicides. Effects of single native cyclodextrin were investigated by varying cyclodextrin concentrations between 10-40 mM. Then the effect of dual cyclodextrin was investigated based on the best separation using single cyclodextrin. The combination of HP-γ-CD with HP-β-CD was tested

3.2.6 Optimization Method of MEKC Parameters for Chiral Separation of Selected Triazole Fungicide Enantiomers

Instrumentations and conditioning of the capillary used same as this previous (chapter 3). Effect of pH was investigated at neutral and basic condition i.e pH 7, 8, and 9, and acidic condition, i.e pH 3, 4, and 5. Phosphate buffer concentration effect was investigated in range 15-50 mM, whereas SDS concentration effect investigated at rang 30-60 mM. Then separation temperature was investigated at range 15-25 ˚C, and separation voltage effect was investigated at -20 kV to -30 kV. The effect of organic modifiers was explored at several organic solvent, namely methanol (MeOH), ethanol (EtOH), n-propanol (n-PrOH), and i-propanol (i-PrOH) at the addition 5-20%. The effect of the addition of mixture of methanol and acetonitrile on enantioseparation of selected chiral agrochemicals using dual CD system was also explored at the addition of % v/v MeOH: can 5:5, 5:10, 10:5, and 10:10.

3.3 Results and Discussion

3.3.1 Screening Cyclodextrin as Chiral Selector

A 25 mM phosphate buffer solution at pH 3 was initially used based on the optimized condition of a previous study, which resulted in the separation of the four enantiomers of propiconazole (Wan Ibrahim, Hermawan, and Sanagi, 2007). Propiconazole is one of the triazole fungicides, and have hydrophobicity close to the selected triazole fungicide used in this study. Accordingly, the background electrolyte (BGE) composition used in this study for screening chiral selector is similar to the one used in the earlier study. Separation using MEKC was performed by adding micelles to the running buffer at 40 mM SDS. To the BGE, methanol: acetonitrile 10%:5% v/v was added as organic modifiers at the same composition used in the previous study (Wan Ibrahim, Hermawan, and Sanagi, 2007

3.3.1.1 Single CD system

Initially, three neutral cyclodextrins (HP-β-CD, HP-γ-CD and γ-CD) were tested individually as the chiral selectors at different concentrations (10, 20, 30, and 40 mM) for the enantioseparation of the selected triazole fungicides. A mixture of the triazole fungicides has ten enantiomers in total i.e. four enantiomers of cyproconazole, four enantiomers of bromuconazole, and two enantiomers of diniconazole. Therefore, a good separation should be shown by the appearance of ten peaks on the electropherogram. Furthermore, resolution, peak height, peak area, and separation time will also be considered to choose the best simultaneous separation of the selected triazole fungicides. An incomplete separation of the triazole fungicide enantiomers was obtained with individual chiral selectors. Furthermore separation using 40 mM HP-β-CD and 40 mM γ-CD gave broad peak and the migration time for all peaks is more than 40 minutes. The electropherogram in several cyclodextrin concentrations are shown in Figure 3.1 for HP-γ-CD, Figure 3.2 for γ-CD and Figure 3.3 for HP-β-CD.

The best separation of mixture of the selected triazole fungicides using HP-γ-CD as chiral selector was achieved at concentration of 30 mM. However, this condition gave eight peaks and resolution was poor because of overlapping signal between some peaks, which is due to the hydrophobicity between selected triazole fungicides being close (Figure 3.1). Actually, separation using 30 mM HP-γ-CD for single standard showed that each enantiomers of the selected triazole fungicides can be separated (Figure 3.4). Thus HP-γ-CD should be suitable as chiral selector for the selected triazole fungicides, but not for the analysis of mixture of the selected triazole fungicides. HP-γ-CD is a derivatized γ-CD because of that HP-γ-CD has an inner cavity diameter around 0.79-0.95 nm (Terabe 1993; Aboul-Enein and Ali, 2003). As known, γ-CD has the biggest inner diameter cavity compared to other native cyclodextrin. Therefore, it can be predicted that all of the selected triazole fungicides will have the chance to form inclusion complex with HP-γ-CD.

The best separation with γ-CD also gave eight peaks i.e the separation using 30 mM γ-CD, but resolution was poor (Figure 3.2). All separation with concentration of 40 mM chiral selector, gave broad and low signal peaks, and analysis time was long i.e. separation with 40 mM HP-γ-CD gave the first signal at 24 minutes and analysis time was 40 minutes, and separation with 40 mM HP-β-CD gave the first peak at more than 40 minute. Accordingly, the best separation was obtained at 30 mM chiral selector (Table 3.1). The best separation using individual chiral selector was obtained with a concentration of 30 mM HP-β-CD solution, where nine peaks were resolved with good resolution (R_s 1.3-14.3). However, diniconazole was not separated using HP-β-CD as in Figure 3.5(c).

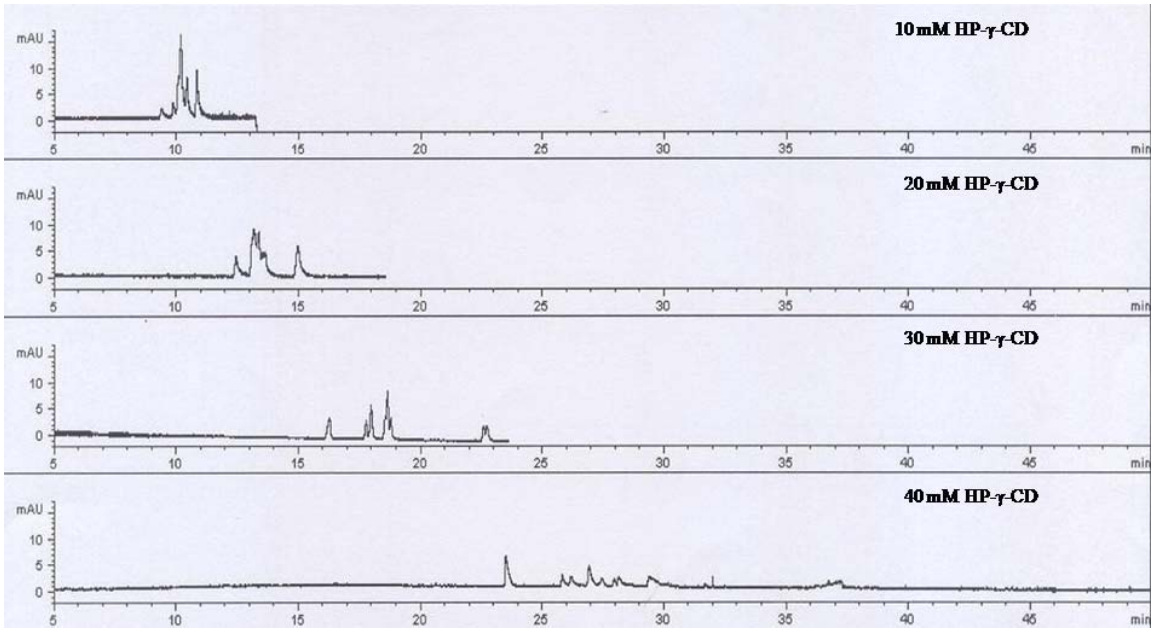

Figure 3.1 Enantioseparation of a mixture of triazole fungicides using CD-MEKC with variation of HP-γ-CD concentration. Other conditions: 40 mM SDS, methanol-acetonitrile 10%:5% v/v in 25 mM phosphate buffer (pH 3.0), Applied voltage 25 kV, temperature 20˚C, and pressure injection at 50 mbar for 1s.

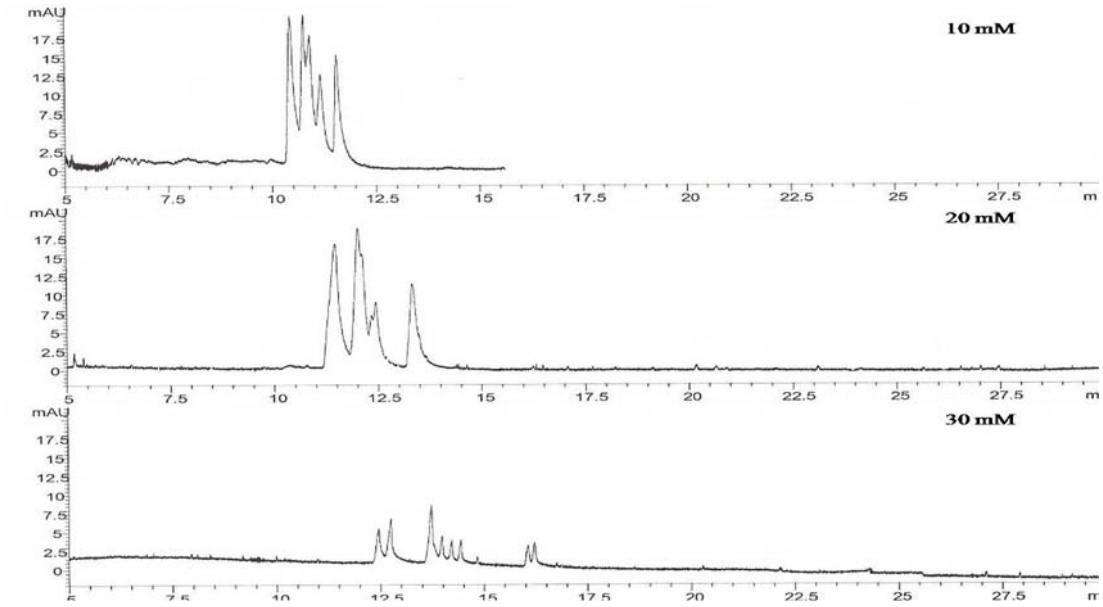

Figure 3.2 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at various γ-CD concentrations. Other condition as in Figure 3.1.

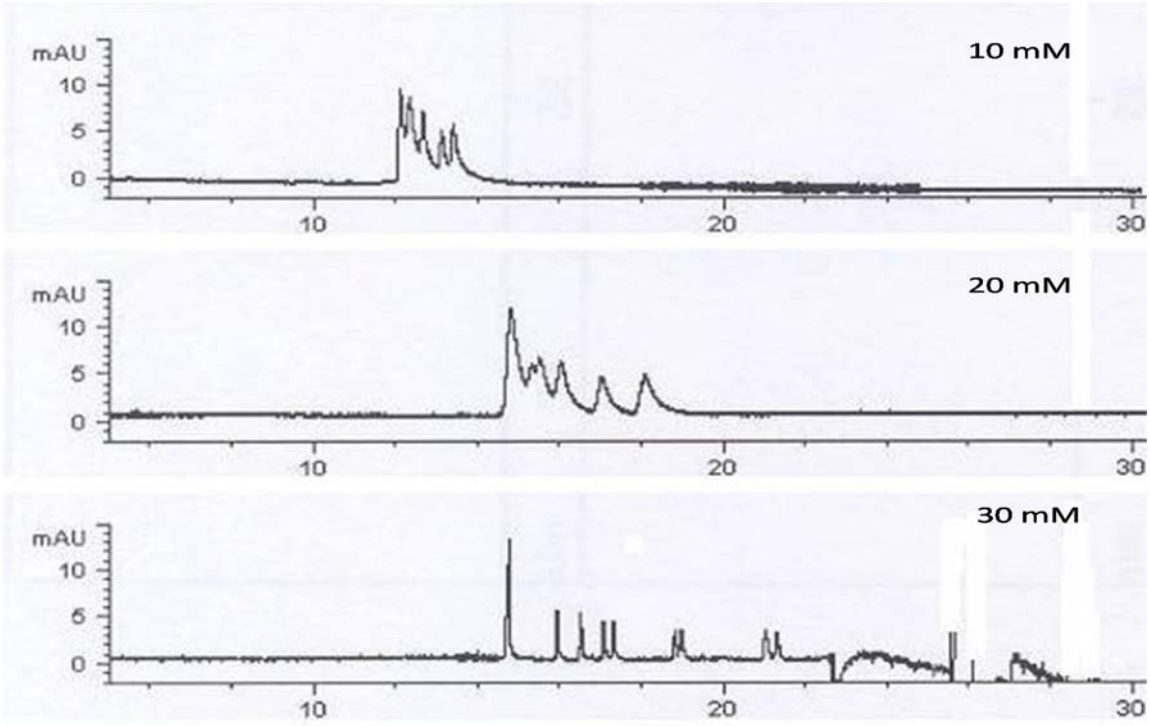

Figure 3.3 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at various HP-β-CD concentrations. Other conditions as in Figure 3.1.

None of the CDs used separated diniconazole bromuconazole and cyproconazole stereoisomers simultaneously. However, the addition of a chiral selector to an electrophoretic system does not guarantee a successful separation of all the stereoisomers. Several intermolecular interactions may participate in the separation of chiral compound, such as Coulombic attraction, Coulombic repulsion, hydrogen bonding, hydrophobic interactions, sterical hindrance, inclusion in the cavity, charge transfer, π-π electron interaction, and dipole-dipole interactions (Vespalec and Boc˘ek, 2000). One of the most important rules for chiral recognition is that the chiral selector must be compatible in size and structure to the racemate; and a minimum of three molecular interactions has to occur (Ingelse, 1997).

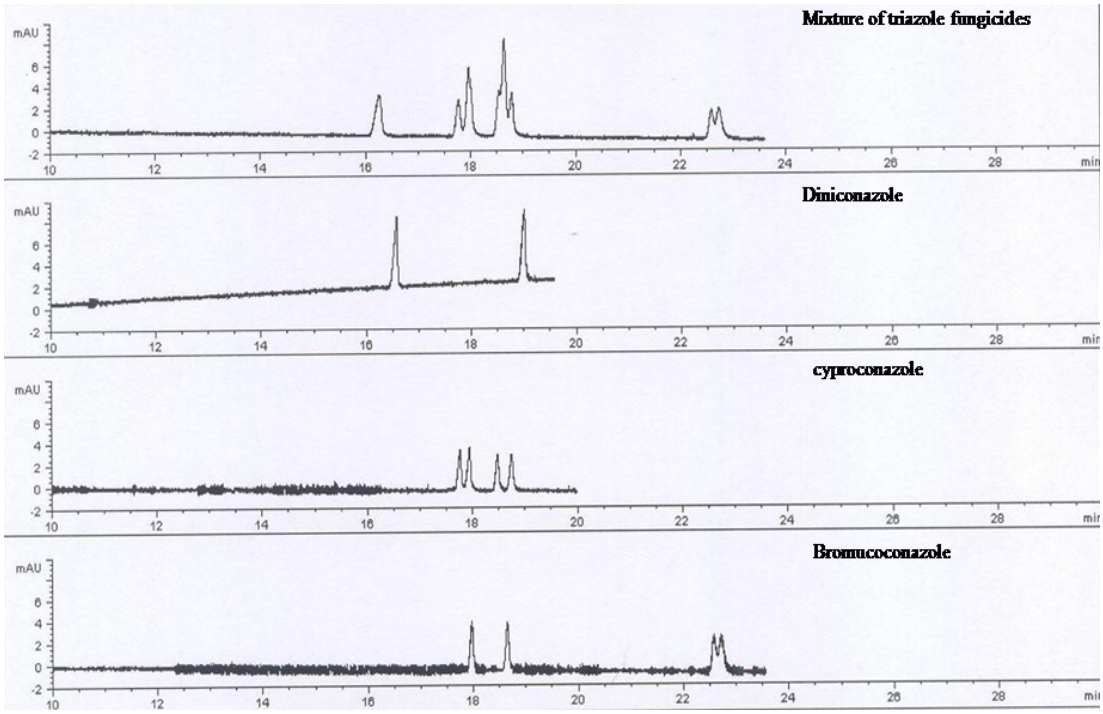

Figure 3.4 Enantioseparation of a mixture of triazole fungicides, and individual triazole fungicide using CD-MEKC with 30 mM HP-γ-CD. Other conditions as in Figure 3.1.

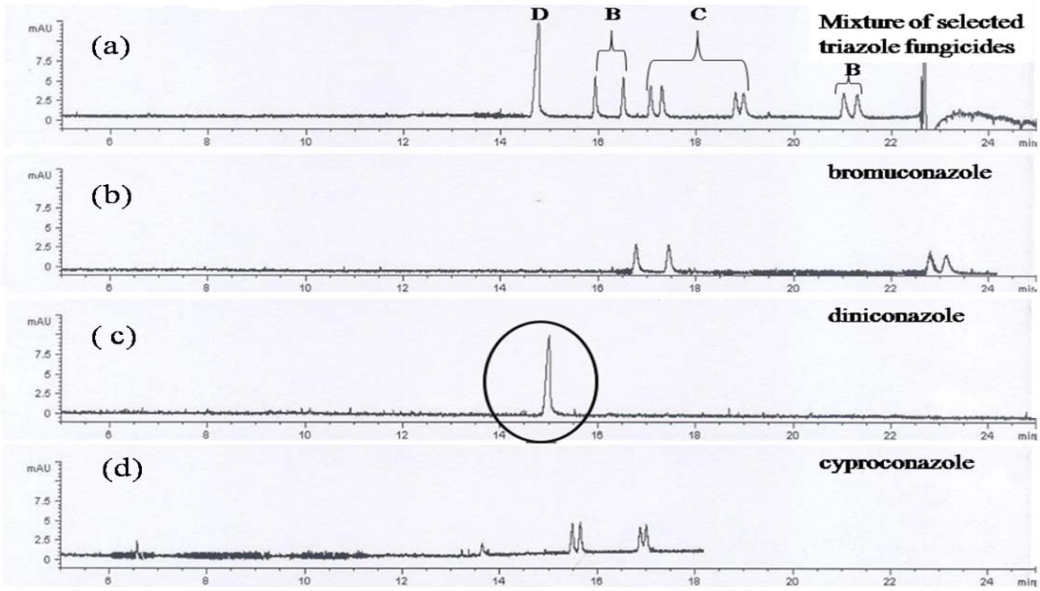

Figure 3.5 Enantioseparation of a mixture of triazole fungicides, and individual triazole fungicide by CD-MEKC at 30 mM HP-β-CD. Other conditions as in Figure 3.1. (D: diniconazole peaks; B: bromuconazole peaks; C: cyproconazole peaks)

Table 3.1 Resolutions (R_s) of enantiomeric separation of selected triazole fungicide using CD-MEKC with different chiral selector at concentration of 30 mM.

Peak	R_s		
	HP-γ-CD	**γ-CD**	**HP-β-CD**
P1-P2	10.07	0.97	9.71
P2-P3	1.01	4.02	6.29
P3-P4	4.02	0.95	5.74
P4-P5	0.33	1.08	2.3
P5-P6	0.69	0.93	13.58
P6-P7	22.53	6.31	1.3
P7-P8	0.66	0.66	14.29
P8-P9	none	none	1.8

P1-P9 indicates peak number in the electropherogram

3.3.1.2 Dual CD system

Since the use of single CD was not able to separate all stereoisomers of diniconazole, bromuconazole, and cyproconazole simultaneously, the use of mixtures of CD was tested. From the three CDs used, HP-β-CD (Figure 3.5) gave better resolution for bromuconazole and cyproconazole on the separation of mixture of selected triazole fungicides, while separation of diniconazole enantiomers gave better resolution on the separation of HP-γ-CD enantiomers (Figure 3.4). Therefore the use of dual CD system containing HP-β-CD and HP-γ-CD should give better separation for mixture of the selected triazole fungicides. For mixture of the selected triazole fungicides, peak signals in single CD separation above 30 mM chiral selector concentration gave broadened peaks and longer migration time. Accordingly in dual chiral selector system, total concentrations of the two chiral selectors were kept at a concentration of 30 mM. Ratio of HP-β-CD to HP-γ-CD used is summarized in Table 3.2.

Initially, dual chiral selector composition containing 15 mM HP-β-CD and 15 mM HP-γ–CD could only separate 9 peaks from the total of ten peaks. Bromuconazole gave three peaks from the four enantiomers. Separation using only HP-β-CD at a concentration of 30 mM gave four peaks of bromuconazole, for this

reason the concentration of HP-β-CD was increased to improve separation of bromuconazole. Thus, HP-β-CD concentration was increased, while HP-γ–CD was decreased. A 20 mM HP-β-CD and 10 mM HP-γ–CD was used, but only eight peaks appeared. Bromuconazole still gave three peaks, and one of them overlapped with one of the peak of cyproconazole enantiomer. Therefore the HP-β-CD concentration was increased to 25 mM to get a complete separation of bromuconazole enantiomers, while HP- γ–CD was decreased to 5 mM. This condition gave a complete separation for all the enantiomers of the studied fungicides and ten peaks appeared. However, R_s for one pair of bromuconazole enantiomer was still poor with $R_s < 1$ (last two peaks) (Figure 3.6). Further optimization was carried out by increasing HP-β-CD concentration and decreasing HP-γ-CD concentration step by step, keeping the CD concentration constant at 30 mM . A summary of the results obtained is shown in Table 3.2. At 1, 2, and 3 mM HP-γ-CD concentration, the R_s of the ten peaks were greater than 1.

Table 3.2 Summary of enantiomeric separation of selected triazole fungicides using CD-MEKC using dual CD system

[HP-β-CD] (mM)	[HP-γ-CD] (mM)	Result
15	15	nine peaks, only three peaks of bromuconazole was observed
20	10	eight peaks, three peaks of bromuconazole was observed and one of them overlap with a peak of cyproconazole enantiomer
25	5	ten peaks: complete separation, $R_s < 1$
26	4	ten peaks: complete separation, $R_s < 1$
27	3	ten peaks: complete separation, $R_s > 1$
28	2	ten peaks: complete separation, $R_s > 1$
29	1	ten peaks: complete separation, $R_s > 1$

Figure 3.6 shows that the peaks of diniconazole enantiomers appeared first followed by one pair of bromuconazole enantiomer, two pairs of cyproconazole enantiomers, and the last is one pair of bromuconazole enantiomer. This observation can be explained based on the hydrophobicities (log P) of each triazole fungicide.

Cyproconazole, bromuconazole, and diniconazole have a log P value of 2.9, 3.24, and 4.3, respectively. So, chromatographically if we have a stationary phase which is more hydrophobic, then the compound that have greater log P will be retained strongly and eluted later. In this study, SDS micelle and CD acts as the pseudostationary phase.

Micelle and CD share similar physicochemical properties, where the interior part of micelle or the CD cavity is relatively hydrophobic, while the outside part is more hydrophilic. Therefore, the larger the values of log P (more hydrophobic) of the solute, the greater its affinity towards the chiral selector (CDs) cavity and therefore strongly retained by micelle. From the log P data, diniconazole has a greater log P value, followed by bromuconazole, and cyproconazole. Thus the order of migration is expected to be cyproconazole, bromuconazole, and diniconazole. Since in this study, separation was conducted at acidic pH, a reversed of migration order was observed.

The electropherogram obtained at different HP-β-CD to HP-γ–CD ratio is shown in Figure 3.7. For diniconazole enantiomers, decrease of HP-γ–CD concentration correlate with a decrease of resolution. Whereas for bromuconazole enantiomers (last two peaks), when HP-γ–CD concentration was decreased, the resolution was increased.

The increase of chiral selector concentration caused the increase of analysis time. Since the cyclodextrins used are neutral, the increasing of analysis time at higher concentration of neutral CD may only be caused by the increasing of viscosity of the BGE. In this study, although the total contentration of CD was kept constant at 30 mM, but Figure 3.7 shows there is effect of the different ratio of dual CD on the analysis time. Initially, no significant difference for the analysis time on separation using 25 mM HP-β-CD:5 mM HP-γ–CD, 26 mM HP-β-CD:4 mM HP-γ–CD, and 27 mM HP-β-CD:3 mM HP-γ–CD, but after that the analysis time increase significantly. Figure 3.8 shows the plot of analysis time for all combination of dual CD concentration. However, the results for all combination of CD concentration give the analysis time below 24 minutes. This result proved that the method in this study

produce better analysis time separation for enantioseparation of diniconazole compared with previous study by Wu, *et al.* (2001).

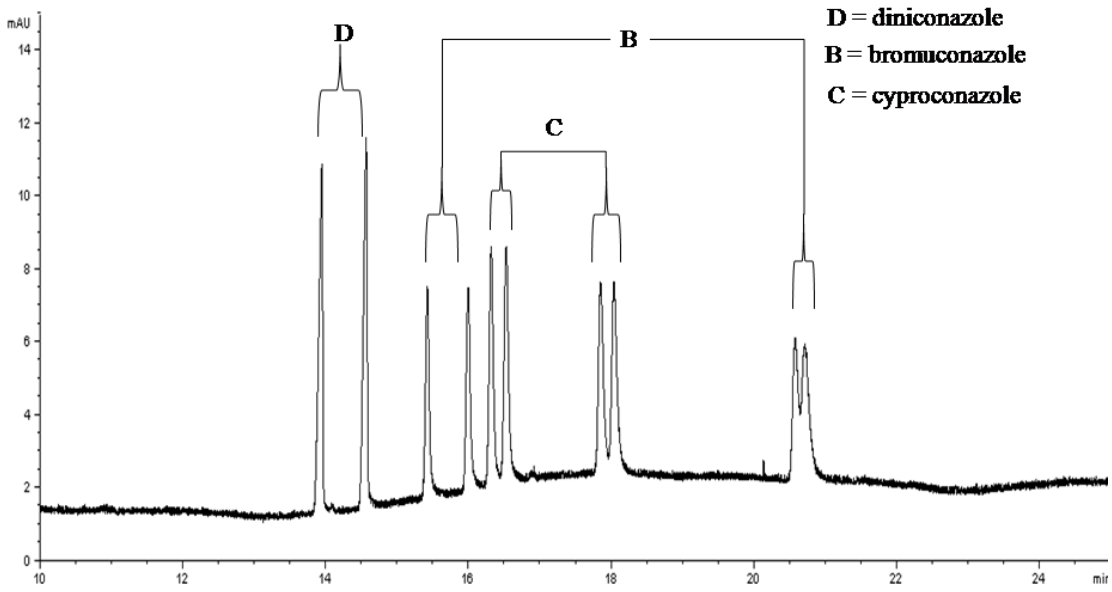

Figure 3.6 A complete separation of a mixture of triazole fungicides by CD-MEKC. Separation solution: 25 mM HP-β-CD and 5 mM HP-γ-CD; other condition as in Figure 3.1.

One of the most important facta to consider in a separation is resolution, R_s. Results for the resolution at various combinations of CD concentrations are summarized in Table 3.3. An increase in the HP-β-CD concentration increased resolution of bromuconazole enantiomers (P9 – P10). Furthermore, a decrease in HP-γ–CD concentration decreased resolution of diniconazole enantiomers (P1 – P2). The effects each CD on the separation in described in the next section (section 3.3.2.3). However, the best condition of separation was taken at 27 mM HP-β-CD : 3 mM HP-γ-CD because on this condition separation gave good R_s with relative short migration time compared to condition of 28 mM HP-β-CD : 2 mM HP-γ-CD and 29 mM HP-β-CD : 1 mM HP-γ-CD, which gave longer migration time.

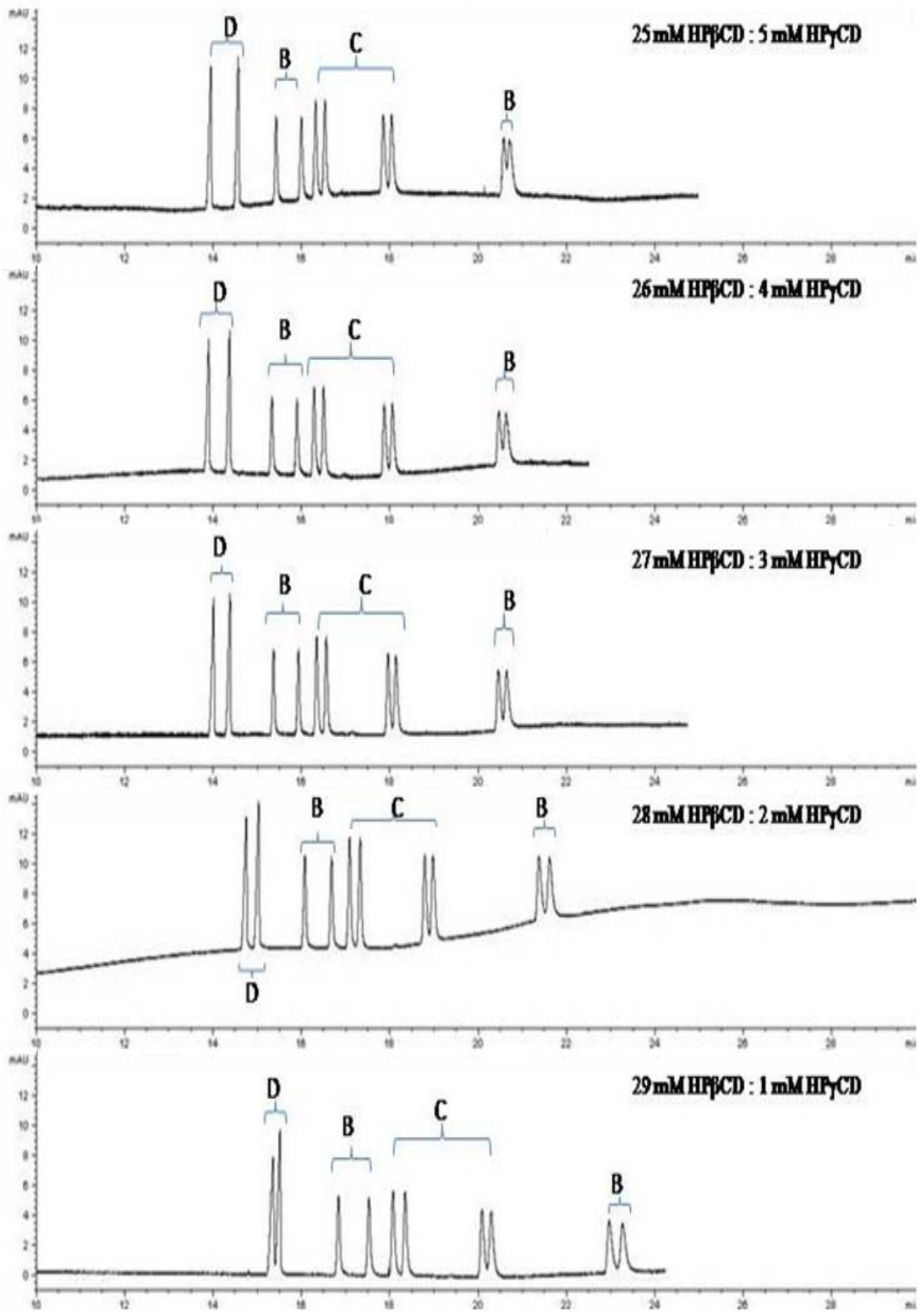

Figure 3.7 Enantioseparation of a mixture of triazole fungicides using CD-MEKC using different ratio of chiral selector concentrations. Separation solution: total concentration of chiral selector was kept constant at 30 mM; other condition as in Figure 3.1 (D: diniconazole peaks; B: bromuconazole peaks; C: cyproconazole peaks)

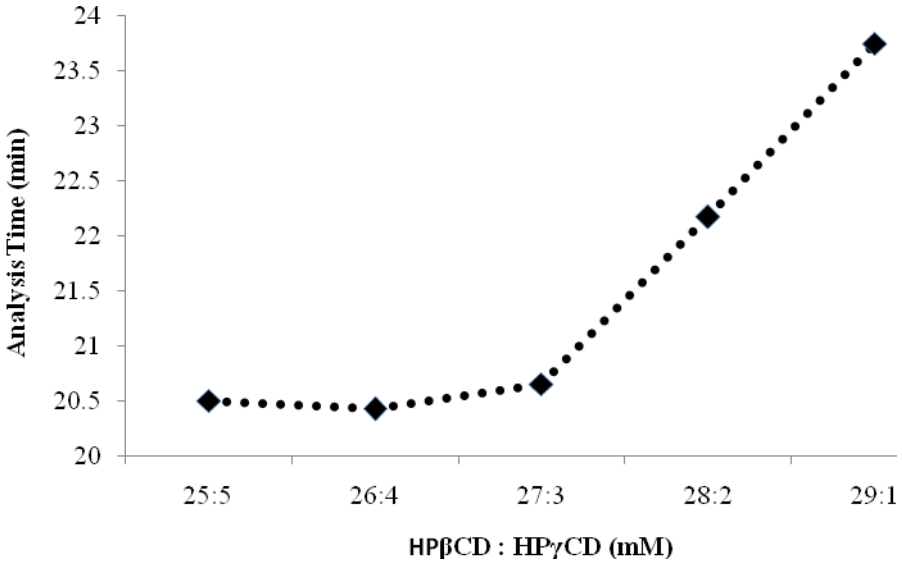

Figure 3.8 Trend of analysis time in enantioseparation of a mixture of triazole fungicides using CD-MEKC at various ratio of HP-β-CD : HP-γ-CD.

Table 3.3 Resolution of enantiomeric separation of selected triazole fungicides using CD-MEKC with dual cyclodextrin systems at various combinations of HP-β-CD : HP-γ-CD.

Peak	R_s				
	25 : 5 [a)]	**26 : 4** [a)]	**27 : 3** [a)]	**28 : 2** [a)]	**29 : 1** [ab)]
P1-P2	6.36	5.04	3.77	2.53	1.32
P2-P3	9.01	10.38	10.62	10.24	12.28
P3-P4	5.85	5.91	6.14	5.96	5.75
P4-P5	3.09	3.77	4.19	3.81	4.72
P5-P6	1.92	2.01	2.11	2.14	2.12
P6-P7	11.06	11.59	12.3	11.95	11.6
P7-P8	1.43	1.39	1.4	1.35	1.25
P8-P9	16.79	16.44	15.88	15.28	14.35
P9-P10	0.71	0.97	1.12	1.35	1.49

[a)]Ratio of [HP-β-CD]: [HP-γ-CD] (mM)

Despite of good resolution, repeatabilities of migration time, peak area, and peak height in terms of relative standard deviation (RSD) must be in the acceptable range. The concentration of each triazole fungicides used is 200 ppm. The maximum RSD value with analyte concentration in range 100-1000 ppm is 3.79 as proposed by EC document SANCO/3030/99 rev.4 11/07/00 (Biocides and Pesticides Unit, NY).

The RSD of migration time on Dual CD system for all peaks of selected triazole fungicides are shown in Table 3.4. As shown in Table 3.4 the RSD (%) for all combination of dual CD concentration used except for 29 mM HP-β-CD : 1 mM HP-γ-CD is lower than the maximum proposed. It's shown that the RSD for most of the combination of dual CD concentration is acceptable.

Table 3.4 The RSD (%) of migration time for all combination of dual CD concentrations used.

Peak	RSD (%, n = 3)				
	25 : 5 [a)]	**26 : 4** [a)]	**27 : 3** [a)]	**28 : 2** [a)]	**29 : 1** [a)]
1	0.3	0.6	1.1	1.1	3.8
2	0.3	0.6	1.1	1.1	3.9
3	0.1	0.4	1.0	1.2	1.7
4	0.1	0.4	1.0	1.3	4.7
5	0.2	0.4	1.0	1.4	5.1
6	0.3	0.4	1.0	1.5	5.2
7	0.5	0.6	0.8	1.4	6.0
8	0.5	0.6	0.8	1.4	6.1
9	0.9	0.8	0.7	1.5	6.9
10	0.9	0.8	0.7	1.6	7.0

[a)] Ratio of [HP-β-CD]: [HP-γ-CD] (mM)

Table 3.5 shows the RSD of peak height for all combinations of dual CD concentration. Good repeatability is only shown with 27: 3 HP-β-CD: HP-γ-CD (mM), where the RSD is lower than the maximum proposed. Furthermore, the RSD of peak area (Table 3.6), where none of the combination of dual CD concentration gives the RSD lower than maximum proposed. The RSD of peak area of 27: 3 of HP-β-CD: HP-γ-CD (mM) and 29: 1 HP-β-CD: HP-γ-CD (mM) is the most acceptable because the RSD is still around 4. From RSD values, the most acceptable combination of dual CD is concentration at 27: 3 of HP-β-CD: HP-γ-CD (mM).

Three of the combinations of CD concentration i.e. 26 mM: 4 mM, 27 mM: 3 mM, and 28 mM: 2 mM of HP-β-CD: HP-γ-CD gave good peak efficiency (N>200,000), as summarized in Table 3.7. The R_s and RSD values showed the optimum ratio of chiral selector concentration was obtained with HP-β-CD: HP-γ-CD at 27 mM: 3 mM. The efficiency, *N*, proved that the mixture of CD at 27: 3 of

HP-β-CD: HP-γ-CD (mM) as the best combination for simultaneous enantioseparation of the selected triazole fungicides.

The results obtained in this work shows that the use of dual CD on the separation of selected triazole fungicides can improve the separation. This results agree with the results obtained by other authors on the simultaneous separation of salbutamol and bupivacaine enantiomers (Wei, Guo, and Lin, 2006), and enantioseparation of pesticides namely imazaquin, diclofop, and imazamethabenz with the use of mixture of DM-β-CD and TM-β-CD (Penmesta, Leidy, Shea, 2007). Thus the use of dual native CD system can obviously improve separation and sensitivity.

Table 3.5 The RSD (%) of peak height for all combination of dual CD concentrations used.

Peaks	RSD (%, n = 3)				
	25 : 5 [a)]	**26 : 4** [a)]	**27 : 3** [a)]	**28 : 2** [a)]	**29 : 1** [a)]
1	2.5	5.6	3.4	5.6	3.0
2	3.8	7.9	0.4	5.8	3.9
3	1.1	4.6	3.5	7.9	10.7
4	0.9	4.3	2.2	8.3	11.1
5	3.0	2.9	2.1	12.1	10.1
6	2.5	2.3	1.4	11.9	10.0
7	1.3	3.4	0.9	11.2	14.5
8	5.5	2.3	0.8	9.0	10.1
9	1.1	3.7	2.7	8.0	11.9
10	2.0	4.3	1.4	8.5	10.3

[a)] Ratio of [HP-β-CD]: [HP-γ-CD] (mM)

Table 3.6 The RSD (%) of peak area for all combination dual CD concentration.

Peaks	RSD (%, n = 3)				
	25 : 5 [a)]	26 : 4 [a)]	27 : 3 [a)]	28 : 2 [a)]	29 : 1 [a)]
1	3.8	9.8	4.8	5.9	2.9
2	4.8	10.4	4.3	6.0	2.6
3	6.5	1.0	2.2	3.3	3.5
4	8.1	0.6	1.9	3.2	4.0
5	8.4	1.8	2.5	0.8	3.6
6	9.2	2.7	2.6	0.4	3.7
7	7.9	1.9	4.0	2.5	4.0
8	8.8	2.7	4.3	2.9	3.7
9	6.1	1.3	3.1	1.7	3.2
10	8.0	11.5	3.0	4.0	4.4

[a)] Ratio of [HP-β-CD]: [HP-γ-CD] (mM)

Table 3.7 Efficiencies (*N*) of selected triazole fungicide enantiomers with different ratio of [HP-β-CD]: [HP-γ-CD].

Peaks	**N**				
	25 : 5 [a)]	**26 : 4 [a)]**	**27 : 3 [a)*]**	**28 : 2 [a)]**	**29 : 1 [a)]**
1	311 955	328 297	314 862	264 328	196 013
2	371 348	408 427	356 499	324 395	296 964
3	422 714	423 686	474 461	420 558	291 184
4	410 561	417 267	457 636	416 323	280 471
5	367 637	375 924	410 862	382 712	268 330
6	358 074	366 186	404 811	388 427	257 551
7	310 254	314 489	343 688	318 549	186 197
8	293 641	288 067	303 227	275 988	204 520
9	238 369	268 626	263 630	257 204	175 722
10	150 006	207 136	228 254	220 372	163 155

[a)] Ratio of [HP-β-CD]: [HP-γ-CD] (mM); * Optimum condition

3.3.2 Optimization of MEKC Parameters for Chiral Separation of Selected Triazole Fungicide enantiomers

The simultaneous enantioseparation of cyproconazole, bromuconazole, and diniconazole using CD-MEKC was successfully achieved using 40 mM SDS, 25 mM phosphate buffer (pH 3.0), 27 mM HP-β-CD, 3 mM HP-γ–CD, and addition of 10%:5% methanol: acetonitrile gave R_s 1.1-15.9, peak efficiency N> 200 000 and separation time of less than 21 min. However the R_s 1.1 is still low, therefore further optimizations of several separation parameters to improve separation performance such as enhance R_s and efficiency was performed. In this study, parameters affecting enantioseparation of selected triazole fungicides such as pH, buffer phosphate concentration, SDS concentration, separation temperature, separation voltage and the use of organic modifiers were explored, so the R_s attainable is expected to be greater than 1.5.

3.3.2.1 Effect of Phosphate Buffer pH

In MEKC, the electrophoretic mobility of an analyte depends on the degree of ionization and the extent of solute-micelle interaction. The buffer pH determines the dissociation of the analytes thus affecting the solute-micelle interactions. The partitioning of the solute between free solutions and pseudostationary phase is also found to be pH dependent (Sekar and Azhaguvel, 2008). In this study, the investigation of separation at different buffer pH is expected to give improvement in the performance of separation, primarily in increasing the resolution. The effect of buffer pH on the chiral separation of propiconazole enantiomers was investigated at different pH, ranging from 7.0 to 9.0 (neutral and basic pH) and from 2.5 to 4.0 (acidic pH). The BGE consist of 27 mM HP-β-CD, 3 mM HP-γ-CD, 40 mM SDS, methanol-acetonitrile 10:5 % v/v, and 25 mM phosphate buffer.

The separation under neutral and basic pH was observed where detection was carried out at the cathodic end of the capillary. The electroosmotic flow (EOF) migrates toward the negative electrode owing to the negative charge of the capillary

surface, whereas the anionic SDS micelle is forced toward the positive electrode by electrophoresis. At neutral and basic pH, the EOF is larger than the electrophoretic migration, and therefore anionic SDS micelle also migrates toward the negative electrode at a retarded velocity (Otsuka and Terabe, 1996). Thus all enantiomers that are distributed in the micelle migrated towards the negative electrode. Enantiomeric separation of selected triazole fungicides at neutral and basic pH is shown in Figure 3.9. The increase in pH value caused an increase of EOF, resulting in the reduction of migration times of analyte and sharper peaks especially for the last two peaks of bromuconazole.

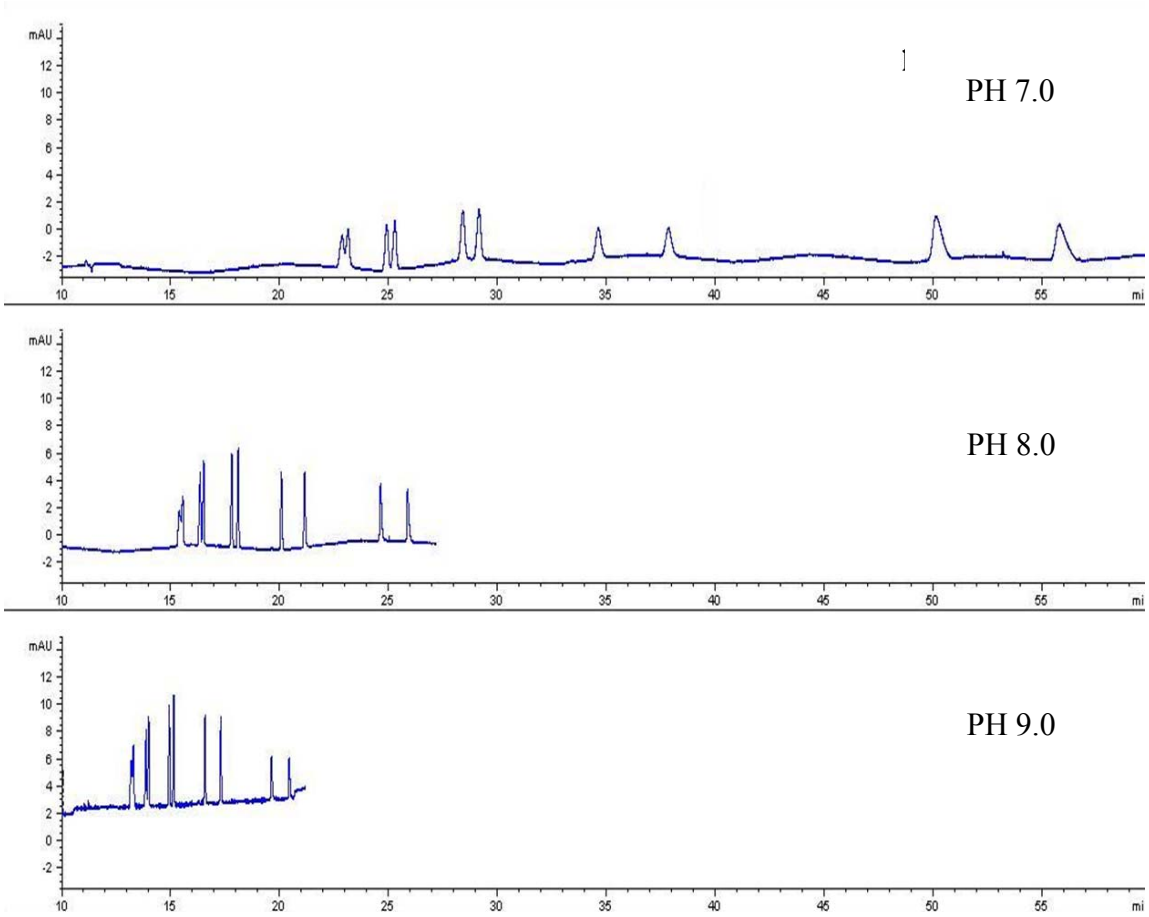

Figure 3.9 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at neutral and basic pH. Separation solution: 27 mM HP-β-CD, 3 mM HP-γ-CD, 40 mM SDS, methanol-acetonitrile 10%:5 % v/v, and 25 mM phosphate buffer; Applied voltage 25 kV, temperature 20˚C, pressure injection at 50 mbar for 1s.

Otherwise, the separation under acidic conditions was observed where detection was carried out at the anodic end of the capillary. Under acidic conditions, the EOF becomes smaller than the electrophoretic velocity of the SDS micelle and the micelle migrates towards the positive electrode (Otsuka and Terabe 1996). As

shown in Figure 3.10, increasing pH resulted in increasing migration times. Separation time at pH 4.0 was too long and therefore separation at pH > 4.0 was not attempted.

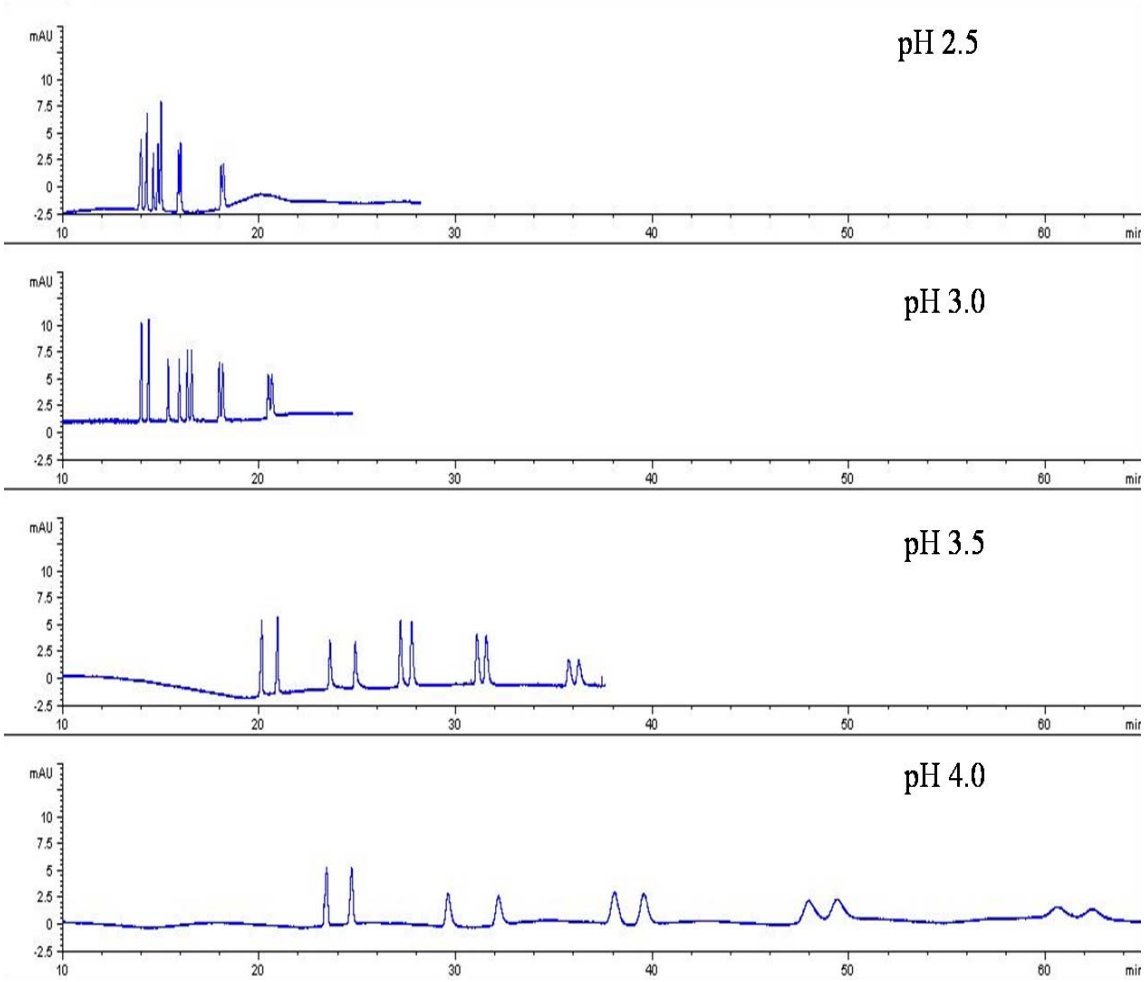

Figure 3.10 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at acidic pH. Composition of separation solution and other conditions as in Figure 3.9.

At neutral and basic pH, (Table 3.8) optimum R_s condition of enantioseparation of the selected triazole funicides was obtained at pH 8.0, while better efficiency of separation (Table 3.9) was obtained at pH 9.0. However at pH 8, resolution between peak 1 and peak 2 is less than 1. R_s of separation is summarized in Table 310 which shows that pH 3.5 is a good condition for the separation, but according to the efficiency (Table 3.11), good condition was obtained at pH 3.0. However, since repeatability of height, area, and migration times at pH 3.0 is better than at pH 3.5, pH 3.0 was chosen as the best condition for separation in acidic pH. Since R_s separartion at acidic pH is better than separation at basic pH, therefore

acidic pH is suitable for enantioseparation of selected triazole fungicides in this study.

Table 3.8 Resolution of separation of selected triazole fungicide enantiomers at neutral and basic pH values

Peak	R_S		
	pH 7.0	**pH 8.0***	**pH 9.0**
P1-P2	0.8	0.74	0.66
P2-P3	5.94	5.27	4.5
P3-P4	1.35	1.35	1.17
P4-P5	10.38	11.27	10.2
P5-P6	2.2	2.62	2.37
P6-P7	13.98	17.6	16.72
P7-P8	6.8	8.9	8.12
P8-P9	18.88	25.59	24.09
P9-P10	6.66	7.92	7.57

[a)] P1-P10 indicates peak number in the electropherogram

Table 3.9 Efficiencies (*N*) of selected triazole fungicide enantiomers separation at neutral and basic pH values

Peak[a)]	N		
	pH 7.0	pH 8.0	pH 9.0*
P1	59 797	61 915	118 554
P2	88 994	133 965	123 818
P3	121 252	256 435	261 447
P4	139 751	340 259	362 709
P5	118 524	344 476	413 599
P6	119 567	431 304	524 153
P7	96 980	430 781	569 009
P8	91 172	429 458	590 822
P9	62 821	405 917	575 853
P10	61 973	379 142	546 937

*Optimum sparation condition

Table 3.10 Resolution of separation of selected triazole fungicides enantiomers at various acidic pH values.

Peak	R_s			
	pH 2.5	**pH 3.0**	**pH 3.5***	**pH 4.0**
P1-P2	2.2	3.77	5.17	4.87
P2-P3	3.06	10.62	15.85	13.78
P3-P4	2.48	6.14	6.75	5.25
P4-P5	1.34	4.19	11.07	9.9
P5-P6	7.65	2.11	2.54	2.15
P6-P7	1.04	12.3	13.5	9.06
P7-P8	17.22	1.4	1.71	1.23
P8-P9	0.86	15.88	13.17	8.63
P9-P10	none	1.12	1.39	1.27

[a)] P1-P10 indicates peak number in the electropherogram
*Optimum sparation condition

Table 3.11 Efficiencies (*N*) of selected triazole fungicides enantiomers separation at various acidic pH values.

No of Peaks	*N*			
	pH 2.5	**pH 3.0***	**pH 3.5**	**pH 4.0**
1	118 009	314 862	262 181	139 713
2	230 768	356 499	283 626	133 362
3	415 419	474 461	262 823	72 272
4	296 632	457 636	248 386	58 359
5	237 267	410 862	251 770	53 454
6	335 258	404 811	234 031	47 796
7	358 205	343 688	222 583	29 013
8	291 314	303 227	192 537	28 310
9	253 376	263 630	165 699	28 443
10	none	228 254	153 834	31 452

3.3.2.2 Effect of Phosphate Buffer Concentration

The effect of phosphate buffer concentration on the resolutions was carried out by using different phosphate buffer concentration at pH 3.0 in the range of 15 to

50 mM. Enantioseparation of mixture of selected triazole fungicides using CD-MEKC with different buffer concentrations is shown in Figure 3.11. The increasing of buffer concentration caused the increasing in migration time of all enantiomers of the selected triazole fungicides. Furthermore, varying of buffer concentration gave various effects on resolution of each enantiomer. However, an increase in the concentration of buffer from 15 to 50 mM caused a significant increase in the R_s for each enantiomer pairs, i.e. diniconazole enantiomer pair (P1-P2), two pairs of bromuconazole enantiomers (P3-P4 and P9-P10), and two pairs of cyproconazole enantiomers (P5-P6 and P7-P8) as shown by shading in Table 3.12. The increasing of buffer concentration caused higher ionic strength of electrophoretic media, because of that the migration time and R_s should be increased (Zheng and Mo 2004). For all separation of selected triazole fungicides enantiomers, buffer concentration at 25 mM gave the optimum R_s.

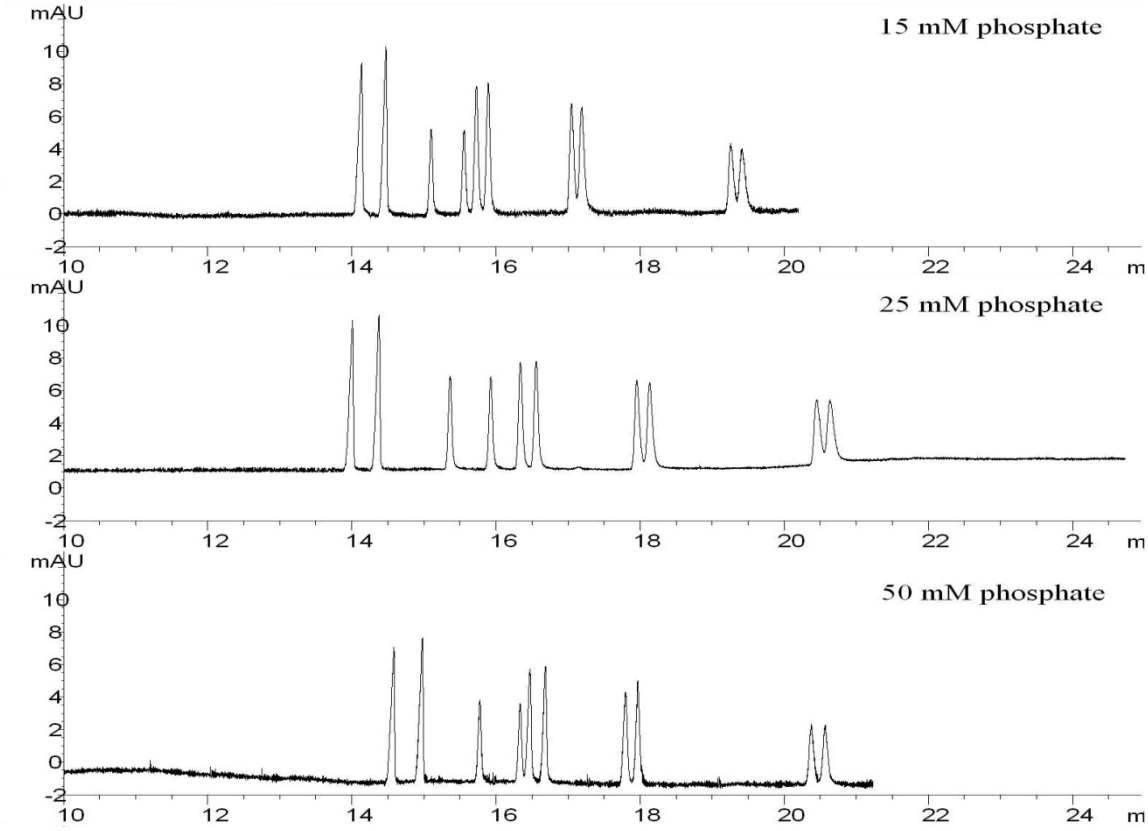

Figure 3.11 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at various phosphate buffer concentrations. Other conditions as in Figure 3.9.

Table 3.12 Resolution of enantiomeric separation of the selected triazole fungicides using CD-MEKC with dual cyclodextrin systems at various phosphate buffer concentrations.

R_s	Phosphate (mM)		
	15	25*	50
P1-P2	3.43	3.77	4.22
P2-P3	7.13	10.62	9.27
P3-P4	5.34	6.14	6.8
P4-P5	1.86	4.19	1.11
P5-P6	1.7	2.11	2.34
P6-P7	11.23	12.3	11.54
P7-P8	1.21	1.4	1.73
P8-P9	16.38	15.88	20.26
P9-P10	1.12	1.12	1.4

3.3.2.3 Effect of SDS Concentration

The SDS concentration effect on the enantioseparation of mixture of selected triazole fungicides was investigated by using 25 mM phosphate buffer pH 3.0 and optimum chiral selector concentration. SDS concentration was varied in the range 30 to 60 mM. Figure 3.12 shows the enantioseparation of selected triazole fungicides by CD-MEKC with different amounts of SDS. Increasing the concentration of SDS leads to decreasing in the migration time for all enantiomers. At acidic conditions, the SDS micelle and the micelle migrates towards the positive electrode by electrophoresis (Otsuka and Terabe 1996). Thus all enantiomers that are distributed in the micelle migrated towards the positive electrode faster with the increasing of SDS concentration.

Varying of the SDS concentration gave effect to the R_s of the separation as shown in Table 3.13. The increasing of the SDS concentration gave decreasing R_s for any enantiomer pairs, i.e. diniconazole enantiomer pair (P1-P2), two pairs of bromuconazole enantiomers (P3-P4 and P9-P10), and two pairs of cyproconazole enantiomers (P5-P6 and P7-P8). However for all enantiomers the best R_s were obtained at 40 mM SDS.

Table 3.13 Resolution of enantiomeric separation of selected triazole fungicides using CD-MEKC with dual cyclodextrin systems at various SDS cocentrations.

R_s	SDS (mM)			
	30	40*	50	60
P1-P2	4.6	3.77	2.23	2.06
P2-P3	6.75	10.62	5.6	5.39
P3-P4	6.91	6.14	3.44	3.3
P4-P5	1.05	4.19	2.16	2.49
P5-P6	2.43	2.11	1.18	1.14
P6-P7	10.91	12.3	8.06	7.7
P7-P8	1.75	1.4	0.94	0.88
P8-P9	19.08	15.88	11.14	10.95
P9-P10	1.56	1.12	0.85	0.8

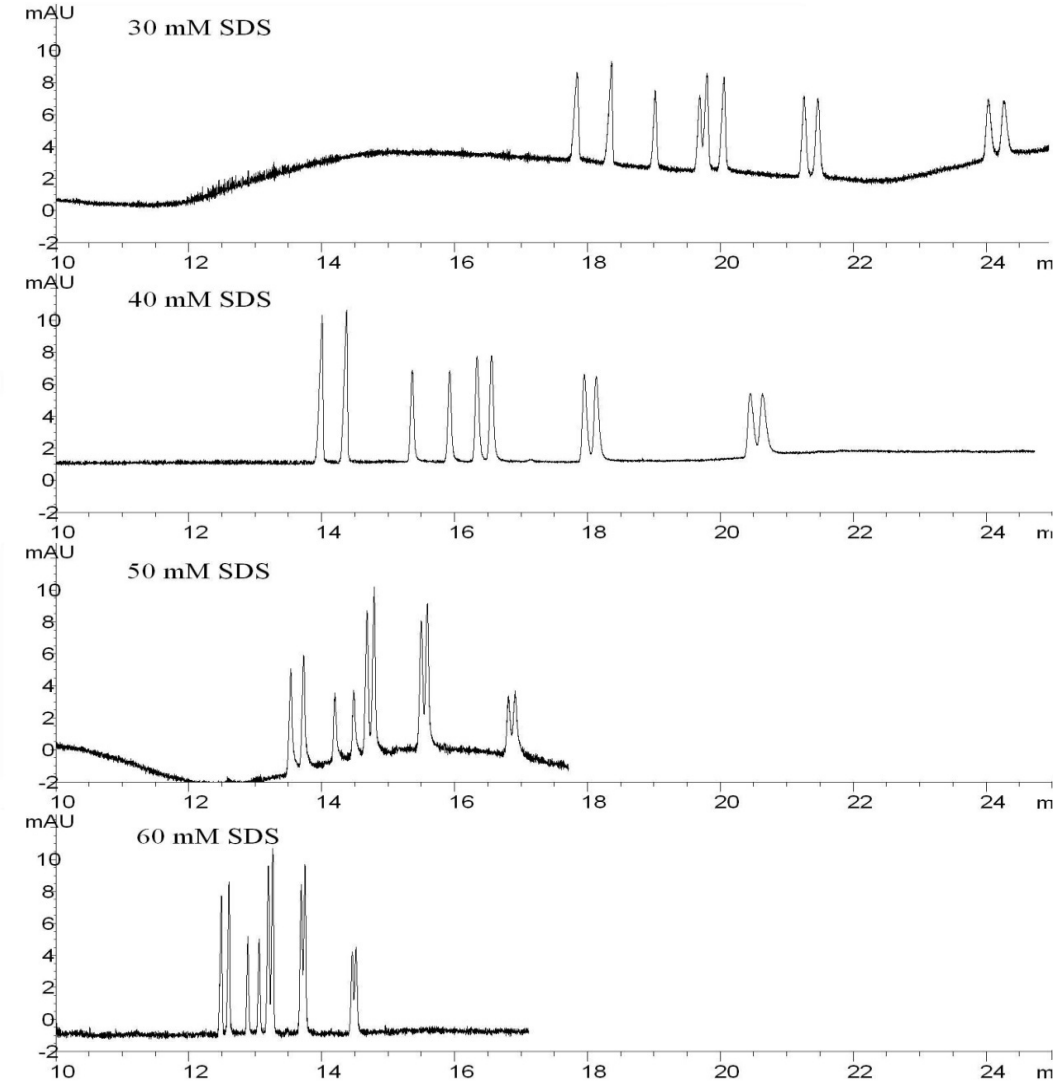

Figure 3.12 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at various SDS concentration (mM). Other conditions as in Figure 3.9.

3.3.2.4 Effect of Separation Temperature

The experiments for the study of effect of separation temperature on the enantioseparation of mixture of selected triazole fungicides were carried out by increasing the temperature from 15ºC to 30ºC. Results separation of selected by CD-MEKC with different temperature is shown in Figure 3.13. An increase in temperature causes a reduction in migration time owing to the decrease in the viscosity. Furthermore, distribution coefficient dependent on temperature, because of that temperature also affects selectivity although not significant (Terabe, 1993). However, incomplete separation is given by separation at 15ºC; only nine peaks observed. In the range of 20-30ºC, the separation was successfully achieved for all enantiomers of mixture of selected triazole fungicides.

Table 3.14 shows the resolution of separation at several temperatures. Separation at 15^{o}C, overlapping of peaks between P4 and P5 was observed. The best resolution was obtained at 20^{o}C, so this temperature was employed for all investigation in this study.

Table 3.14 Resolution of enantiomeric separation of selcted triazole fungicides using CD-MEKC with dual cyclodextrin systems at various temperatures.

R_s	Temperature (^{o}C)			
	15	20	25	30
P1-P2	4.09	3.77	3.79	3.37
P2-P3	6.2	10.62	10.29	11.02
P3-P4	4.31	6.14	5.7	5.31
P4-P5	~	4.19	3.8	4.52
P5-P6	1.54	2.11	1.88	1.66
P6-P7	11.44	12.3	11.86	12.25
P7-P8	1.64	1.4	1.29	1.18
P8-P9	21.29	15.88	17.63	15.47
P9-P10	1.78	1.12	1.02	0.7

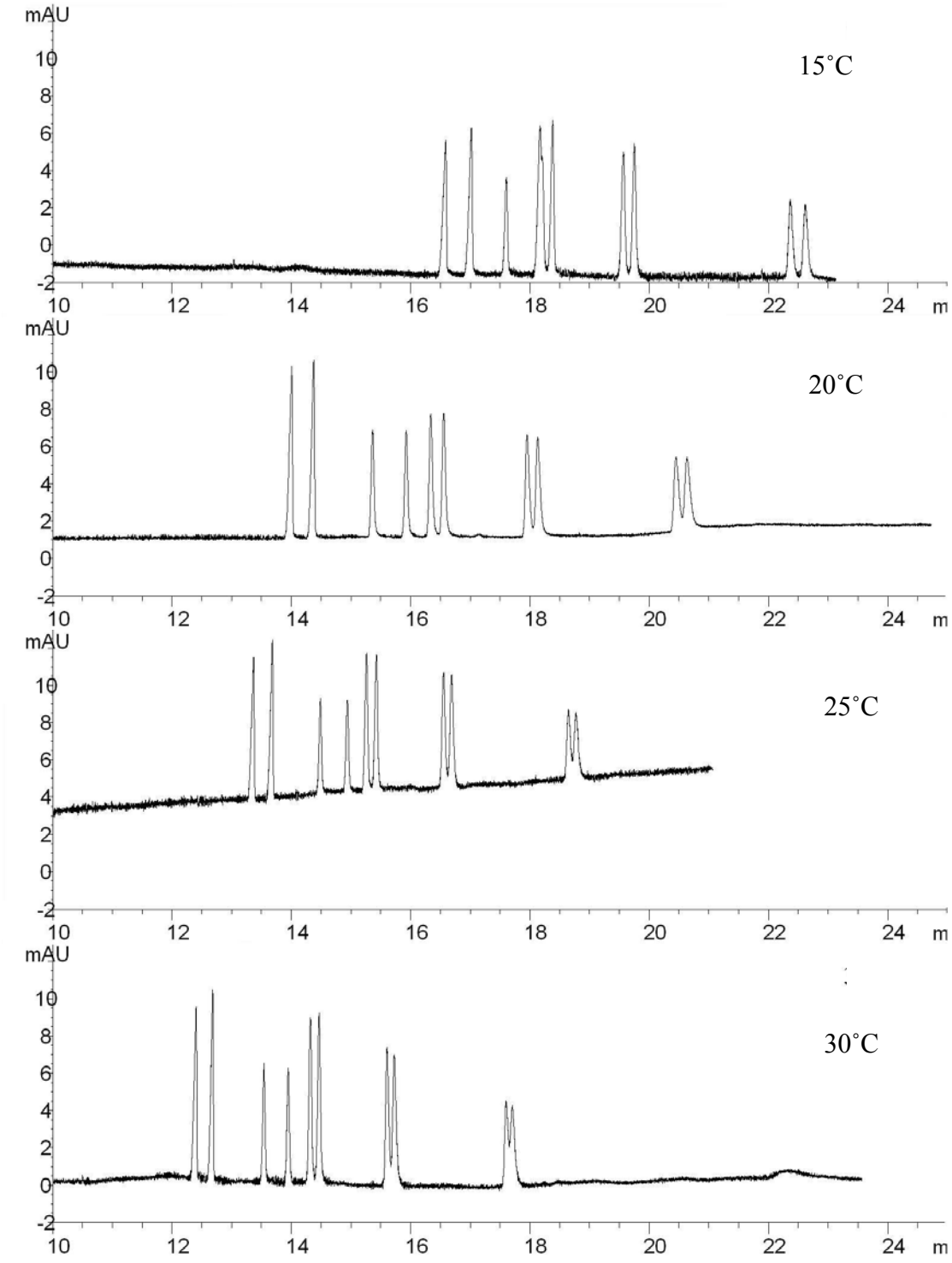

Figure 3.13 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at several temperatures. Composition of separation solution and other conditions as in Figure 3.9.

3.3.2.5 Effect of Separation Voltage

Electroosmotic and electrophoretic mobility are proportional to the field strength, where the use of the higher voltage possible will give the shorter times for separation. The effect of separation voltage on the enantioseparation of selected triazle fungicides was evaluated by varying the applied voltage in the range 20 kV to 30 kV. Figure 3.14 shows the enantioseparation of selected triazole fungicides at various applied voltage. Increasing the voltage reduces analysis time, for every increase 5 kV applied voltage gave the decrease in analysis time to 5 minutes.

The resolutions obtained for separation with different separation voltage are summarized in Table 3.15. The minimal R_s of separation at 30 kV is 0.97, whereas R_s at 25 kV is 1.12, R_s at 20 kV is 1.14. However optimum R_s generally is at 25 kV with relative short analysis time, therefore this separation voltage was selected for the next investigations.

Table 3.15 Resolution of enantiomeric separation of selcted triazole fungicides by CD-MEKC using dual cyclodextrin systems at various applied voltage.

R_s	Applied Voltage (kV)		
	20	25	30
P1-P2	3.62	3.77	3.23
P2-P3	6.92	10.62	7.37
P3-P4	5.84	6.14	4.54
P4-P5	1.14	4.19	1.93
P5-P6	1.87	2.11	1.55
P6-P7	10.49	12.3	9.82
P7-P8	1.39	1.4	1.14
P8-P9	18.17	15.88	15.41
P9-P10	1.26	1.12	0.97

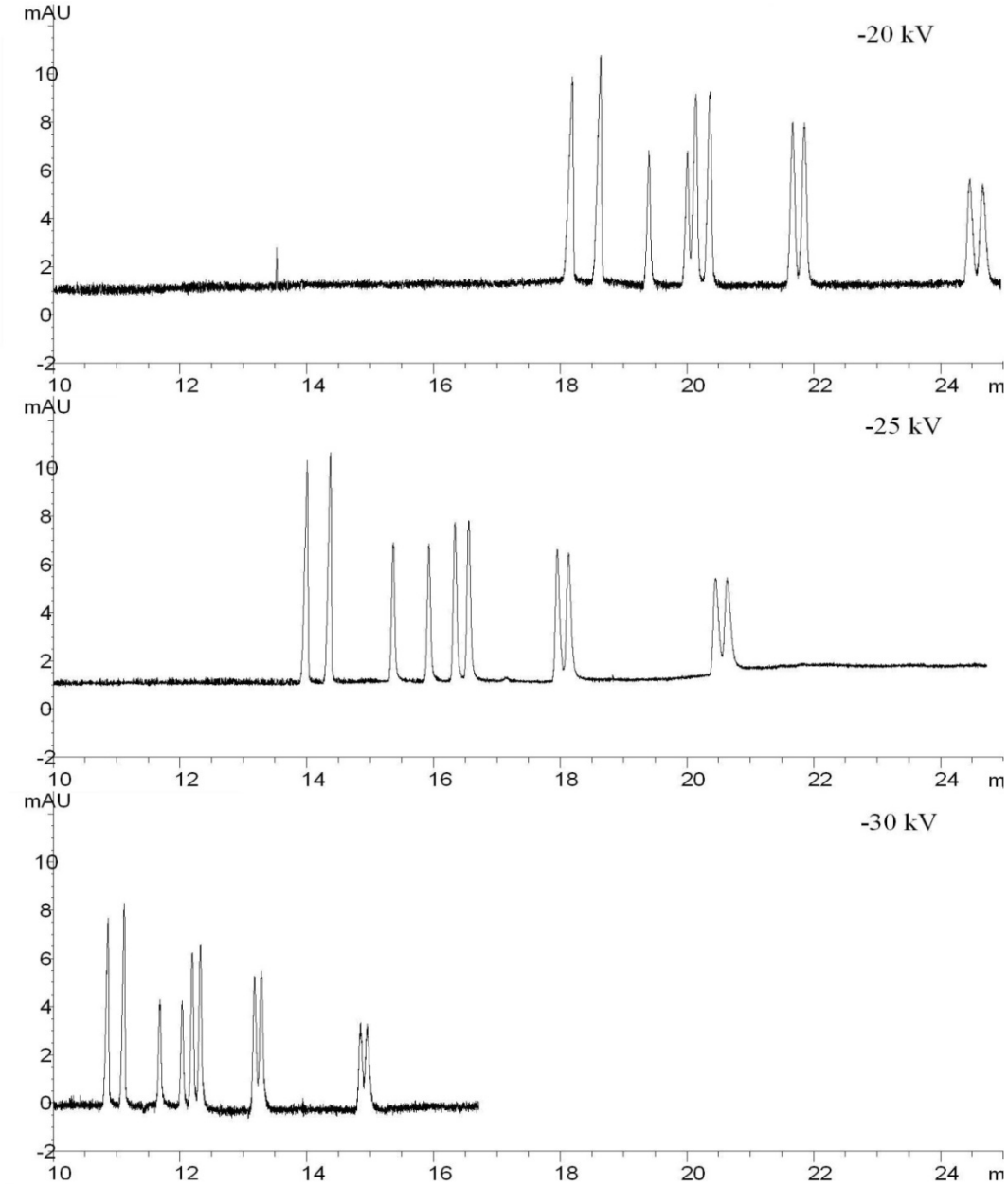

Figure 3.14 Enantioseparation of a mixture of triazole fungicides using CD-MEKC several applied voltages. Other conditions as in Figure 3.9.

3.3.2.6 Effect of Organic Modifier Addition on Separation

Several additives have been used for electrophoretic separation i.e. urea (Yoshinaga and Tanaka, 1995; Wu *et al.,* 2001; Bermudez and Forciniti, 2004), ion-pair reagents (Fanali, et al., 1998; Fanali, 2000), organic solvent (Bretnall and Clarke, 1995; Chen *et al.,* 1995; Van Hove *et al.,* 1996; Dworschak and Pyell, 1999; Zhou *et al.,* 2000; Zhou *et al.,* 2002; Klein *et al.,* 2005; Navarette-Casa *et al.,* 2005;

Denola *et al.,* 2008; Sekar and Azhaguvel, 2008; Kendler, 2009; Wan Ibrahim, et al., 2009;), metal salts (Terabe, 1993; Dworschak and Pyell, 1999) and currently is the addition of silica nanoparticle (Na, *et al*., 2006 and Nilsson and Nilsson, 2006; Wang *et al.,* 2009). However, organic solvents are the most widely used as additive for MEKC (Bretnall and Clarke, 1995; Chen *et al.,* 1995; Van Hove *et al.,* 1996; Dworschak and Pyell, 1999; Thorsteinsdo´ttir *et al.,* 1999; Sekar and Azhaguvel, 2008). Organic solvents are often used as additives to the aqueous buffer in MEKC to reduce the retention factors of strongly bound solutes to the micelles, useful in reducing the electroosmotic flow thus to extend the migration time window and or enhance selectivity (Thorsteinsdo´ttir et al, 1999; Molina and Silva, 2000).

Simultaneous enantioseparation of cyproconazole, bromuconazole, and diniconazole stereoisomers was successfully achieved with the addition of 10% Methanol and 5% acetonitrile as organic modifiers. However *Rs* for one pair of bromuconazole enantiomer was still poor with *Rs* < 1.2. In the absence of any organic modifier, enantioseparation of mixture of selected chiral triazole fungicide produce nine peaks observed (Fig 4.7). A peak overlapped was observed at the fifth peak, i.e between second bromuconazole (B2) and second cyproconazole (C2) enantiomers. Thus the effect of the addition of several different alcohols and acetonitrile on the electrophoretic separation was explored to further increase resolution.

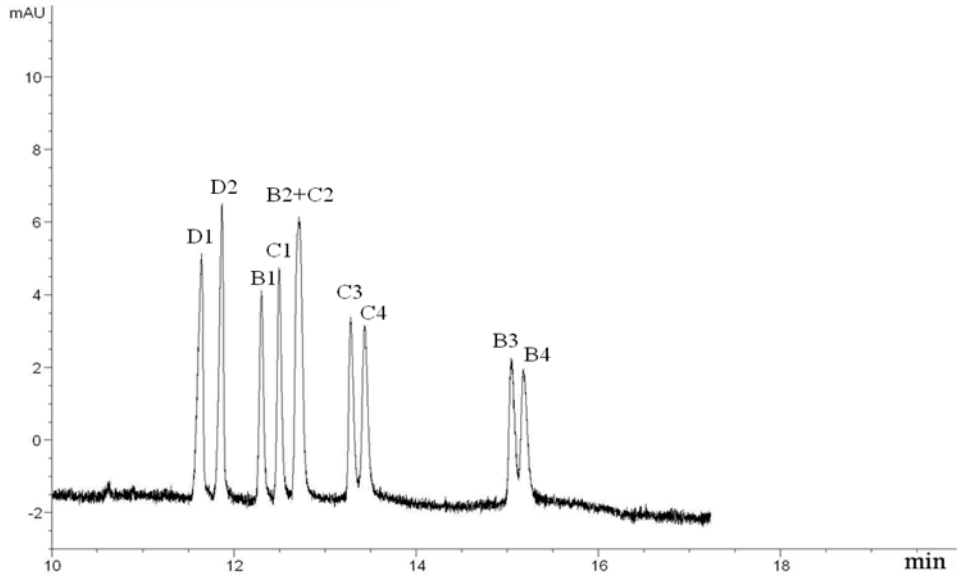

Figure 3.15 Enantioseparation of a mixture of triazole fungicides by CD-MEKC without organic modifiers. Other Conditions: as in figure 3.9.

3.3.6.1.1 Effect of Several Alcohols Addition

Effect of several alcohols namely methanol (MeOH), ethanol (EtOH), *n*-propanol (*n*-PrOH), and *i*-propanol (*i*-PrOH) on the enantioseparation of selected triazole fungicides with dual CD was investigated by using p to 20% level of each alcohol in 5% increments. Generally, an increase addition of several alcohols, namely MeOH, EtOH, *n* -PrOH, and *i*-PrOH, caused the increasing of migration time. The effects of alcohols addition on analysis time are shown in Figure 3.16.

Alcohols used as modifiers in this study are highly polar alcohols, and they are known as typical aqueous phase modifiers, remain mainly outside the micelles (Vanhove, et al., 1996; Lopez-Grio, *et al.,* 1998). Nevertheless, the addition of these alcohols interacts with the micelle surface and has an effect on micelle formation, where formation-destruction of the SDS micelles is highly accelerated by these alcohols (Onori and Santucci, 1992; Lopez-Grio *et al.,* 1998; Berthod and Coque, 2000). Thorsteinsdo´ttir *et al.* (1999) reported that an increase in the several organic modifiers concentration caused an increase of the critical micelle concentration value (CMC); this result in suppression of micelles formation. At acidic conditions, the SDS micelle and the micelle migrate towards the positive electrode by electrophoresis (Otsuka and Terabe, 1996)**.** Thus all stereoisomers that are distributed in the micelle migrated towards the positive electrode faster with increasing SDS concentration owing to higher formation of micelle. On contrary, fewer SDS micelles formation due to high percentage of organic modifier concentration in BGE tends to increase the migration time. However, SDS concentration added was highly more than the CMC value (8.1 mM) (Terabe, 1993)**.** In this study 40 mM SDS was used, so the presence of SDS was more than enough to form micellar phase.

The alcohol molecules incorporate in the micelles with their polar hydroxyl groups in the stern layer and their alkyl chain in the micelle cores (Berthod and Coque, 2000). As a consequence, analyte distribution in micellar phase decreased because of the presence of competition from organic modifier. Thus analytes migrate

slowly. Figure 3.16 shows effect of several alcohols on migration time. As the organic modifier concentration increased, the migration time of the analyte increased due to the increase in viscosity of BGE. More hydrophobic compounds tend to be completely distributed to the micelles (Thorsteinsdo´ttir *et al.,* 1999), therefore alcohols with larger alkyl groups should competes strongly to incorporate in the micelles, so analytes migrate slowly and thus migration time increased e.g. 20% (v/v) MeOH gave a migration time for the last peak at around 27 min, 20% (v/v) EtOH gave a migration time of the last peak at around 31 min, and 20% (v/v) *n*-PrOH gave a migration time for the last peak at more than 35 min. These results prove with the effect of viscosity of each organic modifier, where the higher viscosity value of organic modifiers gave longer migration time. The order of viscosity is *n*-PrOH > EtOH > MeOH (Nikam, *et al.,* 1998), because of that the addition of *n*-PrOH caused longer migration compared to EtOH and MeOH. The use of 20% (v/v) *i*-PrOH in the BGE concentration resulted in lower migration time (< 32 min) compared to *n*-PrOH (> 35 min). This could be due to the fact that *n*-PrOH is more hydrophobic in nature than *i*-PrOH, where the presence of branch in *i*-PrOH molecule caused more water soluble (Fessenden and Fessenden, 1978).

Table 3.16 sumarizes the R_s of enantioseparation of selected triazole fungicides at various percentages of methanols. At the addition of 5% methanol, only 9 peaks were observed (Figure 3.17). There was overlapping between peak 5 and peak 6 (P5-P6). The separation with 15% methanol as organic separation produces Rs 0.95-18.27, with analysis time less than 24 minutes. Figure 3.18 shows the electropherogram of enantioseparation of selected triazole fungicides at various ethanol percentages. Figure 3.18 shows that the addition the addition of 5 and 10% EtOH only produced nine peaks. There was overlapped between P5 and P6, whereas at 10% EtOH peak overlapped was between P4 and P5. R_s of enantioseparation at several percentages of EtOH are summarized in Table 3.17. Over all, the best separation is given by using 15% EtOH with relative short analysis time (< 25 minutes) and range R_s varying from 1.21 to 22.28.

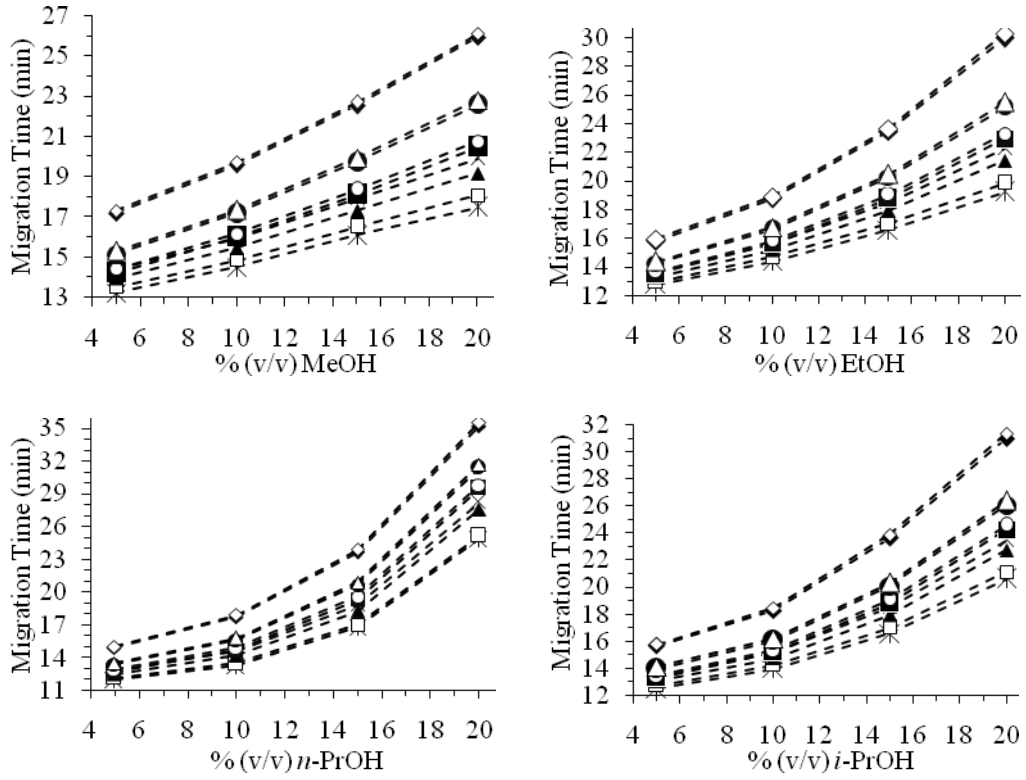

Figure 3.16 Effect of several organic modifiers on migration time of triazole fungicides. ✳ = D1; □ = D2; ▲= B1; ✕ = B2; ■ = C1; ○ = C2; ● = C3; △ = C4; ◆ = B3; ◇ = B4. (D= diniconazole, B = bromuconazole, C = cyproconazole).

Table 3.16 Resolution of enantiomeric separation of selcted triazole fungicides using CD-MEKC with dual cyclodextrin systems at various % v/v of MeOH.

R_s	MeOH (% v/v)			
	5	10	15	20
P1-P2	3.09	3.5	4.12	4.77
P2-P3	5.83	6.25	7.95	8.75
P3-P4	3.64	4.59	6.07	4.26
P4-P5	2.52	0.58	1.85	3.01
P5-P6	*	1.77	2.15	1.48
P6-P7	8.24	9.5	11.3	8.52
P7-P8	1.59	1.6	1.62	1.06
P8-P9	16.61	17.49	18.27	14.78
P9-P10	1.26	1.05	0.95	0.71

*Overlapped peak

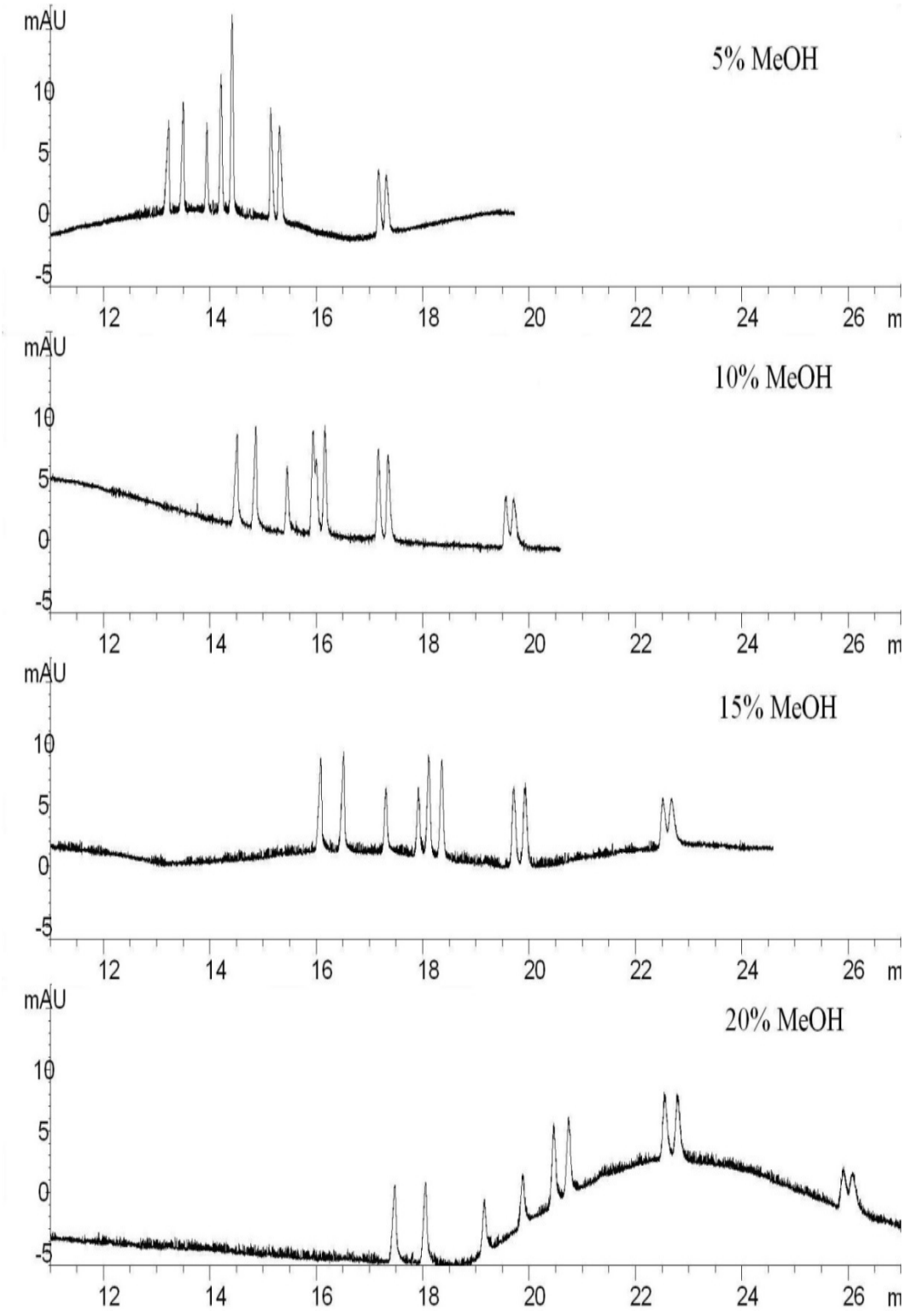

Figure 3.17 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at various MeOH percentages. Other conditions as in Figure 3.9.

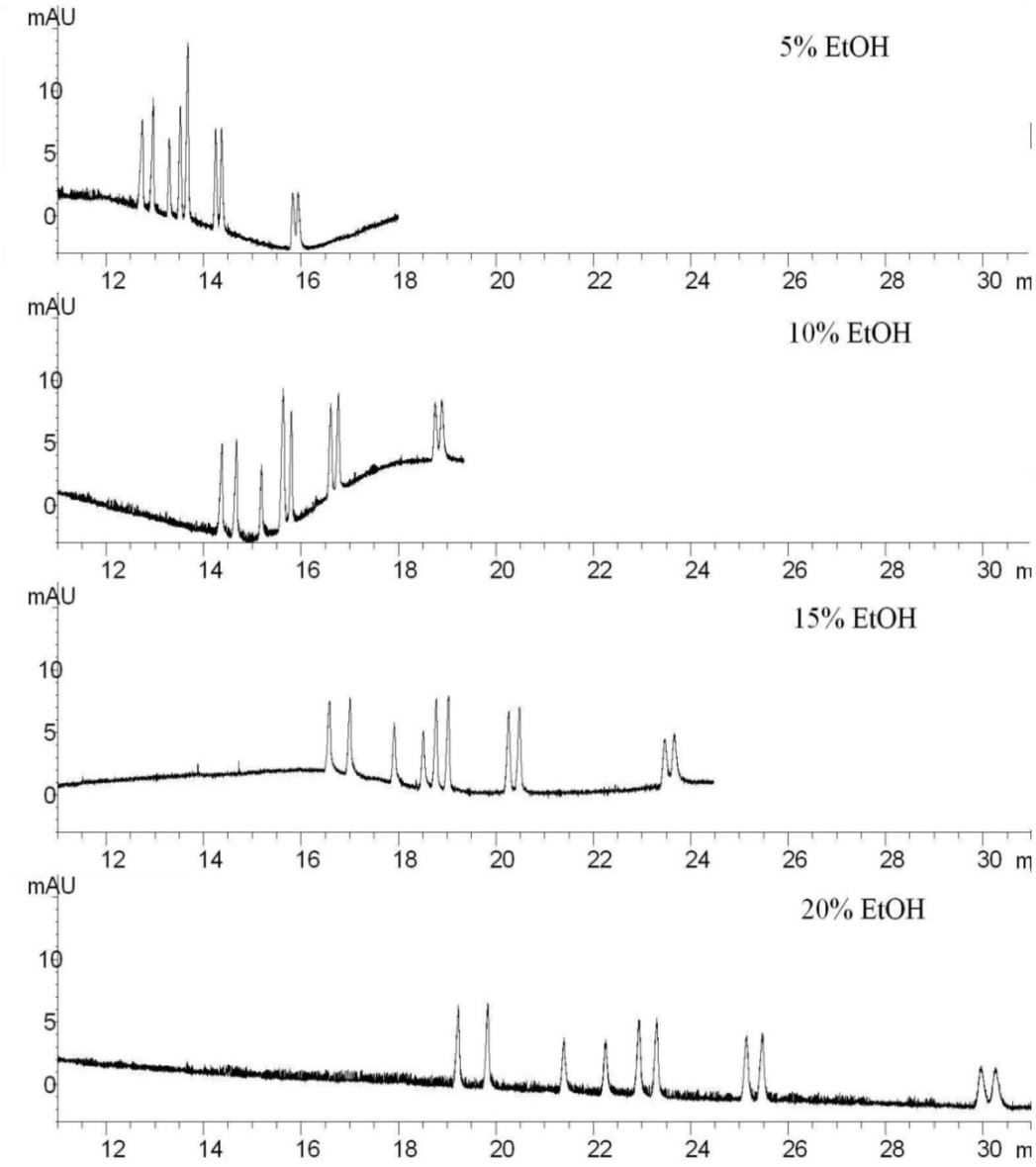

Figure 3.18 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at various EtOH percentages. Other conditions as in Figure 3.9

Enantioseparation of mixture of selected triazole fungicides with addition of *n*-PrOH gave complete separation in the range of 10-20 % (Figure 4.11). Similar to the addition of 5% MeOH, the addition of 5% *n*-PrOH gave nine peaks due to the peak overlapped between P5 and P6. R_s of separation were obtained at various percentages of *n*-PrOH (Table 3.18). The best separation with relative short analysis time and good resolution is given by using 15% *n*-PrOH. The addition of 20% *n*-PrOH also showewd good resolution, but longer analysis time and gave peak produced.

Table 3.17 Resolution of enantiomeric separation of selcted triazole fungicides using CD-MEKC with dual cyclodextrin systems at various percentages of EtOH.

R_s	EtOH (%)			
	5	10	15	20
P1-P2	2.41	3.24	4.22	5.02
P2-P3	4.29	6.06	9.21	12.44
P3-P4	2.89	4.68	5.86	6.49
P4-P5	1.81	~	2.55	4.83
P5-P6	~	1.75	2.4	2.6
P6-P7	6.7	9	11.2	12.53
P7-P8	1.43	1.66	1.93	2.07
P8-P9	15.48	18.8	22.28	23.83
P9-P10	1.05	1.13	1.21	1.21

Table 3.18 Resolution of enantiomeric separation of selected triazole fungicides using CD-MEKC with dual cyclodextrin systems at various percentages of *n*-PrOH.

R_s	*n*-PrOH (%)			
	5	10	15	20
P1-P2	2.27	2.52	2.51	2
P2-P3	4.13	6.58	10.96	12.83
P3-P4	2.69	3.68	4.47	3.19
P4-P5	1.28	0.96	4.03	6.94
P5-P6	~	2.07	2.25	1.68
P6-P7	4.49	7.79	8.91	7.95
P7-P8	1.34	1.78	1.87	1.53
P8-P9	15.77	17.23	16.41	16.56
P9-P10	1.18	1.23	1.16	1.16

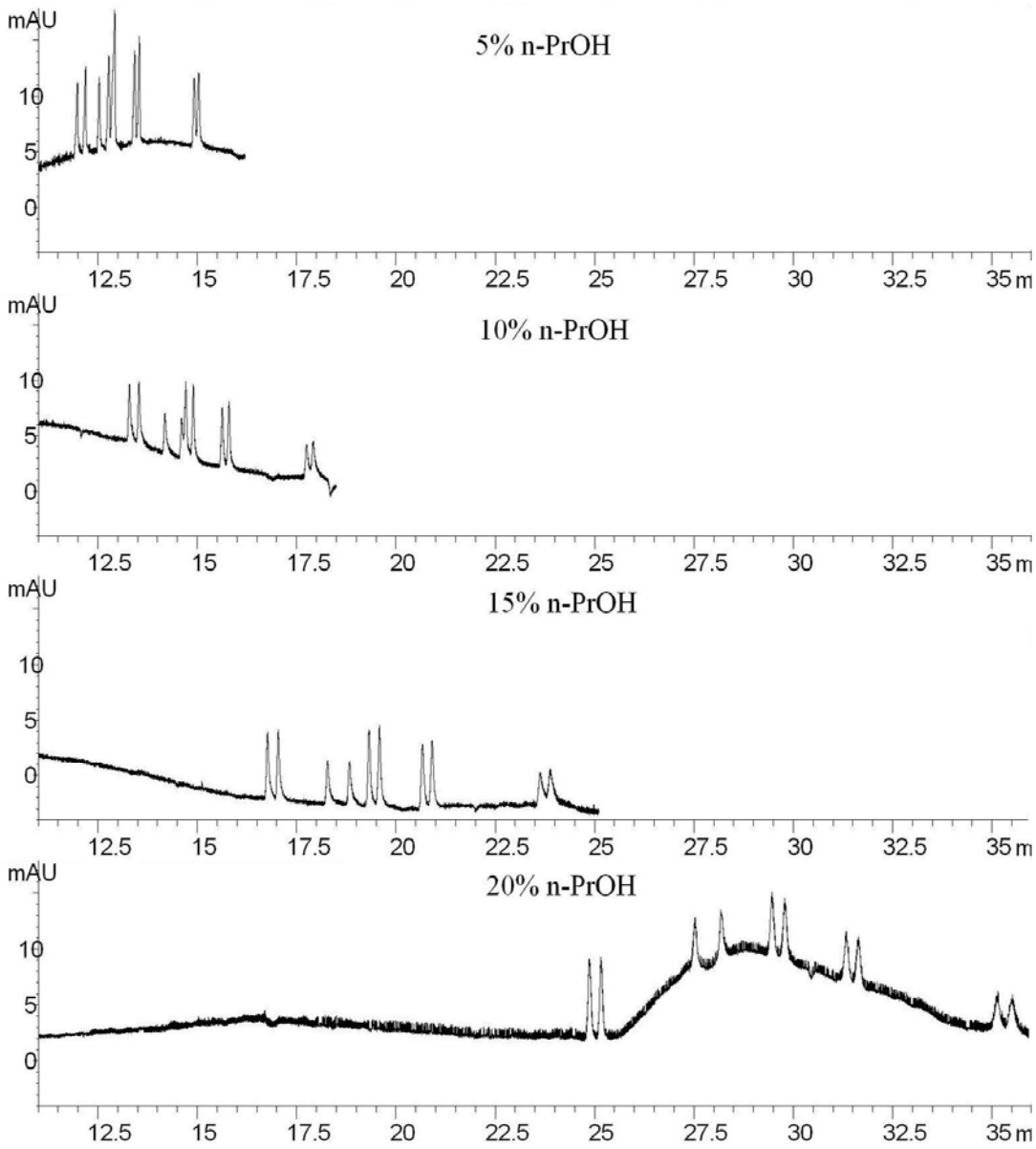

Figure 3.19 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at various percentages of *n*-PrOH. Other conditions as in Figure 3.9

Figure 3.20 shows the enantioseparation from the addition of *i*-PrOH was successfully achieved after the addition 15% *i*-PrOH. The addition of 5% *i*-PrOH gave overlapping peaks at P5, whereas the addition of 10% *i*-PrOH gave overlapping peaks at P4. Resolution of enantioseparation of selected triazole fungicides usng CD-MEKC with dual cyclodextrin systems at various percentages of *i*-PrOH are shown in Table 3.19. The addition of 15% *i*-PrOH and 20% *i*-PrOH gave good resolution

(R_s >1.7). The addition of 15% *i*-PrOH was selected as optimum condition based on peak sharpness shorter analysis time.

Investigation of various kind and percentages of alcohol gave optimum at 15%, except the addition of MeOH, where the resolution obtained was better than in the absence of alcohol. However, addition of 15% *i*-PrOH gave best resolution (R_s >1.7), relatively short analysis time (<25 minutes) and the best efficiency (N > 400,000) (Table 3.20).

Table 3.19 Resolution of enantiomeric separation of selected triazole fungicides usng CD-MEKC with dual cyclodextrin systems at various percentages of *i*-PrOH.

R_s	*i*-PrOH (%)			
	5	10	15*	20
P1-P2	2.63	3.3	4.28	4.88
P2-P3	4.14	5.77	9.88	14.64
P3-P4	2.9	4.67	5.56	5.88
P4-P5	1.74	~	2.87	6.29
P5-P6	~	1.91	2.61	2.76
P6-P7	5.88	7.61	9.95	10.43
P7-P8	1.5	1.68	2.23	2.22
P8-P9	17.58	19.95	26.31	26.3
P9-P10	1.15	1.26	1.74	1.82

Table 3.20 Efficiency, *N* of selected triazole fungicides enantioseparation at the best several alcohols addition.

No of Peaks	alcohol addition		
	15% EtOH	15% n-PrOH	15% i-PrOH
P1	411725	378423	511122
P2	491759	411831	535927
P3	515126	377674	530555
P4	512945	341635	504678
P5	506776	443151	514164
P6	542015	478129	626088
P7	481268	407042	522192
P8	516680	421240	585957
P9	373379	217900	439963
P10	343753	169225	411760

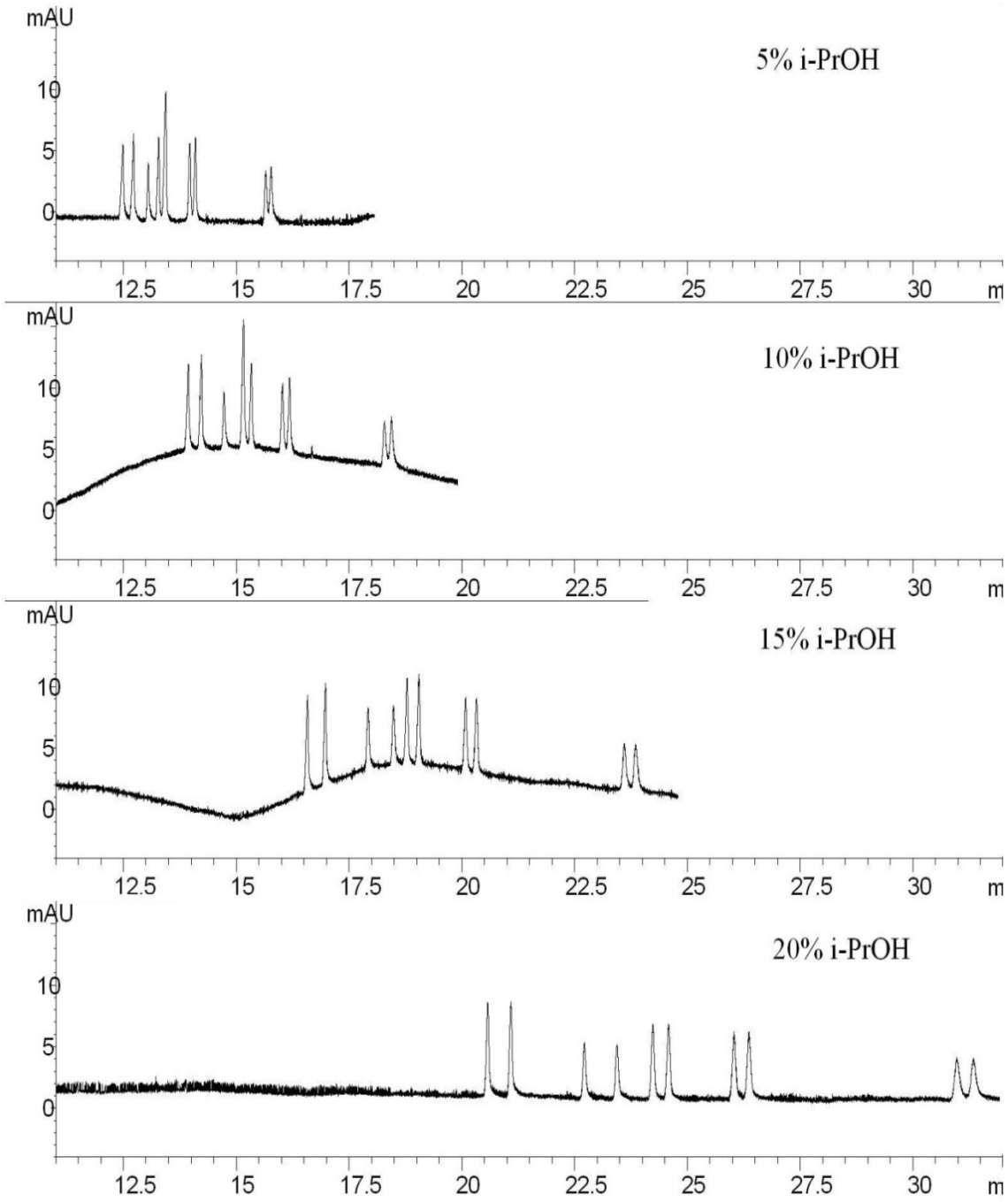

Figure 3.20 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at various percentages of *i*-PrOH. Other conditions as in Figure 3.9

3.3.6.1.2 Effect of Acetonitrile Addition

Acetonitrile is used widely as additive in MEKC. Effect of acetonitrile addition on enantioseparation of selected triazole fungicides was caried out at

optimum BGE concentration and varying acetonitrile percentage in the range 5-20%. Figure 3.21 shows results obtained in the presence of of various percentages of acetonitrile. Similar to the addition of several alcohols, the increasing of percentage of acetonitrile in the BGE tends to longer the analysis time (Figure 3.21). Figure 3.22 show that acetonitrile (AcN) produces shorter migration time (25 min) for several percentages compared to alcoholic solvents (27 to 35 min). As reported by Sekar and Azhaguvel (2008), shorter migration time was observed in the presence of AcN compared with alcoholic solvent caused by AcN as a result of it's tendency to form solvent clusters rather than hydrogen bonding in aqueous buffer.

Thorsteinsdo´ttir *et al.* has proposed a hypothesis that the mobility of SDS micelles decreased at higher acetonitrile content due either to smaller average size or to stronger binding of counter ions to the micelles, as the dielectric constant of the buffer decreased. Resolutions of separation at various percentages of acetonitrile are shown in Table 3.21. There is an optimum acetonitrile concentration (15%, v/v), which gave resolution in the range 1.32-16.67 and relatively short analysis time (< 25 minutes).

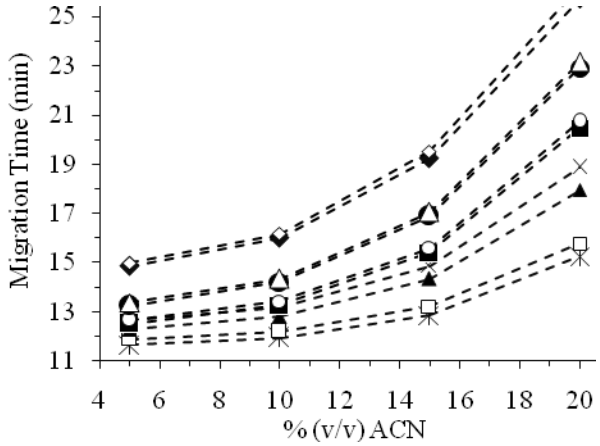

Figure 3.21 Effect of acetonitrile addition on migration time of triazole fungicides. ✳ = D1; □ = D2; ▲= B1; ✕ = B2; ■ = C1; ○ = C2; ● = C3; △ = C4; ◆ = B3; ◇ = B4. (D= diniconazole, B = bromuconazole, C = cyproconazole).

Table 3.21 Resolution of enantiomeric separation of selected triazole fungicides using CD-MEKC with dual cyclodextrin systems at various percentages of AcN.

R_s	AcN addition (%)			
	5	10	15	20
P1-P2	2.64	3.2	4.2	4.4
P2-P3	5.23	7.85	13.38	16.48
P3-P4	3.41	5.2	6.19	5.05
P4-P5	2.1	0.82	5.51	11.09
P5-P6	~	1.92	2.02	1.72
P6-P7	7.75	9.89	11.91	11.49
P7-P8	1.48	1.43	1.32	1.02
P8-P9	16.48	17.75	16.67	10.05
P9-P10	1.31	1.68	2	1.53

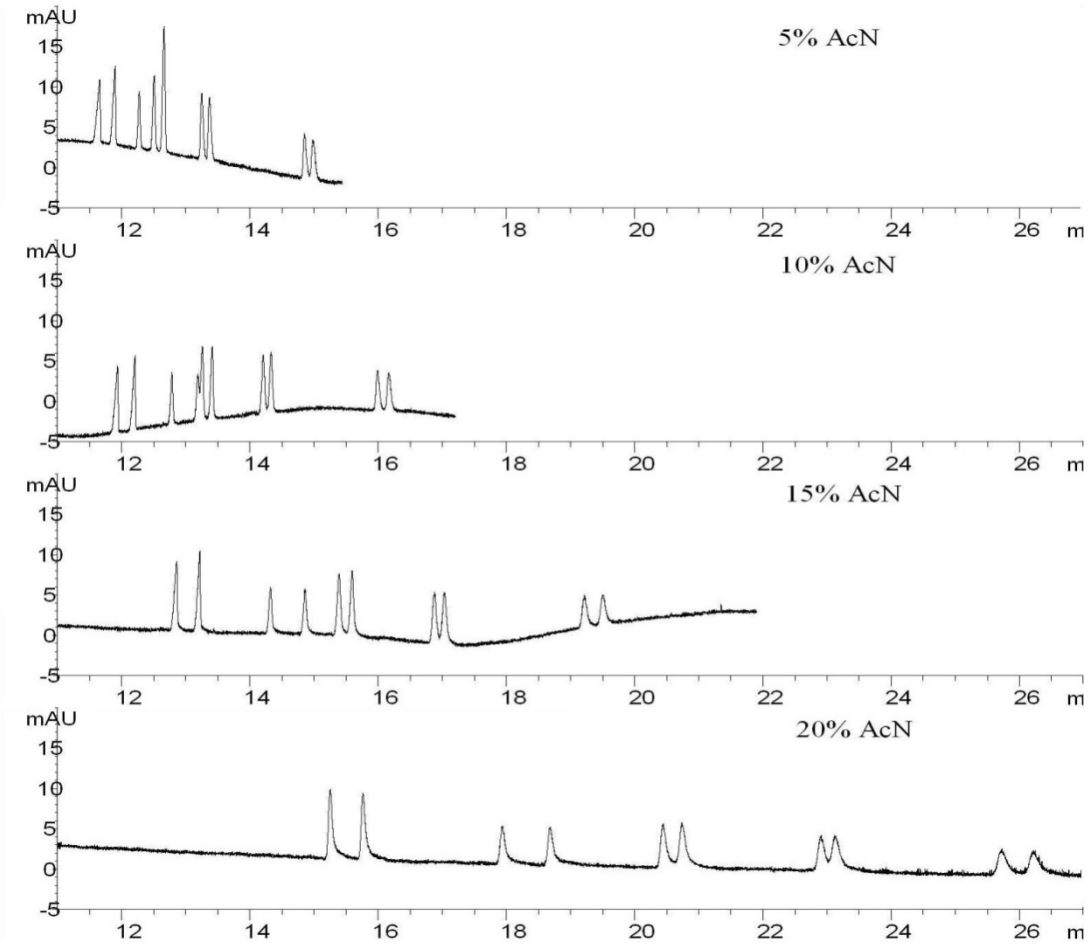

Figure 3.22 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at various percentages of AcN. Separation solution: total concentration of chiral selector was kept constant at 30 mM; other conditions as in Figure 3.9

3.3.6.1.3 Effect of Mix Modifier Addition

The effect of modifier was also investigated at the mixture of organic solvent i.e acetonitrile and methanol. The addition of mixed methanol-acetonitrile was varied from 5%:5% to 10%:10% (v/v). The addition of 5%: 5% v/v MeOH: AcN gave 9 peaks, whereas the other mixed MeOH-AcN gave the separation successfully (Figure 3.23). The higher percentage of organic solvent increased the analysis time. As proposed by Thorsteinsdo´ttir *et al.* (1999) te presence of acetonitrile effect lead to decrease of micellar phase formation. This result corroborates the results of SDS effect concentration, where lower concentration of SDS lead to fewer micelle formation and longer analysis time. Resolutions of separation using mixed MeOH-AcN as organic modifier is shown in Table 3.23. Optimum separation using mixed organic modifier was achieved at 5%: 10% v/v MeOH : AcN, where the resolution is more than 1.52.

Table 3.22 Resolution of enantiomeric separation of selected triazole fungicides using CD-MEKC with dual cyclodextrin systems at various addition of AcN-MeOH.

R_s	%MeOH : % can			
	5:5	10:5	5:10	10:10
P1-P2	3.59	3.77	4.35	5.36
P2-P3	7.97	10.62	12.36	14.88
P3-P4	6.42	6.14	6.5	6.4
P4-P5	~	4.19	4.55	8.65
P5-P6	2.27	2.11	2.21	2.03
P6-P7	11.66	12.3	12.95	13.78
P7-P8	1.75	1.4	1.52	1.35
P8-P9	20.21	15.88	18.76	17.15
P9-P10	1.63	1.12	1.99	1.75

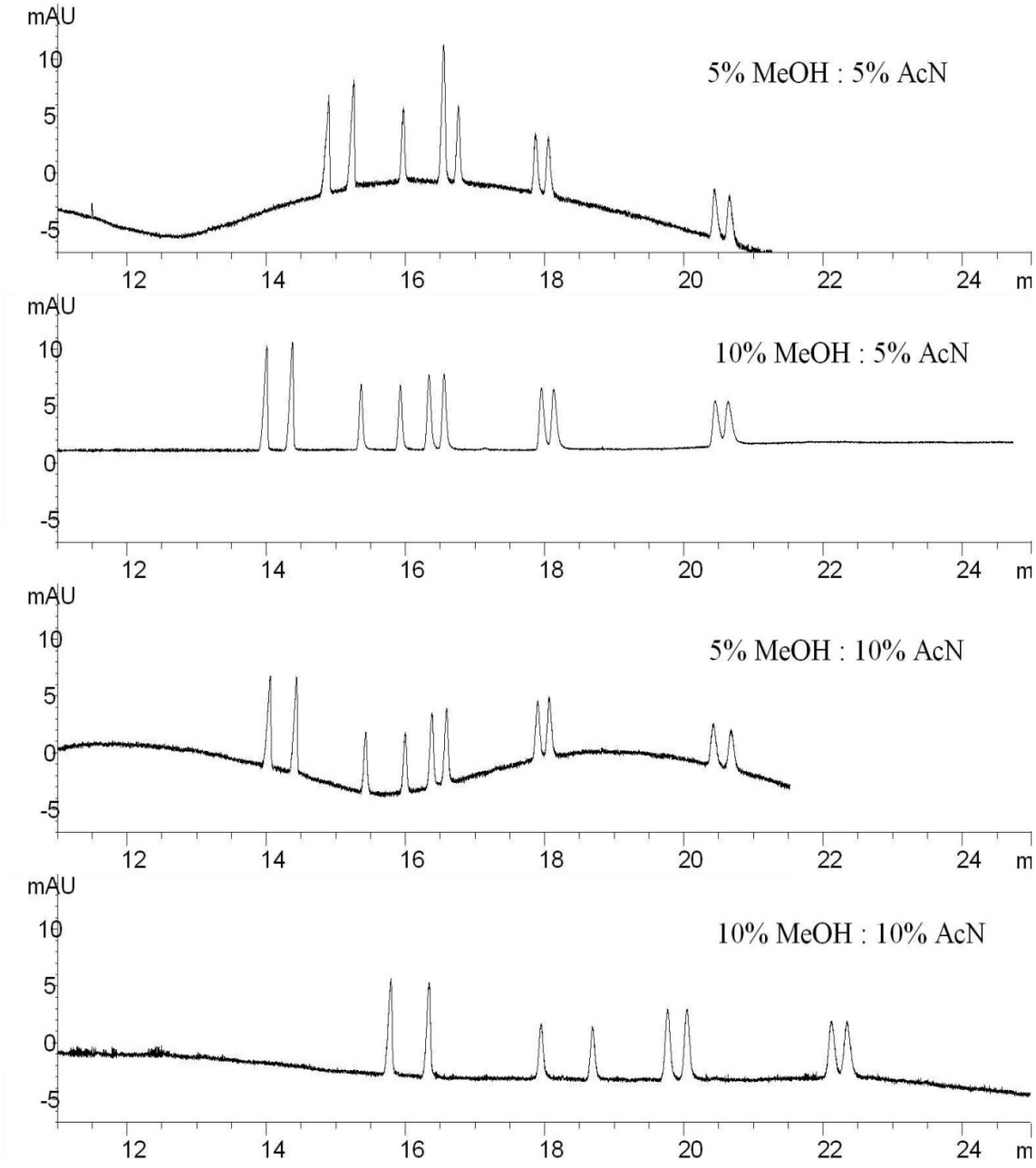

Figure 3.23 Enantioseparation of a mixture of triazole fungicides using CD-MEKC at various mixture of MeOH-AcN additions (%). Other conditions as in Figure 3.9

3.3.6.1.4 The Optimized CD-MEKC Method Performance

The effect of organic modifier addition on resolution can either improve or decrease depending on the type of analyte studied and whether the chiral selector concentration (Fanali, 2000 and Eeckhaut et *al.*, 2004). Several papers reported that

the addition of organic modifiers can improve resolution (Zhou et al., 2000; Zhou *et al.,* 2002; Wan Ibrahim, *et al.*, 2007). In this study, the addition of organic solvent improved the separation. Investigation of several organic solvent addition show optimum separation achieved at the addition of 15% *i*-PrOH, with Rs 17.4-26.31 and relative short analysis time (<25 minutes). It is supported by good separation efficiency, N > 400,000. Furthermore, the enantioseparation of the selected triazole fungicides by using 15% of *i*-PrOH as organic modifiers gave good repeatabilities in the migration time, peak area and peak height were obtained ranging from 0.97% to 1.46%, 2.02% to 5.01% and 0.81% to 3.46%, respectively. EC document SANCO/3030/99 rev.4 11/07/00 the expected RSD values with analyte concentration < 100 ppm is 3.79 (Biocides and Pesticides Unit, NY). As shown in Table 3.23 the RSD (%) is lower than maximum proposed.

Table 3.23RSD of enantioseparation of selected triazole fungicides using CD-MEKC with dual cyclodextrin systems at optimum separation.

Peak	RSD (%), n=3		
	t_M	Peak Area	Peak Height
1	0.97	5.01	3.46
2	0.99	4.89	3.38
3	1.07	2.42	1.47
4	1.10	2.45	2.81
5	1.19	2.39	0.95
6	1.21	2.03	2.24
7	1.27	4.63	2.76
8	1.29	3.34	1.73
9	1.44	2.02	0.81
10	1.46	4.71	1.73

The linearity was measured by constructing the calibration curve of average peak height (n = 3) against the concentration of standards ranged from 50 to 200 mg/L. Calibration lines are linear with the correlation coefficient higher than 0.993 for all enantiomers as summarized in Table 3.24. The LOD was determined by the calibration curve along with the signal-to-noise ratio (S/N) as 3. Slightly poor detectability was observed, i.e 9-15 mg L^{-1} for all enantiomers. It was known that CE technique suffers from poor concentration sensitivity when using UV detection because of the small injection volumes and narrow optical path length.

Table 3.24 Linierity and LOD of enantioseparation of selcted triazole fungicides by CD-MEKC using dual cyclodextrin systems at optimum separation.

	Calibration Curve		LOD
Peak[a]	Equation	R^2	(mg L^{-1})
1	y = 0.0300x + 0.4084	0.9940	15.05
2	y = 0.0316x + 0.2545	0.9949	13.81
3	y = 0.0277x + 0.0876	0.9940	15.08
4	y = 0.0277x + 0.0715	0.9978	9.14
5	y = 0.0330x + 0.4045	0.9958	12.62
6	y = 0.0337x + 0.2405	0.9960	12.33
7	y = 0.0290x + 0.2603	0.9968	11.05
8	y = 0.0294x + 0.2326	0.9976	9.54
9	y = 0.0234x - 0.0476	0.9939	15.12
10	y = 0.0223x + 0.1095	0.9978	9.01

[a]1 - 10 are first to tenth-migrating peaks of simultaneous enantioseparation of selected triazole fungicides, respectively.
[b]Linear range: 50 – 200 mg/L; *y* = peak height; *x* = concentration (mg L^{-1})

3.4 Concluding Remarks

The simultaneous enantioseparation of cyproconazole, bromuconazole, and diniconazole using CD-MEKC was achieved using dual mixture of HP-β-CD and HP-γ–CD. Three neutral cyclodextrins namely hydroxypropyl-β-cyclodextrin (HP-β-CD), hydroxypropyl-γ-cyclodextrin (HP-γ-CD) and γ-cyclodextrin (γ-CD) were tested as chiral selectors at different concentrations ranging from 10 to 40 mM. Effect of several parameters was investigated, i.e pH, phosphate buffer concentration, SDS concentration, temperature separation, applied voltage and organic modifiers addition. However the addition of organic solvent can improve the resolution of the ten selected triazole fungicides enatiomers. At the separation solution with a composition of 40 mM SDS, 25 mM phosphate buffer (pH 3), 27 mM HP-β-CD, 3 mM HP-γ–CD, and the addition of 15 % *i*-PrOH as organic modifier gave the best separation with R_s > 1.7, efficiency, N > 400 000, where highly improve than previous study [20], and separation time less than 25 minutes. The performance of the optimized MEKC using dual CD system gave good linearity (R^2 > 9.993) and repeatability (RSD 0.81-5.01%), therefore considered as an alternative for the chiral analysis of cyproconazol, bromuconazole, and diniconazole.

CHAPTER 4

ENANTIOSEPARATION OF HEXACONAZOLE, PENCONAZOLE, MYCLOBUTANIL, AND TRIADIMEFON WITH HP-γ-CD AS CHIRAL SELECTOR

4.1 Introduction

The separation of enantiomers has become one of the most important fields of modern analytical chemistryespecially for pharmaceutical or agrochemical products since the stereochemistry has a significant influence on the biological activity. Chromatographic methods have been used for enantiomer separations, where chiral stationary phase or chiral columns are often used to achieve optical recognitions (Ellington, *et al.,* 2001; Aboul-Enein and Ali, 2003). Several chiral additives to mobile phases are also used instead of chiral stationary phases in LC (Wang, *et al.,* 2008b). However, the use of chiral stationary phase as well as chiral additives in chromatography method is usually expensive since large amounts of stationary phases or additives are required. Recently, an increasing number of studies have been published concerning the electrophoretic separations of chiral compounds (Grobuschek, *et al.,* 2002; Zhu, *et al.,* 2002; Otsuka, *et al.,* 2003*;* Chankvetadze, *et al.,* 2004; Nevado, *et al.,* 2005; Wan Ibrahim, *et al.,* 2007).

MEKC has shown to be a powerful separation technique for the separation of enantiomers (Schmitt, *et al.,* 1997; Otsuka and Terabe, 2000). The inherent ability of MEKC to provide high separation efficiencies combined with rapid method development and minimal use of expensive chiral reagents makes it an ideal technique for enantiomer separations. Two modes of enantiomers separation are mainly used, namely MEKC using chiral surfactants, and MEKC using CD as chiral selector (CD-MEKC) (Nishi and Terabe, 1996). In CD-MEKC separation mode, the migration behaviors of individual enantiomers are determined by their competitive

distributions into the three "phases" (water, CD, and micelles). The addition of CD to the buffer displaces the distribution of the analytes from the micellar to the water phase as a function of the possible interaction between the water soluble CD and the analytes.

Triazole compounds represent the most important category of fungicides to date. It has excellent protective, curative, and eradicate power toward a wide spectrum of crop diseases. Hexaconazole, penconazole, myclobutanil and triadimefon are commonly used as systemic agricultural fungicides. They have an asymmetrically substituted carbon atom and each compound has two stereoisomers (Fig. 6.1). The baseline enantioseparation of hexaconazole enantiomers was obtained by supercritical fluid chromatography (Torbio, *et al.,* 2004) and normal-phase HPLC method (Wang, *et al.,* 2005). The enantiomeric separation of 14 triazole fungicides including hexaconazole, penconazole, myclobutanil, and triadimefon had been performed using sulfated-b-CD-mediated CE (Wu, *et al.,* 2001). In their work, enantioseparations of most enantiomers were successfully achieved. However, the last enantiomeric peaks of hexaconazole and penconazole were overlapped, with a run time of 36 min for myclobutanil and the LODs were not discussed. To the best of our knowledge, there is no MEKC study being reported on the simultaneous enantioseparation of selected triazole fungicides by CD-MEKC method.

In our previous work (Wan Ibrahim, *et al.,* 2007), a CD-MEKC method for the separation of two enantiomeric pairs of propiconazole was developed. The use of a mixture of 30 mM 2-hydroxypropyl-γ-CD (HP-γ-CD), 50 mM SDS and methanol/ACN 10:5 v/v in 25 mM phosphate buffer solution (pH 3.0) was able to separate two enantiomeric pairs of propiconazole with resolution (Rs) greater than 1.50, peak efficiencies (N) greater than 400 000 and analysis time of less than 16 min. The method used in the current study was adopted from previous study (Wan Ibrahim, *et al.,* 2007) as these selected fungicides belong to the same class of triazole fungicide as propiconazole. The focus of the current work is to obtain a baseline separation of all enantiomers of these three triazole fungicides in a much shorter time than the one previously reported by Wu et al. (Wu, *et al.,* 2001) using sulfated-β-CD, which is a more expensive chiral selector than HP-γ-CD.

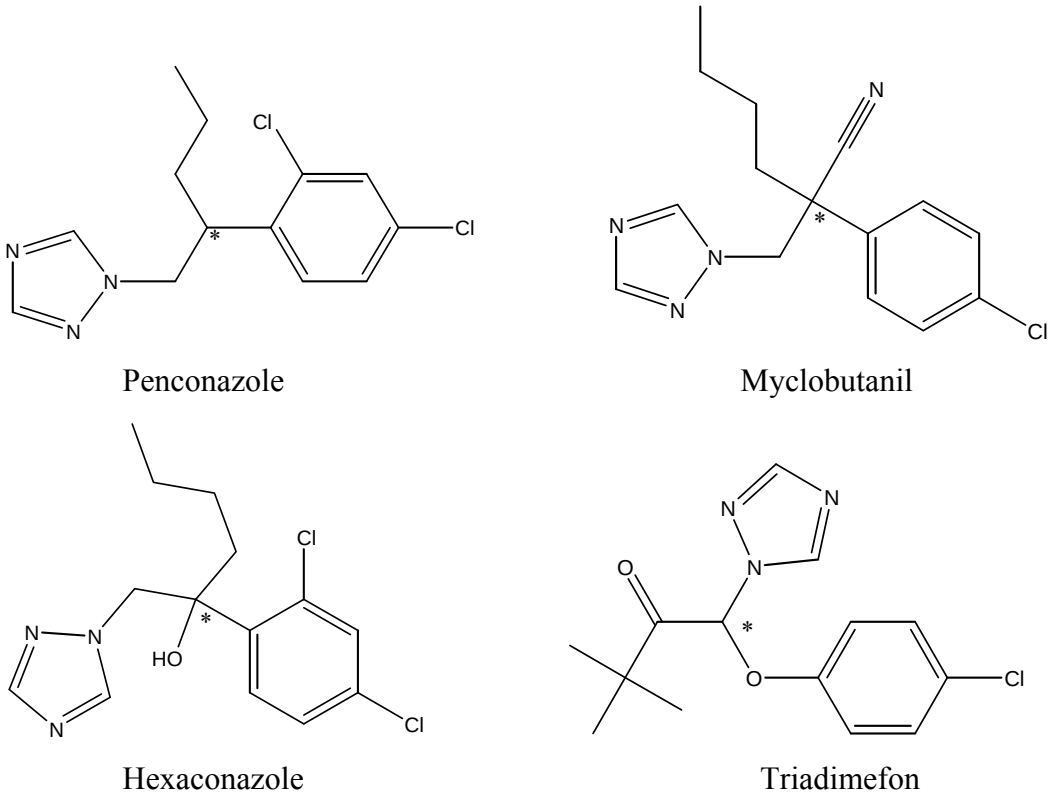

Figure 4.1. Chemical structures of penconazole, myclobutanil, hexaconazole, and triadimefon. (* indicates the position of the chiral center).

4.2 Experimental

4.2.1 Chemicals and Reagents

All triazole fungicides were obtained from Riedel-de Haen (Seelze, Germany). The HP-γ-CD was obtained from Sigma (St. Louis, MO, USA), SDS was obtained from Fisher Scientific (Loughborough, UK), and disodium hydrogen phosphate 12-hydrate was obtained from Riedel-de Haen. All other chemicals and solvents were common brands of analytical reagent grade or better, and were used as received. Water was collected from a Millipore Water Purification System (Molsheim, France).

The stock solutions of the individual triazole fungicides were prepared in methanol in the concentration range 2000 and 6000 mg L^{-1}. Final dilutions (concentrations concentrations in the figures) were prepared by diluting the stock

solution with buffer/separation solution. The separation solutions for CD-MEKC were prepared by dissolving appropriate amounts of SDS and HP-γ-CD in phosphate buffer and adjusting the pH of the buffer with phosphoric acid solution. All running buffers were filtered through a 0.45 nm nylon syringe filter from Whatman (Clifton, NJ, USA).

4.2.2 Instrumentations

All electropherograms were obtained with the Agilent CE system from Agilent Technologies (Waldbronn, Germany), equipped with temperature control and diode array detection (DAD). Separations were performed using an untreated fused-silica capillary of 64.5 cm, 650 μm id (with an effective length of 56 cm to the detector window) obtained from Polymicro Technologies (Phoenix, AZ, USA). Sample introduction was performed hydrodynamically for 1 s at 50 mbar. The detection wavelength used was 200 nm and the capillary temperature was optimized. The separation voltage was maintained at –25 kV. Data were collected and processed on computer using ChemStation software (Agilent Technologies).

4.2.3 Conditioning of the capillary

The new capillary was conditioned by passing 1 M NaOH solution for 10 min followed by washing with deionized water for 10 min and finally equilibrating with an appropriate running buffer for 10 min. At the beginning of each day, the capillary was flushed for 5 min with deionized water, followed by 5 min of 1 N NaOH to activate the silanol groups of the capillary, then followed by 10 min of deionized water, and 5 min of running buffer. Before each sample injection, the capillary was rinsed for 3 min with 0.1 N NaOH, followed by 3 min of deionized water, and 3 min of running buffer.

4.3 Results and Discussion

4.3.1 Simultaneous enantioseparataion of penconazole, myclobutanil and triadimefon.

In this study, simultaneous enantioseparation of three chiral triazole fungicides i.e. penconazole, myclobutanil and triadimefon was investigated using cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) under acidic condition. The method used in the current study was adopted from previous study (Wan Ibrahim, *et al.,* 2007) as these three fungicides belong to the same class of triazole fungicide as propiconazole i.e contain 50 mM SDS and 25 mM phosphate buffer (pH 3.0). In order to achieve the best enantioresolution of these triazole fungicides, the HP-γ-CD concentration range was optimized from 0 to 40 mM in 10 mM increment. Typical enantiomeric separation of the three triazole fungicides by CD-MEKC at different HP-γ-CD concentration is shown in Figure 4.2. The resolutions and efficiencies obtained for triazole fungicides enantiomers at different HP-γ-CD concentrations are presented in Table 4.1.

The results indicate that separation of enantiomers was not achieved with concentration range of HP-γ-CD from 0 to 20 mM. Simultaneous enantioseparation was successfully achieved at 30 mM and 40 mM HP-γ-CD concentration in 25 mM phosphate buffer (pH 3.0) solution containing 50 mM SDS, 10% (v/v) methanol and 5% (v/v) acetonitrile.

It can be seen that there was no chiral resolution for all triazole fungicides at 0, 10, and 20 mM HP-γ-CD. Further increase in the concentration of HP-γ-CD up to 30 mM, increased the Rs significantly to 0.81 (for penconazole), 1.14 (for myclobutanil) and 2.09 (for triadimefon). However, further increase in the concentration of HP-γ-CD up to 40 mM, enantioresolution decreased for myclobutanil (Rs = 0.71) and for triadimefon (Rs = 1.38). While for penconazole, which is more hydrophobic than myclobutanil and triadimefon, Rs increased to 0.90 (Table 4.1). It appears that high concentration of HP-γ-CD favors the resolution of more hydrophobic triazole fungicide whereas lower concentration HP-γ-CD favors

the Rs of less hydrophobic triazole fungicides. The difference of a combination of attractive forces such as hydrogen bonding, hydrophobic interactions, dipole-dipole interactions and M- M interactions between the two enantiomers and the chiral selector (cyclodextrin) plays an important role in the enantiomeric separations (Wang, *et al.,* 2005).

In addition, an increase in the concentration of HP-γ-CD caused a significant increase in the analysis time. In light of these aspects, the optimal concentration for HP-γ-CD was decided to be 30 mM for the simultaneous chiral separation of the three triazole fungicides with peak efficiencies (N) greater than 108 000 (Table 4.2) for all stereoisomers and analysis time of less than 15 min.

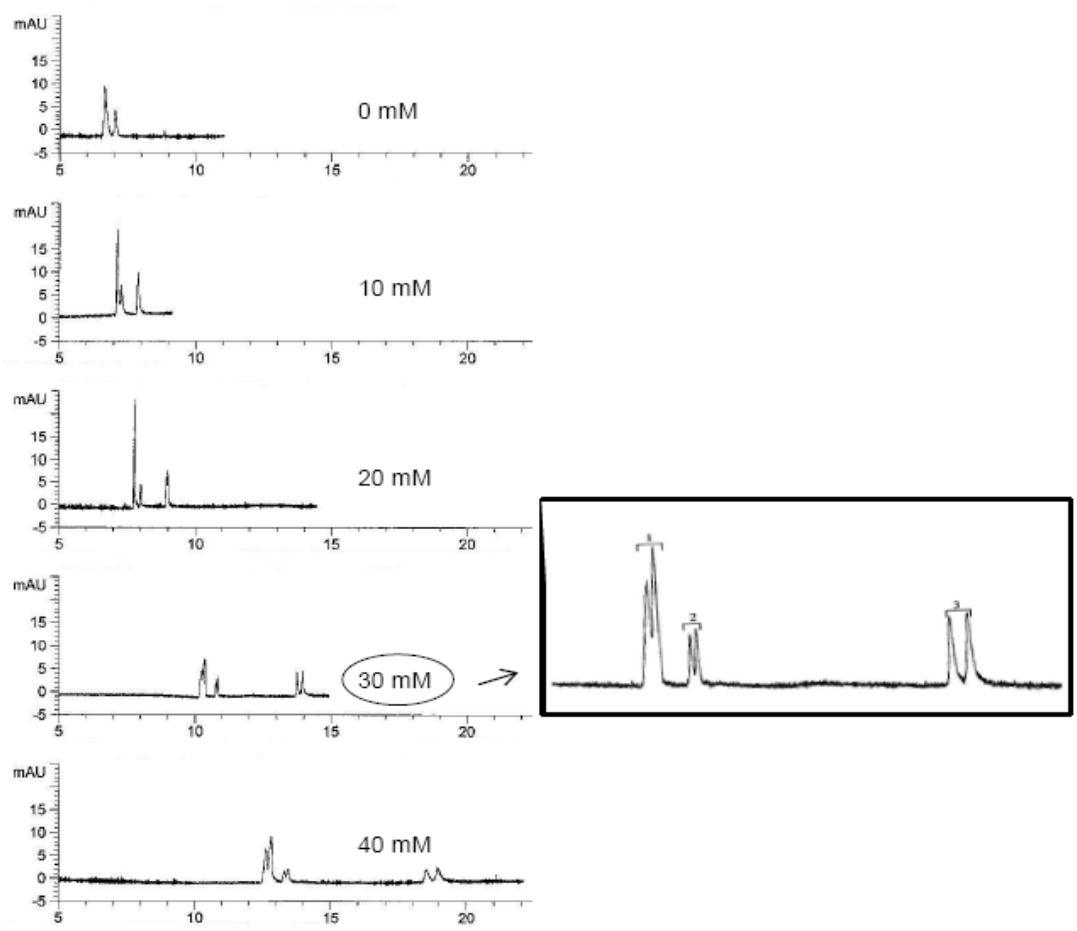

Figure 4.2 Separation of the three triazole fungicides by CD-MEKC with different HP-γ-CD concentrations. Separation solution: 0-40 mM HP-γ-CD, 50 mM SDS, mixed 10% methanol (v/v)-5% (v/v) acetonitrile in 25 mM phosphate buffer solution (pH 3). Sample, 300 mg L-1 standards in water; injected hydrodynamically for 1 s at 50mbar; separation voltage, -30 kV, temperature, 20 ºC, detection wavelength, 200 nm. Peaks: 1. Penconazole 2. Myclobutanil 3. Triadimefon

It is well-known that the principle of separation in MEKC is based on solute partitioning between the micellar phase and the aqueous/solution phase. The migration order for neutral solutes in MEKC is generally relates to the hydrophobicity of analytes (Nishi and Terabe, 1996). In this study, separation of triazole fungicides is performed in the reverse mode MEKC (pH 3.0), which is characterized by a faster moving pseudostationary phase (PS) compared to the EOF. In acidic condition, a slower EOF occurs due to suppressed dissociation of silanol groups at the inner surface of the capillary that reduces the zeta potential. Results indicated that more hydrophobic triazole fungicide (penconazole, log Pow = 3.56) interact more strongly with the micellar phase and thus migrate faster than hydrophilic compounds (myclobutanil, log Pow = 3.16; tridimefon log Pow = 2.77) in this reverse mode MEKC.

Table 4.1. Resolution of triazole fungicide enantiomers by CD-MEKC with different HP-γ-CD concentrations.

[HP-γ-CD]	Rs		
(mM)	P1-P2	M1-M2	T1-T2
0	0	0	0
10	0	0	0
20	0	0	0
30	0.81	1.14	2.09
40	0.90	0.71	1.38

Table 4.2 Efficiency, *N* of triazole fungicide enantiomers by CD-MEKC with different HP-γ-CD concentrations

[HP-γ-CD]	*N*					
(mM)	P1	P2	M1	M2	T1	T2
0	-	-	-	-	-	-
10	-	-	-	-	-	-
20	-	-	-	-	-	-
30	108 513	168 777	461 777	467570	356 866	321 276
40	53 605	65 871	63 348	68 410	57 042	77 68

4.3.2 Simultaneous enantioseparataion of hexaconazole, penconazole, and myclobutanil.

For simultaneous enantioseparation of hexaconazole, penconazole, and myclobutanil was also adopted from previous study (Wan Ibrahim, *et al.,* 2007) i.e using 25 mM phosphate buffer at pH 7.0. However, it was found that the method adopted did not successfully separate all the stereoisomers of these three fungicides. In order to achieve the best enantioresolution of the three triazole fungicides, the HP-γ-CD concentration was first optimized as the conditions adopted from previous study (Wan Ibrahim, *et al.,* 2007) did not successfully produce enantioseparation of these three selected triazole fungicides followed by separation temperature. HP-γ-CD is a nonionic cyclic oligosaccharide consisting of eight glucose units and has numerous chiral recognition centers. It is a derivatized neutral CD and a widely used chiral selector due to the water solubility and low UV absorbance. It has been used as chiral selector in several CD-MEKC methods (Wan Ibrahim, *et al.,* 2007; Otsuka and Terabe, 2000).

The HP-γ-CD concentration was optimized from 0 to 40 mM in 10 mM increment. It can be seen in Fig. 6.3 that there was no chiral resolution for all triazole fungicides at 0, 10, and 20 mM HP-γ-CD. An increase in the concentration of HP-γ-CD to 30 mM produces partial separation of the individual enantiomer. Further increase in the concentration of HP-γ-CD to 40 mM successfully produces a simultaneous enantioresolution of hexaconazole, penconazole, and myclobutanil peaks. In light of these aspects, the optimal concentration for HP-γ-CD was decided to be 40 mM for the simultaneous chiral separation of these three triazole fungicides. It is well known that the migration order for neutral solutes in MEKC is generally related to the hydrophobicity of analytes. In this study, separation of triazole fungicides is performed in the reverse mode MEKC (pH 3.0), which is characterized by a faster moving pseudostationary phase compared to the EOF. In acidic condition, a slower EOF occurs due to suppressed dissociation of silanol groups at the inner surface of the capillary that reduces the zeta potential. Separation at acidic pH performed in previous studies (Wan Ibrahim, *et al.,* 2007) showed that pH 3.0 produced the best results. The results indicate that the more hydrophobic triazole

fungicide (hexaconazole, log P_{ow} = 3.90) interacts more strongly with the micellar phase and thus migrates faster than the less hydrophobic com pounds (penconazole, log Pow = 3.56; and myclobutanil, log Pow = 3.16) (Wan Ibrahim *et al.,* 2008) in this reverse mode MEKC.

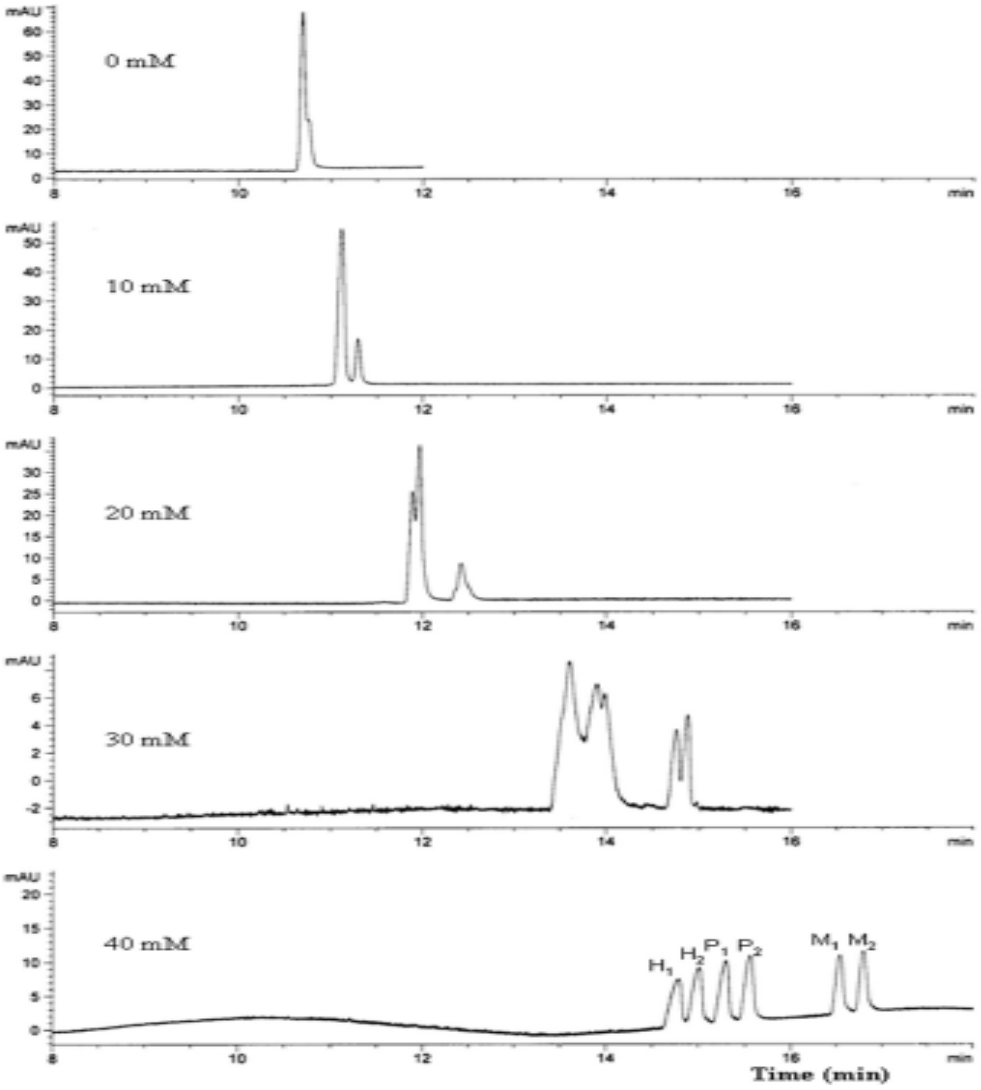

Figure 4.3. Enantiomeric separation of the three triazole fungicides by CD-MEKC at different HP-γ-CD concentrations. Sample, 200 mg/L, injected hydrodynamically for 1 s at 50 mbar; separation solution: 0–40 mM HP-γ-CD and 50 mM SDS in 25 mM phosphate buffer (pH 3.0); capillary, 64.5 cm650 lm id (effective length, 56 cm); applied voltage, –25 kV; detection wavelength, 200 nm, temperature, 258C. Note: 1, 2 are the first- and secondmigrating peaks of triazole fungicides (H: hexaconazole; P: penconazole; M: myclobutanil).

Temperature is an important parameter to control in enantiomeric separations. Variation in the temperature can produce changes in migration times and resolution, thus affecting the enantiomeric separation (Toribio, *et al.,* 2007). In this study, the

effect of varying separation temperature from 25 to 40˚C in 5˚C increments was also investigated in order to obtain the best enantiomeric separation of the three triazole fungicides. Separation was successfully achieved for all enantiomers at this temperature range (Fig. 6.4). Analysis time decreased from 17 to 14 min for the last peak of myclobutanil enantiomer when the temperature was increased from 25 to 40˚C. The results obtained in this work agree with those obtained by other authors (Castro-Puyana, *et al.,* 2006; Denola, *et al.,* 2007) who have found that an increase in temperature causes a reduction in migration time because of the decrease in the viscosity of the buffer solution. However, an increase in the temperature up to 40˚C reduced the peak area, peak height, and peak efficiencies. For thisreason, the optimal temperature was decided to be 35˚C for the best enantiomeric separation of hexaconazole, penconazole, and myclobutanil fungicides by the CD-MEKC method with peak efficiencies (N) greater than 200000, resolutions for all enantiomers (R_s) greater than 1.60 and analysis time within 15 min. This represents a significant saving in time compared to the work of Wu et al. (2001).

The performance of the optimized CD-MEKC system was examined in terms of the linearity, repeatability, and LOD. The results are summarized in Table 4.3. The linearity was measured by constructing the calibration curve of average peak area (n = 3) against the concentration of standards ranged from 50 to 200 mg/L. Calibration curves are linear with the correlation coefficient higher than 0.99 for all enantiomers. Good repeatabilities in the migration time, peak area, and peak height were obtained ranging from 0.45 to 2.83%, 0.46 to 5.23%, and 0.61 to 7.35%, respectively. The LOD for three triazole fungicides, determined by the calibration curve at an S/N of 3, was found to be 4.1–5.6 mg/L. However, the LOD values obtained are modest and insufficient for the analysis of real samples. The maximum residue limits (MRLs) set by Codex Alimentarious Commission (CAC) in tomato, strawberry, and grapes for myclobutanil are 0.3, 1, and 1 mg/kg, respectively while for penconazole in tomato, strawberry, and grapes are 0.2, 0.1, and 0.2 mg/kg respectively. For hexaconazole, no MRL is established or prior MRLs revoked (http://www.codexalimentarious.net/mrls/pestdes/checked on 28 October 2008). It is known that CE technique suffers from poor concentration sensitivity when using UV detection because of the small injection volumes and narrow optical path length. Two

online sample preconcentration techniques namely sample stacking and sweeping, could be used to improve detection sensitivity in MEKC (Kim, *et al.*, 2003; Musijowski, *et al.*, 2006; Juan-Garcia, *et al.*, 2007). A more comprehensive study on the online preconcentration and simultaneous chiral separation of hexaconazole, penconazole, and myclobutanil are currently in progress.

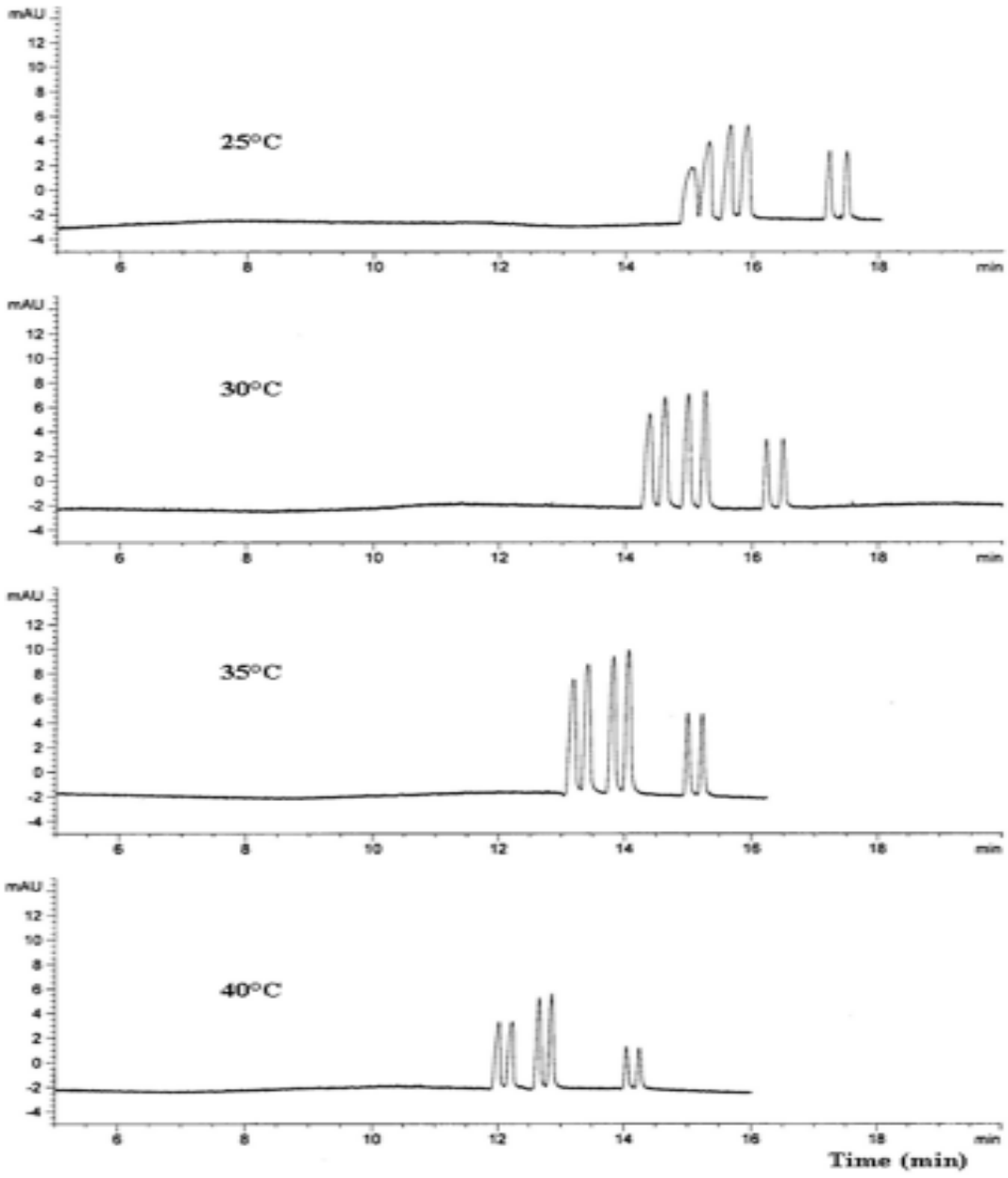

Figure 4.4 Enantiomeric separations of the three triazole fungicides by CD-MEKC at different temperatures. Conditions and notation as in Figure 4.3.

Table 4.3 Linearity, repeatability, and LOD for the triazole fungicides enantiomers in optimized CD-MEKC method.

Peak[a)]	Calibration Curve[b)]		RSD (%)			LOD (mgL^{-1})
	Equation	r^2	t_R	Peak Area	Peak Height	
H1	y = 0.3071x – 1.680	0.9999	0.45	2.19	3.36	4.6
H2	y = 0.3268x – 2.645	0.9991	0.48	0.46	0.61	
P1	y = 0.3235x – 1.960	0.9998	0.79	5.23	7.35	4.1
P2	y = 0.3319x – 1.895	1.0000	0.84	4.50	3.49	
M1	y = 0.2674x – 1.410	0.9977	2.74	2.12	0.61	5.6
M2	y = 0.2761x – 1.830	0.9978	2.83	1.57	1.50	

Conditions as in Fig. 4.3.
[a)] 1, 2 are first- and second-migrating peaks of triazole fungicides (H: hexaconazole; P: penconazole; M: myclobutanil).
[b)] Linear range: 50–200 mg/L; y = peak area; x = concentration (mg/L).

4.4 Concluding Remarks

Simultaneous enantioseparation of the three triazole fungicides was successfully achieved at 30 mM HP-γ- CD concentration in 25 mM phosphate buffer (pH 3.0) solution containing 50 mM SDS, 10% (v/v) methanol and 5% (v/v) acetonitrile with resolutions (*Rs*) between enantiomers for penconazole, *Rs* = 0.81; myclobutanyl, *Rs* = 1.14; triadimefon, *Rs* = 2.09; peak efficiencies (*N*) greater than 108 000 for all stereoisomers and analysis time of less than 15 min. The optimized CD-MEKC system is simple, rapid and minimal sample requirement compared to the conventional chromatographic method (Wang, *et al.,* 2005).

Furthermore the simultaneous enantiomeric separation of hexaconazole, penconazole, and myclobutanil using HP-γ-CD is reported for the first time. The use of a mixture of 40 mM HP-γ-CD and 50 mMSDS in 25 mM phosphate buffer solution (pH 7.0) was able to separate the six enantiomers with resolution (Rs) greater than 1.60, peak efficiencies (N) greater than 200000 and analysis time within 15 min. The repeatabilities in the optimized CD-MEKC method concerning the RSD% (n = 3) for migration time, peak area, and peak height were generally good ranging from 0.45 to 7.35%. Calibration curves were linear with the correlation coefficient higher than 0.99 for all enantiomers. The LOD obtained is modest (4.1–

5.6 mg/L) and insufficient for determining these fungicides in real samples such as tomato, grapes, and strawberry.

CHAPTER 5

ON-LINE PRECONCENTRATION TO IMPROVE SENSITIVITY

5.1 Introduction

Several parameters were optimized for the simultaneous separation of selected triazole fungicide. Since the LOD of analysis selected triazole fungicides was high, preconcentration technique was then used to improve sensitivity of detection, so can be considered as an alternative method for selected triazole fungicides in real sample that need lower LOD.

There are several on-line preconcentration techniques used in capillary electrophoresis, namely stacking techniques (Quirino and Terabe, 1997; Otsuka 2003; Wan Ibrahim, *et al.*, 2007) and sweeping techniques (Otsuka, 2003; Chen, *et al.*, 2004; Huang, *et al.*, 2006; Jen, *et al.,* 2006; and Wan Ibrahim *et al.*, 2007). Several on-line preconcentration techniques are reported for chiral separation of triazole fungicides. Otsuka *et al.* (2003) reported preconcentration of triadimenol by CD-EKC and CD-MEKC. HP-γ-CD was used as chiral selector using CD-EKC, whereas enantioseparation of triadimenol using CD-MEKC used heptakis-6-sulfato-β-cyclodextrin as chiral selector.

On-line preconcentration using stacking and sweeping technique was also reported for the analysis several triazole fungicides namely fenbuconazole, tebuconazole, and propiconazole using HP-γ-CD as chiral selector (Wan Ibrahim, *et al.*, 2007). However the best technique was succesfully acieved using sweeping techniques at acidic pH, where sensitivity enhancement factor up to 100 fold obtained (Wan Ibrahim, *et al.*, 2007). Landers (2008) mentioned, that at favorable condition, sweeping techniques gives high concentration efficiency with improvements up to 1000-fold greater than the conventional injection/separation.

Beacuse of that, in this study was used the sweeping technique was used for preconcentration.

5.2 Experimental

5.2.1 Chemicals and Reagents

Chiral triazole fungicides studied (cyproconazole, bromuconazole, diniconazole, penconazole and myclobutanil), 2-Hydroxypropyl-γ-cyclodextrin (HP-γ-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD), and *i*-propanol (*i*-PrOH)were obtained from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) was obtained from Fisher Scientific (Loughbrough, UK). Disodium hydrogen phosphate 12-hydrate and sodium hydroxide pellets were obtained from Riedel-de Haen (Seelze, Germany). HPLC grade methanol was obtained from J.T. Baker (California, USA). Water was collected from a Millipore Water Purification System (Molsheim, France). All running buffers were filtered through a 0.45 μm nylon syringe filter from Whatman (Clifton, NJ, USA). Stock solutions (2000 mg L^{-1}) of individual triazol fungicide were prepared in methanol. Final sample solutions for sweeping method were prepared by diluting appropriate stock solution by separation solution without micellar phase. For real samples, was used fresh red gtrape and strawberry were obtained from local market in Johor, Malaysia.

5.2.2 Instrumentation

All electropherograms were obtained with the Agilent capillary electrophoresis system from Agilent Technologies (Waldbronn, Germany), equipped with temperature control and diode array detection (DAD). Separations were performed using an untreated fused silica capillary of 64.5 cm × 50 μm i.d. (with an effective length of 56 cm to the detector window) obtained from Polymicro Technologies (Phoenix, AZ, USA). Sample injection was performed hydrodynamically at 50 mbar and injection times were optimized. The detection

wavelength used was 200 nm and the separation voltage was maintained at -25 kV. Separation temperature used was 35°C. Data were collected and processed on computer using ChemStation software (Agilent Technologies). The new capillary was conditioned by passing 1 M NaOH solution for 10 min followed by washing with deionized water for 10 min and finally equilibrating with an appropriate running buffer for 10 min. Between runs, the capillary was washed with 0.1 M NaOH, water, and run buffer for 2 min each. All sample injections were performed in triplicate.

5.2.3 Validation Procedure

The performance of the method was examined in terms of the linearity, repeatability, limit of detection (LOD) and sensitivity enhancement factor (SEF_{area}) or increase in detection sensitivity. Linearity of the optimized method was assessed by constructing the calibration curve of average peak areas (n = 3) against the concentration of standards (at the linear range). The repeatabilities in the migration time, peak area and peak height were recognized in terms of the relative standard deviation (RSD%, n = 3). The LOD was determined by the calibration curve along with the signal-to-noise ratio (S/N) as 3. The SEF_{area} was calculated by comparing (peak area obtained with on-line method / peak area with usual CD-MEKC injection) × dilution factor.

5.3 Results and Discussions

5.3.1 Preconcentration Method For Enantioseparation of Cyproconazole, Bromuconazole, and Diniconazole.

In sweeping preconcentration techniques, the sample solution was prepared by using background solutions (BGS) without micellar phase but similar of conductivity to that BGS and injected hydrodynamically as a long plug (Landres, 2008).In this study, the effect of sample injection time on the chiral separation of selected triazole fungicides enantiomers by sweeping-CD-MEKC was investigated in the range 30 to 150 s. The enantioseparation of these selected triazole fungicides

have an optimum separation at pH 3.0. When a negative voltage for anionic micelle (SDS) is applied, the micelle enters sample zone and collects analytes at the front end of the entering micellar zone until the front end reaches the original boundary between the sample zone and the BGS.

Electropherograms of enantioseparation of selected triazole fungicides by the sweeping-CD-MEKC pH 3.0 with different sample injection time are shown in Figure 5.1. Figure 5.1 shows unsual electropherogram for the enantioseparation of selected triazole fungicides using CD-MEKC, where appearance of peak number six is very high. It is predicted that the peak was interfered by matrix from solvent. Because of that for quantitative analysis is needed the injection of sample solvent as blank.

The effects of the injection time on peak area, peak height and resolutions are given in Figure 5.2. As Figure 5.2a shows, increasing the sample injection from 30 to 120 s resulted an increase in the peak height, and the injection up to 150 s the peak area was decreased. Same trend shown in Figure 5.2b, where the increasing the sample injection from 30 to 120 s increased the peak area, then increasing sample injection time up to 150 s resulted in decreasing peak height. Effect of injection time on R_s was not significant, for all separation shows R_s greater than 1.5 (Figure 5.2c). For this reason, the 120 s was selected for the best sample injection time for sweeping-CD-MEKC, and was then employed in all subsequent investigations for sweeping-CD-MEKC of enantioseparation of selected triazole fungicides.

In this study, the optimized sweeping-CD-MEKC pH 3.0 shows good linearity, repeatability, LODs and sensitivity enhancement factor (SEF) for all selected chiral triazole fungicides as shown in Table 5.1. LOD in sweeping-CD-MEKC increased 30 to 65 fold compared with the conventional CD-MEKC. Table 5.1 shows LOD for diniconazole enatiomers, i.e peak no 1 and 2 is about 0.2 ppm, this results better than previous study, where obtained the LDO of diniconazole enantiomers is 0.49 ppm (Wang, et al, 2005). To the best author knowledge, LOD of two selected triazole fungicides, namely cyproconazole and bromuconazole have never been reported.

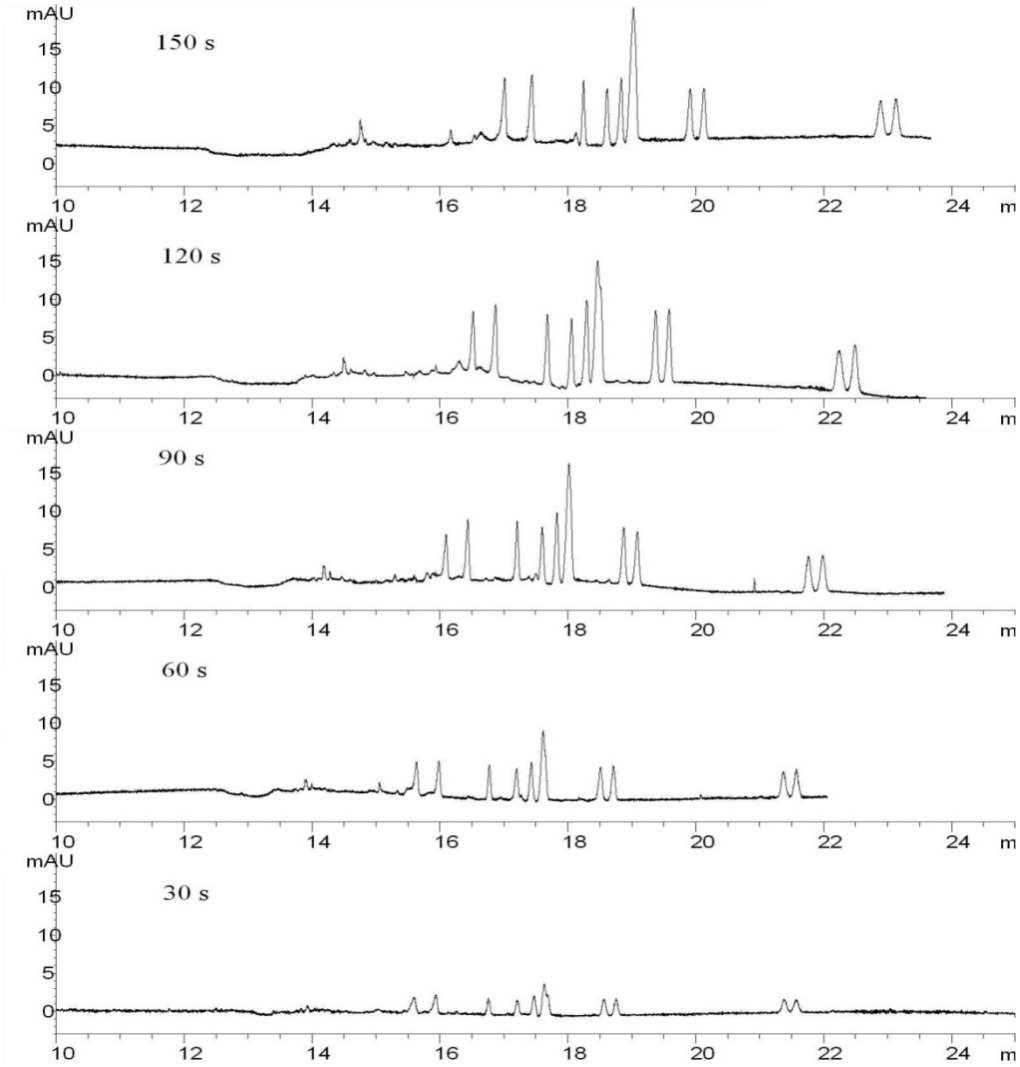

Figure 5.1 Enantioseparation of selected triazole fungicides by sweeping-CD-MEKC at pH 3.0 with different sample injection time at 50 mbar. Sample, 1 mg L^{-1} of each selected triazole fungicides (in buffer solution without micellar phase). Other conditions: 25 mM phosphate buffer (pH 3.0); 40 mM SDS, and 27:3 mM HP-β-CD: HP-γ-CD, applied voltage -25 kV.

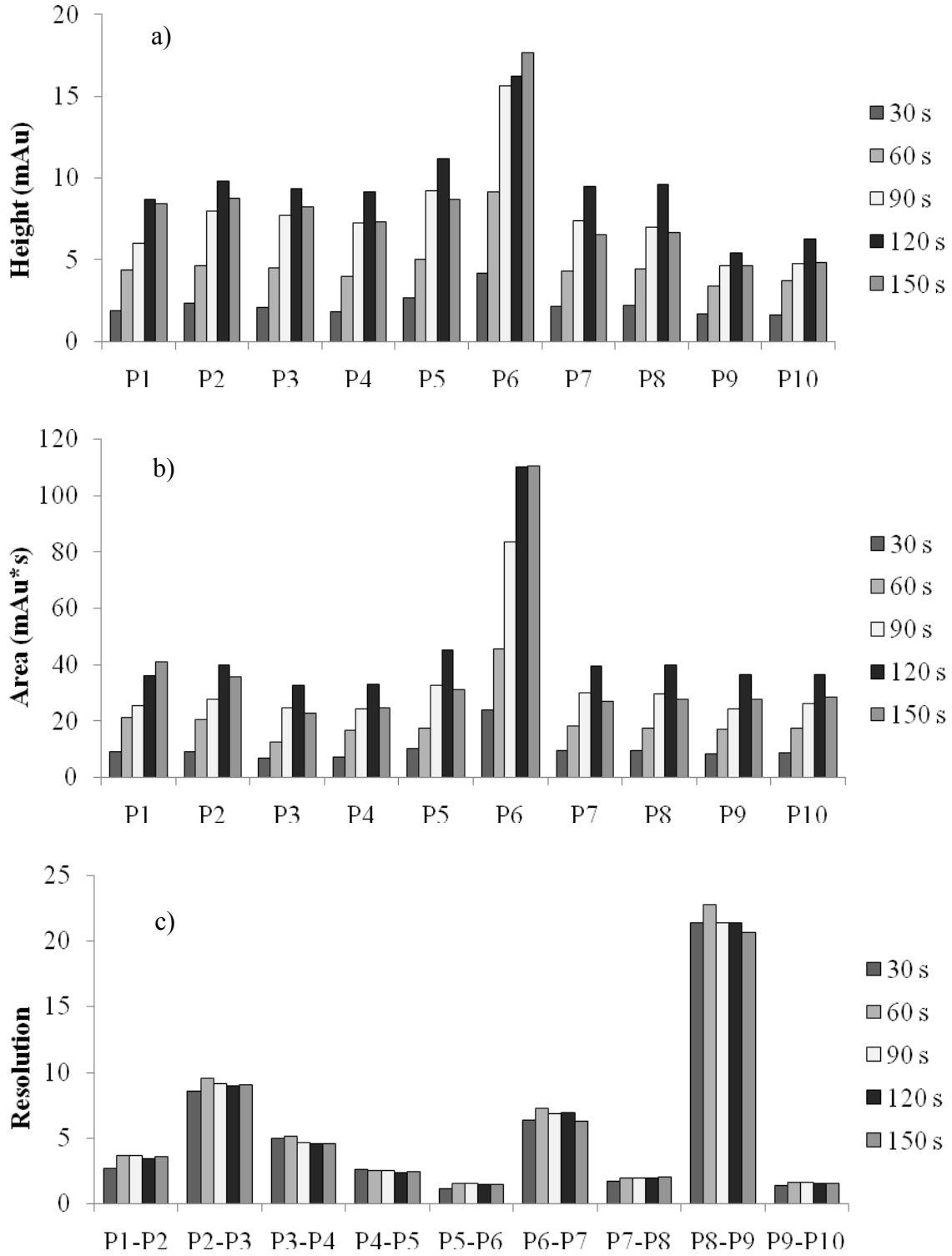

Figure 5.2 Effect of sample injection time on a) peak height, b) peak area and c) resolution of enantioseparation of selected triazole fungicides by sweeping-CD-MEKC. Other conditions are as in Figure 5.1.

Table 5.1 Linearity, repeatability, LODs (S/N = 3) and sensitivity enhancement factor (SEF) of the optimized sweeping-CD-MEKC pH 3.0.

Peak[a]	Calibration Curve		RSD (%, n=3)			LOD	SEF[c]
	Equation[b]	R^2	t_M	Height	Area	(mg L^{-1})	
1	y = 30.5043x + 0.7418	0.9986	0.08	8.44	2.44	0.24	64
2	y = 30.6809x + 0.1375	0.9988	0.21	5.06	1.28	0.21	65
3	y = 32.6530x + 1.1080	0.9980	0.01	4.62	1.90	0.28	54
4	y = 28.8518x + 3.0976	0.9984	0.10	2.97	2.04	0.25	37
5	y = 39.2885x + 4.8829	0.9979	0.12	5.54	2.26	0.29	44
6	y = 41.1486x + 5.8193	0.9977	0.09	2.74	0.97	0.30	41
7	y = 37.2572x + 5.6231	0.9982	0.15	4.75	0.30	0.26	42
8	y = 40.2245x + 2.5529	0.9980	0.14	2.15	1.81	0.28	34
9	y = 26.6712x + 8.5446	0.9977	0.32	5.47	1.43	0.30	51
10	y = 28.2156x + 7.1212	0.9975	0.26	3.37	1.67	0.31	29

Conditions as in Figure 5.1.
[a]1-10 shows first till tenth migrating enantiomers of selected triazole fungicide, are: diniconazole enantiomers peak 1-2, Bromuconazole enantiomers peak 3-4, and peak 9-10, and cyproconazole enantiomers peak 5-8.
[b]Linear range: 0.5-5.0 mg/L; *y* = peak area; *x* = concentration (mg L^{-1}).
[c]SEF_{LOD}: ($LOD_{CD\text{-}MEKC}/LOD_{sweeping\text{-}CD\text{-}MEKC}$).

5.3.2 Preconcentration Method for Enantioseparation of Hexaconazole, Penconazole, and Myclobutanil.

In this present study, stacking with a reverse migrating micelle (SRMM) and sweeping techniques were applied to the CD-MEKC system to enhance detection sensitivity of three triazole fungicide enantiomers. The optimized CD-MEKC conditions (Figure 5.3a) from our previous work were using separation solution: 40 mM HP-γ-CD and 50 mM SDS in 25 mM phosphate buffer (pH 3); capillary, 64.5 cm × 50 μm I.D. (effective length, 56 cm); applied voltage, -25 kV; detection wavelength, 200 nm, and separation temperature, 35ºC.

SRMM was chosen as it requires little sample preparation. To apply the SRMM method to the CD-MEKC system, the conductivity of the sample solution was made lower than that of the separation solution. The sample stock solution was diluted with pure water. Sweeping method was also applied to enhance the

concentration detection sensitivity as these three fungicides are hydrophobic compounds (hexaconazole, log P_{ow} = 3.90 [20]; penconazole, log P_{ow} = 3.56 and myclobutanil log P_{ow} = 3.16) (http://syrres.com/esc/kowwin.htm, downloaded 5 June 2009). To apply the sweeping method to the CD-MEKC system, the conductivity of the sample solution (buffer solution without micellar phase) was adjusted to be same as that of the separation solution (conductivity = 5.40 mS cm^{-1}).

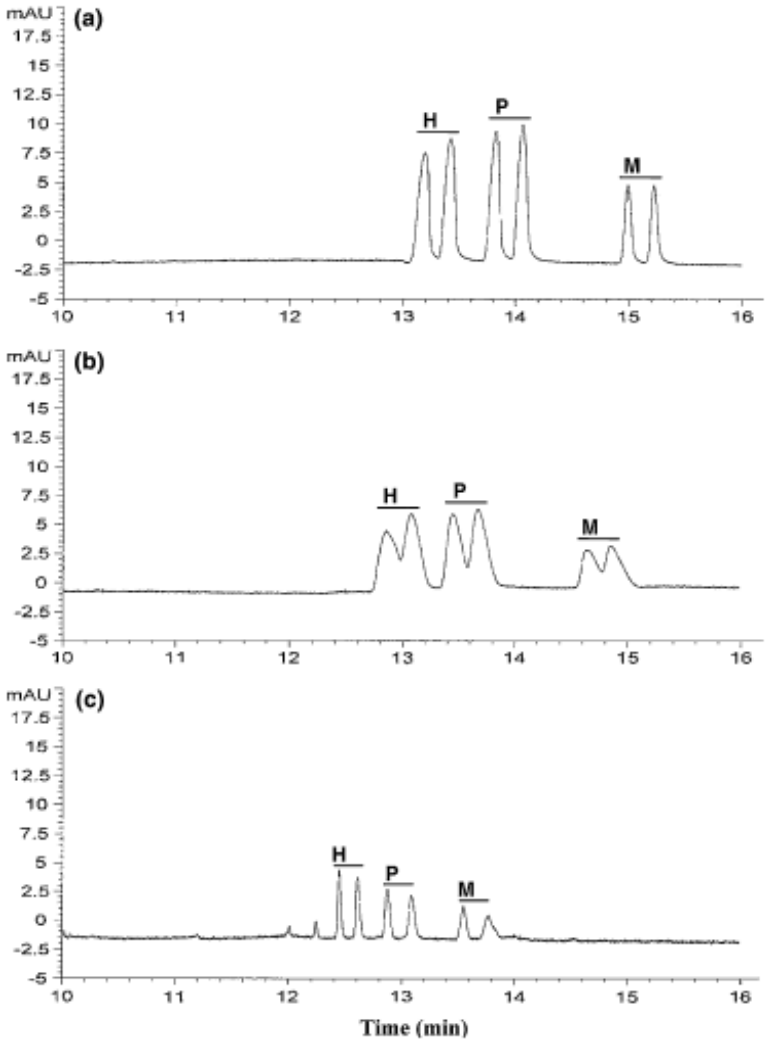

Figure 5.3 Enantiomeric separation of hexaconazole, penconazole, and myclobutanil by the optimized CD-MEKC and on-line-CD-MEKC. Separation solution: 40 mM HP-γ-CD and 50 mM SDS in 25 mM phosphate buffer (pH 3); capillary, 64.5 cm × 50 μm I.D. (effective length, 56 cm); applied voltage, -25 kV; detection wavelength, 200 nm, temperature, 35ºC.(a) CD-MEKC, 200 mg L^{-1} sample in buffer/separation solution, injected for 1 s, at 50 mbar; (b) SRMM-CD-MEKC, 10 mg L^{-1} sample in water, injected for 10 s, at 50 mbar; and (c) Sweeping-CD-MEKC, 0.5 mg L^{-1} sample in buffer solution without micellar phase, injected for 70 s, at 50 mbar.Peaks: hexaconazole (H); penconazole (P); myclobutanil (M).

The sample injection time was first optimized for SRMM method and it was found that the best sample injection time was obtained at 10 s. Higher injection times than 10 s was found to decrease resolutions. A sample injection time of 10 s was employed in all subsequent investigations by SRMM-CD-MEKC. The enantiomeric separation of the three triazole fungicides by the optimized SRMM-CD-MEKC method is shown in Figure 5.3b. The sample injection time was also optimized for sweeping method and it was found that the best sample injection time was obtained at 70 s. This sample injection time was employed in all subsequent investigations by sweeping-CD-MEKC. Increasing sample injection time (higher than 70 s) resulted in decreasing peak resolutions. The enantiomeric separation of the three triazole fungicides by the optimized sweeping-CD-MEKC method is shown in Figure 5.3c.

The performance of the optimized CD-MEKC and on-line preconcentration CD-MEKC methods were examined in terms of the linearity, repeatability, LOD, and sensitivity enhancement factor (SEF). The results are summarized in Table 5.2. As can be seen in Table 5.2, the methods demonstrated a good linearity in the calibration curve (R^2 > 0.995 for CD-MEKC and sweeping-CD-MEKC; R^2 > 0.973 for SRMM-CD-MEKC). The repeatability in the optimized methods concerning the relative standard deviation for migration time, peak area, and peak height for each enantiomer was briefly examined (n = 3). Good repeatabilities in the migration time, peak area and peak height were obtained ranging from 0.45% to 6.96%, 0.46% to 12.61% and 0.61% to 7.73%, respectively.

Compared with the conventional CD-MEKC injection method, SRMM-CD-MEKC enhanced the detection sensitivity of the three triazole fungicides 9- to 10-fold and sweeping-CD-MEKC enhanced it 62- to 67-fold. These results clearly show the possibility of improving the detectability of selected chiral triazole fungicides using SRMM-CD-MEKC and sweeping-CD-MEKC methods. Sweeping-CD-MEKC is better than SRMM-CD-MEKC in terms of the detection sensitivity with a limit of detections (S/N = 3) for the three triazole fungicides ranged from 0.1 to 0.2 mg L^{-1}. The current sweeping-CD-MEKC method provides lower limit of detection for the fungicides compared to a previously reported study of enantiomeric separation of chiral triazole pesticides by HPLC (Wang *et al.,* 2005). This is consistent with other

publications that sweeping technique is a powerful on-line sample preconcentration technique that improves the concentration sensitivity of CE (Wan Ibrahim *et al.*, 2007).

Table 5.2 Linearity, repeatability, LODs (S/N = 3) and sensitivity enhancement factor (SEF) for the three triazole fungicides in the optimized CD-MEKC and on-line-CD-MEKC

	CD-MEKC	SRMM-CD-MEKC	Sweeping-CD-MEKC
Linearity range (mg L^{-1})	50 - 200	5 - 20	0.5 - 3
R^2	0.9978 - 0.9998	0.9735 – 0.9986	0.9959 – 0.9986
RSD (%, n=3)			
Migration Time	0.45 – 2.83	3.04 – 6.96	2.39 – 3.90
Peak Area	0.46 – 5.23	6.80 – 12.61	1.96 – 6.15
Peak height	0.61 – 7.35	1.62 – 7.73	2.80 – 6.64
LOD (mg L^{-1})	4.1 – 5.6	1.2 - 4.0	0.1 – 0.2
SEF_{area}		9 - 10	62 - 67

5.4 Concluding Remarks

Sweeping preconcentration was performed on simultaneous enantioseparation of cyproconazole, bromuconazole, and diniconazole at pH 3. In this study, sweeping preconcentartion techniques could improve the detection sensitivity 30 to 60 fold for all enantiomers. Repeatabilities in the migration time, peak area and peak height in terms of the relative standard deviation were good ranging from (0.08-0.32)%, (0.03-2.44)%, and (2.13-8.44)% respectively. The LOD for all enantiomers is 0.3 mg L^{-1} on average. The LODs values for dniconazole enantiomers are better than previous study (Wang, 1998). The average recoveriesin grape sample achieved between 50 to 115% with RSDs ranging from 4.26 % to 13.05% (n = 3). Whereas recoveriesin strawbery sample good achieved between 60.61 to 85% with RSDs ranging from 4.90 % to 15.09% (n = 3).

Furthermore, SRMM-CD-MEKC and sweeping-CD-MEKC methods could be used to increase detection sensitivity and simultaneous chiral separation of hexaconazole, penconazole and myclobutanil. The results indicated that sweeping-CD-MEKC method gave the best detection sensitivity with a limits of detection (S/N = 3) for the selected triazole fungicides ranged from 0.1 to 0.2 mg L^{-1}. The online preconcentration method developed using sweeping CD-MEKC for the three triazole fuingicides gave a much higher detection sensitivity increase (~70-fold) compared to the one attained by Otsuka et al. using both sweeping and stacking with a reverse migrating pseudostationary phase for triadimenol, a fungicide from the triazole fungicide class, separation i.e. ~10-fold. This is first report on the on-line preconcentration and chiral separation of hexaconazole, penconazole and myclobutanil by CD-MEKC in only one injection. The developed sweeping-CD-MEKC method can be used as a preliminary study for further study on the analysis of the selected chiral triazole fungicides on real samples using MEKC with UV detector.

CHAPTER 6

DETERMINATION OF BINDING CONSTANT OF DINICONAZOLE AND TRIADIMEFON WITH HP-γ-CD BY MICELLAR ELEKTROKINETIC CHROMATOGRAPHY

6.1 Introduction

Cyclodextrins (CDs) are non reducing oligosacahrides cyclic consisting of D-glucopyranose units bonded through α-1,4 linkages. CDs have been effectively employed for chiral separation and for improvement of a large number of separation processes in many chromatographic techniques, either as mobile-phase additives or as stationary phase or stationary phase additives (Cserhati and Forgacs, 2003). CDs or their derivatives are the most successful and useful chiral selectors for enantiomeric separation, especially for the separation of enantiomeric drugs (Chen, and Weber, 2008).

Chiral selector interacts with the analyte forming transient diastereoisomers. Enantiomers are resolved if these diastereoisomers possess different stability constants. Selectivity of CD is based on their cavity (Huang, *et al.,* 2007). Thus CDs are capable of forming inclusion compexes with compounds having a size compatible with the dimensions of the cavity. CDs are typical host molecules, can include other 'guest' molecule i.e. several of molecules having the size of one or two benzene rings, or even larger compounds, which have a side chain of comparable size to form inclusion complexes (Gu and Pan, 1999). The formation of CD complexes improves physical, chemical and biological properties of the guest molecular.

As the quantitative description of the inclusion equilibrium between CD and guest molecule, the inclusion constant reflects the strength of the binding force

between them. So, the inclusion constant is an important and basic parameter to the applications of CD.

Numerous methods have been reported for the determination of inclusion constant or formation complex constant cyclodextrins with some analytes which includes spectroscopic method i.e. differential scaning calorimetry (Chen, Wu, and Chen, 2003); nuclear magnetic resonance method (Imai, *et al.*, 1984); phase-solubility technique (Zughul and Badwan, 1997); liquid chromatography (Uekama, Hirayama, and Irie, 1978)., electrochemistry i.e potentiometric (Funasaki, Nagaoka, and Hirota, 2005)., coulometric (Gu and Pan, 1999). Determination of inclusion constant of CDs by capillary electrophoresis have been reported using elektrokinetic chromatography (Martin-Biosca, Garc'ya-Ruiz, and Marina, 2000), and capillary zone electrophoresis (Jiao, *et al.*, 2008). In this study, micellar elektrokinetic chromatography (MEKC), is used to simultaneous determine inclusion constant or binding constant of diniconazole and triadimefon enantiomers using hydroxypropyl-γ-cyclodextrin simultaneously.

6.2 Experimental

6.2.1 Chemicals and Reagents

Diniconazole, triadimefon, and 2-Hydroxypropyl-γ-cyclodextrin (HP-γ-CD) were obtained from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) was obtained from Fisher Scientific (Loughbrough, UK). Disodium hydrogen phosphate 12-hydrate and sodium hydroxide pellets were obtained from Riedel-de Haen (Seelze, Germany). HPLC grade methanol was obtained from J.T. Baker (California, USA). Water was collected from a Millipore Water Purification System (Molsheim, France). Stock solutions (2000 mg L^{-1}) of individual triazole fungicide were prepared in methanol. Final dilutions were prepared by diluting the stock solution with water to 200 mg L^{-1}. The separation solutions for CD-MEKC were prepared by dissolving appropriate amounts of SDS, cyclodextrin, methanol and acetonitrile in phosphate buffers and adjusting the pH of the buffer with phosphoric

acid solution. All running buffers were filtered through a 0.2 μm nylon syringe filter from Whatman (Clifton, NJ, USA)

6.2.2 Instrumentations

All experiments were carried out on Agilent capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany), equipped with a diode array detector. Data acquisition and system control was carried out by the 3D-ChemStation Software by Agilent Technologies. Separations were performed using an untreated fused silica capillary of 64.5 cm total length, 56 cm effective length, 75 μm i.d for separation under neutral condition and 50 μm i.d for separation under acidic condition. Sample injections were performed hydrodynamically at a constant pressure of 50 mbar for 1s. The separation runs were performed at a constant applied voltage of +20 kV for separation under neutral condition and -30 kV for separation under acidic condition at several temperatures.

6.2.3 Conditioning of the capillary

At the beginning of each day, the capillary was flushed for 5 min with deionized water, followed by 5 min of 1 N NaOH to activate the silanol groups of the capillary, then followed by 10 min of deionized water, and 5 min of running buffer. Before each sample injection, the capillary was rinsed for 3 min with 0.1 N NaOH, followed by 3 min of deionized water, and 3 min of running buffer.

6.2.4 Data Treatments

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The inclusion complexes binding constants for each enantiomer–HP-γ-CD pair were calculated, from electrophoretic mobilities, using linier plotting approaches by least-square methods. From the experimental data, the electrophoretic mobility of each enantiomer (μ_i) was determined by the following equation:

$$\mu_i = \frac{lL}{V}\left(\frac{1}{t_i} - \frac{1}{t_o}\right) \quad \text{..... (1)}$$

where L and l are the total capillary length and the length to the detector, respectively, V is the run voltage, t_i is the migration time, and t_0 is the migration time of methanol, used as neutral marker to correct changes in solution viscosity caused by variations in CD concentration. At acidic conditions, the migration time of methanol is very slow, thus t_0 in acidic conditions was neglected. Experimental data and parameters were calculated using Excel from Microsoft Office 2007. Binding constants can be determined using the equation:

$$K[CD] = \left(\frac{\mu_f - \mu_i}{\mu_i - \mu_c}\right) \quad \text{..... (2)}$$

where K is the binding constant, [CD] is the concentration of cyclodextrin that was added in the separation, μ_f and μ_c are the electrophoretic mobilities of the free and complexed analyte, and μ_i is the analyte mobility at the cyclodextrin concentration [CD]. μ_c is measured from the analyte mobility at very high concentrations of CD, but its measurements is difficult or imposible due to the difficulty of finding suitable CD markers and reaching saturating conditions (Martin-Biosca, Garc'ya-Ruiz, and Marina, 2000). Equation (2) can be rearranged to avoid the measurements of μ_c, following the equation below:

$$\frac{1}{(\mu_i - \mu_f)} = \frac{1}{K[CD](\mu_c - \mu_f)} + \frac{1}{(\mu_c - \mu_f)} \quad \text{..... (3)}$$

6.3 Results and Discussion

6.3.1 Determination Kf Value of Diniconazole with HP-γ-CD under neutral pH.

The choosing of the type and concentration of the chiral selector is the most important step in developing a chiral separation method. In this study, HP-γ-CD was chosen based on previous study that was successfully used in the enantioseparation of fungicides (Wan Ibrahim, *et al.*, 2007). Figure 6.1 shows the electropherogram of

diniconazole enantiomers separation at 20˚C. It can be observed that migration times decreased when HP-γ-CD concentration was increased. This phenomena show the differences with separation using cyclodextrin by EKC, where the increase of cyclodextrin concentration caused the increase of migration time, due to the increase in the viscosity of the solution (Martin-Biosca, Garc'ya-Ruiz, and Marina, 2000). In CD-MEKC method, the mobility was not only caused by viscosity of the solution, but the distribution of the analyte with CD and micellar phase. Separation under neutral pH causes all solutes to migrate towards the negative electrode by electrophoresis (Otsuka and Terabe 1996). The presence of higher concentration of cyclodextrin may decreased the distribution of analyte in micellar phase, thus the analyte will migrate faster toward the negative electrode.

Table 6.1 shows the K_f value of diniconazole with HP-γ-CD at several temperatures. Temperature is an important factor in controlling chiral recognition processes (Jiao, *et al.*, 2008). Data obtained shows good linearity. However, all K_f value obtained has a negative value. It's a unique phenomenon, where the value of formation constant shows a negative value. In this case it may be caused by the behavior of analyte in CD-MEKC under neutral condition that migrate slower at high concentration of cyclodextrin. For that reason, the determination of diniconazole enantiomers by CD-MEKC under acidic condition was then explored.

Table 6.1 K_f value of diniconazole enantiomers with HP-γCD at several temperatures under neutral condition.

Diniconazole Enantiomer	Equation[a]	R^2	K_f (M^{-1})
15˚C			
E1	y = 0.0033x - 0.0217	0.9964	-6.57
E2	y = 0.0035x - 0.0177	0.9959	-5.06
20˚C			
E1	y = 0.0030x - 0.0186	0.9998	-6.20
E2	y = 0.0031x - 0.0112	0.9990	-3.61
25˚C			
E1	y = 0.0025x - 0.0113	0.9992	-4.52
E2	y = 0.0026x - 0.0081	0.9975	-3.11

[a] $y = \frac{1}{(\mu_i - \mu_f)}$, $x = \frac{1}{[CD]}$

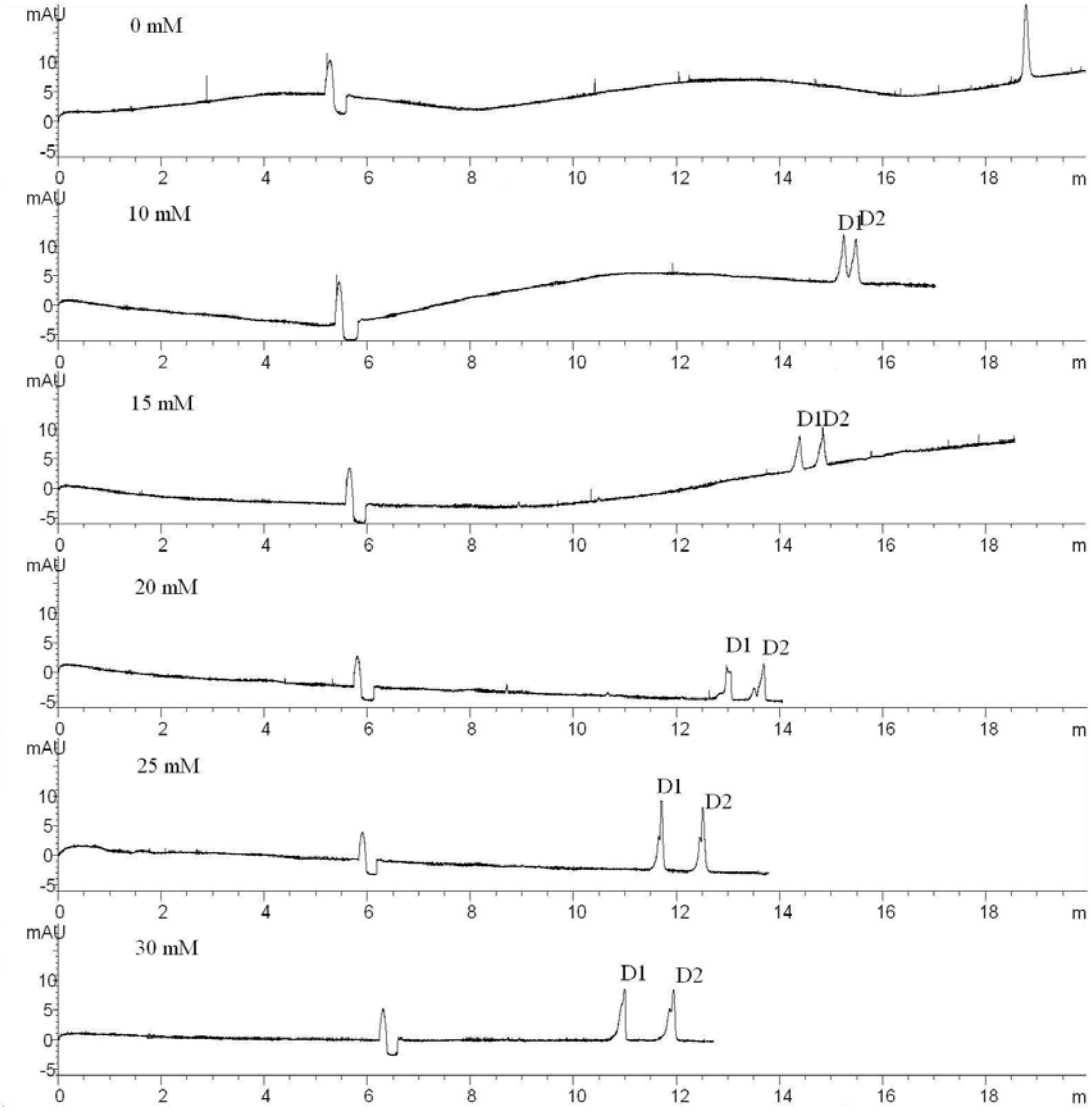

Figure 6.1 Electropherogram of the separation of diniconazole enantiomers by CD-MEKC at several HP-γ-CD concentrations. Other conditions: 25 mM phosphate buffer (pH 7.0); 40 mM SDS, applied voltage +20kV, Temperature 20˚C. Injection at 50 mbar for 1s. Effective length of Capillary 56 cm (i.d. 75 μm)

6.3.2 Determination of Kf Value of Diniconazole and Triadimefon with HP-γ-CD under acidic pH

The determinatio of K_f value of diniconazole enantiomers- HP-γ-CD under acidic condition was investigated and include the determinataion of K_f value of triadimefon enantiomers-HP-γ-CD simultaneously. Figure 6.2 shows the electropherogram of eantioseparation of diniconazole and triadimefon enantiomers with various HP-γ-CD concentrations at 20˚C. The K_f values of diniconazole with

HP-γ-CD at several temperatures are summarized in Table 6.2, whereas K_f value of diniconazole with HP-γ-CD are summarized in Table 6.3. Both Kf values of diniconazole enantiomers-HP-γ-CD and K_f values of triadimefon enantiomers-HP-γ-CD shows negative values.

Table 6.2 K_f value of diniconazole enantiomers with HP-γ-CD at several temperatures under acidic condition.

Diniconazole Enantiomer	Equation[a]	R^2	K (M^{-1})
20˚C			
E1	y = 0.0049x - 0.0323	0.9924	-6.59
E2	y = 0.0041x - 0.0158	0.9468	-3.85
25˚C			
E1	y = 0.0035x - 0.0174	0.9955	-4.97
E2	y = 0.0034x - 0.0124	0.9921	-3.65
30˚C			
E1	y = 0.0036x - 0.0110	0.9918	-3.06
E2	y = 0.0032x - 0.0082	0.9962	-2.56

[a] $y = \frac{1}{(\mu_i - \mu_f)}$, $x = \frac{1}{[CD]}$

Table 6.3 K_f value of triadimefon enantiomers with HP-γ-CD at several temperatures under acidic condition.

Triadimefon Enantiomer	Equation	R^2	K (M^{-1})
20˚C			
E1	y = 0.0036x - 0.0128	$R^2 = 0.9898$	-3.56
E2	y = 0.0027x - 0.0075	$R^2 = 0.9952$	-2.78
25˚C			
E1	y = 0.0027x - 0.0074	$R^2 = 0.9951$	-2.74
E2	y = 0.0027x - 0.0067	$R^2 = 0.9946$	-2.48
30˚C			
E1	y = 0.0026x - 0.0053	$R^2 = 0.9929$	-2.04
E2	y = 0.0033x - 0.0043	$R^2 = 0.9681$	-1.30

[a] $y = \frac{1}{(\mu_i - \mu_f)}$, $x = \frac{1}{[CD]}$

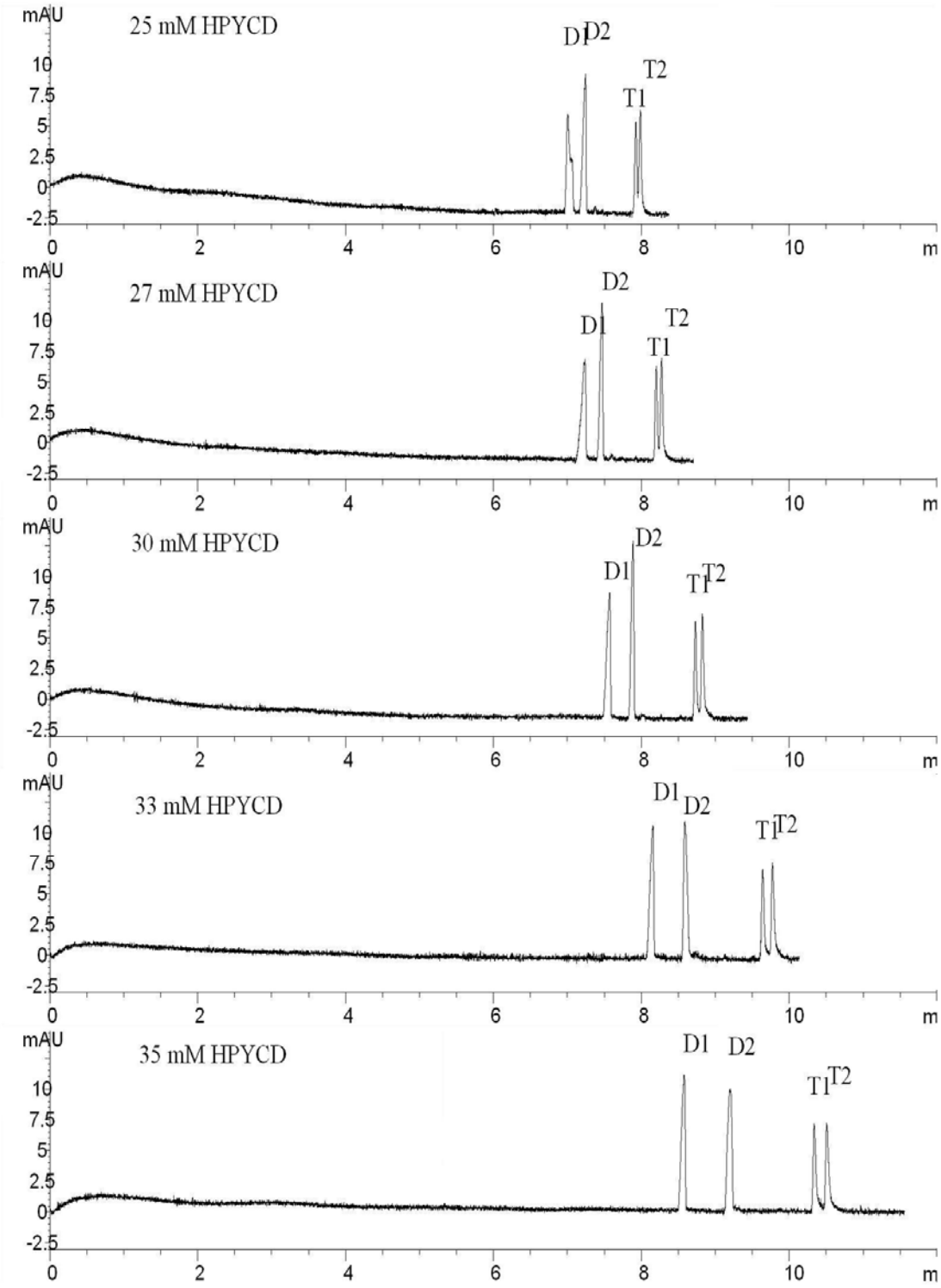

Figure 6.2 Electropherogram of the separation of diniconazole and triadimefon enantiomers by CD-MEKC at several HP-γ-CD concentrations. Other conditions: 25 mM phosphate buffer (pH 3.0); 40 mM SDS, applied voltage -20kV, Temperature 20˚C.Injection at 50 mbar for 1s. Effective length of Capillary 56 cm (i.d. 50 μm)

6.4 Concluding Remarks

Complex formation constant, K_f, of diniconazole-HP-γ-CD either under neutral or under acidic at several temperatures was investigated. All condition gave a negative value of K_f of diniconazole-HP-γ-CD. K_f of triadimefon-HP-γ-CD was also investigated under acidic condition. However a negative value of K_f was obtained. As we know formation constant value should be a positive value. There is factor that caused a negative value obtained i.e. unsuitable of formula with the investigation condition. To the best authors' knowledge, determination of K_f value has never been reported using MEKC method. Over all can be concluded that the formula in Eq.1 and Eq.2 is unsuitable to determine K_f value using MEKC method, because in MEKC, micellar phase gave high effect to complex formation of analyte with cyclodextrin.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

Cyclodextrin-modified micellar elektrokinetic chromatography has been developed and used for the separation of enantiomers of triazole fungicide (cyproconazole, bromuconazole, diniconazole, hexaconazole, penconazole, triadimefon, and myclobutanyl). Micellar electrokinetic chromatography (MEKC) method was able to simultaneous separate cyproconazole, bromuconazole, and diniconazole enantiomers using dual CD system. Effects of several parameters in MEKC were explored to improve the performance of separation. The use of a mixture of 27 mM HP-β-CD, 3 mM HP-γ-CD, 40 mM SDS and the addition 15% (v/v) of *i*-PrOH as organic modifier in 25 mM phosphate buffer solution (pH 3.0) was able to separate two pairs of cyproconazole and bromuconazole enantiomers, and one pair of diniconazole enantiomer with resolution (R_s) greater than 1.7, peak efficiencies (N) greater than 400 000 and better analysis time (< 23 min) compared with previous study for cyproconazole analysis using SFC (30 min) (Toribio, *et al.*, 2004), and analysis of diniconazole using CE (30 min) (Wu, *et al.*, 2001). However, detectability observed was poor in this CD-MEKC method, with LOD for each compounds is 15 mg L^{-1}.

Simultaneous enantioseparation of the three triazole fungicides was successfully achieved at 30 mM HP-γ- CD concentration in 25 mM phosphate buffer (pH 3.0) solution containing 50 mM SDS, 10% (v/v) methanol and 5% (v/v) acetonitrile with resolutions (Rs) between enantiomers for penconazole, Rs = 0.81; myclobutanyl, Rs = 1.14; triadimefon, Rs = 2.09; peak efficiencies (N) greater than 108 000 for all stereoisomers and analysis time of less than 15 min. Whereas the simultaneous enantiomeric separation of hexaconazole, penconazole, and

myclobutanil using HP-γ-CD was achieved with the use of a mixture of 40 mM HP-γ-CD and 50 mMSDS in 25 mM phosphate buffer solution (pH 3.0) with resolution (Rs) greater than 1.60, peak efficiencies (N) greater than 200000 and analysis time within 15 min.

In order to improve detection sensitivity, sweeping as an on-line preconcentration technique was investigated. Sweeping preconcentration was performed at optimum condition, i.e. at pH 3.0. In this study, sweeping preconcentartion techniques could improve the detection sensitivity 30 to 60-fold for all enantiomers. Repeatabilities in the migration time, peak area and peak height in terms of the relative standard deviation were good ranging from (0.08-0.32)%, (0.03-2.44)%, and (2.13-8.44)% respectively. The LOD for all enantiomers is 0.3 mg L^{-1} on average. The LODs values for diniconazole enantiomers are better than previous study by Wang (1998). The SRMM-CD-MEKC and sweeping-CD-MEKC methods could be used to increase detection sensitivity and simultaneous chiral separation of hexaconazole, penconazole and myclobutanil. The results indicated that sweeping-CD-MEKC method gave the best detection sensitivity with a limits of detection (S/N = 3) for the selected triazole fungicides ranged from 0.1 to 0.2 mg L^{-1}. The online preconcentration method developed using sweeping CD-MEKC for the three triazole fuingicides gave a much higher detection sensitivity increase (~70-fold) compared to the one attained by Otsuka et al. using both sweeping and stacking with a reverse migrating pseudostationary phase for triadimenol, a fungicide from the triazole fungicide class, separation i.e. ~10-fold. This is first report on the on-line preconcentration and chiral separation of hexaconazole, penconazole and myclobutanil by CD-MEKC in only one injection. The developed sweeping-CD-MEKC method can be used as a preliminary study for further study on the analysis of the selected chiral triazole fungicides on real samples using MEKC with UV detector.

Furthermore complex formation constant, K_f, of diniconazole-HP-γ-CD either under neutral or under acidic at several temperatures was investigated. K_f, of triadimefon-HP-γ-CD was also investigated under acidic condition. However a negative value of K_f was obtained either for complex of triadimefon- HP-γ-CD or

complex of diniconazole- HP-γ-CD. To the best authors' knowledge, determination of K_f value has never been reported using CD-MEKC method. Over all it can be concluded that the formula in Eq.1 and Eq.2 is unsuitable to determine K_f value using CD-MEKC, because in MEKC, micellar phase gave high impact to complex formation of analyte with cyclodextrin.

7.2 Future Directions

In this study, sweeping preconcentartion techniques only could improve the detection sensitivity 30 to 60 fold for all enantiomer. For this reason, there is need to investigate the other preconcentration technique i.e. high conductivity sample stacking mode (HCSSM)-MEKC. To increase the recoveries of selected triazole fungicide, salting out effect with sodium chloride might help especially for the more polar triazole fungicides. Then is needed to prepare proper model for K_f determination using CD-MEKC for triazole fungicides.

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APPENDICES

APPENDIX A
PUBLICATIONS

Wan Aini Wan Ibrahim**,** Susanti A. Warno, Hassan Y. Aboul Enein, Dadan Hermawan and M. Marsin Sanagi. (2009). Simultaneous enantioseparation of cyproconazole, bromuconazole, and diniconazole enantiomers by cyclodextrin-modified micellar electrokinetic chromatography. *Electrophoresis***,** 30, 1976-1982.

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APPENDIX B

PRESENTATIONS

Wan Aini Wan Ibrahim, Susanti, M. Marsin Sanagi, Dadan Hermawan and Hassan Y. Aboul Enein. (2009). Electrophoretic separation of chiral agrochemicals using neutral cyclodextrin based dual selector system. *Poster presented at Second International Conference and Workshops on Basic and Applied Sciences & Regional Annual Fundamental Science Seminar 2009.* 2nd- 4th June 2009, Johor Bahru, Malaysia.

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