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IMMOBILIZATION OF CYCLODEXTRIN FOR THE DETERMINATION OF BENZIMIDAZOLE FUNGICIDES

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พัชรวรรณ จงมีวาสนา: การตรึงไซโคลเดกซ์ทรินเพื่อใช้ในการตรวจวัดปริมาณสารฆ่าราเบนซิมิดาโซล (IMMOBILIZATION OF CYCLODEXTRIN FOR THE DETERMINATION OF BENZIMIDAZOLE FUNGICIDES) อ. ที่ปรึกษา : อ. ดร. มัญชุมาส เฮงสกุล, อ.ที่ปรึกษาร่วม : รศ. ดร. เปี่ยมสุข พงษ์สวัสดิ์ ; 103 หน้า. ISBN 974-13-0762-4.

ไซโคลเดกซ์ทรินเป็นโอลิโกแซคคาไรด์รูปวงแหวนที่มีความสำคัญอย่างมากในเทคโนโลยีเกี่ยวกับ Inclusion complex ซึ่งมีการนำไซโคลเดกซ์ทรินไปประยุกต์ใช้ในด้านเคมีวิเคราะห์ โดยเฉพาะอย่างยิ่งการพัฒนาเป็นโมเลกุลที่สามารถใช้ตรวจวัด (molecular sensor) สารบางชนิด สำหรับงานวิจัยนี้ ได้เสนอวิธีการนำไซโคลเดกซ์ทรินที่ตรึงบนไมโครไตเตอร์เพลทมาใช้เพื่อตรวจวิเคราะห์สารฆ่าราเบนซิมิดาโซล โดยแข่งขันกับตัวติดตามเบนซิมิดาโซล (benzimidazole tracer) ในการจับกับไซโคลเดกซ์ทริน (competitive benzimidazole tracer encapsulation) จากการทดลอง divinylsulfone สามารถใช้ตรึงบีตา-ไซโคลเดกซ์ทรินบนผิวอะมิโนไมโครไตเตอร์เพลทได้ และบีตา-ไซโคลเดกซ์ทรินนั้นยังคงสามารถจับกับสารที่ต้องการตรวจสอบได้ โดยการทดสอบกับตัวติดตามเบนซิมิดาโซลที่เป็นคอนจูเกตระหว่าง 2-succiamidobenzimidazole และ horseradish peroxidase เมื่อหาภาวะที่เหมาะสมในการเกิดเอนแคปซูเลชัน (encapsulation) ของตัวติดตามเบนซิมิดาโซล จากการทดลองพบว่าการใช้ ตัวติดตามเบนซิมิดาโซลความเข้มข้น 2.5 μg/ml บ่มในไมโครไตเตอร์เพลทที่ตรึงโดยใช้สารละลายไซโคลเดกซ์ทริน 5% (w/v) นาน 1 ชั่วโมง ที่ 25 °C และใช้ 0.1 M acetate/citric acid buffer, pH 5 เป็นบัฟเฟอร์สำหรับล้างการจับแบบไม่จำเพาะ

เมื่อทำการทดสอบวิธีการดังกล่าวเพื่อประยุกต์ใช้ โดยการนำไปตรวจวิเคราะห์สารมาตรฐานคาร์เบนดาซิมและสารสกัดจากองุ่นที่เติมสารมาตรฐานคาร์เบนดาซิม พบว่าวิธีการดังกล่าวสามารถนำไปใช้ในการตรวจวิเคราะห์สารคาร์เบนดาซิมได้ โดยสามารถตรวจพบสารคาร์เบนดาซิมในสารสกัดจากองุ่นได้ที่ประมาณ 3 ppm เป็นความเข้มข้นต่ำสุดที่ทำให้เกิดการยับยั้งทั้งหมดของการเอนแคปซูเลชันของตัวติดตามเบนซิมิดาโซล ซึ่งวิธีการดังกล่าวสามารถนำไปใช้ในการตรวจสอบเบื้องต้นเพื่อทดสอบการตกค้างสารคาร์เบนดาซิม หรือสารอื่นที่ใกล้เคียงในภาวะจำเพาะกับสารนั้นได้ ถึงแม้ว่าวิธีการนี้จะยังไม่สามารถตรวจวัดปริมาณสารคาร์เบนดาซิมได้และมีค่าการตรวจวัดต่ำสุด สูงกว่าวิธี ELISA อย่างไรก็ตามวิธีนี้สามารถปรับปรุงให้มีประสิทธิภาพมากขึ้นและสามารถตรวจวัดปริมาณได้ในงานวิจัยต่อไป

ภาควิชา.....-.....ลายมือชื่อนิสิต.....

สาขาวิชา.....เทคโนโลยีทางชีวภาพ.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2543.....ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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PACHARAWAN JONGMEEVASANA : IMMOBILIZATION OF CYCLODEXTRIN FOR THE DETERMINATION OF BENZIMIDAZOLE FUNGICIDES. THESIS ADVISOR : MANCHUMAS HENGSAKUL, Ph.D. THESIS CO-ADVISOR : ASSOC. PROF. PIAMSOOK PONGSAWASDI, Ph.D. 103 pp. ISBN 974-13-0762-4.

Cyclodextrins (CDs), the cyclic oligosaccharides play an important role in the inclusion complex technology. Its application in analytical chemistry with both chromatographic and non-chromatographic methods is remarkably increased, especially, the development of CDs as molecular sensors. In this study, the use of immobilized CD on microtiter plate and the competitive benzimidazole tracer encapsulation approach were employed and developed for the determination of benzimidazole fungicides. A specialized technique for CD immobilization on a microtiter plate was first developed. The result indicated that CD could be immobilized on microtiter plate by the use of divinylsulfone as a cross-linking reagent and the immobilized CD could entrap benzimidazole tracer. The optimization of benzimidazole tracer for the encapsulation into immobilized CD was determined. It was found that the optimal condition was the use of 2.5 μg/ml benzimidazole tracer for the encapsulation into amino microtiter plate coated with 5% β-CD, incubated for 1 hour at 25 °C and 0.1 M acetate/citric acid buffer, pH 5 as washing buffer.

The capability test of competitive benzimidazole tracer encapsulation was examined by the use of carbendazim standard and spiked sample solution. The result indicated that the approach could be used for carbendazim detection in grape extract at approximately 3 ppm, which was the minimum concentration completely inhibited benzimidazole tracer encapsulation. Although, the approach showed the lower sensitivity than ELISA method, it could be available for screening purpose and could be further improved for quantitative determination of carbendazim and other related compounds under specific condition.

Department……-……..Student's signature……
Field of study……Biotechnology……..Advisor's signature……
Academic year……2000……Co-advisor's signature……..

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ABBREVIATIONS

A	absorbance
BNZ	benzimidazole
CD	cyclodextrin
°C	degree Celsius
DVS	divinylsulfone
ELISA	enzyme linked immunosorbent assay
g	gram
μg	microgram
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
LOD	limit of detection
LOQ	limit of quantitation
mA	milliampare
μl	microlitre
M	molar
ml	millilitre
mM	millimolar
MRLs	maximum residue limits
nm	nanometer
PBS	phosphate buffer saline
PAGE	polyacrylamide gel electrophoresis
SAB	2-succiamidodenzimidazole
SDS	sodium dodecyl sulfate
TMB	tetramethylbenzidine
V	volt
w/v	weight by volume
WSC	water soluble carbodiimide

CHAPTER I

INTRODUCTION

1.1 Cyclodextrins

Cyclodextrins (hereinafter referred to CDs or CD) are cyclic oligosaccharides having the structure of a hollow truncated cone with hydrophobic cavity (*Figure 1*). There are primary hydroxyl groups on the narrow rim of the cyclodextrin cavity and secondary hydroxyl groups on the wider rim. Because of the presence of the hydrophilic hydroxyl groups existing at the two ends of the cavity, CDs are soluble in water, whereas the interior cavity is relatively hydrophobic. The most common CDs are α-, β-, and γ-CD, which consist of six, seven and eight glucopyranose units, respectively (*Table 1*). In aqueous solution, the hydrophobic environment of the cavity enables CDs to form inclusion complexes with water-insoluble compound. Inclusion complexes are defined as entities comprising two or more molecules, in which one of the molecules (the host) totally or partially encloses a guest molecule by physical forces only (Luong, *et al*. 1995). Their complexation ability has been attributed to four factors: (1) Van der Waals interactions, (2) hydrogen bonding, (3) displacement of high-energy water molecules from the cavity and (4) release of strain energy of the CD on inclusion of a guest molecule. These physical interactions can be considered as molecular encapsulation. The selectivity and sensitivity of the complexation are strongly dependent on the analyte (inclusate) geometry and functional group orientation (Gong and Zhang, 1996). The unique ability of cyclodextrins to entrap certain molecules in their molecular cavities offers remarkable effects in stabilizing and solubilizing lipophilic, unstable substances without the formation of chemical bonds and without changing their structures. The complexation technique has been used successfully over the past 25 years mainly in pharmaceutical industry, food technology and agriculture. In recent years, the application of CDs in analytical chemistry has been increasing remarkably, in both chromatographic and non-chromatographic uses. In order to illustrate the application of cyclodextrins in analytical uses, *Table 2* shows many forms of CDs such as free, immobilized and polymer forms of native and modified CDs used in this application. Moreover, CD supramolecules are particularly interesting to be used as sensing molecules. The schematic structures of some CDs supramolecules are shown in *Figure 2*.

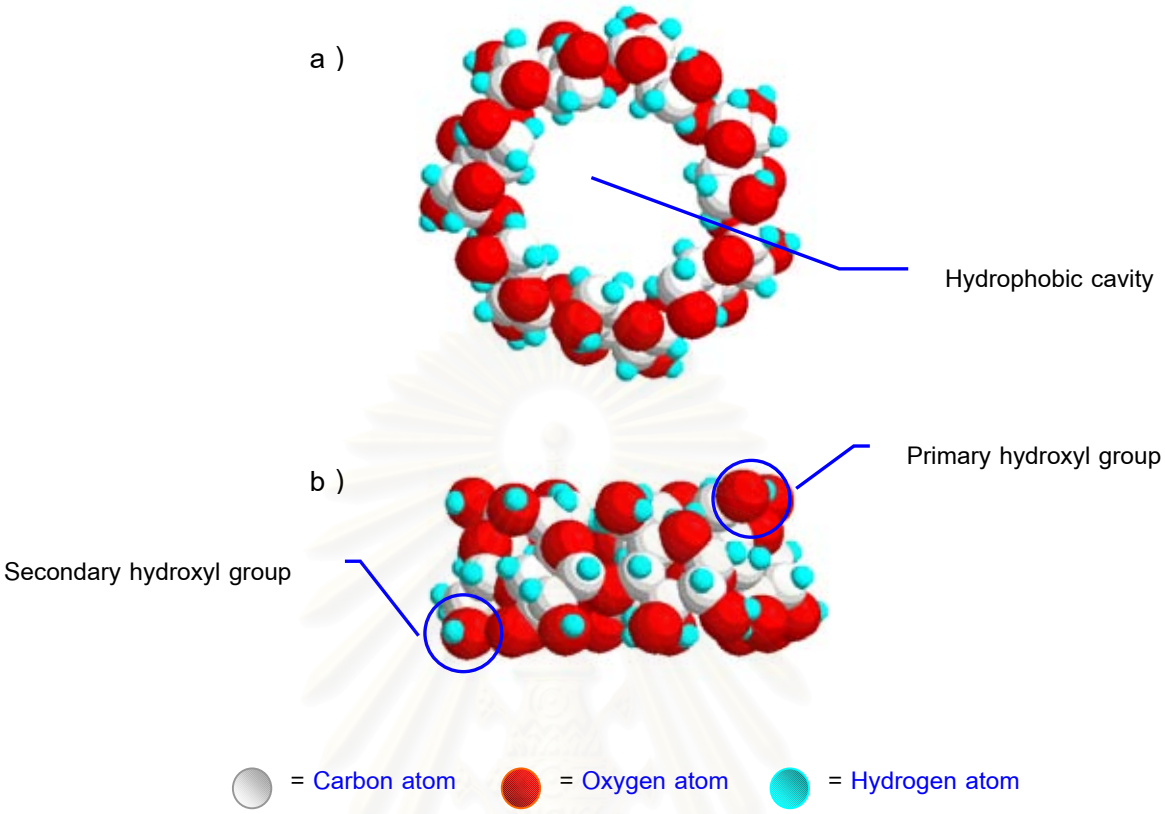
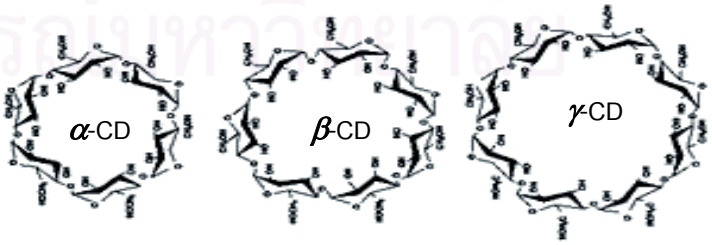


Figure 1. The schematic structure of β-cyclodextrin: ***a. top view and b. side view***

Table 1. Characteristics and dimension of cyclodextrins

	Cyclodextrin (CD)		
Characteristic	α-CD	β-CD	γ-CD
Glucopyranose units	6	7	8
Percentage solubility in water(w/v)	14.5	1.8	23.2
Molecular weight	972	1135	1297
Molecular structure			

(Szejtli,1988 and Szejtli,1990)

Table 2. Application of cyclodextrins in analytical uses

Cyclodextrins(CDs)	Published studies and patents
Chromatographic uses	
1. Native CDs	
1.1 Immobilized β-CD	1. Retention time behaviors of some aromatic compounds on chemically bounded cyclodextrin silica phase in liquid chromatography. (Fujimura, Uedo and Ando, 1983)
1.2 Immobilized α- and β-CD	2. Examination of the enantioselectivity of wall- immobilized cyclodextrin copolymers in capillary gas chromatography. (Tanng, Zhou and Armstrong, 1994)
2. Modified CDs	
2.1 Free form of sulfobutyl ether-β-CD and methyl-β-CD	1. Cyclodextrin- modified capillary electrophoresis: Determination of polycyclic aromatic hydrocarbon in contaminated soil. (Brown *et al.*,1996)
Non-Chromatographic uses	
1. Native CDs	
1.1 β-CD polymer	1. Determination of trace amounts of manganese by beta-cyclodextrin polymer solid phase spectrophotometry using 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol. (Jiang *et al.*, 1999)
1.2 Immobilized CD	2. Cyclodextrin-based optosensor for the determination of warfarin in waters.(Badia and Diaz-Garcia,1999)
2. Modified CDs	
2.1 Fluorescent CD polymer	1. Fluorescent polymer labeled conjugates and intermediates. US patent 56,661,040 (Huff *et al.*, 1997)
2.2 Immobilized ethyl-β-CD	2. Determination of tetracyclines with a modified β-cyclodextrin base fluorosensor. (Gona and Zhang *et al.*, 1997)
2.3 Supramolecular CD	3. A supramolecular chemosensor for aromatic hydrocarbons. (Mortellaro and Nocera *et al.*, 1996)
	4.Guest-induced colour changes and molecule-sensing abilities of p-nitrophenol-modified cyclodextrins. (Matsushita *et al.*, 1997)

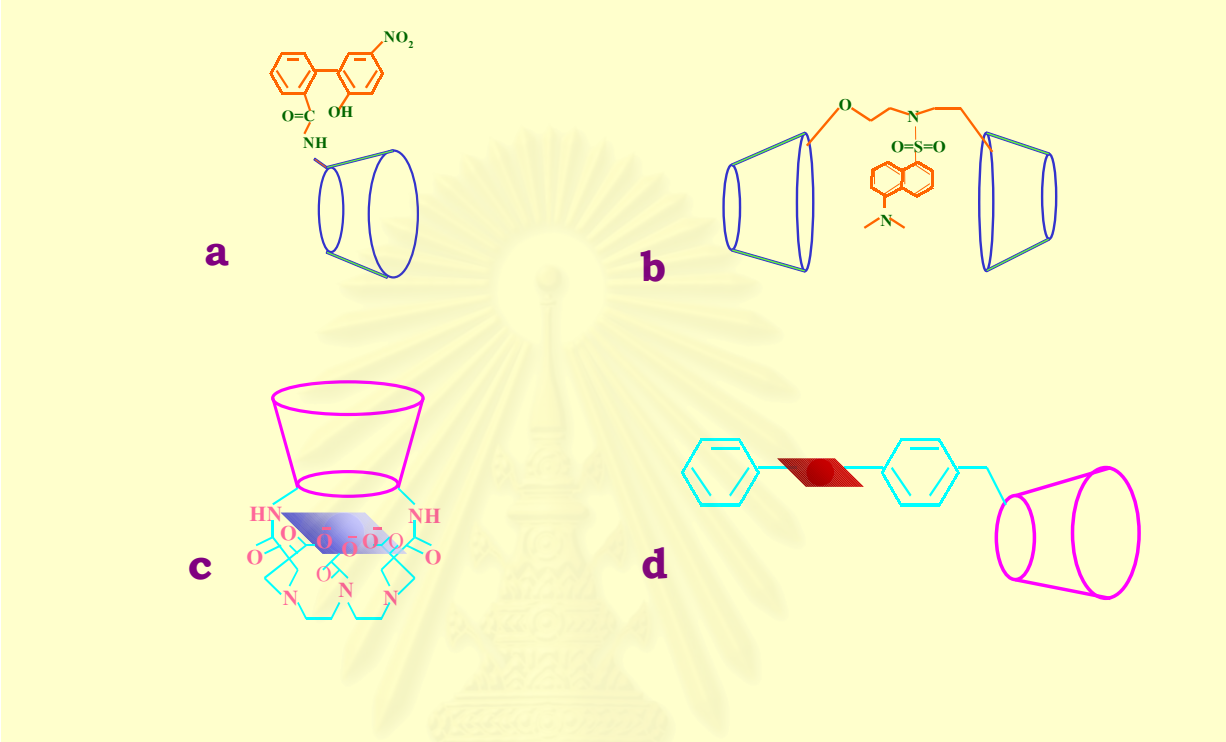


Figure 2. Schematic structure of some supramolecular cyclodextrins: *The truncated cone is used to represent CD structure.*

a) p-nitrophenol modified-CD

b) Fluorescent-CD-dimer

c) Photoactive metallo-CD; lanthanide ion was present as

d) Luminescent CD; rubidium (II) was present as

CD supramolecules are CDs modified with chromophore, fluorophore, photoactive metal complexes or other easily detecting functional groups in order to be used as sensor for detecting certain organic compounds. *Figure 2a* shows the schematic structure of CD modified with chromophore (*p*-nitrophenol). In aqueous solution, it changes from yellow to colorless when guest presents in its cavity (Matsushita *et al*., 1997). While, CD dimers connected with fluorophore (*Figure 2b*) responded to the presence of steriods with an increase or decrease of the fluoresence intensity depending on the substitution pattern of the steriod (De Jong. *et al*., 2000). The luminescent CD is CD with lanthanide ion (terbium or europium 3^+ ion) at the bottom of CD bucket (*Figure 2c*). When the benzene fills the CD bucket, the lanthanide ion can harvest light photons by adsorption and pass the energy from the bottom of the bucket to " light up" a bright red or green luminescence, depending on whether the lanthanide ion is europium or terbium, respectively (Mortellaro and Nocera, 1996). *Figure 2d* displays another type of luminescent CD, CD with Ru (II) complex exhibit luminescence that is quenched by guest binding (Magennis *et al*., 1999). All of these applications mentioned above obviously indicated that CDs are attractive molecules to be used as molecular sensor for determining some aromatic compounds.

1.2 The immobilization of cyclodextrins

The application of immobilized CDs are commonly used in both chromatography and non chromatography. In the field of chromatography, the attention is paid to the use of immobilized CDs as the stationary phase. Therefore, various methods for CDs immobilization on supports have been prepared. Silica is the main support used for CDs immobilization as chiral stationary phases in gas and liquid chromatographic column (He and Zhao, 1993). Generally, most of immobilization methods are achieved by introducing spacer arm between CDs and the support as shown in *Figure 3a*. CD was bonded to silica gel by the use of 3-aminopropyl-trimethoxysilane and this modified CD was used to detect retention behavior of some aromatic compound in LC (Fujimura, Ueda and Ando, 1983). Moreover, CDs could be immobilized on wall of fused–silica capillary by branched-attachment of organosilane oligomers for enantioselectivity in GC capillary (Tang, Zhou and Armstrong, 1994) as shown in *Figure 3b*. In the field of non-chromatography, although CDs immobilized directly on polystyrene bead as biospecific adsorbents for removal of toxins was suggested as shown in *Figure 3c* (He and Zhao, 1993).

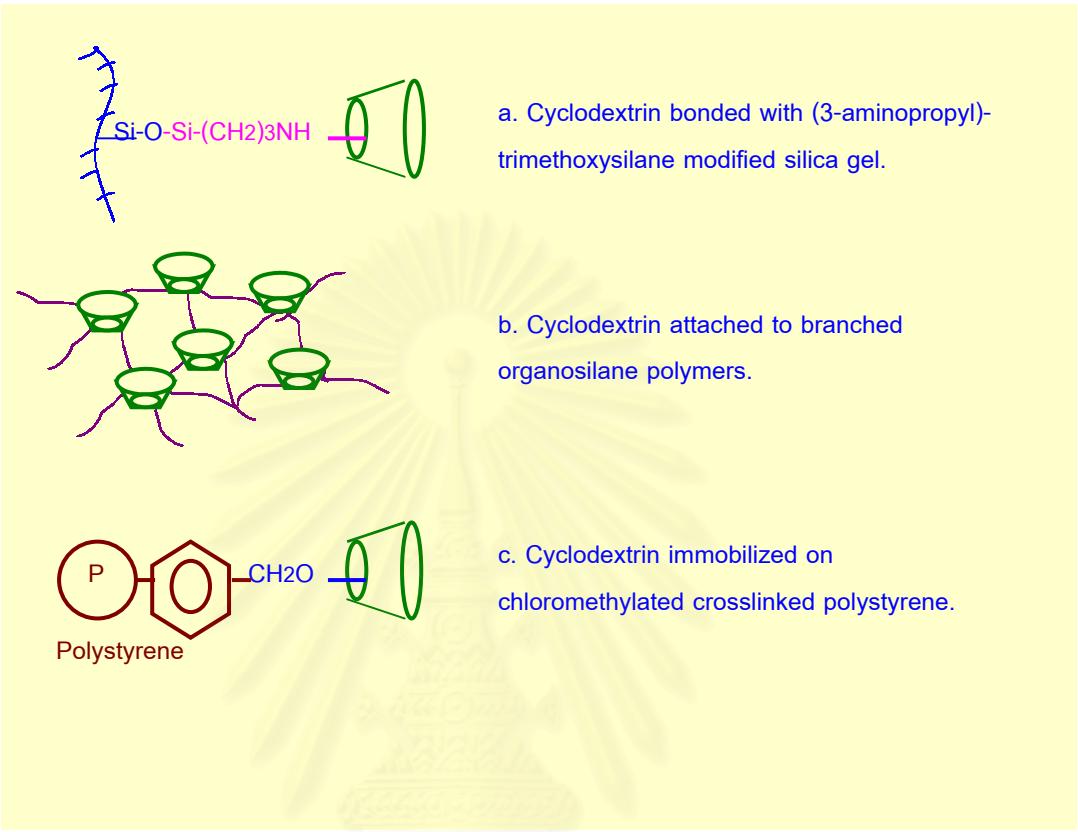


Figure 3. Schematic structure of immobilized CD: *the truncated cone was used to represent CD structure.*

The CD immobilized on silica gel by introducing spacer arm was also commonly used as recognition phase based fluorosensor (Gong and Zhang. 1997) and optosensor (Badia and Diaz-Garcia. 1999) for the determination of tetracyclines and warfarin (pesticide), respectively.

1.3 Benzimidazole fungicides

Benzimidazole fungicides are systemic agrochemicals with fungitoxin activity e.g. benomyl, thiophanate-methyl, carbendazim and thiabendazole (***Figure 4***). Benomyl (methyl (1-(butylcarbamoyl)-1*H*-benzimidazole-2-yl) carbamate) is one of the most wildely used systemic fungicides, but its distribution in the environment is still not clearly understood. The major reason is that it is difficult to trace the behavior of benomyl that rapidly decomposes to carbendazim (methyl-2-benzimidazolecarbamate, MBC) once dissolved in water (Clemons and Sisler, 1969; Peterson and Edginton, 1970). Due to its extensive use and carcinogenic activity, the determination of benomyl and its main product compound carbendazim have been the objectives of several studies in environmental water, soil extract and crops. Thiophanate-methyl is a benzimidazole precursor. It also is converted to carbendazim under natural and artificial conditions. Low recoveries of thiophanate-methyl, especially from crop samples, are due to its degradation during analytical procedures. Therefore, thiophanate-methyl is usually determined as carbendazim after quantitative conversion. Moreover, tolerance levels for thiophanate-methyl and benomyl are expressed as carbendazim limits (Hiemstra *et al*., 1995). The degradation of benomyl and thiophanate-methyl to carbendazim is shown in ***Figure*** 5. Thiabendazole (2-(4-thiazolyl)-1*H*-benzimidazole, TBZ) is not only used as a postharvest fungicide to control green mold and blue mold, but also used as a benzimidazole anthemintic drugs for animals. Because thiabendazole is relatively safe with acceptable daily intake (ADI) established by the World Health Organization (WHO) of 0.3 mg/kg of body weight per day (Brandon *et al.*1993). Benzimidazole fungicides are systemic fungicides widely used for pre and postharvest control of fruits and vegetables fungal diseases. Fungicides play a major role in food protection and quality preservation. Therefore, the monitoring of these fungicide residues is crucial for proper assessment of exposure to fungicides through food.

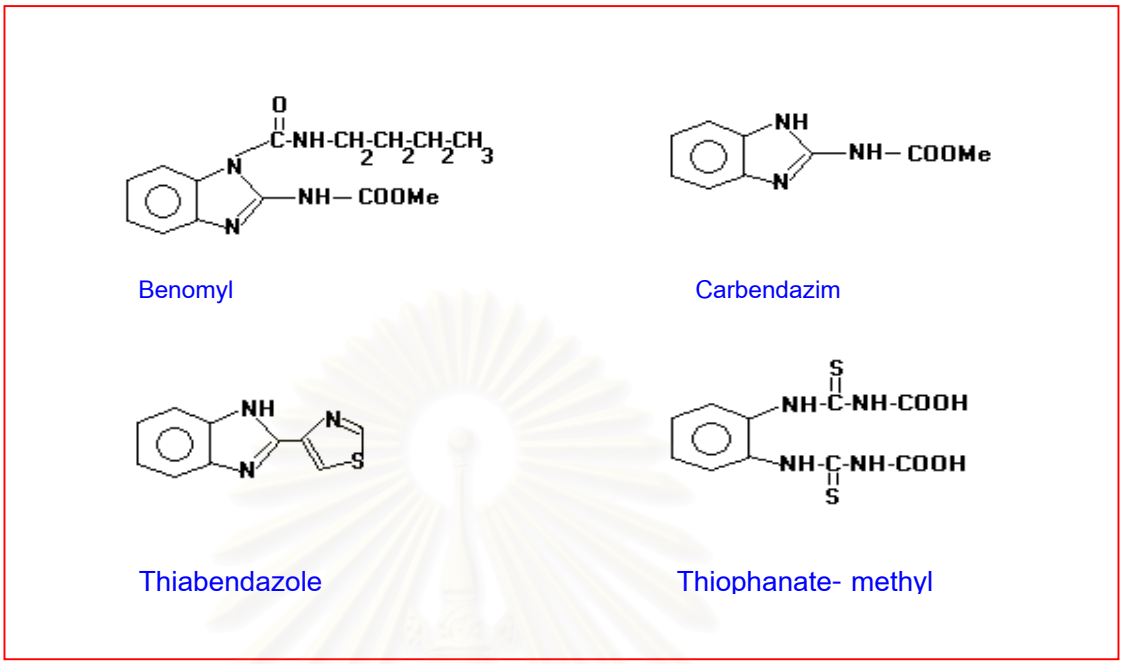


Figure 4. Schematic structure of benzimidazole fungicides

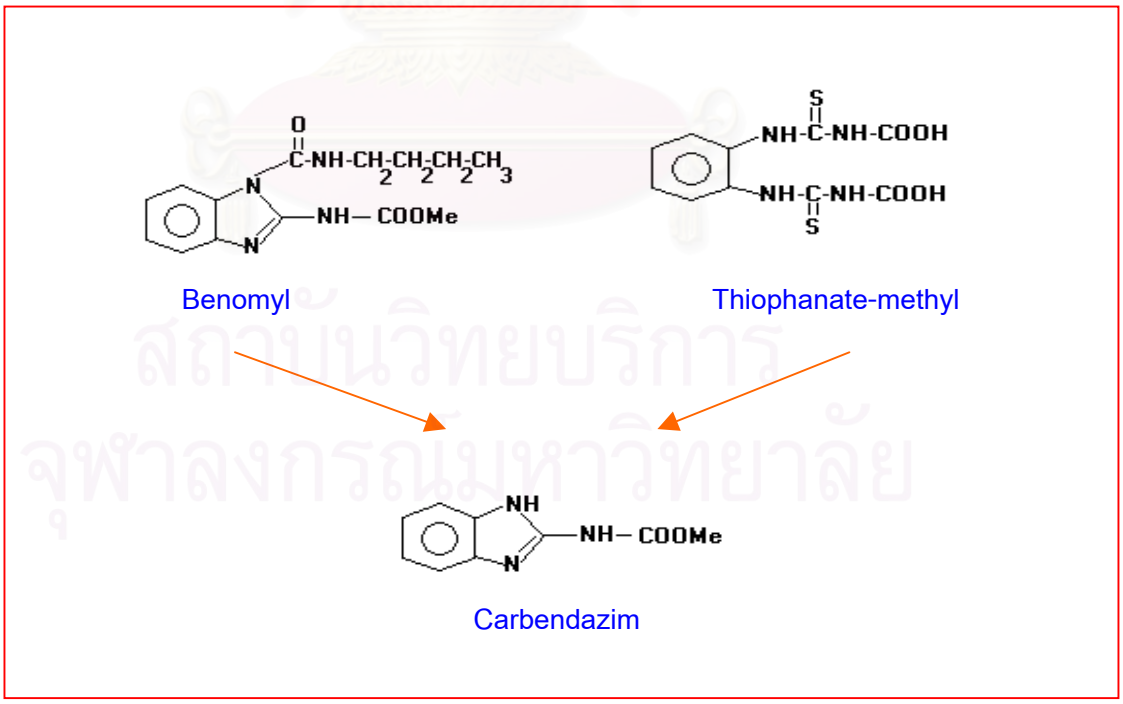


Figure 5 Carbendazim is the degraded product of benomyl and thiophanate-methyl.

1.3.1 Maximum residue limits of benzimidazoles

Maximum residue limits (MRLs) is the maximum concentration of an agrochemical permitted legally in a foodstuff. MRLs have been set by government agencies in order to regulate the use of agrochemicals that affect their consumers' health from harmful levels of them on foods. Factors, such as good agricultural practice (GAP), acceptable dietary intake (ADI)* and recommendations by the Codex Alimentarius Commission** are taken into account in setting MRLs. Benzimidazole is one of agrochemicals with veterinary drug residue limits and pesticides residues limits (MRLs) in many foods. The example of benzimidazole MRLs in crops accepted by the World Food Agriculture Organization, the World Health Organization and CODEX are shown in *Table 3 and 4*. Noticeably, most fruits and vegetables in the tables show benzimidazole fungicides MRLs in the range of 0.5-10 mg/kg. Therefore, the limit of detection and quantification for the benzimidazole determination should correspond to MRLs. In Thailand, there are no specific benzimidazole fungicides MRLs in any crops, although the major benzimidazole fungicide, carbendazim is one of top five fungicides imported to Thailand between the year 1995-1999 as shown in *Figure 6*. That is because there are no preliminary studies of benzimidazole fungicide residues in crops for setting MRLs.

1.3.2 Determinations of benzimidazole fungicides

Although, the standard method established in Pesticide Analytical Method Vol.1 (PAM I) for the determination of benzimidazole fungicides is chromatographic method (Givydis and Walter, 1990). The determination of benzimidazole fungicides still has been continuously developed in many studies. The conventional and available methods to quantify benomyl and carbendazim in food usually employ high performance liquid chromatography (HPLC) and enzyme linked immunosorbent assay (ELISA). The development of HPLC methods mainly focuses on the clean up step in order to increase the sensitivity and decrease time consuming. The examples are the automated method for clean up and determination of benzimidazole and thiabendazole in table ready foods (Levene, *et al* 1998) and the selective clean up applicable to aqueous acetone extracts for the determination of carbendazim and thiabendazole in fruits and vegetables by HPLC with UV detection (Muccio,*et al* 1999).

***Acceptable dietary intake (ADI)-**the daily dosage of a chemical, which, during an entire lifetime, appears to be without appreciable risk on the basis of all the facts known at the time. These are agreed upon for each agrochemical by FAO and WHO at their regular Joint Meeting on Pesticide residues.

** **Codex Alimentarius Commission**–an international body established by the FAO and WHO,with the objective of upgrading and simplifying international food regulations.Codex MRLs have been set for some agrochemicals in a range of crops,and several countries accept Codex MRLs in the absence of their own.

Table 3. Proposed maximum residue limits for carbendazim from various foodstuff [a]

Commodity	MRL (mg/kg)	Application	Commodity	MRL (mg/kg)	Application
Apricot	10c	B,C	Mushrooms	1	B,C,T
Asparagus	0.1d	B,T	Nectarine	2	B
Avocado	0.5	B	Onion, bulb	2	C,T
Banana	1c	B,C,T	Peach	10c	B,C,T
Barley straw and fodder, dry	2	B	Peanut	0.1d	B,C
Bean fodder	50	C	Peanut fodder	5	B,C
Beans, dry	2	B	Peppers	5	C
Berries and other small fruit	5	B,C,T	Pineapple	20c	B
Brussel sprouts	0.5	B	Plums	2c	B,C,T
Broad bean	2	T	Pome fruit	5c	B,C,T
Carrot	5c	C,T	Potato	3c	B,C
Cattle meat	0.1d	B	Poultry meat	0.1d	B,T
Celery	2	B,C	Rape seed	0.05d	C
Cereal grains	0.5	B,C,T	Rice straw and fodder, dry	15	B,C,T
Cherries	10c	B,C,T	Sheep meat	0.1d	B
Citrus fruits	10c	B,C,T	Soya bean, dry	0.2	C
Coffee beans	0.1d	C	Soya bean fodder	0.1d	C
Common bean	2	C	Squash, summer	0.5	B
Cucumber	0.5	B,C,T	Sugar beet	0.1d	B,C,T
Eggs(poultry)	0.1d	B,T	Sugar beet leaves on tops	10	B,C,T
Egg plant	0.5	C	Swede	0.1d	C
Gherkin	2	C,T	Sweet potato	1	B
Hops, dry	50	C	Taro	0.1d	B
Lettuce, head	5	B,C,T	Tomato	5	B,C,T
Mango	2	B	Tree nuts	0.1d	B
Melons, except watermelons	2c	B,C	Wheat straw and fodder, dry	5	B
Milk	0.1d	B	Winter squash	0.5	B

[a] From: FAO/WHO (1999b)

[b] B= benomyl; C= carbendazim; T= thiophanate-methyl

[c] MRL based on post-harvest use

[d] At or about the limit of detection

Table 4. Pesticide residues in food (MRLs) CODEX 1999

Thiabendazole / Commodity	MRL (mg/kg)	Application	Thiophanate-methyl / Commodity	MRL (mg/kg)	Application
Apple	10	f	Cereal grains	0.1	f
Milks	0.1	f	Cherries	10	f
Pear	10	f	Chicken meat	0.1	f
Potato	15	f	Citrus fruits	10	f
Poulty meat	0.05	f	Currant black	5	f
Strawberry	3	f	Gooseberry	5	f
Witloof chicory (sprouts)	0.05	f	Grape	10	f
Cattle muscle, liver	0.1	v	Lettuce, head	5	f
Pig muscle, liver	0.1	v	Mushroom	1	f
Sheep muscle, liver	0.1	v	Plums(including prunes)	2	f
Goat mucle, liver	0.1	v	Rasberries, red, black	5	f
			Strawberry	5	f
			Sugar beet leave and tops	5	f
			Tomato	5	f

f = used as fungicide

v = used as veterinary drug

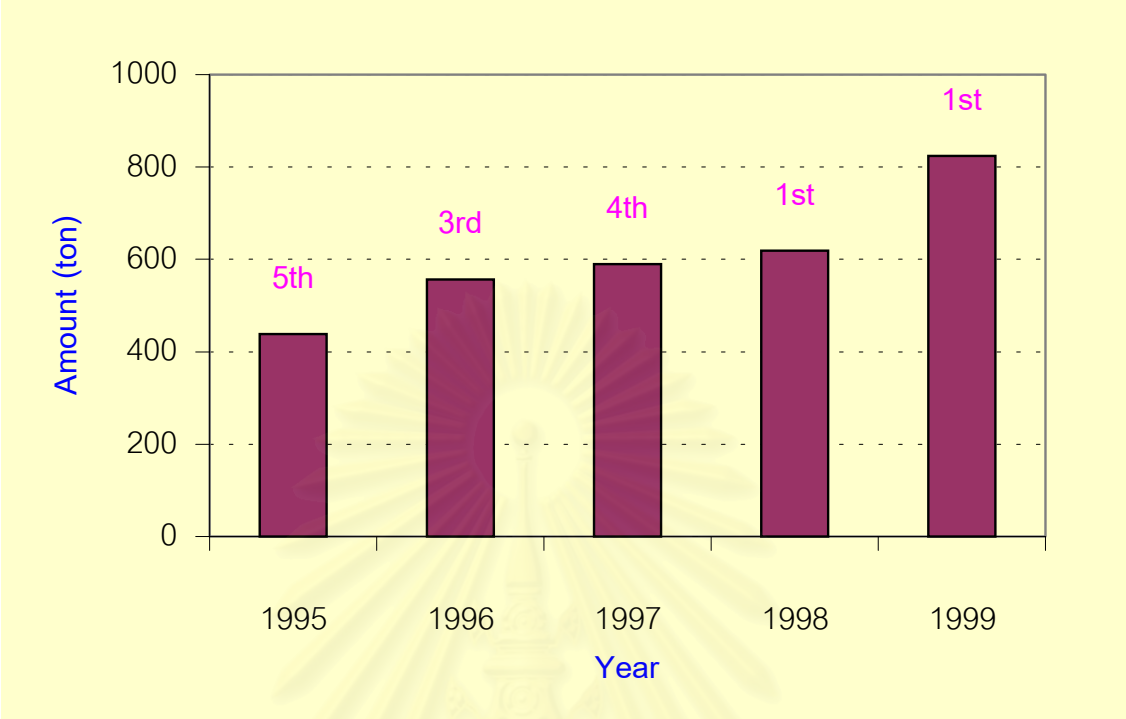


Figure 6 Amounts of carbendazim imported into Thailand between 1995-1999: *The amounts of carbendazim and the comparative order of carbendazim and other fungicides reported by Agricultural Regulatory Division, Department of Agriculture.*

The ELISA methods are widely used for the determination of benzimidazole fungicides because they have advantages over HPLC methods. They are easier to perform and they are rapid and sensitive, especially, when they have been developed to be ELISA Kit. These kits are commonly used for monitoring benzimidazole residues in many foods. The examples are benomyl kits used for the determination of carbendazim in wine (Bushway, *et al*. 1993), in bulk fruit juice concentrates (Bushway, *et al*. 1994) and benzimidazole (BNZ) Kit used for screening benzimidazole residues in bovine liver (Bradon, *et al*.1998). *Table 5* shows the chronological technique developments for the determination of benzimidazole fungicides.

1.4 The approach for the determination of benzimidazoles by immobilized CD

There are some disadvantages of both chromatographic and ELISA methods for the benzimidazole determination. Chromatographic techniques are expensive, require clean-up step with large amount of dangerous solvent and time consuming. Whereas, ELISA techniques enable a quicker, more sensitive and field adaptable assay but antibodies immobilized on a microtiter plate can't be reused because they are denatured by stop solution used for stopping enzyme activity and ELISA kits are rather expensive because they have to be imported. Therefore, the application of immobilized CDs as sensing molecules instead of antibodies for the determination of benzimidazole is very interesting. We then developed the approach based on competitive-inhibition enzyme immunoassay (Kaufman and Clower. 1991) as shown in *Figure 7*. The figure shows steps of aromatic compounds determination by immobilized CD method modified from ELISA principle. Firstly, CD was immobilized on microtiter plate surface as antibody. Secondly, benzimidazole and benzimidazole-tracer solutions were added into wells with immobilized CD for competitive encapsulation. Thirdly, unbound molecules were washed and then benzimidazole tracers encapsulated into CDs cavities were examined by enzyme-substrate reaction. Finally, the enzyme-substrate reaction was stopped by the addition of stop solution and the absorbance was then read and the amount of benzimidazole could be quantified by using benzimidazole calibration curve.

The present work thus focuses on the development of the immobilized CD system for the detection of benzimidazole through ELISA technique. Although, the specificity and affinity between CD and guest are lower than that of antibody and antigen. For screening purpose, it is probable that immobilized CD under optimal condition could be used for determining a group of guests with structure similarity, such as benzimidazole fungicide group.

Table 5. Analytical techniques for the determination of benzimidazole fungicides

Analytical technique	Type of benzimidazole and sample	LOD or LOQ
Liquid chromatography		
●Ion-pairing LC with UV and fluorescence detection	Thiabendazole, thiophanate-methyl and carbendazim in foods (Gilvydis and Waters, 1990)	LOQ = 0.1 ppm
●Automated solid phase extraction clean up and online LC with UV and fluorescence detection	Carbendazim and thiabendazole in fruits and vegetables (Hiemstra *et al*., 1995)	LOD = 0.05 ppm
●Reversed- phase LC with UV detection	Carbendazim and thiabendazole in orange and grape (Tharsis *et al*., 1997)	LOD = 0.06 ppm
●Automated method for clean up and LC determination with UV and fluorescence	Benomyl and thiabendazole in table-ready foods (Levine *et al*., 1998)	LOQ=0.1 ppm for Benomyl and 0.01 for thiabendazole
ELISA		
●Indirected ELISA	Carbendazim and thiabendazole in some foods (Newsome and Collins, 1987)	LOD=0.35 ppm for benomyl and 0.03 ppm for thiabendazole
●Indirected ELISA (Monoclonal antibodies)	Thiabendazole in potatoes and apple (Bradon *et al*., 1993)	LOD~0.2 ppm
●Competitive inhibition (Benomyl kit)	Carbendazim in wine (Bushway *et al*., 1994)	LOQ = 5 ppb
●Competitive inhibition (Benomyl kit)	Carbendazim in bulk fruit juice concentrates (Bushway *et al*., 1994)	LOQ = 10 ppb
●Competitive inhibition (Benzimidazole and thiabendazole kit)	Benzimidazole and thiabendazole residues in bovine liver (Brandon *et al*., 1998)	LOD = 0.7 ppb for benzimidazole and 0.2 ppb for thiabendazole

LOD = Limits of detection and LOQ = Limits of quantity

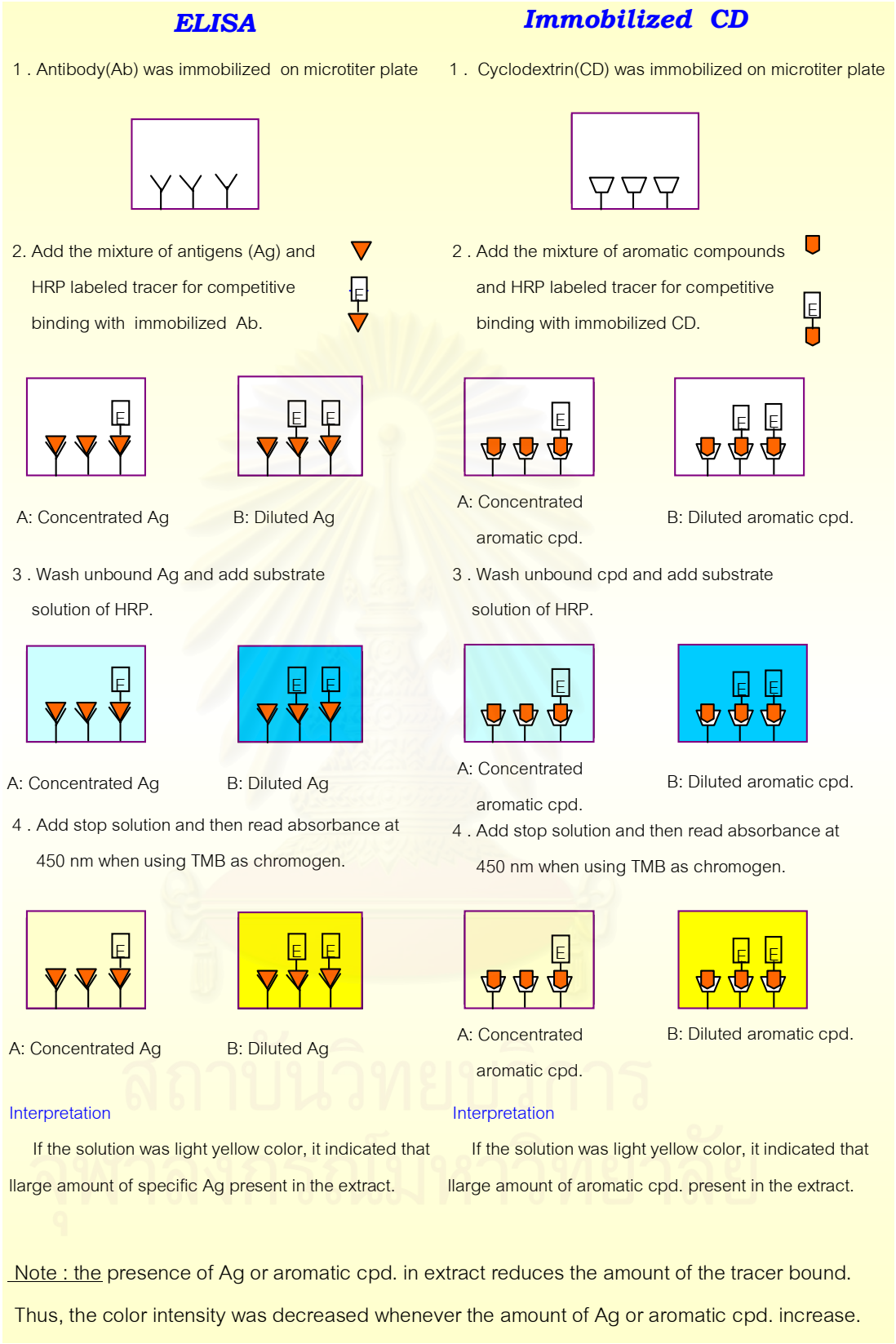


Figure 7 Illustration of ELISA and immobilized CD principle for the determination of aromatic compounds: *Major steps consists of* ***1****) Antibody or CD coating on well,* ***2****) Sample extract and enzyme-tracer loading,* ***3****) Unbound molecule washing and enzyme-substrate reaction performing and* ***4****) the reaction stopping and the resultant color measuring.*

1.5 The objectives of this thesis

The aims of this study cover the followings:

1. To synthesize benzimidazole tracer
2. To immobilize CD on polystyrene microtiter plate surface.
3. To test the capability for encapsulation of immobilized CD by using benzimidazole tracer.
4. To test the capability of CD immobilized on microtiter plate for the determination of benzimidazole fungicides.
5. To test the stability of CD immobilized on microtiter plate for reusing purpose.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Analytical column for HPLC: Ultracarb 5 C8 150X4.60 mm, Phenomenex, U.S.A.

Analytical balance: - Sartorius MC 210S, Scientific Promotion Co., Ltd.,Germany
- Sartorius LC 6200S, Scientific Promotion Co., Ltd.,Germany
- Mettler AE 6200S, Mettler-Toledo, Switzerland

Autopipette: Pipetman, Gilson, France and Finnpipette digital multichannel pipette, Labsystem Inc., Findland

Desalting column: Hitrap™ Sephadex-G25, Phamacia Biotech, U.S.A.

Electrophoresis apparatus: Hoefer Mighty Small, Phamacia Biotech, U.S.A.

ELISA microtiter plate: CovaLink NH, Nunc, Denmark

ELISA microtiter plate: Cloning, U.S.A.

ELISA microtiter plate reader: Titertek multiskan plus plate reader: Labsystems Ins.,Findland

Fluorescence detector: SpectraSystem FL2000, Thermo Separation Products, U.S.A.

Homogenizer: Ultraturax T 25, IKA-Labortechnik, Germany

HPLC: ContaMetric 4100 pump, Thermo Separation Products, U.S.A.

PH meter: PHM 83 Autocal pH meter, Radiometer, Denmark

Rotary tube shaker: Rollordrum TC-7, New Brunswick Scientific Co, U.S.A.

Rotary vacuum evaporator: Rotavapor-RE, Buchi, Switzerland.

Spectrophotometer: Jenway 6405 UV/VIS, Jenway Ltd, U.K.

UV detector: SpectroMonitor 3200, Thermo Separation Products, U.S.A.

Vortex: Geine model K-550-GE, Sciencetific Industries, U.S.A.

2.2 Chemicals

2-Aminobenzimidazole, Fluka, Switzerland

Carbendazim, Chem Service,U.S.A.

Cyclodextrins: -β-cyclodextrin, Sigma, U.S.A.

-hydroxypropyl-β-cyclodextrin, Bioresearch Corporation of Yokohama, Japan

-methyl-β- cyclodextrin, American Maize-Product Company, U.S.A.

1-(3-(Di-methylamino)propyl)-3-ethylcarbodiimide hydrochloride, Sigma, U.S.A.

Divinylsulfone, Aldrich, Germany

Succinic anhydride, Fluka, Switzerland

3, 3′, 5, 5′ Tetra methyl benzidine, Sigma, U.S.A.

Tween 20, Sigma, U.S.A.

Urea hydrogen peroxide (carbamide peroxide), Sigma, U.S.A.

Other common chemicals were obtained from Merck, Fluka or Sigma.

2.3 Buffers and reagents

Mobile phases for HPLC: Acetonitrile : 0.01 M Potassium hydrogen phosphate (40%:60%)

Loading buffer for the encapsulation:

-5 mM Phosphate buffer saline (PBS) containing 0.15 M NaCl, pH 7

-0.1 M Sodium acetate/ citric acid buffer, pH 2.5 and 5

Washing buffer for the encapsulation:

- 5 mM Phosphate buffer saline (PBS) containing 0.5 M NaCl, pH 7

- 5 mM Phosphate buffer saline (PBS) containing 0.15 M NaCl and 0.3% Tween 20, pH 7

- 0.1 M Sodium acetate/ citric acid buffer containing 0.5 M NaCl, pH 5

- 0.1 M Sodium acetate/ citric acid buffer containing 0.3% Tween 20, pH 5

CD coating reagent:

- 0.5 M Sodium carbonate buffer, pH 11

- 1 M Tris solution

Washing reagent for plate reusing: 0.1 M Sodium hydroxide: dilute 1:100 before use

2.4 Enzyme

Horseradish peroxidase (VI A), Sigma, U.S.A.

2.5 Synthesis of benzimidazole tracer

Benzimidazole tracer was a conjugate of 2-succiamidobenzimidazole (SAB) and horseradish peroxidase (HRP). The unique characteristics of this molecule were that it could be encapsulated into CD owing to the hydrophobicity of 2-succiamidobenzimidazole and the occurred inclusion complex could be investigated by horseradish peroxidase activity assay.

2.5.1. Synthesis of 2-succiamidobenzimidazole (SAB)

A solution of 2-aminobenzimidazole (13.3 g, 0.1 mol) and succinic anhydride (10.0 g, 0.1 mol) in acetonitrile (400 ml) was stirred at ~40°C for 3 hours. The heavy precipitate was filtered on a medium porosity Buchner filter and washed thoroughly with distilled water. The solid was then suspended in boiling methanol (500 ml) and filtered. After being washed with a further portion of boiling methanol (500 ml), the solid was air-dried. The product was identified and confirmed by fourier transform nuclear magnetic resonance spectroscopy (FT-NMR) and mass spectrometry (MS) (Newsome and Shields, 1981).

2.5.2. Conjugation of 2-succiamidobenzimidazole and horseradish peroxidase

The tracer consisting of 2-succiamidobenzimidazole coupled to horseradish peroxidase (HRP) was synthesized as the following procedure. 1-(3-(Di-methylamino)propyl)-3-ethylcarbodiimide hydrochloride (56.4 g) in distilled water (0.5 ml) was added to a stirred solution of HRP (5 mg) in 5 mM phosphate buffer saline (PBS) containing 0.15 M NaCl, pH 7. After that 2-succiamidobenzimidazole (2.3 mg) dissolved in dilute NaOH (1.0 ml), pH 10-11 was added and adjusted pH to 7 with dilute HCl. Gentle stirring was continued for 20 hours at room temperature by the use of rotary tube shaker (Newsome and Shields, 1981). The conjugate was then purified by Sephadex-G25 (Hitrap™ desalting column) and eluted with 5 mM PBS, containing 0.15 M NaCl, pH 7. Aliquots of the conjugate solution were stored at -20°C.

2.6 Characterization of benzimidazole tracer (SAB-HRP conjugate)

2.6.1. The maximun absorption of benzimidazole tracer

Native HRP and benzimidazole tracer (modified HRP) were dissolved in 5 mM PBS containing 0.15 M NaCl, pH 7 with the absorbance of 0.6 and 0.4 at 405 nm, respectively, before the maximum absorption was scanned in the range of 250 - 450 nm.

2.6.2. The molecular weight of benzimidazole tracer

The molecular weight of benzimidazole tracer was determined by SDS-PAGE with 12.5 % gel. The denaturing gel containing 0.1% (w/v) SDS in 12.5% (w/v) separating and in 3.0% (w/v) stacking gel was performed and Tris-glycine buffer, pH 8.8, containing 0.1% SDS was used as electrode buffer according to Bollag and Edelstein (1991). Samples to be analyzed such as benzimidazole tracer and horseradish peroxidase dissolved in buffer should be

dialyzed overnight against distilled water. After that samples were treated with sample buffer containing 0.5% SDS and boiled for 10 minutes prior to application to the gel. The electrophoresis was performed at constant voltage of 120 V. per slab, on Hofer electrophoresis unit from cathode towards the anode. When the electrophoresis was completed, the gel was stained with 0.025% Coomassie blue R-250 in 40%(v/v) methanol and 7% (v/v) acetic acid for at least 1 hour. The slab gel was destained with a solution of 40% methanol and 7% of acetric acid for 1 hour followed by several changes of destaining solution until gel background was clear.

2.6.3 The enzyme activity of benzimidazole tracer

The activity of native HRP and benzimidazole tracer was examined and compared by using the method for HRP activity determination, according to Bos *et. al*. (1981). Native HRP and benzimidazole tracer solutions with the absorbance of 0.6 and 0.4 at 405 nm, respectively, in 5 mM PBS, containing 0.15 M NaCl, pH7 were diluted to 1: $2x10^{-5}$. Four hundred microlitre of substrate solution, containing 0.1 mg/ml of tetramethylbenzidine (TMB) and 0.1 mg/ml of urea hydrogen was added into a glass cuvette and followed by the addition of 100 μl of native HRP or benzimidazole tracer solutions. The absorbance at 655 nm was observed in the range of 0-120 seconds for kinetic measurement. One unit of enzyme is the amount of enzyme, which oxidizes 1 μmole of TMB per minute.

2.7 Immobilization of β-CD on microtiter plate

In this study, divinylsulfone was used as a crosslinker for covalent immobilization of β-CD on amino polystyrene microtiter plate surface. The procedure for the immobilization of CD was modified from the method for carbohydrate immobilization on amino polystyrene microtiter plate surface (Hatakeyama *et al*., 1996).

Three hundred microlitre of 5%(v/v) divinylsulfone in 0.5 M carbonate buffer, pH 11, was added into wells and incubated at room temperature (around 25° C) for 1 hour. Wells were washed for 3 times with 300 μl of 0.5 M carbonate buffer before 300 μl of β-CD solution (1%, 2.5% and 5%) in 0.5 M carbonate buffer was added and incubated at room temperature for 18 hours. The excess CD was removed by washing with 300 μl of distilled water and incubated for 1 hour, 3 times. Another 300 μl of distilled water (around 40° C), incubated for 15 minutes, 6 times, was used for washing to ensure the excess CD was removed. After that 320 μl of 1 M Tris was added and incubated for 1 hour in order to block active vinyl groups. Wells were washed 3

times with 320 μl of distilled water and another 3 times with 300 μl of 0.1 M sodium acetate/citric acid buffer, pH 5. The immobilized CD microtiter plate was air dried and kept in desiccator at room temperature.

2.8 Capability test of immobilized β-CD on microtiter plate for benzimidazole tracer encapsulation

The immobilized β-CD on microtiter plate surface could be examined by using benzimidazole tracer as guest molecule for the encapsulation. Benzimidazole tracer was used in order to ensure if CD was immobilized on the surface and still able to encapsulate guest into its cavity. The procedure is as follows.

Fifty microlitre of 1-10 μg/ml benzimidazole tracer in 0.1 M acetate/citric acid buffer, pH 5, was added into wells and incubated for 1 hour at room temperature (25° C). The solution was discarded and wells were washed twice with 300 μl 0.1 M acetate/citric acid buffer, pH 5, containing 0.5 M NaCl (washing buffer) and followed by 3 washes of 0.1 M acetate/citric acid buffer, pH 5. The enzyme activity was determined by adding 100 μl of substrate solution, containing 0.1mg/ml tetramethylbenzidine and urea hydrogen. After incubating for 2 min, 50 μl of 2 M sulfuric acid was added in order to stop the reaction. Sulfuric acid caused a blue to yellow color change in wells. The absorbance at 450 nm was then read.

2.9 Optimization for conditions of benzimidazole tracer for encapsulating into immobilized CD

There are many factors that could affect the encapsulation of benzimidazole tracer into immobilized CD. Therefore, in order to know the best condition for the tracer encapsulation, various factors were investigated and the procedure was the same as in protocol 2.8

2.9.1 Types of washing buffer

In this experiment, two types of buffer, phosphate buffer saline pH 7 and acetate buffer pH 5 were used. Both of them containing 0.3% Tween and that containing 0.5 M NaCl were examined for their effects on specifically and nonspecifically bound molecules of benzimidazole tracer on the surface.

2.9.2 Concentration of β-CD

The concentrations of CD solution used for the immobilization may affect the amount of CD immobilized on the surface. In this experiment, different β-CD concentrations (1%, 2.5% and 5% w/v) were used for immobilization of β-CD on microlitre plate and then used for benzimidazole tracer encapsulation.

2.9.3 Concentration of benzimidazole tracer (HRP conjugate)

High concentration of tracer used for encapsulation into immobilized CD may increase nonspecifically bound molecules on the surface and cause variability of tracer signal. So in this experiment, 50 μl of 1, 2.5, 5 and 10 μg/ml of tracer concentration were used for encapsulation into immobilized CD.

2.9.4 Types of CD

Some properties of β-CD derivative were found to be different from those of native one. So different types of β-CD were used to coat on the surface. This may affect amounts of tracer that could encapsulate into the immobilized CD. In this experiment, β-CD, hydroxypropyl-β-CD and methyl-β-CD were then explored.

2.10 Capability test of immobilized CD and benzimidazole tracer for detecting carbendazim standard

The competitive encapsulation between carbendazim standard and benzimidazole tracer was studied by observing the effect of benzimidazole tracer concentrations and pH of carbendazim solution loaded for encapsulating into CD. The procedure was performed as follows.

2.10.1 Preparation of benzimidazole tracer

An aliquot of benzimidazole tracer solution was diluted to 1200 μg/ml in PBS containing 0.15 M NaCl as stock solution and it was continuously diluted in 0.1 M acetate/citric acid, pH 5 to get 1.2 and 2.5 μg/ml working solution.

2.10.2 Preparation of carbendazim standard

The carbendazim stock standard solution was prepared in methanol at concentration of 200 μg/ml. The solution was then diluted to 50 μg/ml in 0.1 M acetate/citric acid buffer, pH 2.5 and 5.0 and it was diluted further in the same buffer to get 1-7 μg/ml as the working solution.

2.10.3 Determination of carbendazim by competitive benzimidazole tracer encapsulation.

Fifty microlitre of carbendazim standard solution was loaded into CD immobilized wells and incubated for 1 hour at room temperature (25°C). Unbound carbendazim was removed by washing twice with 250 μl of 0.1 M acetate/citric acid buffer, pH 5. After that 50 μl benzimidazole tracer at concentration of 1.2 and 2.5 μg/ml was added into the same wells and incubated for 1 hour at room temperature. The solution was discarded and wells were washed twice with 250 μl of 0.1 M acetate/citric acid buffer, pH 5, containing 0.5 M NaCl (washing buffer) and followed with 3 washes of 0.1 M acetate/citric acid buffer, pH 5. The enzyme activity was determined by adding 100 μl of substrate solution, containing 0.1mg/ml tetramethylbenzidine and urea hydrogen. After incubating for 2 min, 50 μl of 2 M sulfuric acid was added in order to stop the reaction. Sulfuric acid caused a blue to yellow color change in wells. The absorbance at 450 nm was then read.

2.11 Capability test of immobilized CD and benzimidazole tracer for detecting carbendazim in fruits

The study of immobilized CD and benzimidazole tracer capability for detecting carbendazim in fruits consisted of three parts. The first part was to determine carbendazim in fruit samples, for example grape and apple, by HPLC method in order to determine whether there were carbendazim residues. The second part was to check the interference of sample matrix that may affect the competitive determination system by using the sample which carbendazim residues were not detected by HPLC and the last part was to determine carbendazim by competitive benzimidazole encapsulation into immobilized CD on microtiter plate.

2.11.1. HPLC method for determining bezimidazole residues in sample

This method was modified from Givydis and Waters (1990), consisted of sample preparation, extraction and LC determination as follows.

2.11.1.1 Sample preparation

Inedible portions such as seeds in grape and apple were removed and the edible portion was homogenized with blender.

2.11.1.2 Sample extraction

Fifty grams of prepared sample was weighed into 300 ml beaker. 100 ml methanol was added and the mixture was homogenized for 2 minutes with the Ultraturax T25. The mixture was then filtered with suction through shark skin paper in bucher funnel, and rinsed with additional 50 ml methanol. The filtrate was transferred to 500 ml separatory funnel, and followed by the addition of 10 ml of 1 N hydrochloric acid and 100 ml of 1% (w/v) sodium chloride solution. The mixture was extracted twice with 100 ml of dichloromethane, and shaken for 1 minute each time. Combined dichloromethane layer extracts in 500 ml round-bottom flasks were evaporated just to dryness using vacuum rotary evaporator with 30°C water bath. This was acidic partition (aqueous methanol layer was kept in separatory funnel for basidic partition). After the portion was evaporated, residues were redissolved in 5 ml methanol and filtered through 0.45 μm before injected to LC system.

Aqueous methanol phase was drained from separatory funnel into beaker and separatory funnel was rinsed with 10 ml portion of water. Adjust pH of solution to 7.5-8 using sodium hydroxide (5 M and 1 M) and 1 M hydrochloric acid, as necessary (strong alkaline condition during adjustment should be avoided). Solution was returned to separatory funnel, 1 ml of saturated sodium chloride solution was added. The mixture was extracted with two 100 ml portions of dichloromethane. Combined extracts were evaporated to dryness in rotary vacuum evaporator, and prepared as before for LC determination.

2.11.1.3 LC operation

The column used was the reverse phase Ultracarb 5 C8 (150×4.6 mm). Fluorescence detector was connected in tandem with UV detector. The wavelength of UV detector was set at 278 nm. Excitation and emission wavelengths of fluorescence detector were

set at 284 nm and 314 nm, respectively. The system was equilibrated with mobile phase containing 40% of acetonitrile and 60% of 0.01M potassium hydrogen phosphate at flow rate of 0.5 ml/min for at least 30 minutes before using. When the system was not in use for shorter periods, 70% acetonitrile was rinsed at least 1.5 hour before stopping to prevent salt deposition and 50% methanol in water was used instead when not in use for extended periods.

2.11.1.4 LC determination

Twenty microlitre of sample solution was injected into LC system using operating conditions. Chromatographic response (peak retention times, heights, and/or areas) of standard and sample solutions was determined and the residue amount was calculated. If further dilutions were necessary, methanol was used as diluent.

2.11.1.5 Recovery study of LC method

50 g of prepared apple and grape with no carbendazim residues were spiked with 1 ppm of carbendazim standard. Then they were extracted and determined as *2.11.1.2 and 2.11.1.4,* respectively.

The recovery was calculated by the equation of

$$\text{Recovery (\%)} = \frac{\text{Amount of carbendazim determined}}{\text{Amount of carbendazim spiked}} \times 100$$

(PAM,1991)

2.11.2 The effect of sample matrix on the determination of carbendazim in samples by using benzimidazole tracer and immobilized CD on microtiter plate

The sample matrix of grape was checked whether there are other substances in the sample apart from carbendazim that could be encapsulated into CD cavity when they were incubated in the same condition as carbendazim standard. So the sample with no carbendazim residues (as checked by HPLC) was performed as follows. 25 g samples without carbendazim residues were extracted with 25 ml dichloromethane, pH was adjusted to 7.5-8 using sodium hydroxide (5 M and 1 M) and 1 M hydrochloric acid, as necessary. The mixture was shaken for 3 minutes and filtered through shark skin paper. 5 ml filtrate was pipetted into 15 ml graduate tube

and then 5 ml of 0.1 M sodium acetate/ citric acid buffer, pH 2.5 or 5 (loading buffer) was added. Two-layer solution was observed. The bottom layer (dichloromethane layer) was evaporated by passing nitrogen gas until the bottom layer disappeared. The upper layer (1g/ml sample-extracted solution) was diluted in loading buffer to get 0.1, 0.25, 0.5 g/ml and then it was used for the encapsulation in replace of carbendazim standard described in protocol 2.10.3.

2.11.3 The determination of carbendazim in samples by using benzimidazole tracer and immobilized CD on microtiter plate

The diluted grape extract in 2.11.2 that showed the lowest interference was spiked with carbendazim standard at 1-10 ppm loaded into immobilized CD wells in order to determine carbendazim residues by the competitive encapsulation as the method used for carbendazim standard determination (protocol 2.10.3).

2.12 Stability test of immobilized CD on microtiter plate for reusing purpose

CD is a stabilized molecule in which guests could be well encapsulated in its cavity under optimal conditions. However, there are some conditions that could make guest release from CD cavity. This experiment aims at finding conditions for regeneration of immobilized CD, thus the microtiter plate could be reused.

After the activity of tracer was determined as in section 2.9.3 or 2.10.3. The used immobilized CD plate for carbendazim determination was washed 3 times with 300 μl of distilled water and followed by 2 washes with 300 μl of 1mM sodium hydroxide, incubated for 15 minutes each time to remove guest from CD's cavity. After that the plate was washed 3 times with 300 μl of distilled water and followed by 3 washes with 250 μl 0.1 M acetate/citric acid buffer, pH 5. Finally, immobilized microtiter plate was air-dried and kept in desiccator at room temperature when not in use.

The immobilized CD microtiter plate prepared as above was reused for the test of the benzimidazole tracer encapsulation and the determination of carbendazim as in the protocol 2.9.3 and 2.10.3, respectively.

CHAPTER III

RESULTS

The study of cyclodextrin immobilization on microtiter plate for the determination of benzimidazole fungicides consisted of 7 major steps; 1) Synthesis of benzimidazole tracer, 2) Immobilization of CD on microtiter plate, 3) Capability test of immobilized CD for benzimidazole tracer encapsulation, 4) Optimization for benzimidazole tracer encapsulation into immobilized CD, 5) Capability test of immobilized CD and benzimidazole tracer encapsulation for the detection of carbendazim standard, 6) Capability test of immobilized CD and benzimidazole tracer encapsulation for the detection of carbendazim in fruits and 7) Stability test of immobilized CD on microtiter plate.

3.1 Benzimidazole tracer synthesis

Benzimidazole tracer is an important molecule in this study because we need a molecule that could be encapsulated into CD and could also be used to investigate the amount of immobilized CD on microtiter plate surface. Moreover, benzimidazole encapsulated into immobilized CD could be detected by the competition of benzimidazole and benzimidazole tracer for the encapsulation. Benzimidazole tracer is a conjugate of 2- succiamidobenzimidazole (SAB) and horseradish peroxidase (HRP). The unique characteristics of this molecule are that it can be encapsulated into CD cavity as an inclusion complex owing to the hydrophobicity of SAB and the occurred inclusion complex could be investigated by horseradish peroxidase actvity assay. Besides, SAB chemical structure is similar to carbendazim as shown in ***Figure*** 8. Due to the hypothesis above, SAB was then synthesized and examined for its properties.

3.1.1 Synthesis of 2-succiamidobenzimidazole (SAB)

SAB was synthesized from 2-aminobenzimidazole (13.3 g) and succinic anhydride (10 g) as shown in *Figure* 9. The product synthesized from the anhydride method was 12.8 g (55% yield). In order to confirm that it was truly SAB, some properties of synthesized product were examined. In the first step, the maximum absorption spectrum of the product was determined and compared to those of parent compounds. It was found that the maximum absorption peak of the product was 284 nm and that of 2-aminobenzimidazole was 278 nm but

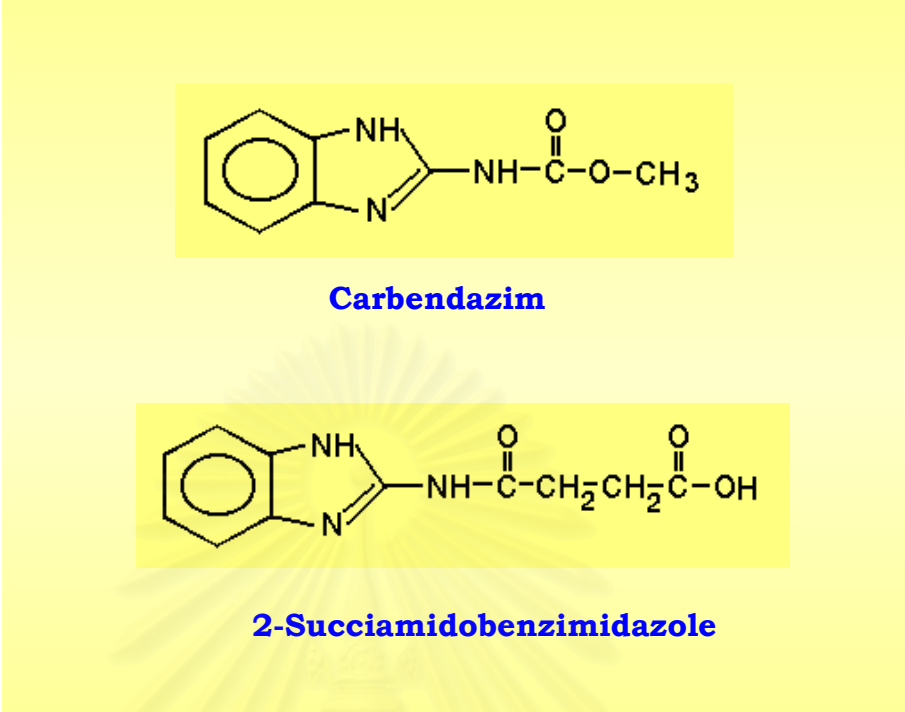


Figure 8 Chemical structures of carbendazim and 2-sacciamidobenzimidazole

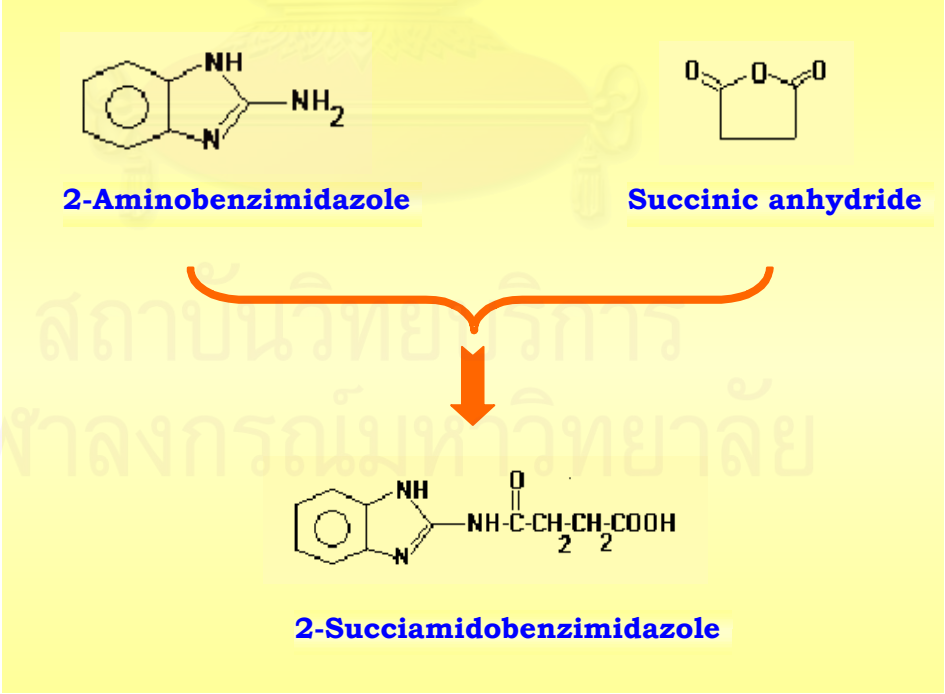


Figure 9 Synthesis of 2-aminobenzimidazole (SAB) from 2-aminobenzimidazole and succinic anhydride

there was no maximum absorption of succinic anhydride (*Figure* 10). In the second step, functional groups of the product were determined by NMR (Scientific and Technological Research Equipment Centre, CU). Analysis of functional groups were according to ^{1}H and ^{13}C NMR correlation chart (Silverstein, Bassier and Morrill, 1981). The ^{1}H NMR spectrum in *Figure* 11 showed that the product molecule consisted of –CH in aromatic ring and –NH groups and the ^{13}C NMR spectrum in *Figure* 12 also showed that SAB consisted of – CH in aromatic ring, $-CH_2$ and –COOH groups. In the final step, the product was determined for molecular mass by TOF-MS (National Science and Technology Development Agency). It was shown that the molecular mass of the synthetic product was 233 daltons (*Figure* 13). The NMR spectra and the mass obtained confirmed that the product was SAB.

3.1.2. Conjugation and purification of benzimidazole tracer (SAB-HRP conjugate)

SAB and HRP were conjugated as benzimidazole tracer by 1-(3-(Di-methylamino) propyl)-3-ethylcarbodiimide hydrochloride activation for 20 hours as described in 2.5.2. The conjugate was then purified using Sephadex G-25 desalting column to remove excess SAB from the conjugate. According to the maximum absorption peak of SAB which was at 284 nm and HRP were at 280 and 405 nm (*Figure* 14), the absorbance at 280 and 405 nm was then read to detect SAB and HRP molecules, respectively. Although the absorbance at 284 nm has an interfering effect from 280 nm, the resulting purification profile is shown in *Figure* 15. Fraction no.3-5, which contained benzimidazole tracer, were pooled and examined for various properties and compared with the native HRP.

3.2. Characterization of benzimidazole tracer (SAB -HRP conjugate)

The biochemical properties including absorption spectra, the molecular weight and the activity of SAB-HRP conjugate were determined and compared with the native HRP in order to prove that it was SAB-HRP conjugate.

3.2.1 The maximum absorption of benzimidazole tracer

The absorption spectrum in the range of 250 - 450 nm was examined and compared to that of the native HRP. It was found that the maximum absorption peak of both native HRP and SAB-HRP were at 280 and 405 nm but the A_{405} / A_{280} ratio of HRP conjugate was lower than that of native HRP. This means that at the same A_{405} of both HRP and HRP conjugate, the A_{280} of HRP conjugate was higher than that of the native one (*Figure* 16).

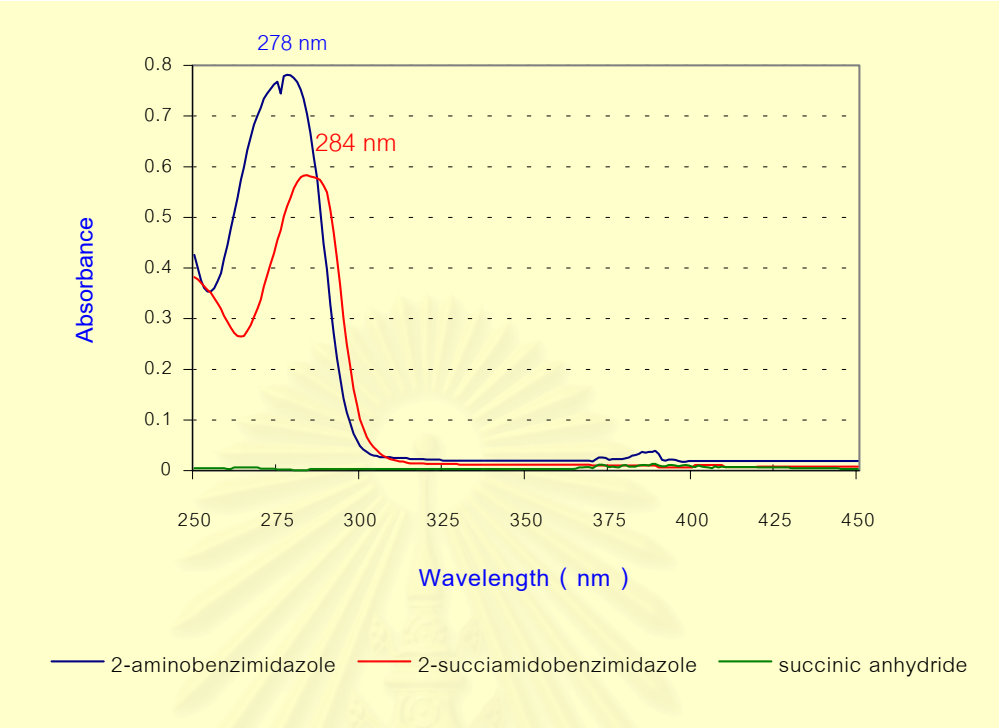


Figure 10 Absorption spectra of 2-aminobenzimidazole, 2-succiamidobenzimidazole (SAB) and succinic anhydride

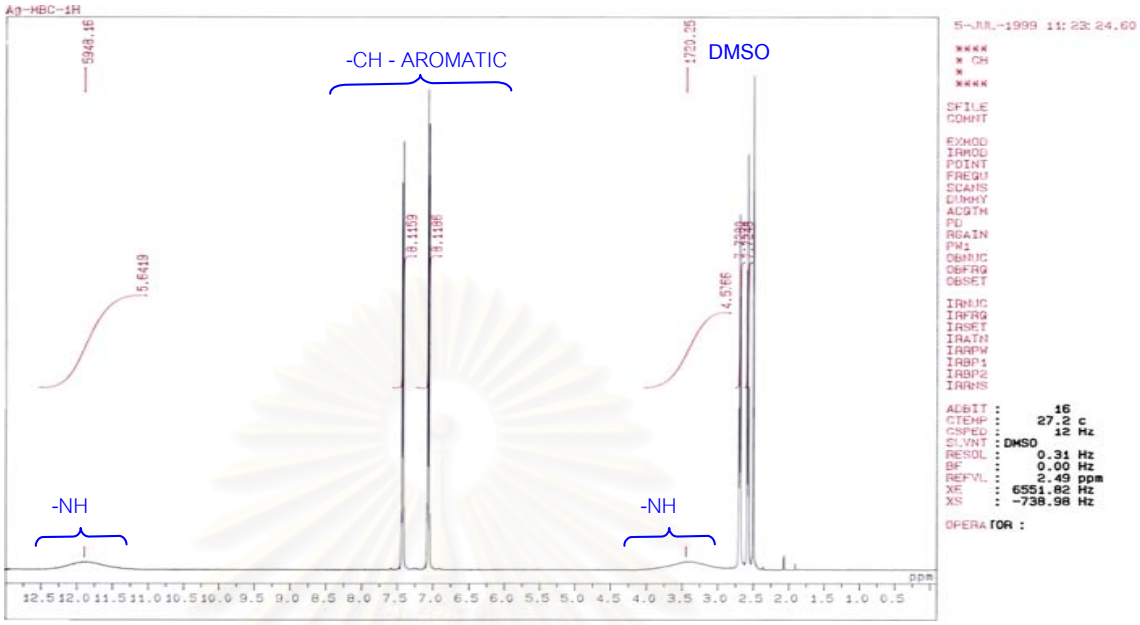


Figure 11 ***^{1}H NMR spectrum of 2-succiamidobenzimidazole*** *: SAB was dissolved in DMSO for the determination*

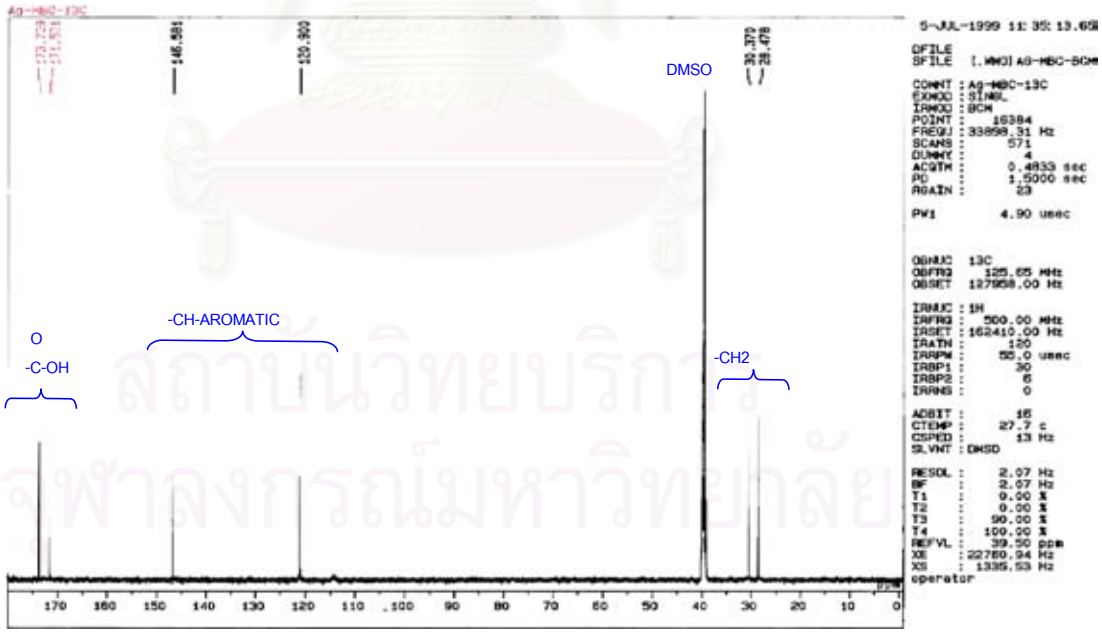


Figure 12 ***^{13}C NMR spectrum of 2-succiamidobenzimidazole:*** *SAB was dissolved in DMSO for functional group determination.*

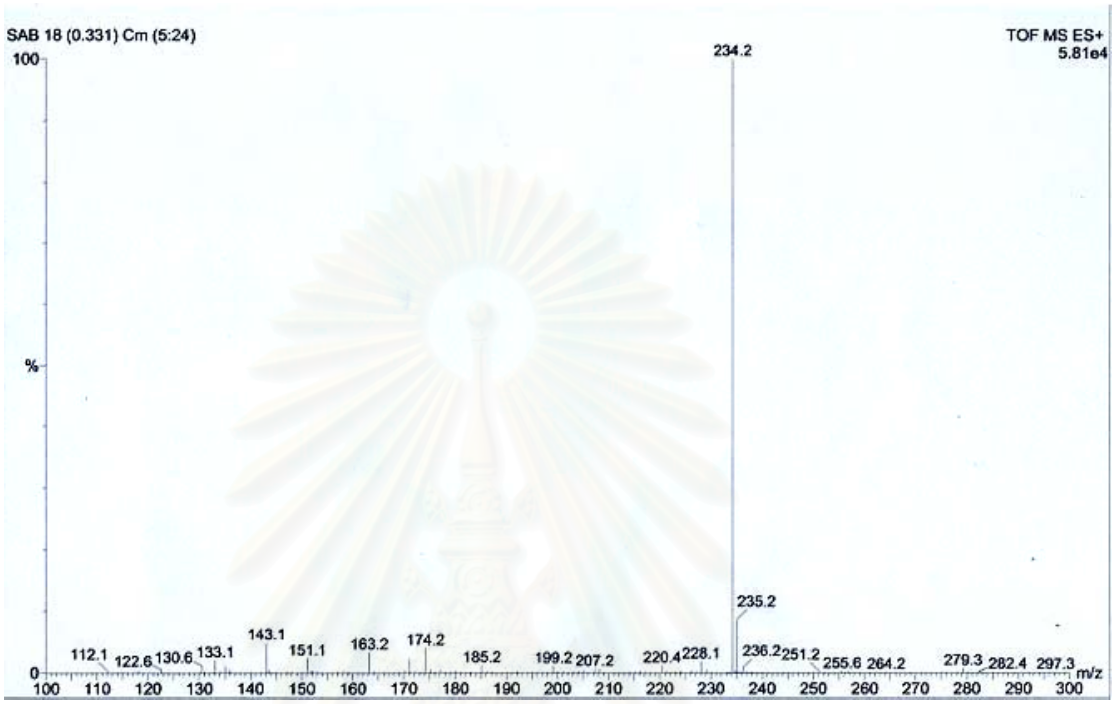


Figure 13 ESI-TOF MS spectrum of 2-succiamidobenzimidazole (M PLUS H): *SAB was dissolved in acetonitrile for mass determination.*

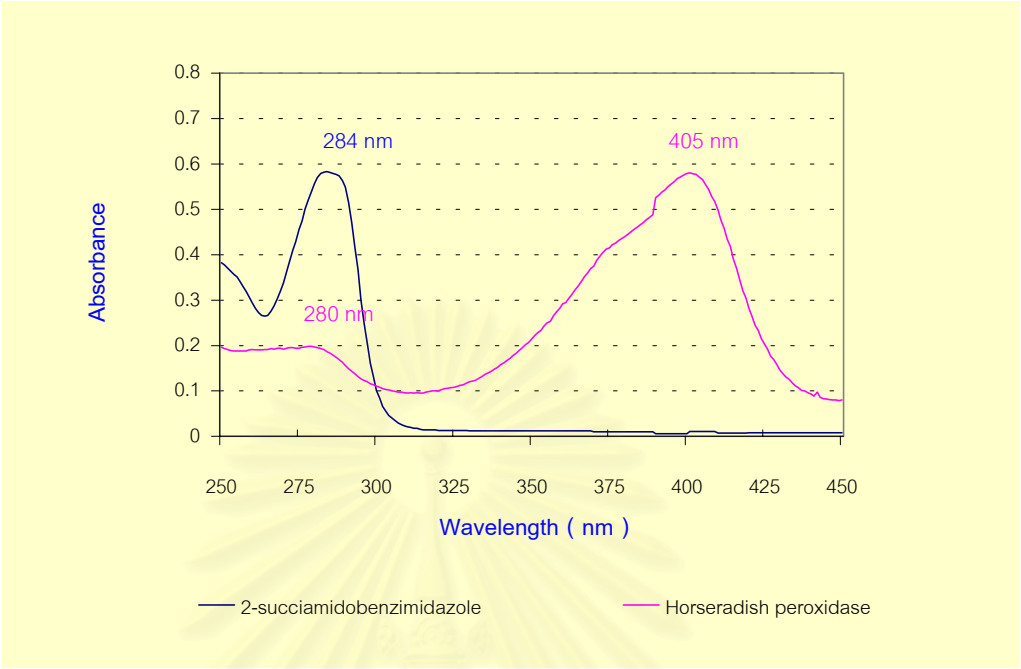


Figure 14 Apsorption spectra of 2- succiamidobenzimidazole (SAB) and horseradish peroxidase (HRP

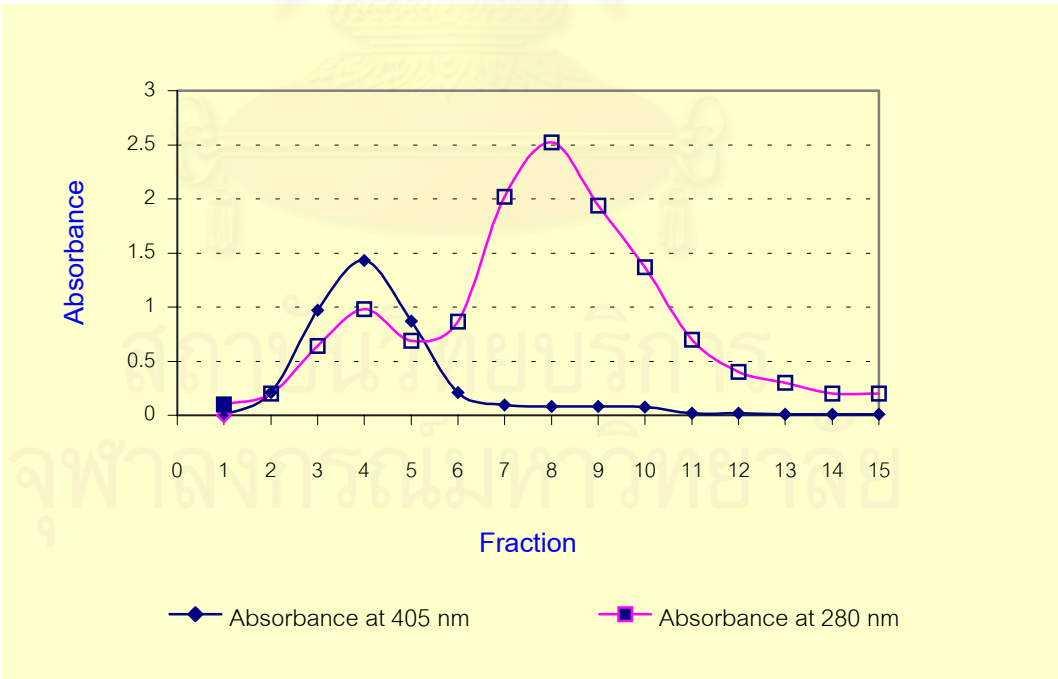


Figure 15 Purification profile of SAB-HRP conjugate: *Use of Sephadex G-25 as desalting column and 5 mM phosphate buffer containing 0.15 M NaCl, pH 7 as an eluent.*

3.2.2 The molecular weight of benzimidazole tracer

The molecular weight of HRP and HRP-conjugate were determined by SDS-PAGE as shown in ***Figure*** 17. The molecular weight of HRP-conjugate was estimated by calibration with standard proteins with known molecular weights (***Figure*** 18). The result showed that the modified HRP was slightly larger than the native one. The approximate molecular weight of HRP and HRP-conjugate was 39 and 41 kD, respectively.

3.2.3 The activity of benzimidazole tracer

The comparative study of native HRP and SAB-HRP conjugate activity was examined spectrophotometrically using urea hydrogen and tetramethylbenzidine as HRP substrate (***Figure*** 19). The result showed that the specific activity of HRP-conjugate and HRP was found to be 7,200 and 9,000 Unit/mg, respectively. The specific activity of HRP-conjugate was 80% compared to the native one in 0.1 M acetate/citric acid buffer, pH 5.

3.3. The immobilization of cyclodextrin on microtiter plate

In this study, we attempted to immobilize CD on microtiter plate surface in order to use it to determine benzimidazole fungicides. There have yet been no reports on the immobilization of CD on microtiter plate. Divinylsulfone is a bifunctional cross-linking agent which is well suited for the attachment of compound containing hydroxyl or amino groups to hydroxyl matrices with simple procedure. Moreover, there is a previous study using the divinylsulfone for carbohydrate immobilization on amino polystyrene microtiter plate surface (Hatakeyama *et al*., 1996). Therefore, divinylsulfone was used to cross-link an amino group of polystyrene microtiter plate and a hydroxyl group of CD to form covalent immobilization as shown in ***Figure*** 20.

3.4 Benzimidazole tracer encapsulation

After CD was immobilized on microtiter plate surface, the SAB-HRP conjugate as benzimidazole tracer was used to determine the amount of immobilized CD and testing the ability of CD for guest encapsulation, simultaneously.

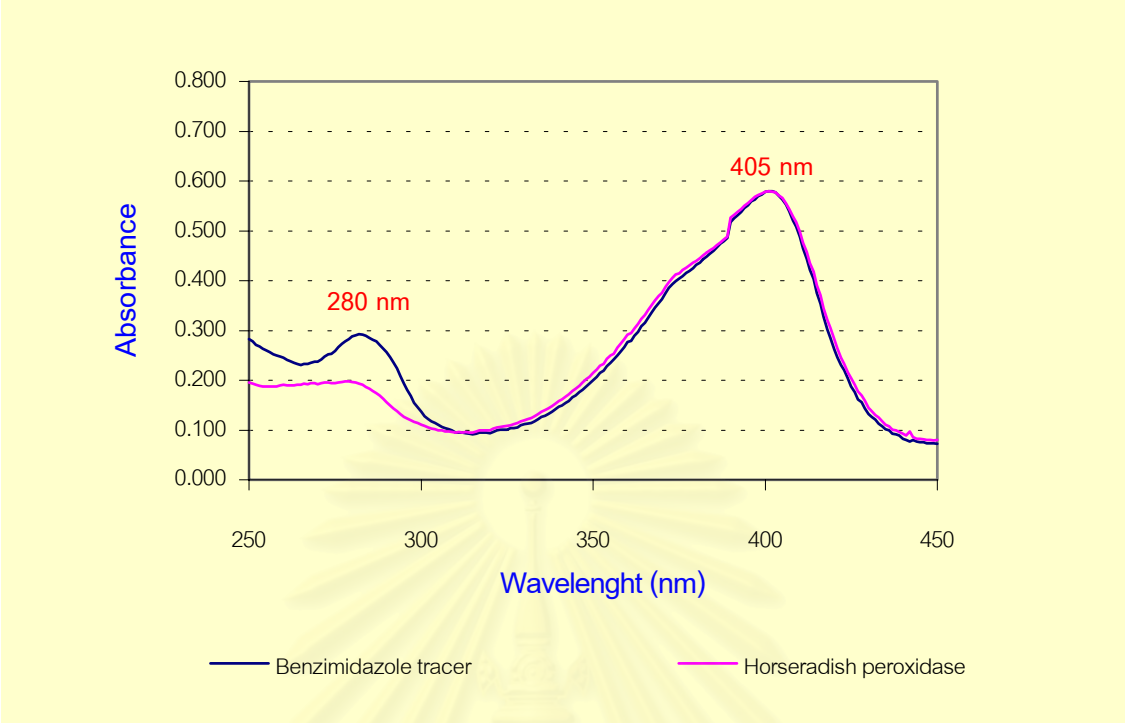


Figure 16 Absorption spectra of horseradish peroxidase and benzimidazole tracer (SAB - HRP conjugate)

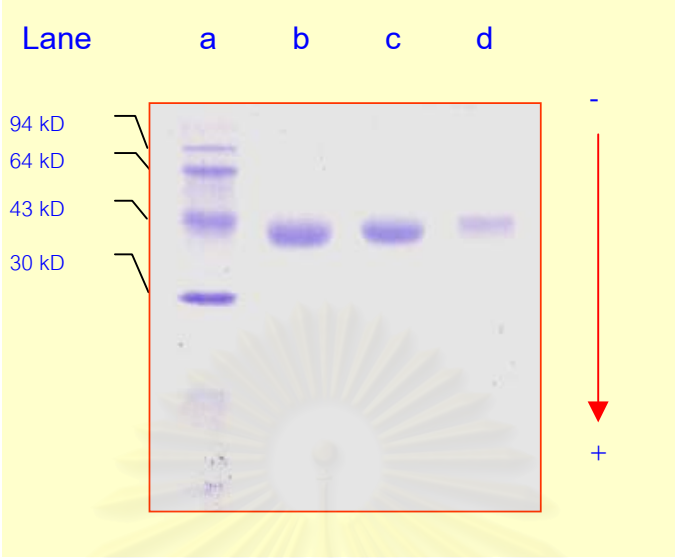


Figure 17 SDS gel electrophpresis of horseradish peroxidase and Benzimidazole tracer (SAB-HRP conjugate): *Lane a) Molecular weight protein markers b) Horseradish peroxidase c) Benzimidazole tracer and d) Ovalbumin*

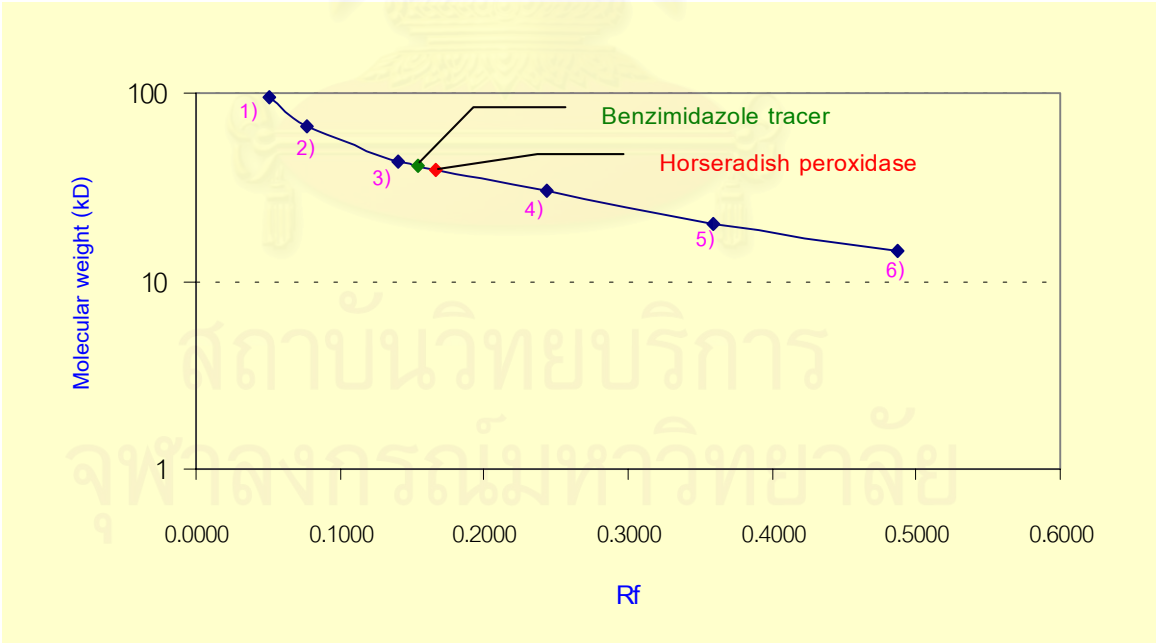


Figure 18 Determination of the molecular weight of horseradish peroxidase and benzimidazole tracer: *1) Phosphorylase (94 kD), 2) BSA (64 kD), 3) Ovalbumin (43 kD), 4) Carbonic anhydase (30 kD), 5) Soybean trypsin inhibitor (20.1 kD) and 6) α-lactabumin (14.1 kD).*

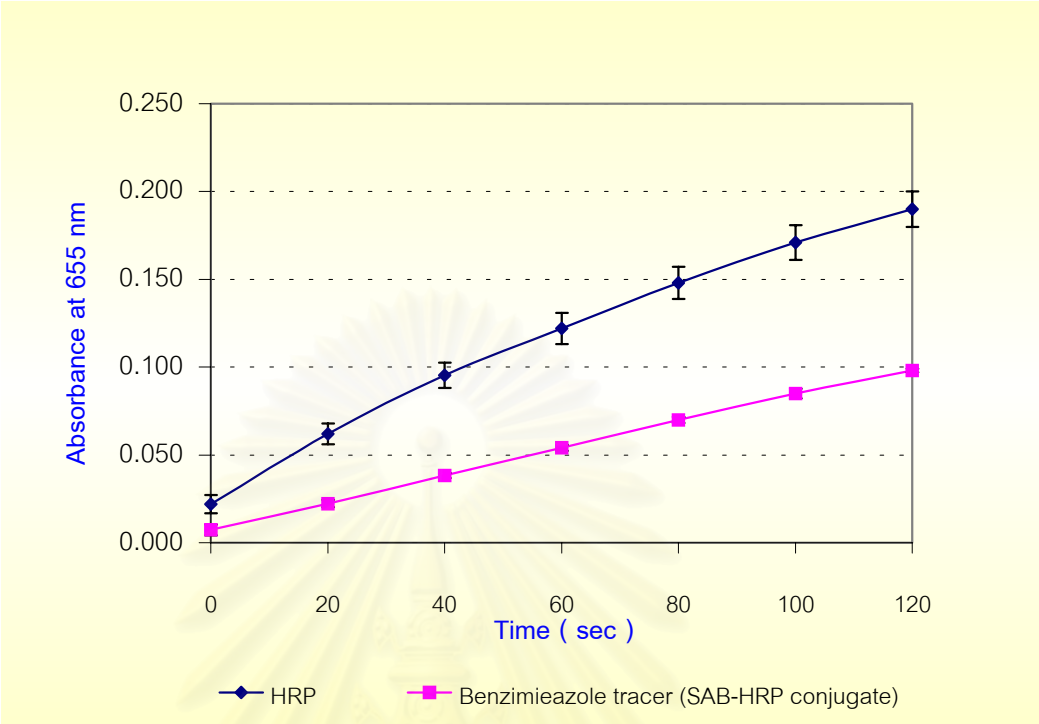


Figure 19 Activity assay of benzimidazole tracer in comparision with horseradish peroxidase: *The assay was performed in 0.1 M acetate/citric acid buffer, pH 5*

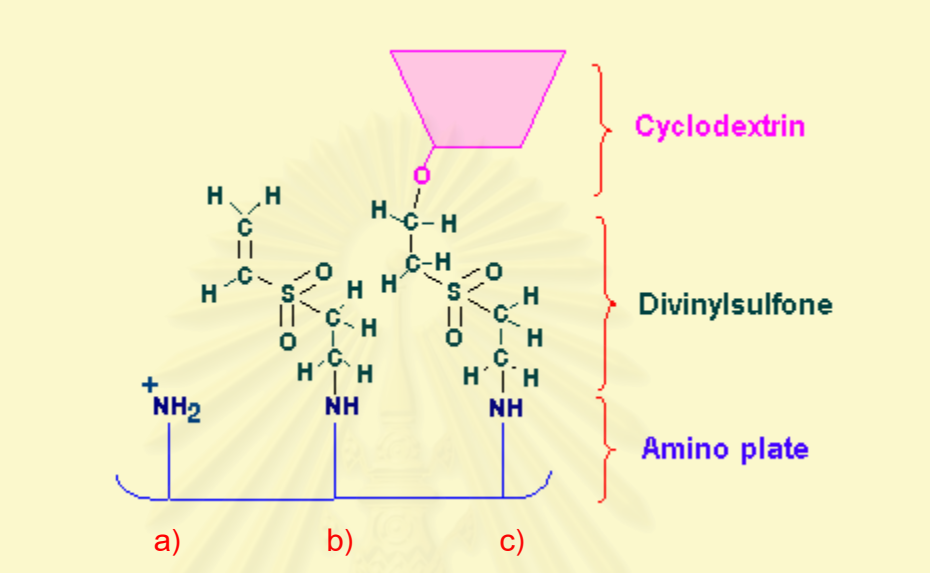


Figure 20 The scheme of the immobilization of CD on amino microtiter plate by the use of divinylsulfone as the cross-linking reagent:
a) Amino surface , b) Amino surface treated with divinylsulfone (DVS) and c) Amino surface treated and coated with divinylsulfone and CD (DVS/CD).

At the beginning, the comparative study of native HRP and HRP-conjugate binding behavior on amino, adsorbed CD (CD), divinylsulfone (DVS) and immobilized CD (DVS/CD) surfaces were investigated. Each surface was examined and compared in order to prove that CD was covalently immobilized on microtiter plate. The amino and divinylsulfone treated surfaces were used to investigate the adsorption of HRP and benzimidazole tracer on the surfaces. While, the adsorbed and immobilized CD surfaces were used to investigate the specific binding of benzimidazole tracer that SAB was encapsulated into CD cavity. After benzimidazole tracer was incubated with various types of surfaces and washed with different types of washing buffer, the HRP activity was then determined. A variety of washing solution was used here to reduce the non-specific adsorption of protein that could be bound on to these surfaces. The amount of HRP activity bound on to each surface is shown in *Figure* 21. Two main results were found, the first one was that HRP activities bound on all surfaces washed with all three types of buffer were much lower than HRP-conjugate activities. Especially, the HRP activities non-specifically bound on CD activated surface was found to be very low. The other finding was that different types of washing buffer had different effect on the amount of HRP-conjugate bound on to the surfaces.

With an amino plate surface, it was found that a buffer containing 0.3% Tween gave the lowest HRP-conjugate activities and PBS with salt did not reduce the adsorption of the HRP-conjugate (no difference between PBS containing high and low salt). On the contrary, the signal of HRP-conjugate activity on divinylsulfone treated (DVS) surface was the highest when the surface was washed with PBS, pH 7, containing 0.3% Tween and lowest when the plate was washed with PBS containing salt (no difference between PBS containing high and low salt).

For the adsorbed (CD) and immobilized (DVS/CD) CD surface, it was found that PBS containing low salt concentration did not reduce the non-specific binding of protein whereas PBS containing high salt or Tween showed less HRP-conjugate activity bound on to both surfaces. Noticeably, the washing buffer, PBS, pH 7, containing 0.5 M NaCl, could make the difference of HRP-conjugate signal between divinylsulfone treated (DVS) surface (without CD) as non-specific adsorption and both adsorbed (CD) and immobilized (DVS/CD) surfaces as specific binding of HRP conjugation.

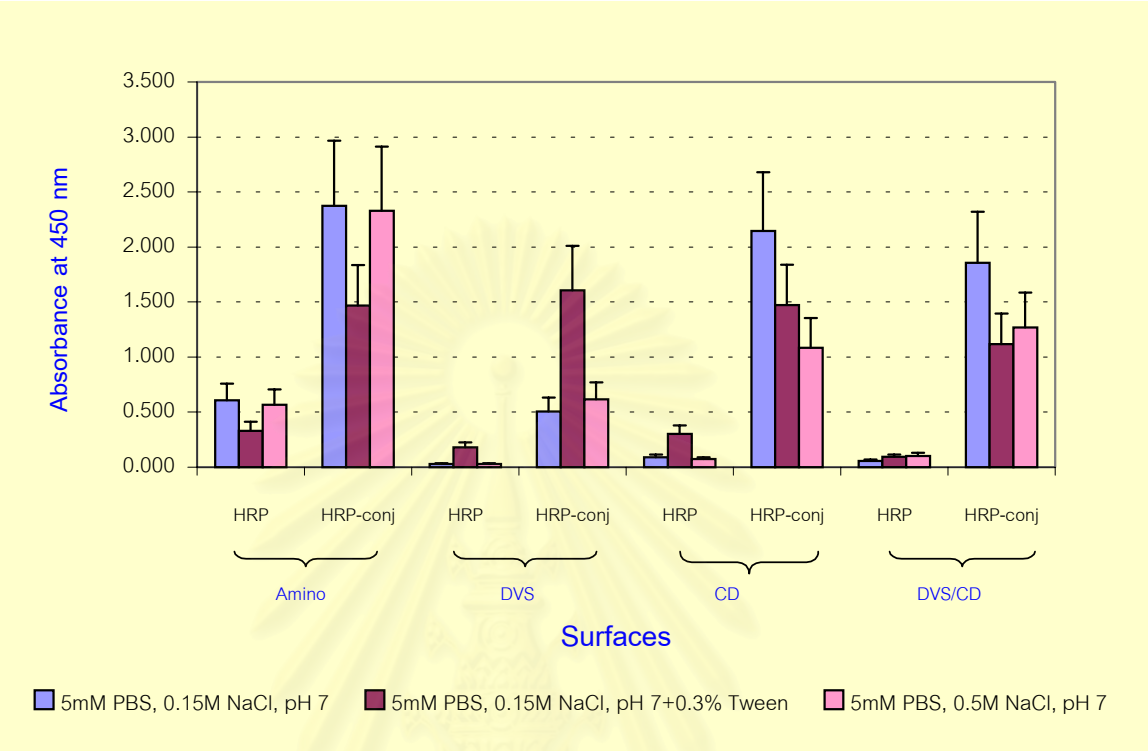


Figure 21 The effect of washing buffer on horseradish peroxidase and benzimidazole tracer bound onto surfaces: *2.5% beta-cyclodextrin was coated on amino plate surface. 10 µg/ml of HRP and benzimidazole tracer was used for encapsulation and washed with different types of washing buffers. (These values were from two separate determinations)*

From these results, the next experiments were then designed to find the optimal condition for benzimidazole tracer encapsulation. This should distinguishably exhibit the difference in HRP-conjugate activity immobilized on CD activated and CD adsorbed surfaces and should also give the lowest signal of divinylsulfone treated (DVS) surface.

3.4.1 The effect of washing buffer types on benzimidazole tracer encapsulation

The effect of washing buffer types on the non-specifically and specifically bound tracer was examined. Many types of washing buffers such as 5 mM PBS, pH 7, containing 0.15 M NaCl or 0.5 M NaCl and or 0.3% Tween and 0.1 M acetate/citric acid buffer, pH 5, containing 0.5 M NaCl and or 0.3% Tween. The result was that the differences of HRP-conjugate activity bound on to divinylsulfone treated (DVS) surface (without CD) and both of immobilized CD (DVS/CD) and adsorbed CD (CD) surfaces were shown in ***Figure*** 22.

The different signal between immobilized (DVS/CD) and adsorbed (CD) surfaces was the highest after washing with acetate buffer, pH 5,containing 0.5 M NaCl and the runner-up was found after washing with phosphate buffer, pH 7, containing 0.15 M NaCl. On the contrary, there were no differences between both surfaces after washing with acetate buffer, pH 5 or phosphate buffer, pH 7, containing 0.5 M NaCl. Noticeably, HRP-conjugate activities bound on to three surfaces were not different and showed the lowest activity when they were washed with either PBS or acetate buffer, containing 0.3% Tween.

As the results, 0.1 M acetate buffer, pH 5,containing 0.5 M NaCl was selected as washing buffer for further experiments, because it showed the highest difference in HRP-conjugate activity bound on to adsorbed and covalently immobilized CD surfaces and also gave the lowest non-specific adsorption of HRP-conjugate on divinylsulfone treated surface where CD was not added.

3.4.2 The effect of β-CD concentrations on benzimidazole tracer encapsulation

The effect of CD concentration used for the immobilization was observed in order to find the lowest concentration that could display high specific binding of benzimidazole tracer.The result was showed in ***Figure*** 23. It illustrates signals occurred in divinylsulfone treated (DVS), immobilized (DVS/CD) and adsorbed (CD) surfaces of the amino and normal polystyrene plate.

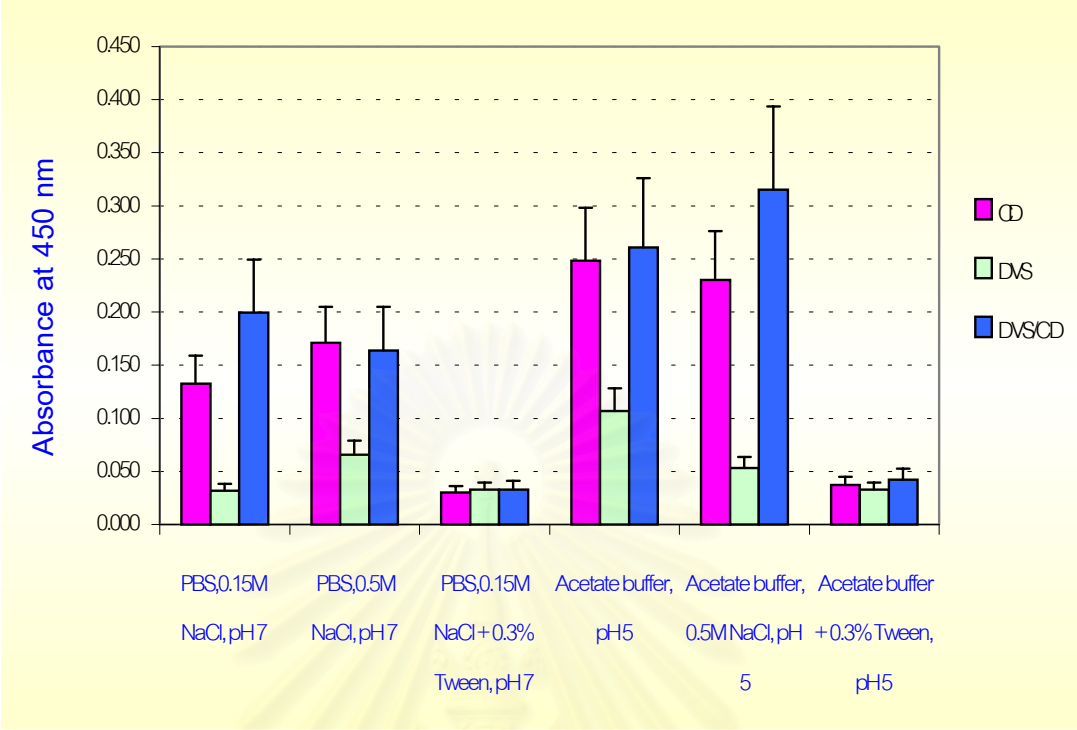


Figure 22 The effect of washing buffer on benzimidazole tracer binding into adsorbed and immobilized CD surfaces: *5% beta-cyclodextrin was coated on amino plate surface. 2.5µg/ml benzimidazole tracer was used for encapsulation and washed with different types of washing buffers. (These values were from two separate determinations)*

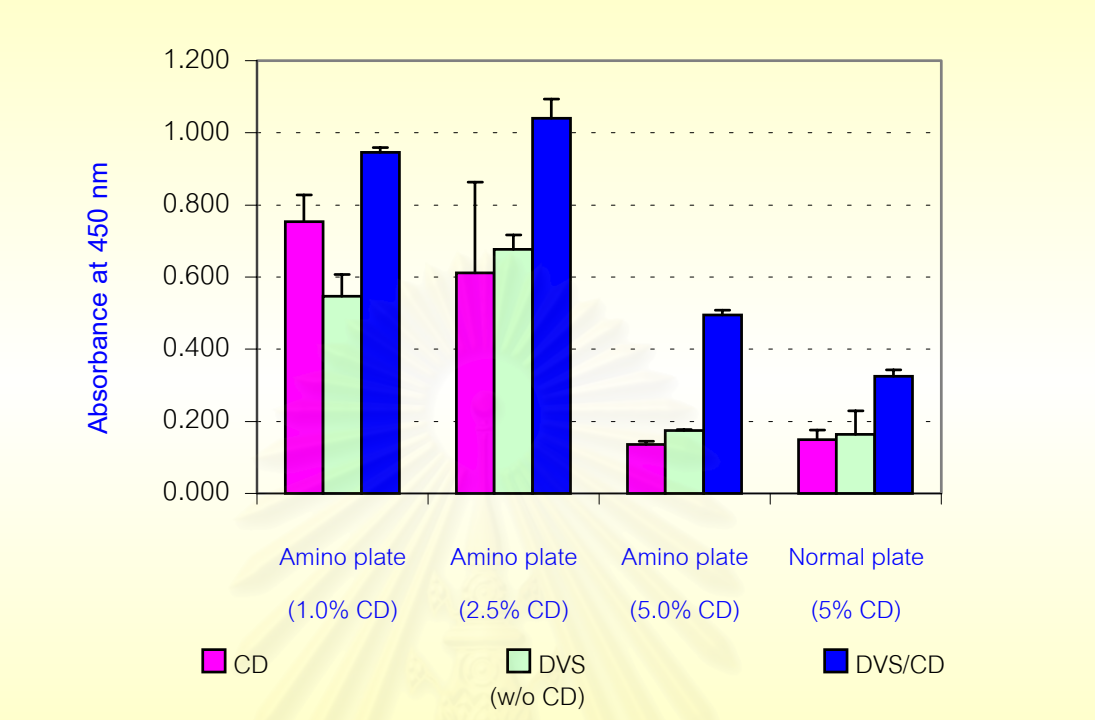


Figure 23 The effect of CD concentration on benzimidazole tracer binding onto adsorbed and immobilized CD surfaces: *1.0, 2.5 and 5% beta-cyclodextrin were coated on amino plate surfaces and 5% beta-cyclodextrin was coated on normal plate surface. 2.5µg/ml benzimidazole tracer was used for competitive encapsulation and 0.1 M acetate/citric acid buffer, containing 0.5 M NaCl, pH 5 was used as washing buffer. (These values were from two separate determinations)*

It was found that the signal of adsorbed (CD) and immobilized (DVS/CD) CD surfaces of amino plate coated with 1.0% and 2.5% were slightly different although the CD concentration used for the immobilization was about twice higher. Moreover, the signal of adsorbed CD and immobilized (DVS/CD) surfaces of amino plate was much lower but the signal difference between adsorbed CD and immobilized (DVS/CD) surfaces was found to be higher than that of amino plate coated with 1.0% or 2.5% CD. Besides, the signal of both adsorbed and the divinylsulfone treated surfaces of amino plate coated with 5% CD was only 30% to that of bound molecules specifically. The signal of adsorbed and divinylsulfone treated surface of both amino plate and normal plate coated with 5% CD was comparable, while the signal of the immobilized CD obtained from amino plate coated 5% CD was higher. It could be considered that CD could be immobilized on amino plate. Therefore, 5% CD solution was used for CD immobilization on microtiter plate in the next experiment.

3.4.3 The effects of benzimidazole tracer concentrations on its encapsulation

Different concentrations of the HRP-conjugate were used for encapsulation in order to find the optimal concentration of tracer for the encapsulation. Moreover, the enacapsulation was performed on surfaces coated with 1%, 2.5% and 5% CD. Ideally, The optimal tracer concentration should display the difference between immobilized CD and adsorbed on the surface with the lowest non-specific binding. From the result in *Figure* 24, it was found that the signal of all surfaces increased when the tracer concentration increased. It was clearly observed that the signal of the adsorbed CD and immobilized CD of the amino plate coated with all CD concentration were different while it could not be seen in the normal plate. Noticeably, the signal difference between adsorbed and immobilized CD surfaces almost the same although different concentrations of CD were used. It can also be seen that 2.5 μg/ml of benzimidazole tracer showed clear difference between adsorbed and immobilized CD, especially in amino plate coated with 5% CD. Therefore, the concentration of benzimidazole tracer at 2.5 μg/ml was then chosen for the next experiment.

3.4.4 The effect of CD types on benzimidazole tracer encapsulation

In order to know the effect of CD types on the degree of immobilization and the capability of immobilized CD for the guest encapsulation, different types of CD including β-CD, methyl-β-CD and hydroxypropyl-β-CD were used.

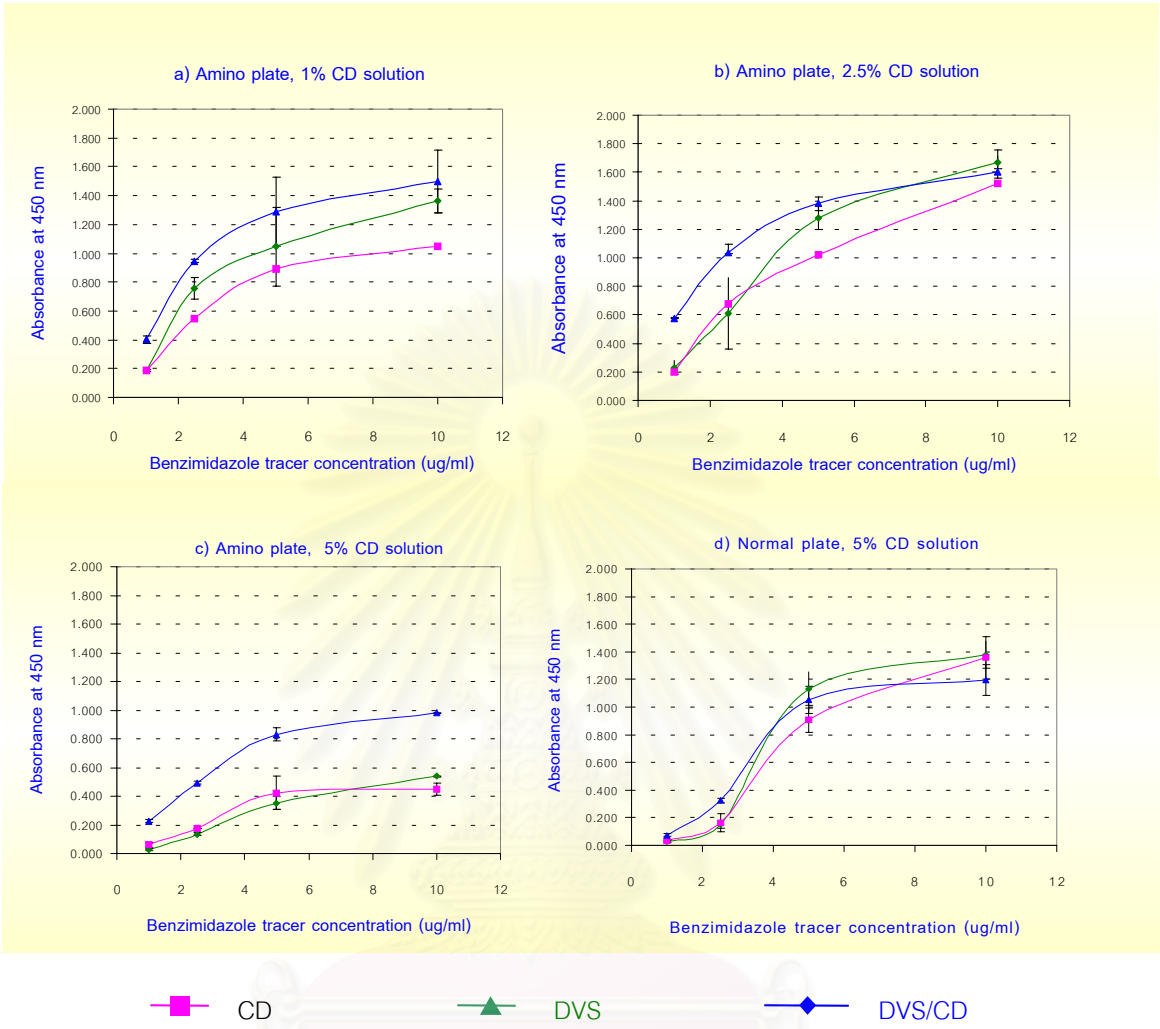


Figure 24 The effects of benzimidazole tracer concentration on it's binding onto adsorbed and immobilized CD surfaces: *a), b) and c) Amino plate coated with 1.0, 2.5 and 5.0 % beta-cyclodextrin on the surfaces, respectively. d) Normal plate coated with 5.0 % beta-cyclodextrin. 1-10 µg/ml benzimidazole tracer was used for competitive encapsulation and 0.1 M acetate/citric acid buffer, containing 0.5 M NaCl, pH 5 was used as washing buffer. (These values were from two separate determinations)*

After the encapsulation, the tracer signals bound to the immobilized (DVS/CD), adsorbed (CD) and divinylsulfone treated (DVS) surfaces were observed. It was found that amino plate coated with β-CD showed the highest difference between adsorbed (CD) and immobilized (DVS/CD) surfaces, while some differences could be observed from the amino plate coated with methyl β-CD. On the contrary, there was no difference of both surfaces could be observed from the amino plate coated with hydroxypropyl-β-CD as shown in *Figure* 25. From the results, β-CD was the most suitable for benzimidazole tracer encapsulation on microtiter plate.

3.5 Determination of carbendazim standard by competitive benzimidazole tracer encapsulation

According to the previous experiments, the optimal condition for the encapsulation of benzimidazole tracer was as follows. The optimal immobilized surface was the amino surface activated with divinylsulfone and coated with 5% β-CD and the optimal concentration of benzimidazole tracer was 2.5 μg/ml benzimidazole tracer. For further study, we attempted to apply the use of immobilized CD on microtiter plate for standard carbendazim determination by the competitive benzimidazole tracer encapsulation. The competitive benzimidazole tracer encapsulation was performed based on benzimidazole tracer concentration and buffer pH for carbendazim determination. The pattern of carbendazim standard was observed when carbendazim diluted in buffer pH 5 and the tracer at the concentration of 1.2 and 2.5 μg/ml were used for competitive encapsulation. It was found that the tracer signal of adsorbed and immobilized CD surfaces was lower when carbendazim concentration was higher in both cases as shown in *Figure* 26. The signal of adsorbed CD surface obtained form1.2 and 2.5 μg/ml of tracer were lower than that of immobilized CD surfaces. In addition, the signal of adsorbed CD surface obtained form 1.2 μg/ml of tracer shows almost constant signal (lower than 0.1 A_{405} Unit) from 2 μg/ml of carbendazim. *Figure* 27 showed the pattern of carbendazim standard which was observed when carbendazim was dissolved and diluted in loading buffer of pH 2.5 and 5.0 and 2.5 μg/ml of tracer was used for competitive encapsulation.

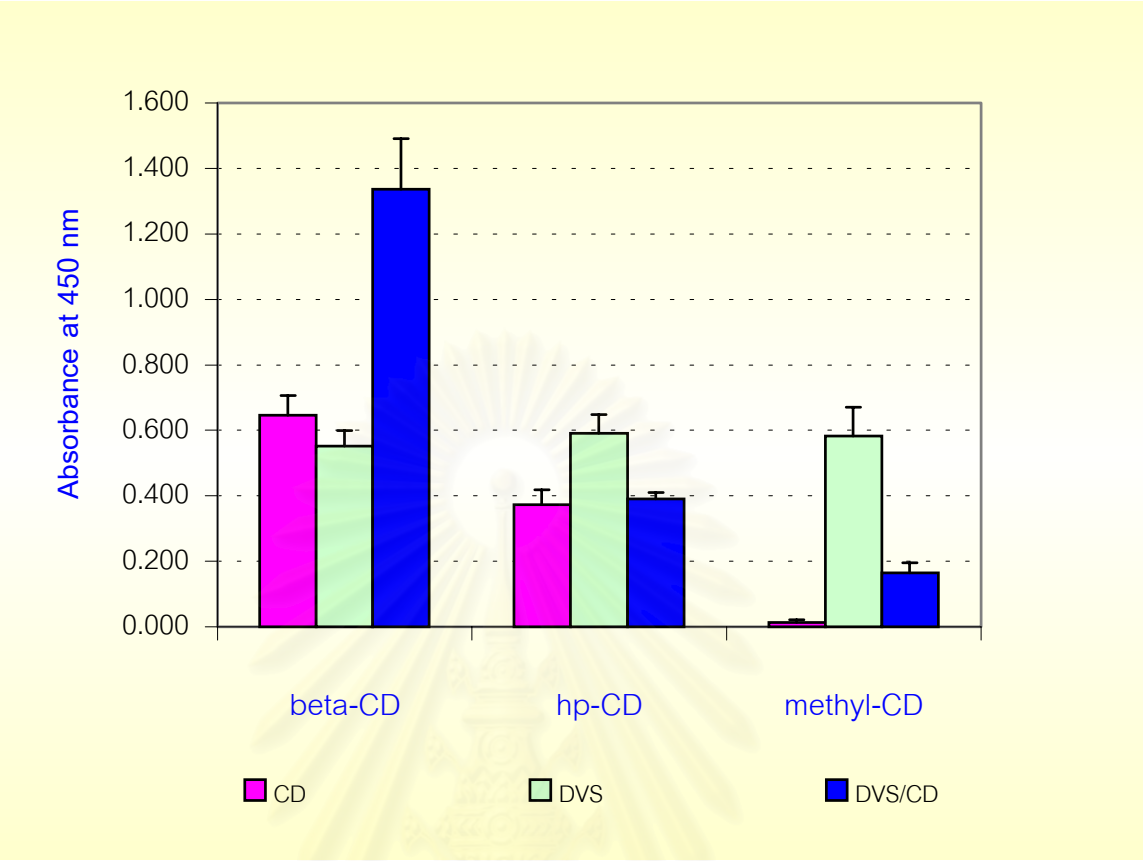


Figure 25 The effect of CD types on benzimidazole tracer binding onto adsorbed and immobilized CD surfaces: *5% of β-, hydroxypropyl β- and methyl β-cyclodextrin were coated on amino plate surfaces. 2.5 μg/ml benzimidazole tracer was used for competitive encapsulation with 0.1 M acetate/citric acid buffer, containing 0.5 M NaCl, pH 5 as washing buffer. (These values were from two separate determinations)*

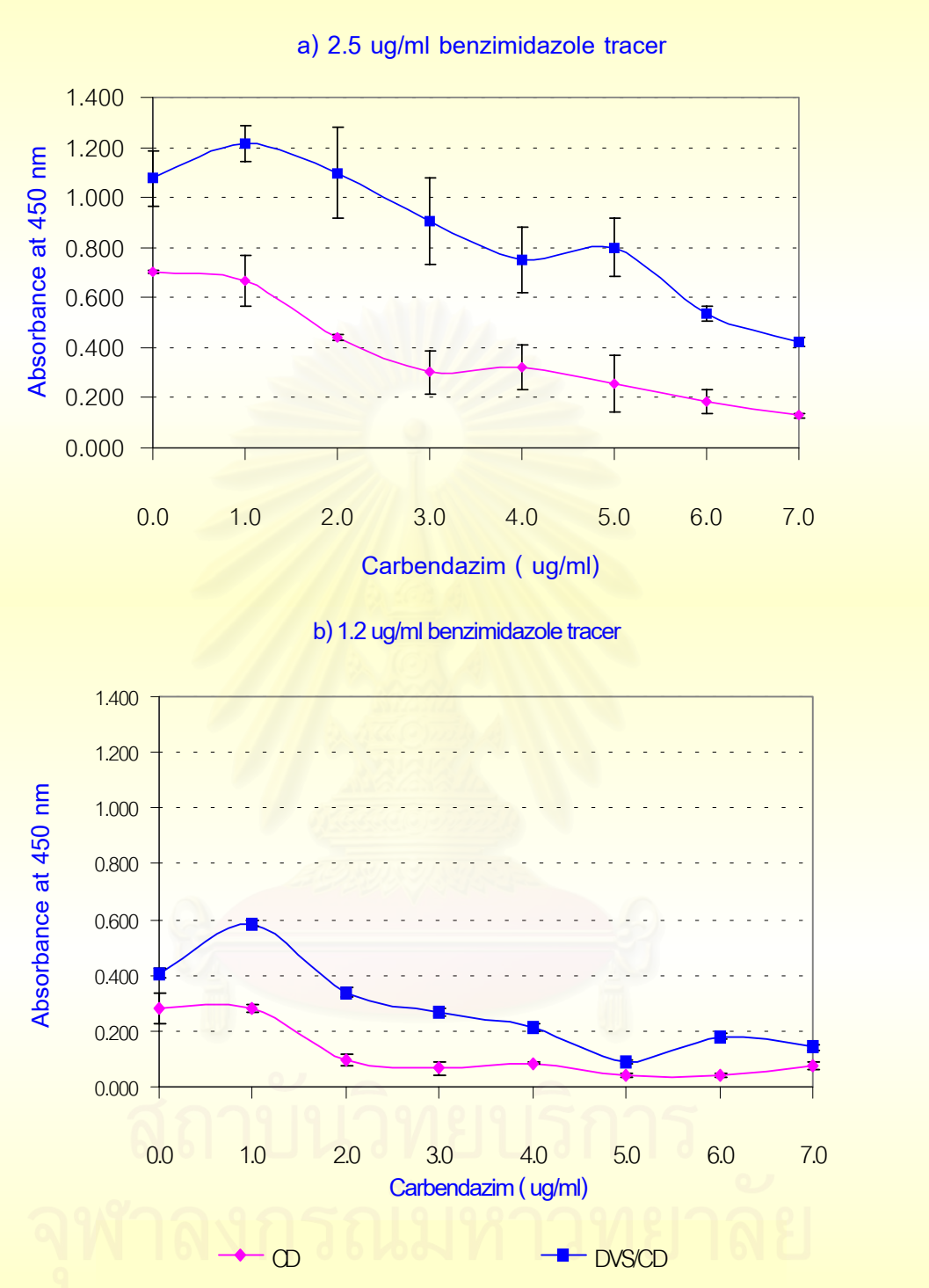


Figure 26 Effect of carbendazim on competitive inhibition of benzimidazole tracer encapsulation into adsorbed and immobilized CD surfaces: *a) 2.5 µg/ml benzimidazole tracer and b) 1.2 µg/ml benzimidazole tracer was used for competitive encapsulation into 5% CD solution coated surfaces and carbendazim was diluted in acetate/citric acid buffer, pH 5. (These values were from two separate determinations)*

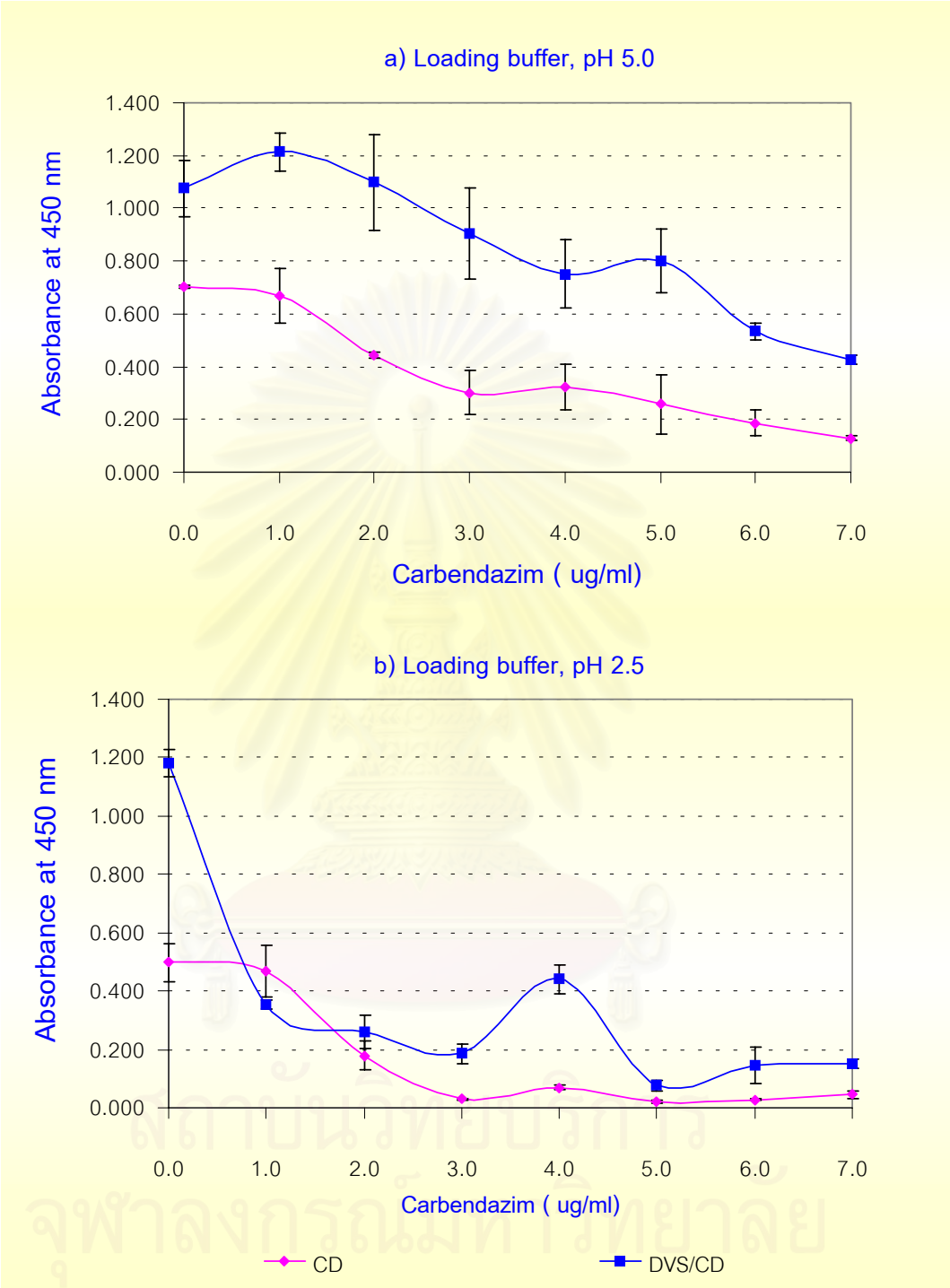


Figure 27 Effect of carbendazim on competitive inhibition of benzimidazole tracer encapsulation into adsorbed and immobilized CD surfaces : *a) Carbendazim diluted in loading buffer, pH 5 , b) Carbendazim diluted in loading buffer, pH 2.5 with benzimidazole tracer at 2.5 µg/ml was used for competitive encapsulation. (These values were from two separate determinations*

The result showed that tracer signal was lower when carbendazim concentration was higher in both adsorbed and immobilized CD surfaces loaded with carbendazim at pH 2.5 and 5.0. Noticeably, the tracer signal obtained from carbendazim loading at pH 2.5 was more rapidly decreased than the signal obtained from carbendazim loading at pH 5.0 when carbendazim concentration increased. This occurred in both adsorbed and immobilized CD surfaces. Moreover, almost constant signal (lower than 0.1 A_{405} Unit) was observed when the carbendazim concentration was in the range of 3.0-7.0 μg/ml *(Figure* 27b). However, the comparative study of the loading buffer pH 2.5 and 5.0 was examined in the next experiment.

3.6 Capability test of immobilized CD and benzimidazole tracer for carbendazim determination in fruits

The study of immobilized CD and benzimidazole tracer capability for the determination of carbendazim in fruits consisted of three parts. First, it was necessary to set the negative control: to measure carbendazim residues in fruit samples contained no carbendazim as detected by HPLC. Secondly, the fruit samples were determined to check the interference of sample matrix that may affect the competitive determination system. Thirdly, the limit of detection (LOD) of this method was determined. So the conventional method for the determination of carbendazim residues in food such as HPLC method was necessary to investigate the samples at first.

3.6.1 LC determination

Apple and grape were samples to be used for carbendazim determination by UV and fluorescence detectors of HPLC. The maximum wavelength of absorption of carbendazim is ~280 nm and the excitation and emission wavelenght is at 280 nm and 310 nm, respectively (Gilvydis and Walters, 1990). Fruits were extracted with methanol and partitioned into dichloromethane after acidification and again after subsequent alkalization of the extract. It was found that there were no carbendazim residues detected in either acidic or basidic extracted solution. Carbendazim peak should show up at the R_t of 3.5 min in both UV and fluorescense spectra. Carbendazim standard was eluted at 3.527 in UV (***Figure*** 28-a) and 3.597 in Fluorescense spectra (***Figure*** 29-a). In acidic extracted solution of apple and grape, there were peaks at around 3.2 and 3.4 min detected by UV detector (***Figure*** 28 b and d) but they could not be detected by fluorescence detector (***Figure*** 29 b and d). On the contrary, in basidic extracted solution, there were no interferences detected by both UV and fluorescence detectors (***Figures*** 28 and 29, c and e).

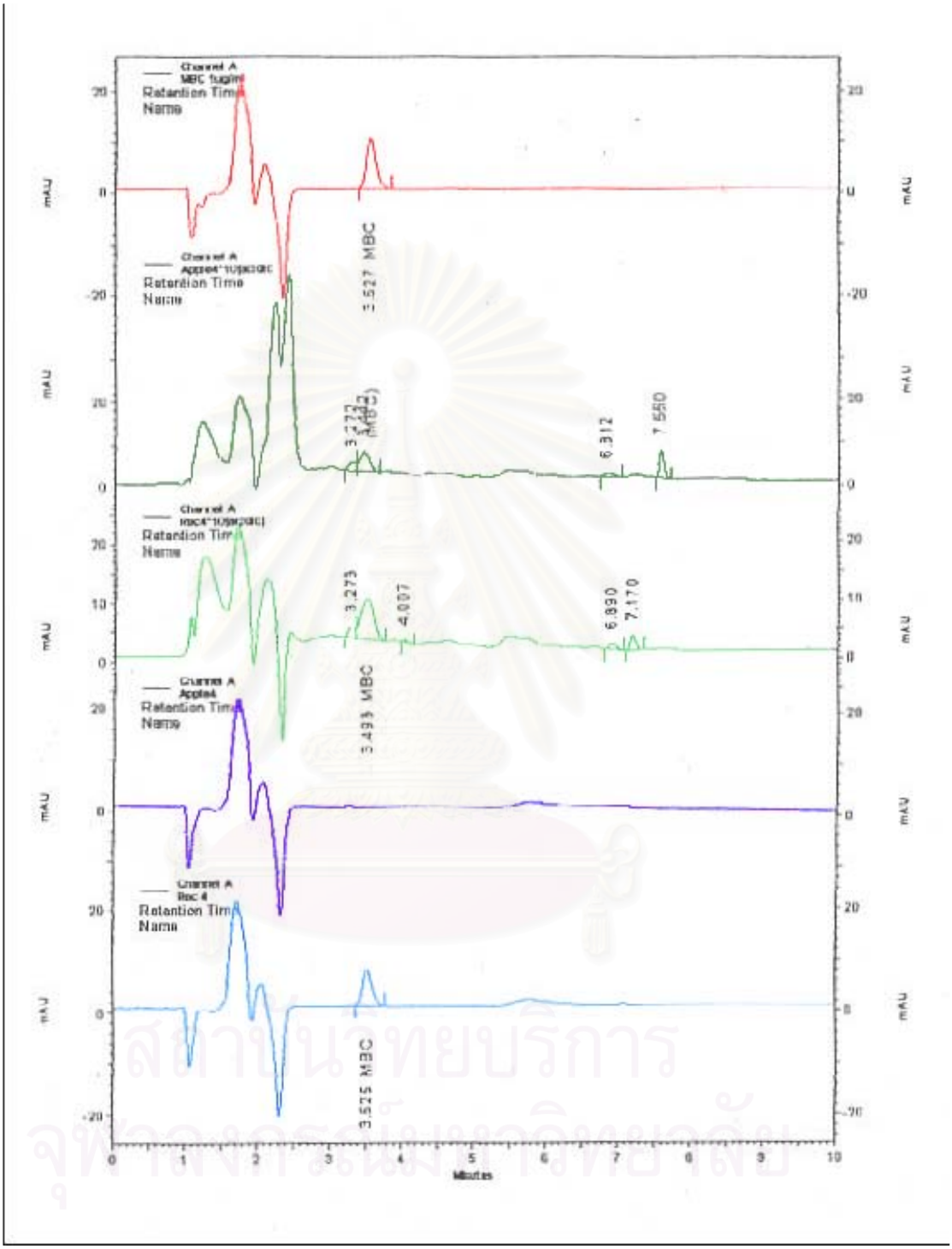


Figure 28 LC determination of carbendazim in fruit extracts by UV detector: a*) 1µg/ml carbendazim,* ***b****) 0.1 g/ml apple, acidic extracted solution,* ***c****) 1.0 g/ml apple, basidic extracted solution,* ***d****) 0.1 g/ml grape, acidic extracted solution and* ***e****) 1.0 g/ml grape, basidic extracted solution*

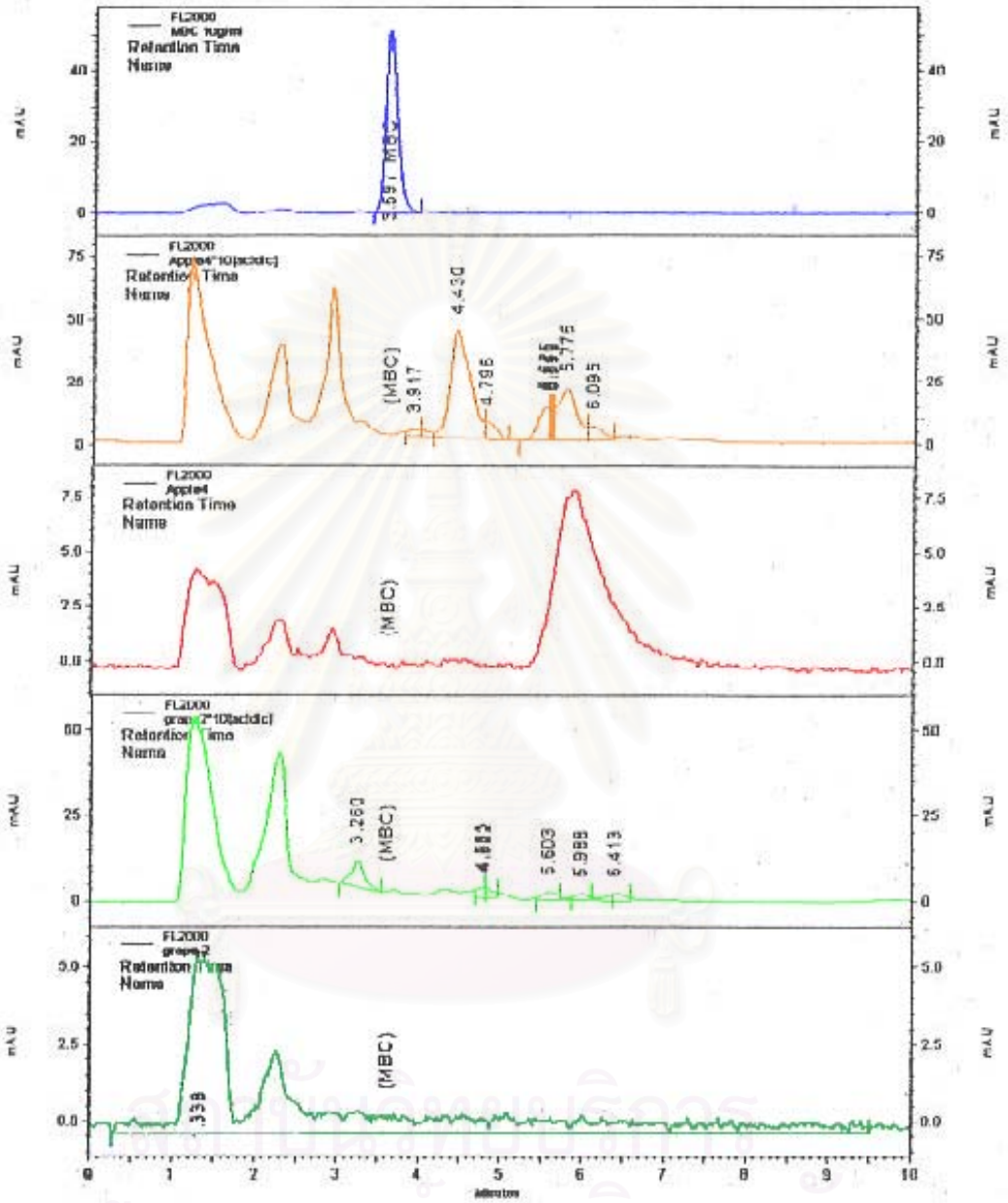


Figure 29 LC determination of carbendazim in fruits by fluorescence detector: a*) 1µg/ml carbendazim,* ***b****) 0.1 g/ml apple, acidic extracted solution,* ***c****) 1.0 g/ml apple, basidic extracted solution ,* ***d****) 0.1 g/ml grape, acidic extracted solution and* ***e****) 1.0 g/ml grape, basidic extracted solution*

3.6.2 The recovery study of HPLC method

Although the LC method used for carbendazim residues determination in sample is the standard method recommended in Pesticide Analytical Method (PAM. Volumn I, 1991), the analytical data should be quality controlled in order to ensure they were reliable and acceptable. Therefore, in this study, the accuracy of the method was determined in term of the percent recovery by analysis of known added amounts of carbendazim. The precision was also determined and represented by coefficient of variation (%CV).

Apple and grape extracted solution spiked with carbendazim standard at 1-ppm level were determined for carbendazim residues. It was found that the recovery of acidic extracted solution was much lower than basidic extracted solution in both sample types detected by UV and fluorescence detector (*Figure* 30-33). *Table* 6 shows that in apple, the recovery of acidic and basidic extracted solution by UV detector was 10.2% and 69.6% and was 3 .8% and 68.4% for fluorescence detector , respectively. In grape, the recovery was in the same pattern as in apple. The recovery of acidic and basidic extracted solution of UV detector was 6.0% and 62.0% and was 4.8% and 61.9% for fluorescence detector, respectively. The statistical summary of recoveries of carbendazim from fortified samples was shown in *Table* 7. Average recovery determined by UV and fluorescence detectors was 79.8% and 72.3% in apple and 67.2% and 66.8% in grape, respectively. The %CV of the determination of carbendazim standard by UV detector was lower than 2% and fluorescence detector was lower than 3%.

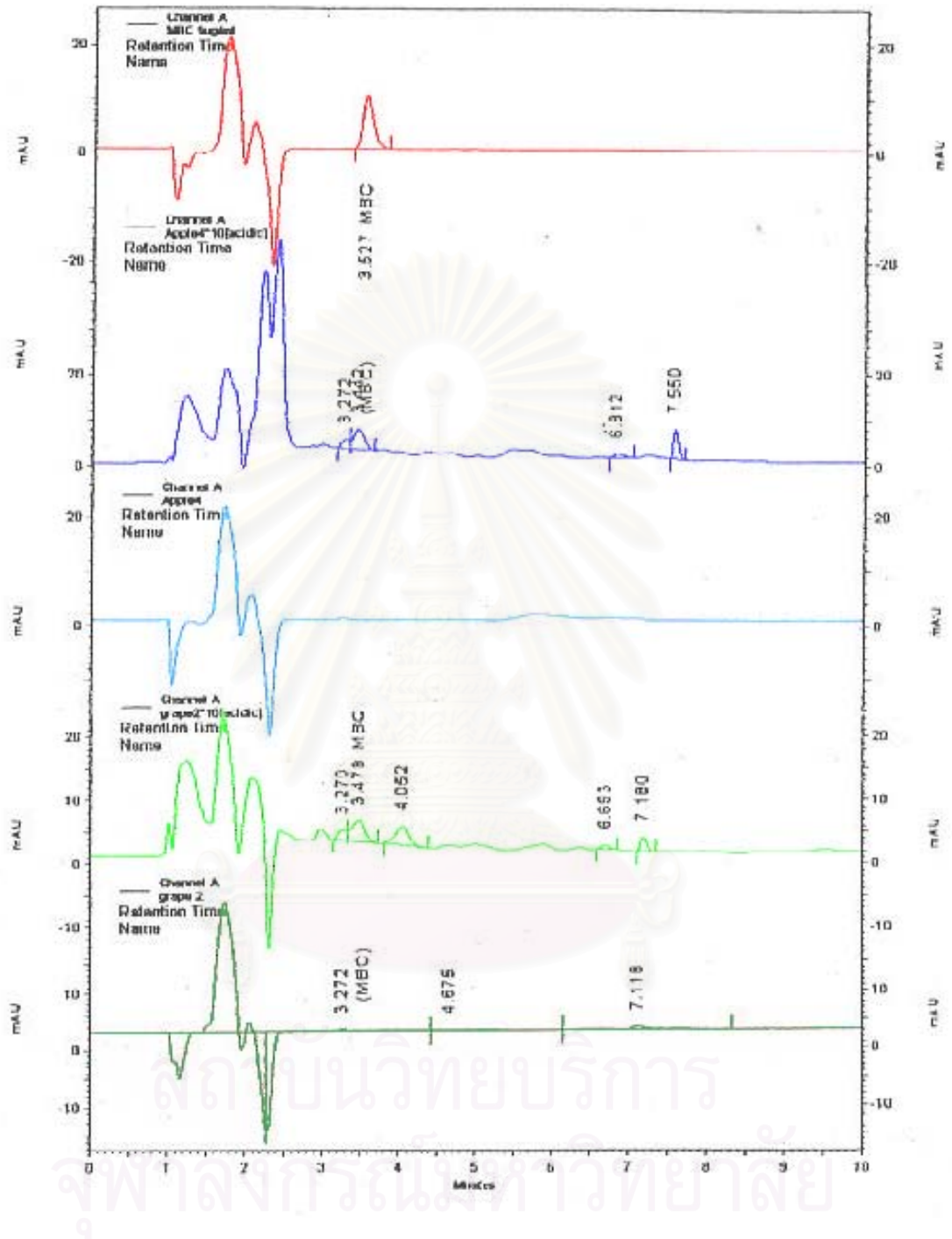


Figure 30 Recovery study of carbendazim determination in apple extract by LC with UV detector: a*) 1µg/ml carbendazim,* ***b****) 0.1 g/ml apple, acidic extracted solution,* ***c****) 1 ppm spiked apple, acidic extracted solution,* ***d****) 1.0 g/ml apple, basidic extracted solution and* ***e****) 1 ppm spiked apple, basidic extracted solution*

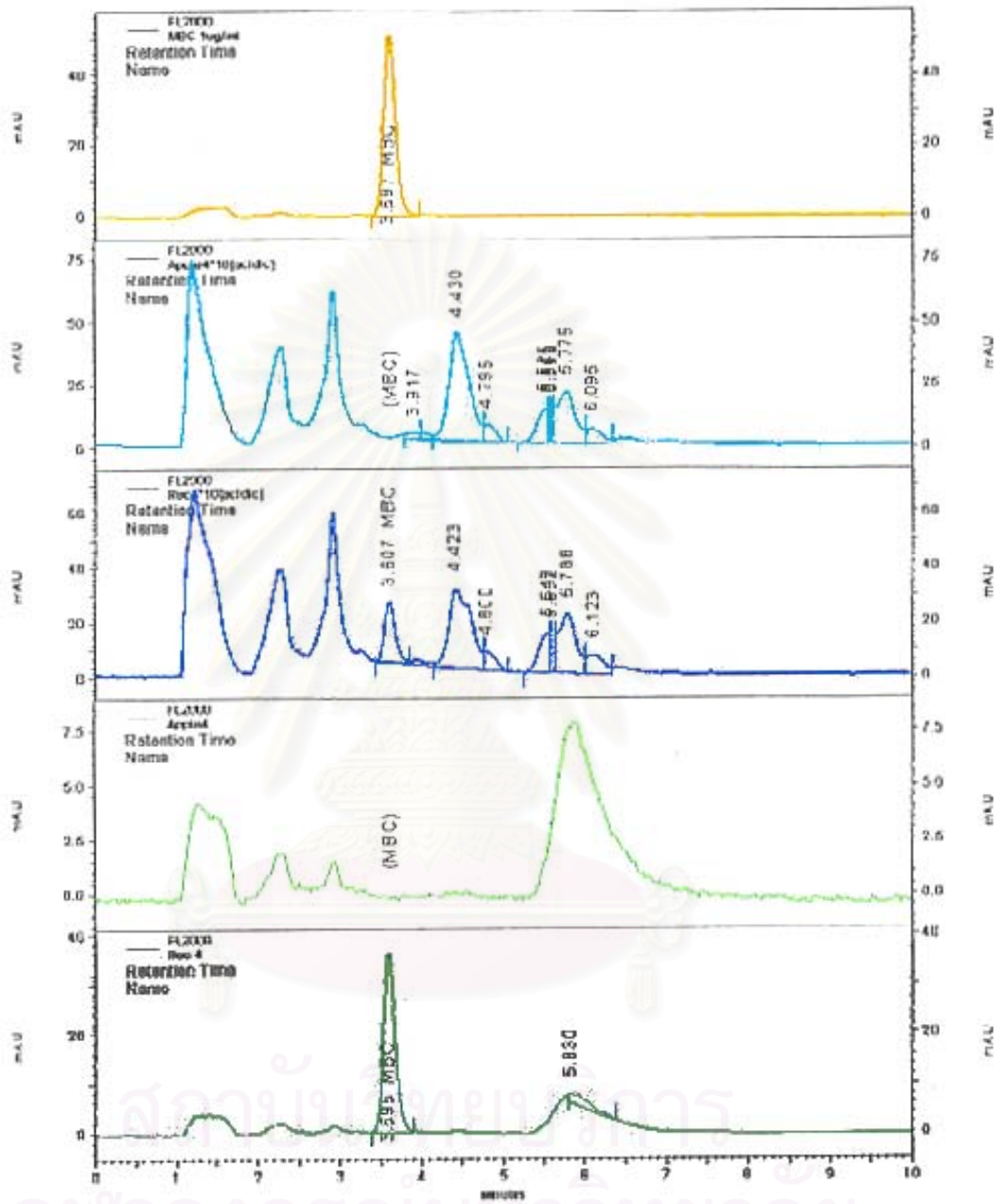


Figure 31 Recovery study of carbendazim determination in apple extract by LC with fluorescence detector: a*) 1µg/ml carbendazim,* ***b****) 0.1 g/ml apple acidic extracted solution,* ***c****) 1 ppm spiked apple, acidic extracted solution,* ***d****) 1.0 g/ml apple, basidic extracted solution and* ***e****) 1 ppm spiked apple, basidic extracted solution*

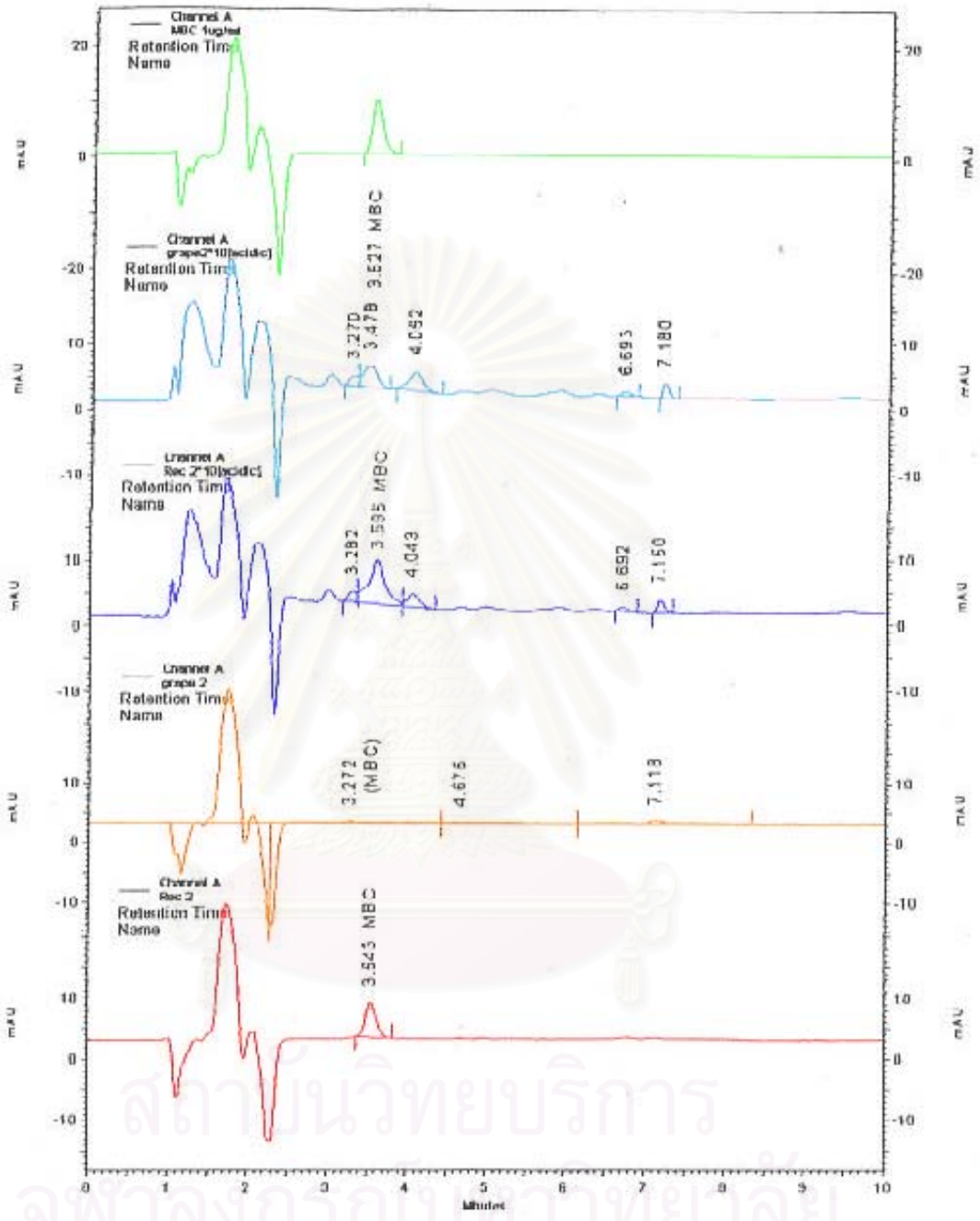


Figure 32 Recovery study of carbendazim determination in grape extract by LC with UV detector: a*) 1µg/ml carbendazim,* ***b****) 0.1 g/ml apple, acidic extracted solution,* ***c****) 1 ppm spiked apple, acidic extracted solution,* ***d****) 1.0 g/ml apple, basidic extracted solution and* ***e****) 1 ppm spiked apple, basidic extracted solution*

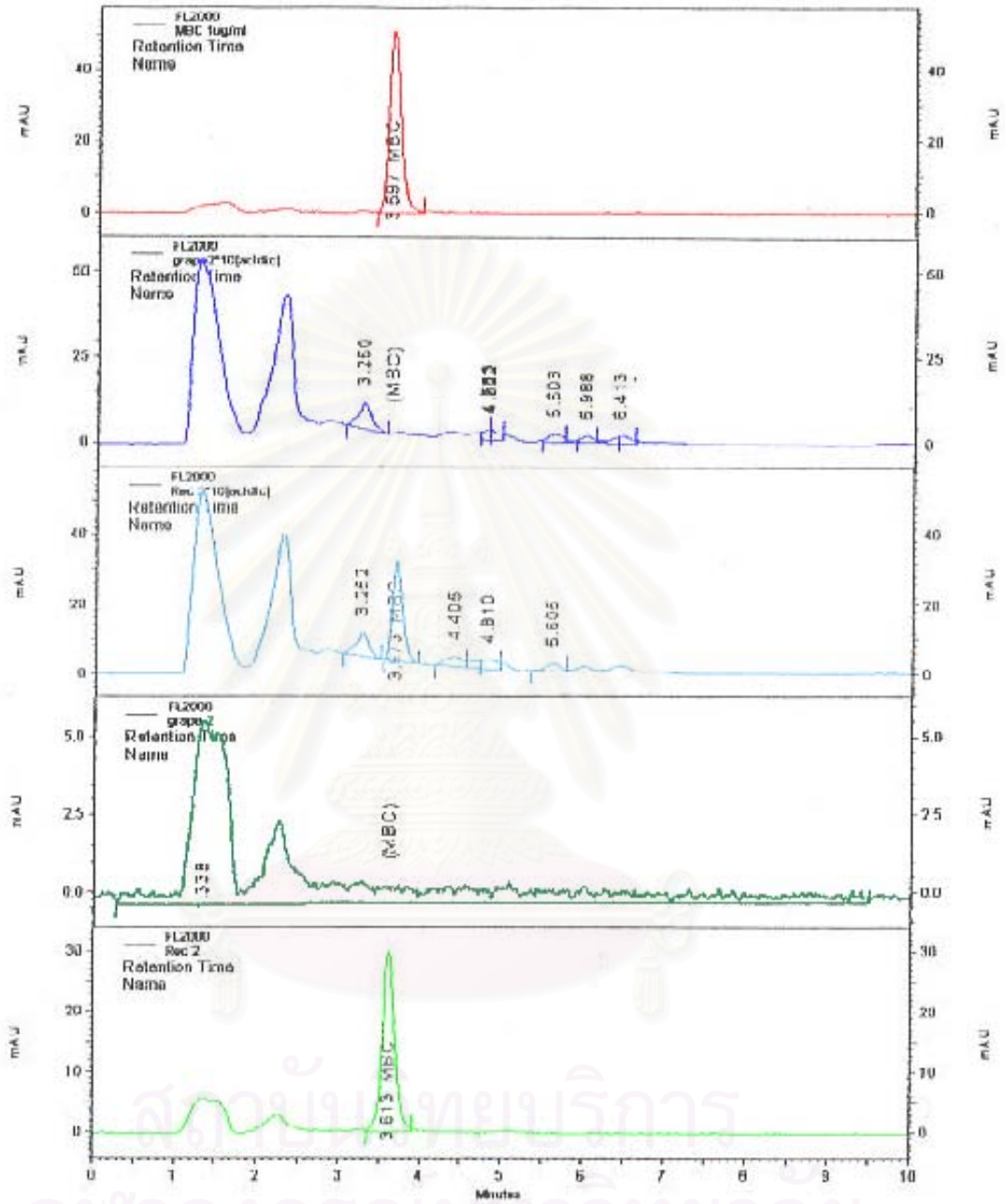


Figure 33 Recovery study of carbendazim determination in grape extract by LC with fluorescence detector: a*) 1µg/ml carbendazim,* ***b****) 0.1 g/ml apple acidic extracted solution,* ***c****) 1 ppm spiked apple, acidic extracted solution,* ***d****) 1.0 g/ml apple, basidic extracted solution and* ***e****) 1 ppm spiked apple, basidic extracted solution*

Table 6 Percent recovery of carbendazim through HPLC method

Types	Carbendazim Added (ppm)	Recovery of carbendazim (%)					
		Acidic partition		Basidic partition		Total	
		UV	Fluorescence	UV	Fluorescence	UV	Fluorescence
Grape	1.0	6.0	4.8	61.2	62.0	67.2	66.8
Apple	1.0	10.2	3.8	69.6	68.4	79.8	72.3

The percent recoveries are averaged from two separate experiments.

Table 7 Statistical summary of carbendazim recovery from fortified fruits

Statistics	Grape		Apple	
	UV	Fluorescence	UV	Fluorescence
Average (%)	*67.2*	*66.8*	*79.8*	*72.3*
SD (%)	*1.1*	*1.8*	*0.9*	*2.1*
CV (%)	*1.6*	*2.8*	*1.2*	*2.9*

3.6.3 The effect of sample matrix on the determination of carbendazim in sample by competitive benzimidazole tracer encapsulation

The concentration of grape extracted-solution at 0.1, 0.25, 0.5 and 1 g/ml was used for the competitive encapsulation with 2.5 μg/ml benzimidazole tracer. It was determined in order to study the effect of sample matrix on the system of competitive benzimidazole tracer encapsulation under specific conditions. The result showed that the signal of both adsorbed and immobilized CD surface decreased when the concentration of extracted solution increased as shown in ***Figure*** 34. Noticeably, the signal difference between adsorbed and immobilized CD surface was observed. The difference was formed to be pronounce when high sample concentration was used and vice versa. Both signal of adsorbed and immobilized CD surface obtained from sample extracted solution loaded at pH 5 was more rapidly decreased than obtained from sample loaded at pH 2.5. It seemed that the lowest signal of pH loading at 5.0 occurred at 0.5 g/ml of grape extracted-solution while that of pH loading 2.5 occurred at 1.0 g/ml. Moreover, it was found that at 0.1 g/ml of grape extracted-solution showed close signal to that obtained from the surface when there was no matrix added. From ***Figure*** 34, it can be concluded that the sample matrix could inhibit the encapsulation of benzimidazole tracer.

3.6.4 The determination of carbendazim in grape by competitive benzimidazole tracer encapsulation

In the previous experiment, it was found that at 0.1 g/ml of grape extract showed the lowest interference. So, it was spiked with carbendazim standard 0, 0.1, 0.3, 0.5 and 1.0 μg/ml to get the level of carbendazim residues in sample at 0, 1, 3, 5 and 10 ppm. It was found that the signal obtained from sample loaded at pH 2.5 showed rapid decrease of both immobilized and adsorbed CD surface, and the signal was nearly zero when the concentration of carbendazim was in the range of 0.3 – 1.0 μg/ml. On the contrary, the signal obtained from sample loaded at pH 5.0 showed the fluctuated signal but it tended to decrease when the concentration of carbendazim was increased (***Figure*** 35).

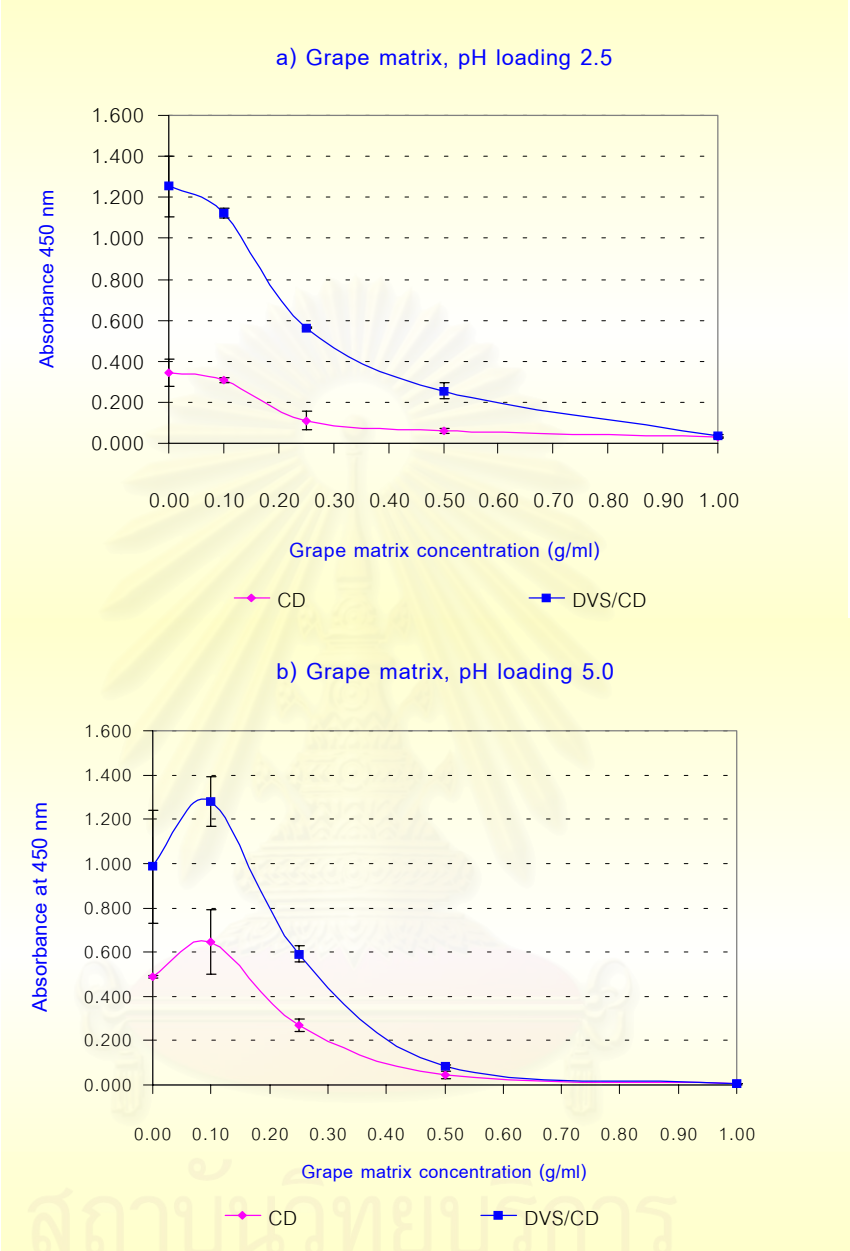


Figure 34 Effect of grape matrix on the inhibition of 2.5 µg/ml benzimidazole tracer encapsulation: **a) Grape extracted-solution was loaded at pH 2.5 and b) Grape extracted-solution was loaded at pH 5.0.** *(These values were from two separate determinations)*

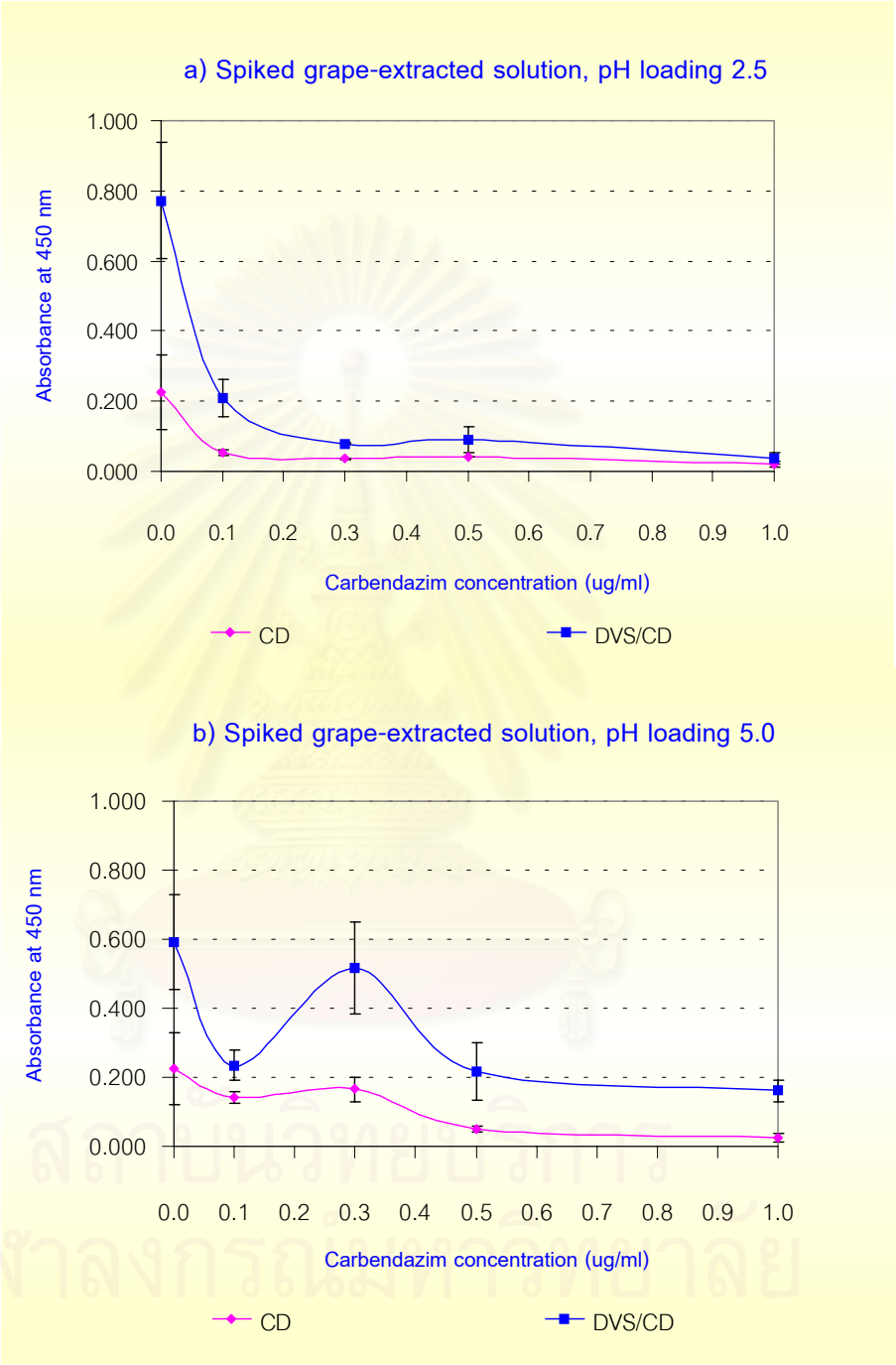


Figure 35 Effect of carbendazim spiked in grape soultion on the inhibition of 2.5 µg/ml benzimidazole tracer encapsulation: **a) Grape extracted-solution was loaded at pH 2.5 and b) Grape extracted-solution was loaded at pH 5.0.** *(These values were from two separate determinations)*

3.7 Stability test of immobilized CD on microtiter plate for reusing purpose

Due to the fact that there are some reports suggested that CD polymer or membrane could be reused after entraping some guests. We tried to find the optimal condition for removing guest out of the immobilized CD cavity and still be able to entrap guest molecules. To remove the entrapped molecules, plate was washed with water and 1mM NaOH. Then it was reused for the encapsulation on all of adsorbed CD, divinylsulfone treated and immobilized CD surfaces and the signals of benzimidazole tracer were then observed. When the amino plate coated with 1%, 2.5% and 5% CD and 1–10 μg/ml benzimidazole tracer concentration were used for the encapsulation as protocol 2.9.3. It was found that the tracer signal of the first and second encapsulation of amino plate coated with 1% CD were almost comparable on both adsorbed CD and divinylsulfone treated surfaces in the range of 1-10 μg/ml benzimidazole tracer concentration. While, the signal of HRP-conjugate on immobilized CD surface was the same when the benzimidazole tracer concentration was only in the range of 1-2.5 μg/ml (***Figure*** 36). On the contrary, the signal difference of both encapsulations obviously occurred in immobilized and adsorbed CD surfaces of amino plate coated with 2.5% CD but the signal difference of both encapsulations tended to be lower when the tracer concentration was lower. While the signal of both encapsulations of divinysulfone treated surface seemed to be no different (***Figure37)*** . However, the signal difference of both encapsulations of amino plated coated with 5% CD when 1-10μg/ml benzimidazole tracer was used, showed close signal in both adsorbed CD and divinylsulfone treated surfaces. While, that of amino plated coated with 5% CD on immobilized CD surface was close when the benzimidazole tracer concentration used in the range of 1-5μ g/ml (***Figure38)***. Noticeably, the signal obtained from the use of 2.5 μg/ml of benzimidazole tracer for the first and secondary encapsulation on amino surface coated with 5% CD seemed to be the most repeatable. So, the washing procedure used in this study was effective for reusing the amino plate coated with 5% CD when 2.5 μg/ml benzimidazole tracer was used for the encapsulation.

Furthermore, the result was consistent with followings when carbendazim standard in 0.1 M acetatate/citric acid buffer, pH 2.5 was loaded into amino plate coated with 5.0% CD and 2.5 μg/ml benzimidazole tracer was used for the encapsulation as the protocol 2.10.3. It was found that the tracer signal of the first and the secondary encapsulation was almost the same in both

adsorbed CD and immobilized CD surfaces (*Figure* 39). Whereas, that of amino plate loaded with carbendazim standard in 0.1 M acetate/citric acid buffer at pH 5.0, it showed the significant signal difference of the first and the second use in both immobilized CD surfaces (*Figure* 40).

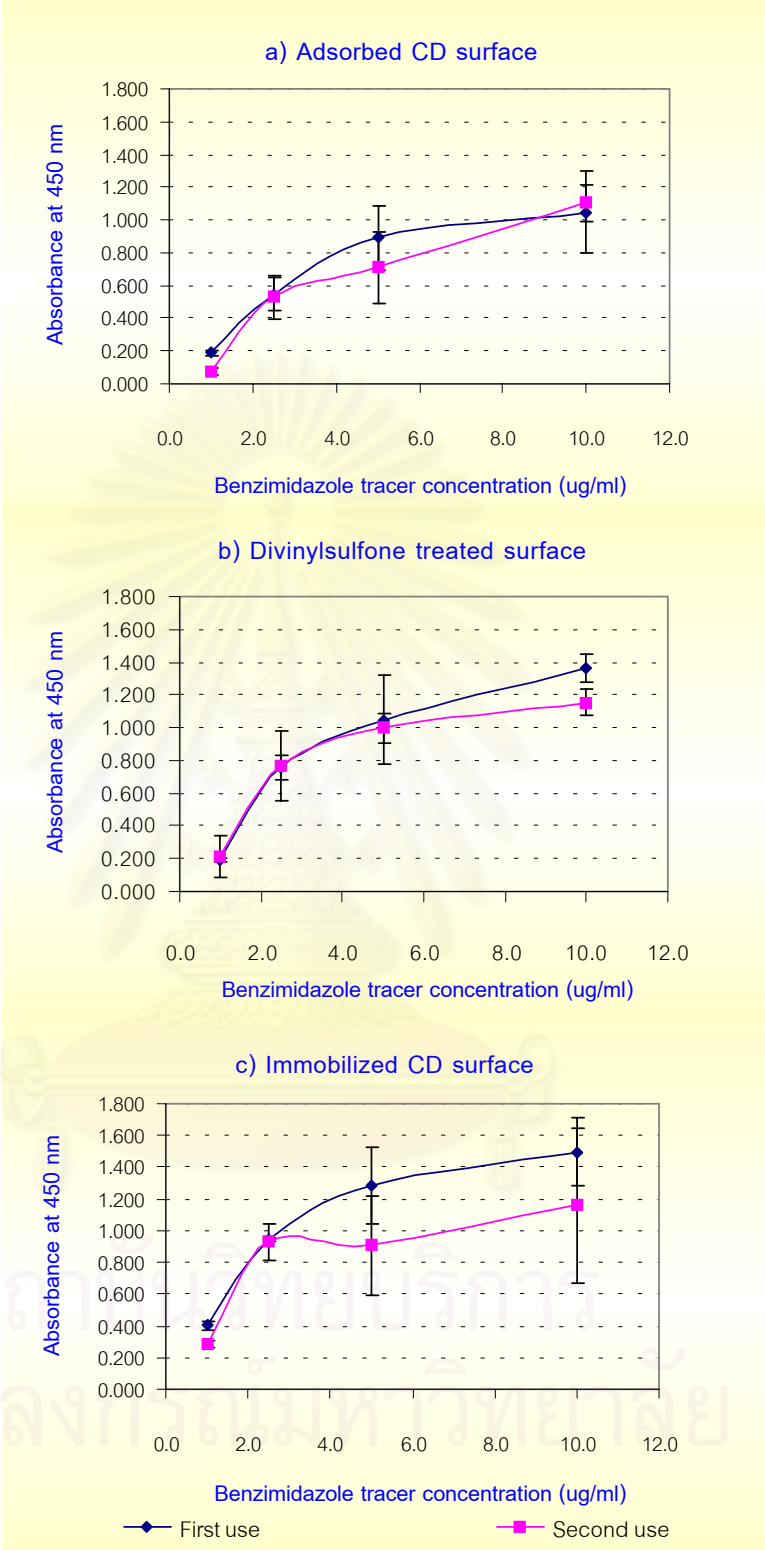


Figure 36 Reuse of amino plate coated with 1.0 % CD : *Benzimidazole was loaded at pH 5.0, a) Adsorbed CD surface (CD), b) Divinylsulfone treated surface (DVS) and c) Immobilized CD surface (DVS/CD). (These values were from two separate determinations)*

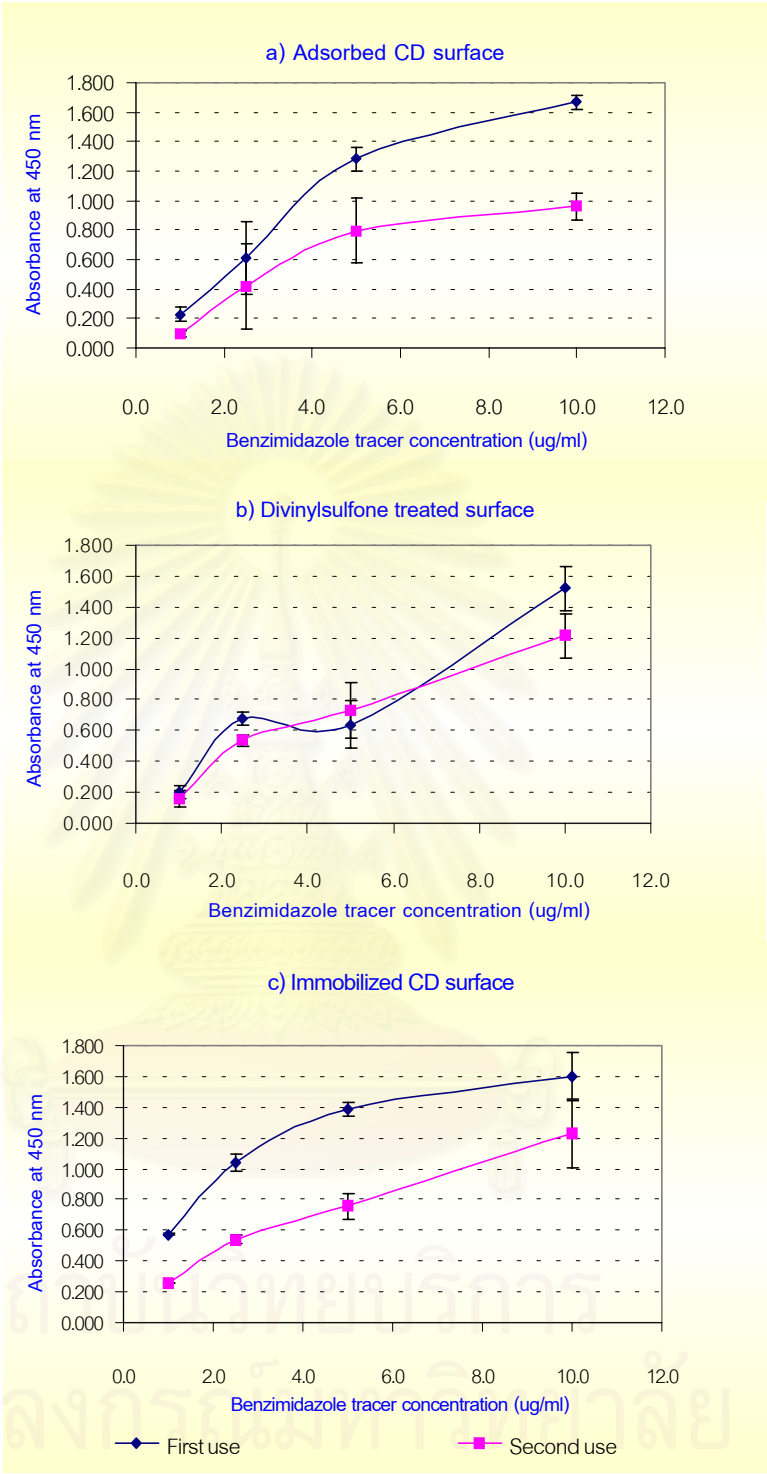


Figure 37 Reuse of amino plate coated with 2.5 % CD : *Benzimidazole was loaded at pH 5.0, a) Adsorbed CD surface (CD), b) Divinylsulfone treated surface (DVS) and c) Immobilized CD surface (DVS/CD). (These values were from two separate determinations)*

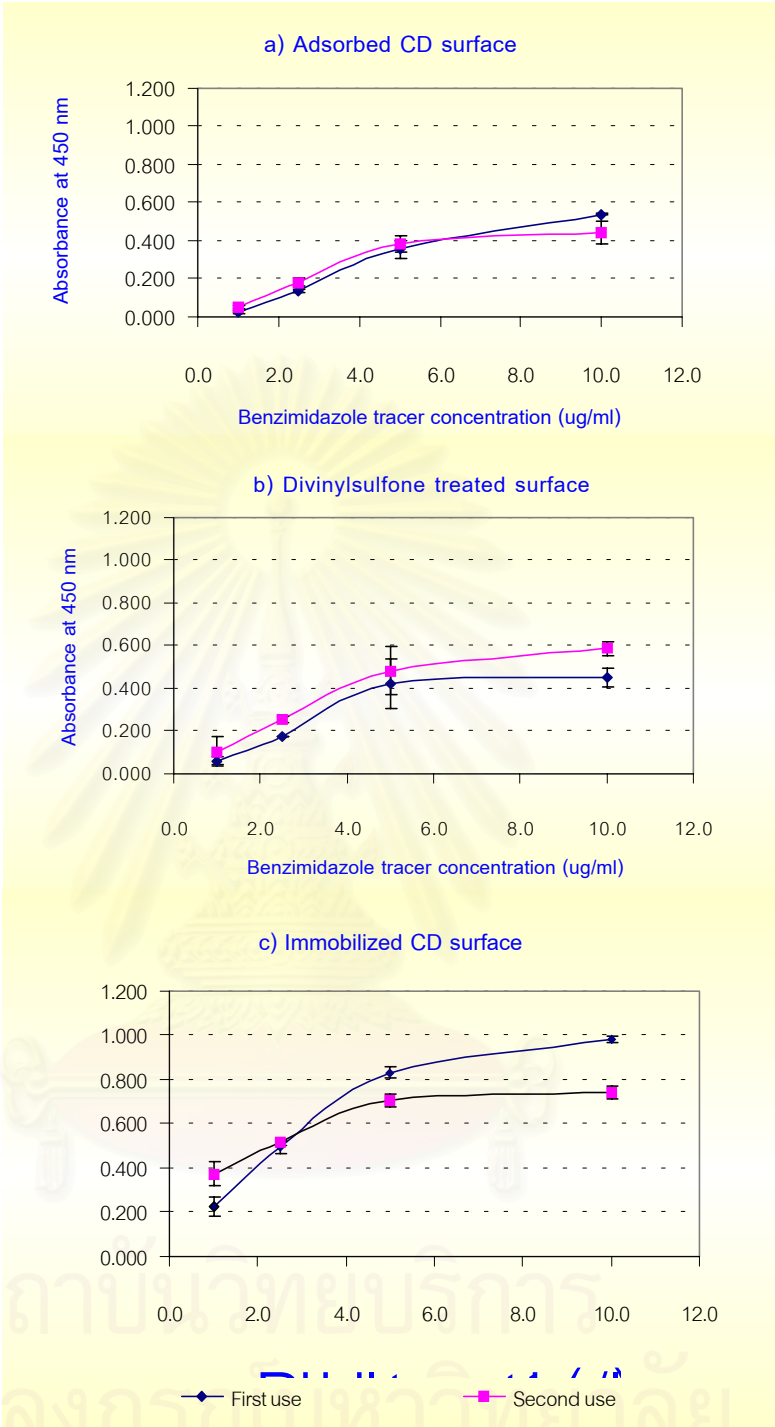


Figure 38 Reuse of amino plate coated with 5.0 % CD : *Benzimidazole was loaded at pH 5.0, a) Adsorbed CD surface (CD), b) Divinylsulfone treated surface (DVS) and c) Immobilized CD surface (DVS/CD). (These values were from two separate determinations)*

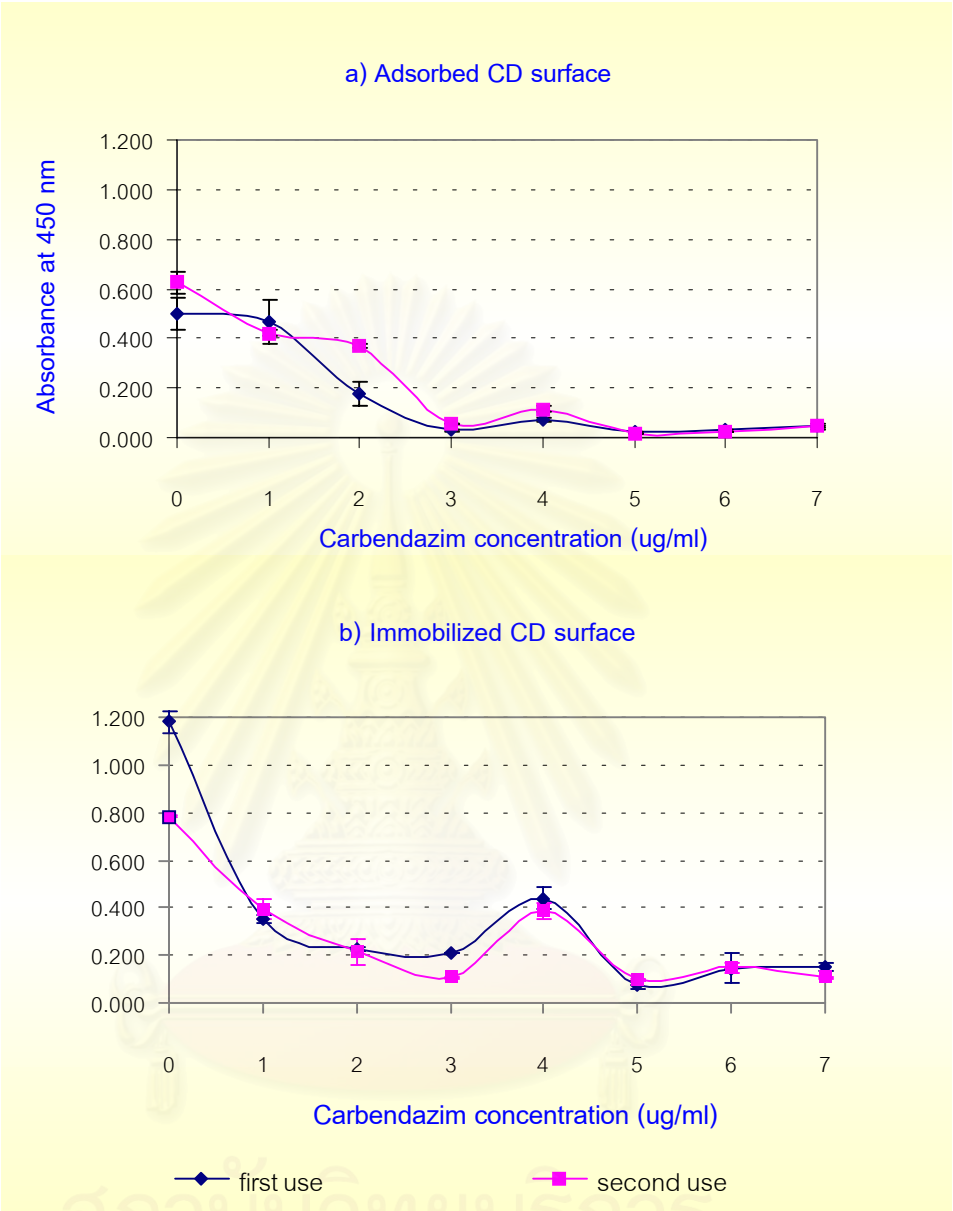


Figure 39 Reuse of amino plate coated with 5.0 % CD in which carbendazim standard in 0.1 M acetate/citric acid buffer, pH 2.5 was loaded: *2.5 µg/ml benzimidazole tracer was loaded at pH 5.0, a) Adsorbed CD surface (CD) and b) Immobilized CD surface (DVS/CD). (These values were from two separate determinations)*

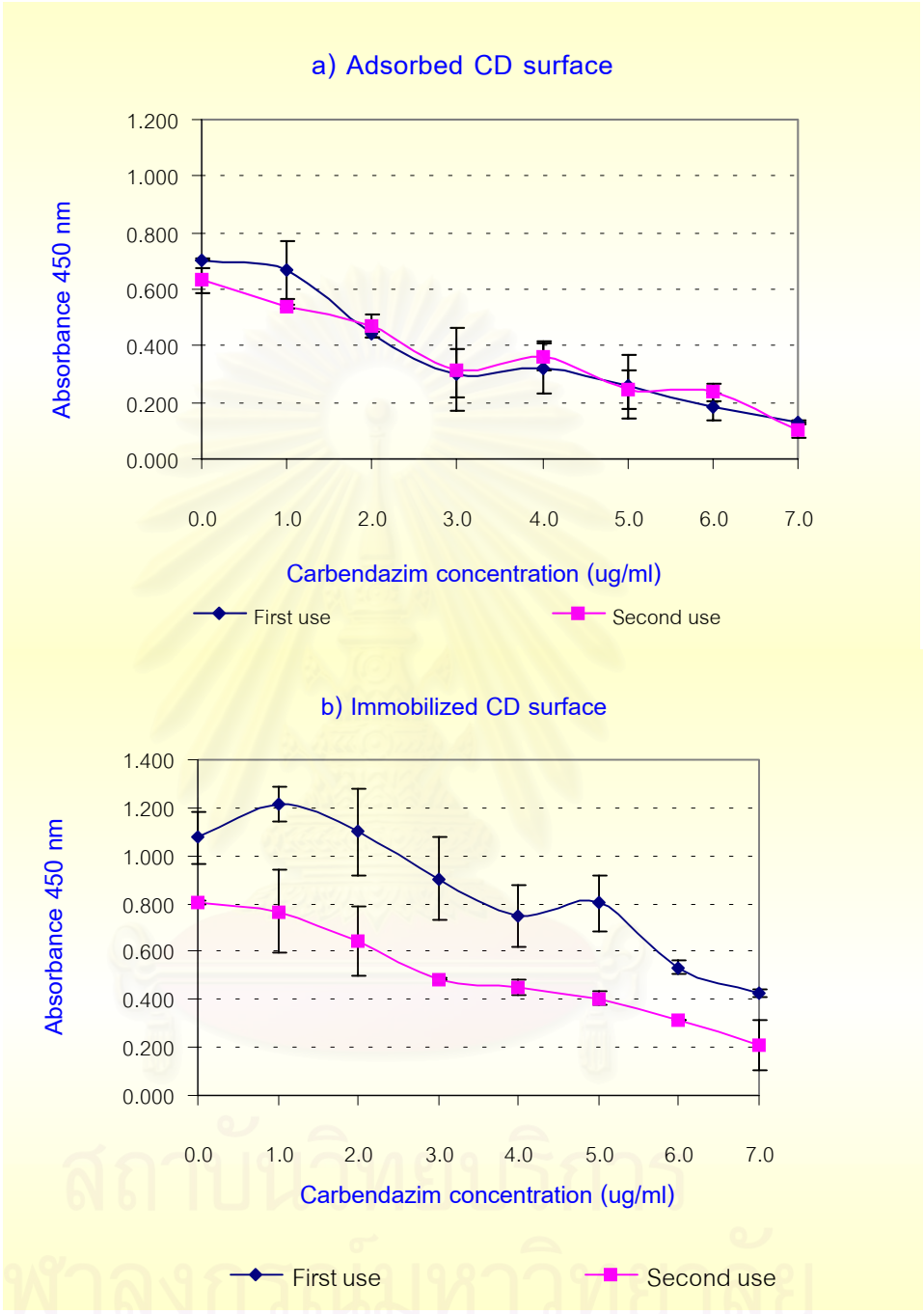


Figure 40 Reuse of amino plate coated with 5.0 % CD in which carbendazim standard in 0.1 M acetate/citric acid buffer, pH 5.0 was loaded: *2.5 µg/ml benzimidazole tracer was loaded at pH 5.0, a) Adsorbed CD surface (CD) and b) Immobilized CD surface (DVS/CD). (These values were from two separate determinations)*

CHAPTER IV

DISCUSSION

The present work involves the immobilization of cyclodextrin (CD) on microtiter plate for the determination of benzimidazole fungicides. The aims were to immobilize CD on microtiter plate and apply the use of the immobilized CD for the determination of benzimidazole molecule. The affinity of CDs and various pesticides has been reported (Szejlti, 1988) but there were no reports on the use of immobilized CD for the detection of benzimidazole fungicides.

4.1 Benzimidazole tracer synthesis

In ELISA method used for the determination of benzimidazole fungicides, 2-succiamidobenzimidazole (SAB) is always a major hapten linked to ovalbumin or bovine serum albumin (BSA) as the immunogen and conjugated to horseradish peroxidase (HRP) as benzimidazole tracer (Newsome and Shields, 1981 and Newsome and Collins, 1987). Due to the similarity of SAB and carbendazim structures, the antibody obtained from SAB-immunogen immunization also can react to carbendazim and SAB molecule of benzimidazole tracer can compete with carbendazim to bind the antibody.

In this study, the modified ELISA technique was developed for benzimidazole determination by using CD in replace of the antibody. SAB was conjugated to horseradish peroxidase (HRP) as benzimidazole tracer in order to compete with carbendazim to encapsulate into CD cavity. The amount of the encapsulated tracer was investigated by observing HRP activity.

4.1.1 Synthesis of 2- succiamidobenzimidazole

The product synthesized from anhydride method was determined as 2-succiamidobenzimidazole (SAB) because the wavelength of maximum absorption was different from those of the parent compounds. Secondly, the NMR spectra showed that it consisted of –NH, -COOH, $-CH_2$ and –CH in the aromatic ring which was the functional groups of SAB. Moreover, mass spectra showed that the molecular mass of SAB was 233 (M plus H as

234) which was indentical with the value reported by Newsome and Shield (1981) and in good agreement with the calculated value of 239 from its chemical structure.

4.1.2 Conjugation of 2- succiamidobenzimidazole and horseradish peroxidase

The conjugation of two compounds by the carbodiimide method requires the presence of an amino and a carboxyl group for the direct formation of peptide bond. In most cases the amino groups involved in the reaction are lysyl residues of protein carrier and carboxyl groups are contributed by hapten. In the field of immunology, the main use of carbodiimide has been in the conjugation of weakly immunogenic or nonimmunogenic compounds to large carrier protein or synthetic antigen, due to the fact that preparation procedure is simple and easy to perform. (Bauminger and Wilchek, 1980). According to the presence of a carboxyl group in SAB and amino groups in HRP, they could be conjugated by water-soluble carbodiimide activation. After the conjugation was performed, data from biochemical characterization of the product supported that it was SAB-HRP conjugate.

4.2 Biochemical properties of benzimidazole tracer

After the conjugation and purification of SAB-HRP conjugate, its biochemical properties including the maximum absorption, HRP molecular weight and activity were determined. All properties were compared to those of the native HRP in order to confirm that it was SAB-HRP conjugate. For maximum absorption, it was found that the maximum absorption (λmax) of both HRP occurred at 280 and 405 nm but the A_{405} / A_{280} ratio of HRP conjugate was lower than that of the native one. The higher absorption at 280 nm which reflects to lower A_{405} / A_{280} ratio of the conjugate should be explained by the overlapping peaks of HRP at 280 nm with SAB at 284 nm. The result thus confirmed that SAB was conjugated with HRP molecules.

When molecular weight were determined, SDS-PAGE gel showed slight difference in molecular weight between native (39 kD) and conjugated HRP (41 kD). The molecular weight of conjugated HRP depended on the amount SAB bound. SAB is a small molecule with molecular mass of 233. It could be conjugated with HRP by carbodiimide activation requiring amino group of amino acid side chain and carboxyl group of SAB. According to the study by Bauminger, Kohen and Lindner (1974), they reported that in most cases the amino groups involved in the

reaction are lysyl residues. Therefore, the amount of SAB molecules that could be bonded also depended on the amounts of lysine in HRP. In addition, the amino acid sequence reported by Welinder (1979) showed that HRP consisted of 308 amino acids with arginine, histidine, tyrosine and leucine as the important residues in the catalytic active site and there were 6 molecules of lysines in HRP (***Figure*** 41). Moreover, the three dimensional structure model of HRP was displayed in ***Figure*** 42. In theory the difference of molecular weight between conjugated and native HRP should be approximately 1400 kD if SAB was bound to all of 6 lysine positions. As a result, it is rather difficult to determine such difference in molecular weight by SDS-PAGE.

In most cases, after HRP was chemically modified, its activity was partially lost because of extreme condition used during conjugation or enzyme conformational changes after conjugation. Therefore, SAB-HRP conjugate activity should be examined before using as benzimidazole tracer. It was found that SAB-HRP conjugate showed 80% activity to that of the native HRP. It could be implied that the carbodiimide method was a mild and suitable method for the conjugation. In addition, the positions of lysines which SAB was linked to HRP did not affect much on HRP activity.

HRP is one of various enzymes suitable for use in enzyme-immunoassay (EIA) (Schuurs and Weemem, 1977). Colorimetric determination of HRP activity is also important in EIA. *o*-Phenylene diamine (OPD) and 2,2′-azino-di(3-ethyl-benzothiazoline-sulfonate)(ABTS) are most commonly used as chromogenic peroxidase substrates with satisfactory color sensitivity (Wolters *et al*.1976, Michal, Mollering and Siedel. 1983). However, both compounds were found to be mutagenic in Ames test (Ames *et al*.1975 and Voogd *et al.* 1980). The alternative chromogen, 3,3′,5,5′ tetramethylbenzidine (TMB), was shown to be desirably noncarcinogenic and non mutagenic (Hoolland *et al*., 1974 and Garner *et al*., 1975). Moreover, the intensity of color development for the end point determination in EIAs is clearly superior to that obtained with OPD (Bos *et al*., 1981). The oxidation of TMB by HRP in the presence of hydrogen peroxide can be presented as in ***Figure*** 43 (Cattaneo and Luong, 1994).

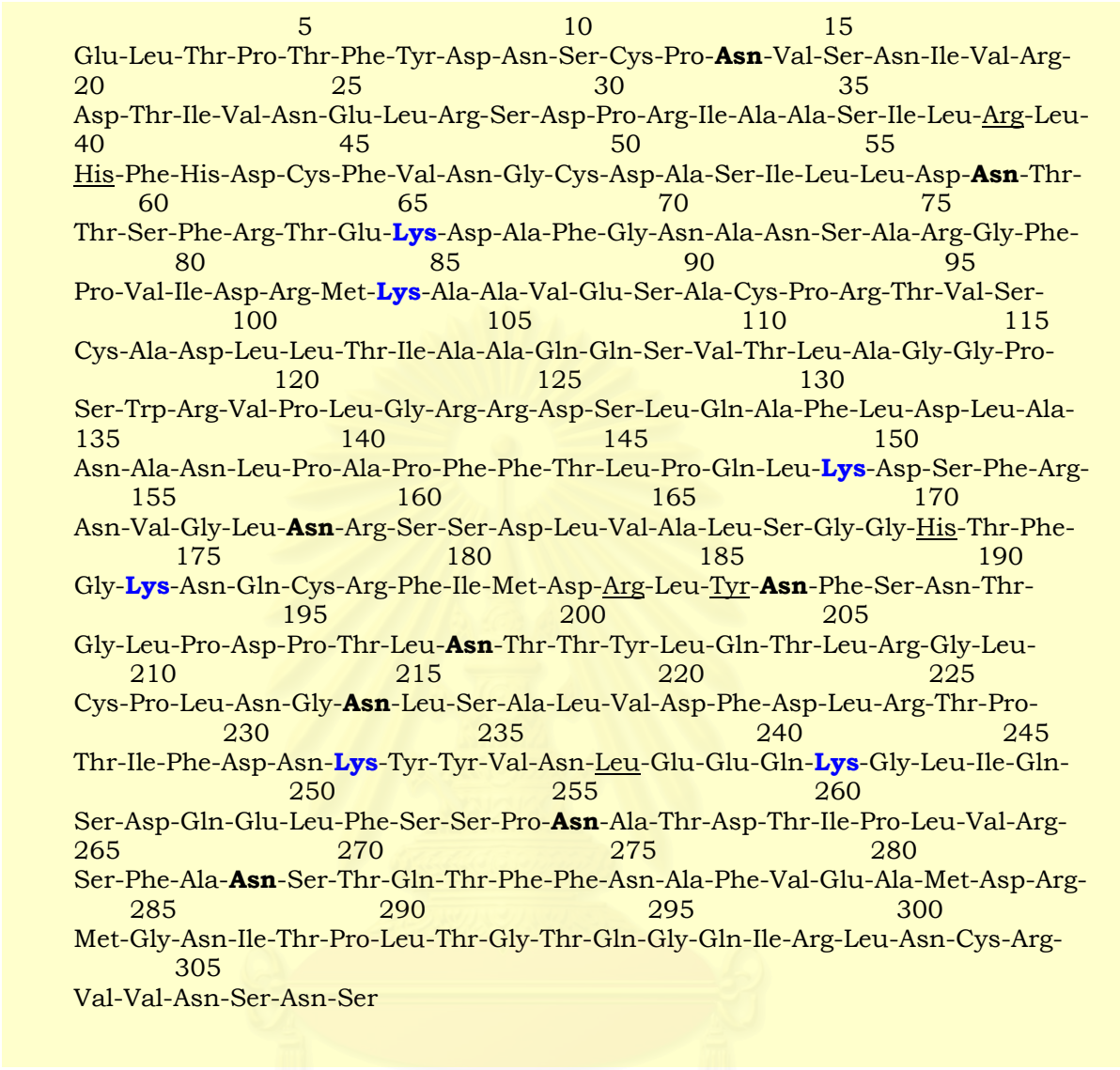


Figure 41 Amino acid sequence of horseradish peroxidase isoenzyme C. *Glycosylation sites are indicated in bold, important residues of the catalytic active site are underlined and lysine residues are blue. Standard three-letter code is used to represent amino acids (Welinder, 1979).*

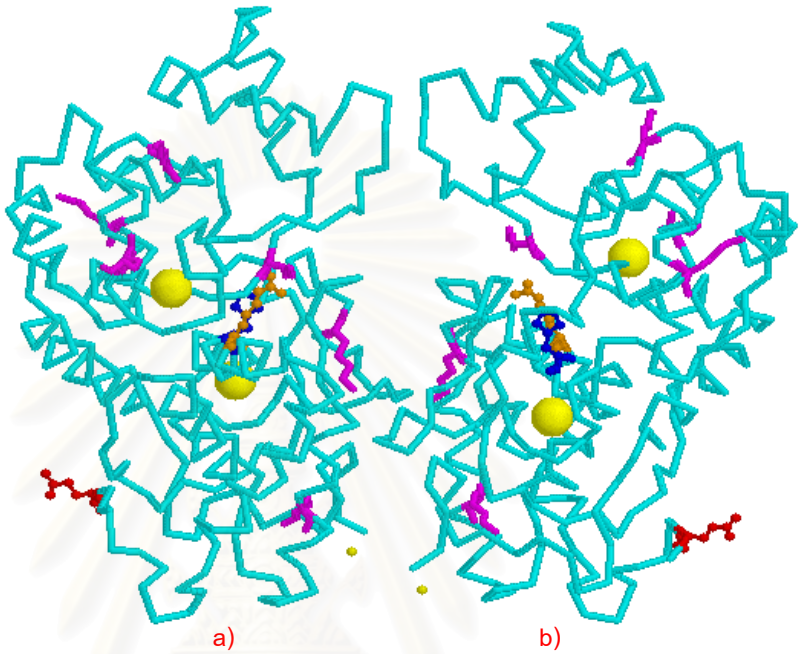


Figure 42 The molecular model of HRP *showing catalytic active site, arginine in brown and histidine in blue, lysine residues, the positions where SAB could be linked are shown in pink and HRP contains 308 amino acids with two calcium ions( ): a) front view and b) back view*

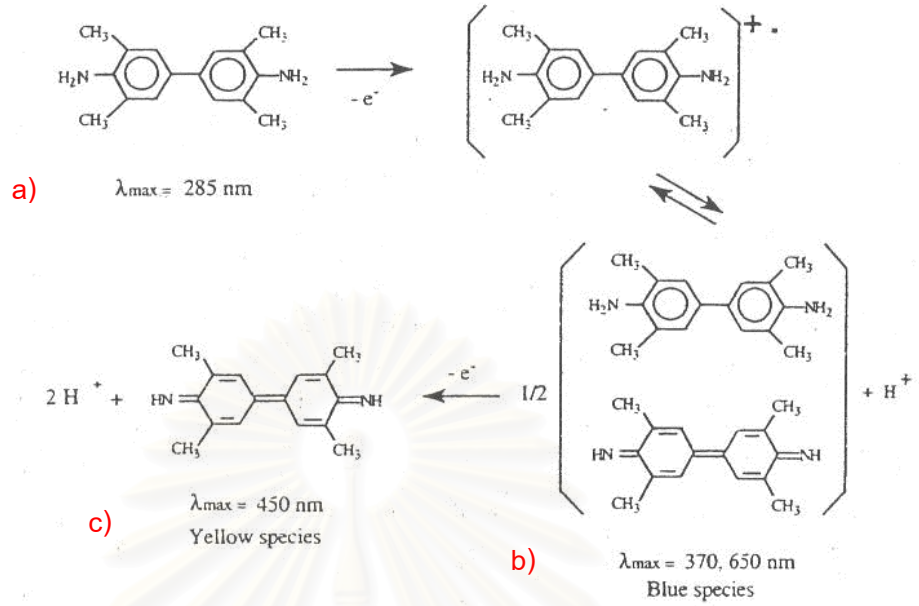


Figure 43 Schematic structure of tetrametylbenzidine(TMB): *a) normal form (TMB), b) the first oxidized product (TMB•TMB^{2+}) and c) the secondary oxidized product (TMB^{2+})*

The maximum absorption of TMB is at 284 nm with the absorption coefficient of 18,350 $M^{-1}cm^{-1}$, in acetate buffer, pH 5.0. Whenever it becomes the first oxidized product ($TMB•TMB^{2+}$), it turns to be blue species with the absorption coefficient 35,800 $M^{-1}cm^{-1}$ at 650 nm. While the secondary product (TMB^{2+}) is the yellow one with the absorption coefficient of 67,300 $M^{-1}cm^{-1}$ at 450 nm. Because of its high absorption coefficient and safeness, TMB was used as chromogenic substrate for HRP activity determination in this study.

4.3 The immobilization of CD on microtiter plate

Microtiter plate is commonly used as solid phase in immunoassays. It is almost composed of polystyrene designed as 96 well microtiter plate used in ELISA procedures. Generally, the biomolecules are simply immobilized on such hydrophobic polymer surfaces by passive adsorption (Gregorious, *et.al.* 1995). The simple adsorption is affected by the physical properties of plastic surface and this may increase the variation between ELISAs (Shekarchi *et. al*. 1984). So the method involves covalent coupling of antigen molecules to the plastic surface has been reported to be more effective for strong binding of various molecules prosessing primary amino groups.

In this study, we used CovaLink NH, a microwell plate with secondary amino groups bound to its surface as a solid-phase for the immobilization of CD. According to the structure of CD that consists of both primary and secondary hydroxyl groups on the rims of its cavity, CD can be linked to the amino plate with the use of divinylsulfone. Because divinylsulfone was shown to be used as a crosslinking agent for attachment of compounds containing amino or hydroxyl groups to hydroxylic matrices (Porath, 1974).

The procedure of CD immobilization on microtiter plate consisted of 3 steps. First, divinylsulfone would lead to the formation of vinylsulfonyl ethyl ether on the amino polymer with cross-linking as a side reaction. It would then couple to hydroxyl groups of CD. Next excess CD was removed by washing with water and unreacted vinyl groups were blocked by the addition of 1 M Tris. Although the procedure was simple, it took a long time to process. The incubation of CD immobilization took 18 hours. When 5% CD was used for the immobilization, CD became crystalline on microtiter plate surface. In order to remove excess CD, the plate was washed many times with warm water and this step took about 6 hours. The adsorbed CD remained on

the surface rather stable even though it was excessively washed for reusing purpose. However, the cross-linkage of divinylsulfone was unstable in alkaline solution (amino link was unstable above about pH 8 and hydroxyl link at about pH 9 or 10). Consequently, used microtiter plate washed with 1 mM NaOH for removing guest should be incubated with a short period of time and then rewashed rapidly with acetate/citric acid pH 5. Furthermore, the orientation of immobilized CD on the surface is random because we could not control which CD's hydroxyl groups to bond to the plate surface. Both primary and secondary hydroxyl groups could be probably bound. When a cyclodextrin molecule is bound by its secondary rims, the larger opening to the hydrophobic cavity is hindered. As a result, it is difficult for guest molecule to enter the CD cavity. For the suggestion of improvement of the CD immobilization on microtiter plate, it could be done in two ways. The first one is the use of CovaLink NH microtiter plate and carboxymethyl CD for the immobilization. The carboxymethyl CD, β-CD substituted with carboxymethyl in primary rim, could be activated by carbodiimide (WSC) to form peptide bond with amino groups on CovaLink NH plate. However, the CovaLink NH microtiter plate cost is very expensive (700 baths/plate). In order to reduce the cost, the normal plate could be used for CD immobilization by the modification of the method used for polystyrene microwells amination (Zammatleo *et al*., 1996). This procedure involves 3 steps: the oxidation of polystyrene by permanganate ($KMnO_4$) followed by the activation with carbodiimide and coupling to lysine or amino-CD can be used in this step instead of lysine for CD immobilization (***Figure*** 44). Amino-CD could be synthesized by the method of Matsumoto, Noguchi and Yoshida (1998).

4.4 Benzimidazole tracer encapsulation

After CD was immobilized onto amino polystyrene microtiter plate surface, it was used for benzimidazole tracer (HRP conjugate) encapsulation in order to test whether CD was immobilized or adsorbed on the surface and CD was still capable to encapsulate guest molecule. The result showed that HRP activities corresponding to bound benzimidazole tracer, were found in both immobilized and absorbed CD surfaces. It could be indicated that CD was bound on amino surface by both adsorption and covalent immobilization by the use of divinylsulfone crosslinker after the non-specific binding of benzimidazole tracer was reduced.

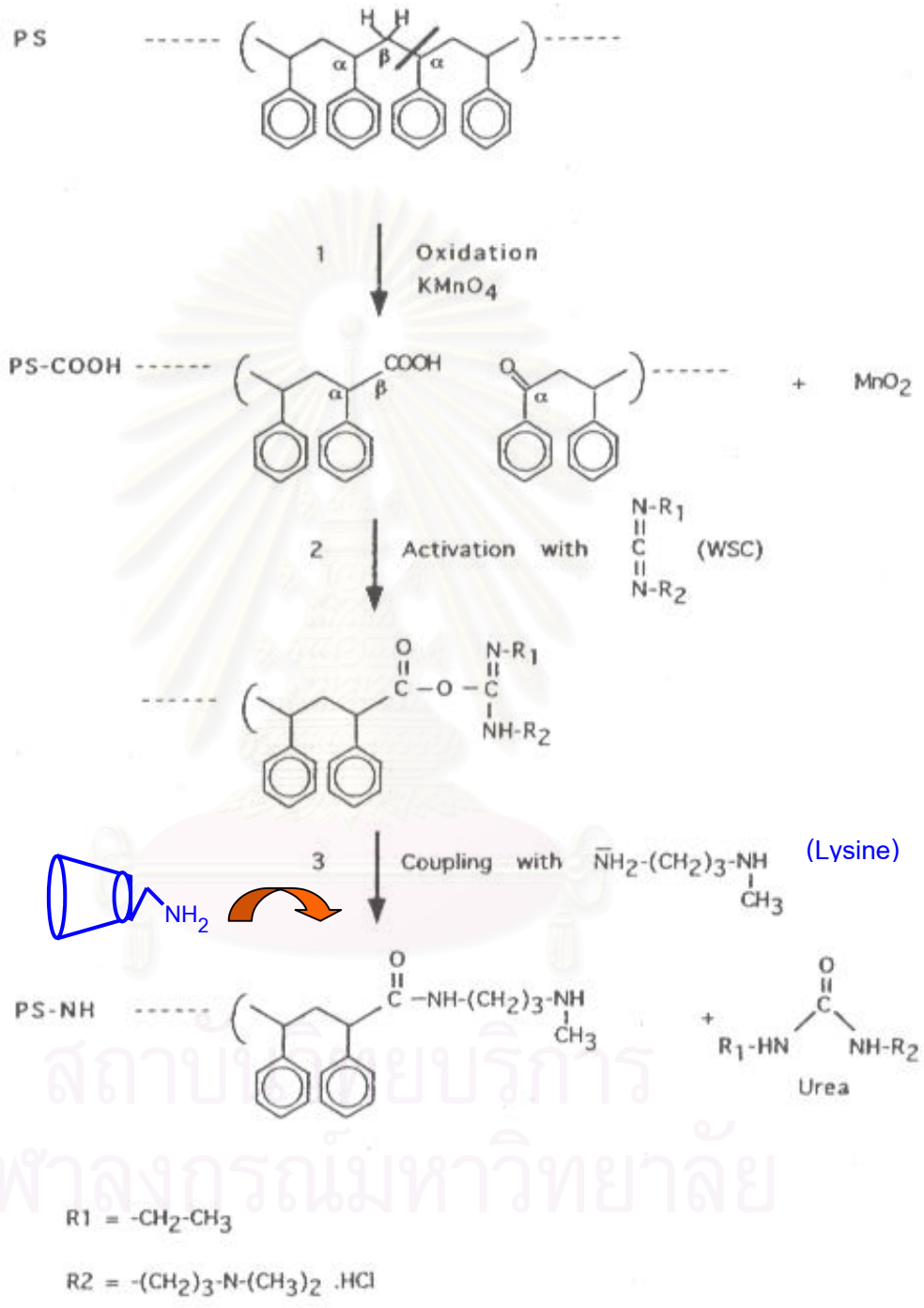


Figure 44 Schematic protocol for the amination of polystyrene surface: *amino CD presented as truncate cone could be used instead of lysine in the third step of the polystyrene amination (Zamatleo et al. 1996)*

At the beginning, in order to know whether behaviors of benzimidazole tracer and native HRP for binding on surfaces were different. They were loaded into amino (without CD), adsorbed CD, divinylsulfone treated (DVS w/o CD) and immobilized CD (DVS/CD) surfaces and the signal of HRP was observed after excess HRP was removed.

It was found that the signal of bound benzimidazole tracer was much higher than that of native HRP on amino surface washed by all types of washing buffers (Figure 22). The signal observed from amino surface was considered as non-specific binding of benzimidazole tracer which bound on the surface by passive adsorption and this was compared with the signals obtained from adsorbed and immobilized CD surfaces. Moreover, when the buffer containing salt was used, it could make the difference of benzimidazole tracer signal among adsorbed CD, immobilized CD and divinylsulfone treated surfaces. On the contrary, there were no significant differences of native HRP among those surfaces (Figure 21). From the result, it could be indicated that there were some different properties between benzimidazole tracer and native HRP molecules and also indicated that benzimidazole tracer could be encapsulated into CD, while native HRP could not. Therefore, the next experiments were designed to find the condition showing the signal differences between encapsulated and adsorbed benzimidazole tracer and investigate the factors affected the encapsulation of tracer.

4.4.1 The effect of washing buffer types on benzimidazole tracer encapsulation

The divinylsulfone treated (DVS) surface was assigned for the investigation of non-specific binding of benzimidazole tracer in replace of amino surface. The result in *Figure* 22 shows that it could be well eliminated by the use of buffer containing 0.3% Tween. According to the property of Tween that it may disrupt the hydrophobic interaction, the adsorption of benzimidazole tracer on the DVS treated surface may be hydrophobic. Unfortunately, it also interrupted the specific binding of guest encapsulated into CD cavity. This may be due to the fact that SAB was encapsulated into CD by its size and hydrophobic interaction. So when buffer-containing Tween was used, the tracer signals were the lowest in all surfaces. Furthermore, CD and DVS/CD surfaces were assigned for the investigation of the amount of CD that was adsorbed or covalently immobilized on microtiter plate surfaces, respectively. There were two buffers showed the signal difference between CD and DVS/CD surfaces with low adsorption of

tracer on the DVS surface. One was PBS containing 0.15 M NaCl, pH 7.0 and the other was acetate/citric acid buffer containing 0.5 M NaCl, pH 5.0. This could be explained by the assumption that the salt solution might disrupt the electrostatic interaction of tracer adsorbed on the surface whereas the specific binding of benzimidazole tracer still remained. Noticeably, the tracer signal of surfaces washed by acetic acid buffer containing 0.5 M NaCl, pH 5.0 were higher than that of surfaces washed by PBS containing 0.15 M NaCl, pH 7.0. It may be because the inclusion complex was stable in acidic solution. So in neutral solution SAB-HRP may be released from CD cavity. This result showed consistency with another result in that there was no signal difference between CD and DVS/CD surfaces when washed with higher salt solution at neutral pH (PBS containing 0.5 M NaCl, pH 7.0. It may because the high salt solution may interrupt the weak formation of inclusion complex.

Furthermore, there were some reports suggested that hydroxy acid, especially citric and tartaric acids were found to increase the solubility of beta-CD (Fenyvesi *et al*. 1999 and German *et al*. 1995). It was also suggested that the effect relies on the capability of the hydroxy acids to modify the intramolecular hydrogen bond system involving the secondary hydroxyl groups of CDs and/or affect their interaction with the surrounding water molecules. Moreover, the hydroxy acid also found to increase the formation of inclusion complex by forming CD multicomponent complexes. For example, beta-CD multicomponent complexes of *cis*-ketoconazole (KC), an imidazole antifungal agent, with tartalic or citric acid, 2,200- and 80- fold KC solubility enhancements were achieved in comparison to the drug itself and to the binary complex, respectively (Chiesi *et al*. U.S. Patent 5,855,916). Fenyvesi *et al*. (1999) suggested that it was because the drugs that elicited the most outstanding solubility improvement were strongly bound to the CD cavity even though they were in ionized from. Their basic center and hence the charge were indeed located quite far away from the part of molecule included. So the complexing ability was not significantly compromised. The hydroxy acid was kept in the proximity of external rim of CD by a concerted mechanism. It involves the binding of the hydrophobic part of the drug and simultaneous formation of a strong ion pair (Redenti,Szente and Szejtli, 2000).

4.4.2 The effect of β-CD concentration on benzimidazole tracer encapsulation

In this experiment, normal polystyrene plate was also used for comparative observation with amino plate coated with and without the use of divinylsulfone as a crosslinking reagent. From the result in ***Figure*** 23, the tracer signal found in the adsorbed CD and divinylsulfone treated surface of both amino and normal plates were almost the same (see plate coated with 5% CD). It indicated that β-CD could adsorb on both amino and normal plate or the tracer could non-specically bind to both surfaces, equally. The signal of DVS/CD surface of normal plate was higher than that of the adsorbed CD surface. It may because some divinylsulfone molecules could be bound on normal surface although it did not contain any amino group. Therefore, the extra signal was obtained from the reaction of adsorbed divinylsulfone linked with CD. Among groups of amino plate coated with various CD concentrations. The signal difference between adsorbed and immobilized amino plate coated with 2.5% CD was slightly higher than that of amino plate coated with 1% CD. While the amino plate coated with 5% CD showed the highest signal difference between adsorbed and immobilized CD surface with the lowest adsorption of benzimidazole tracer occurred in DVS surface. The results indicated that the high CD concentration used could increase the amount of immobilized CD. However, the signal of 5% CD coated plate was lower than those of 1% and 2.5%. When 5% CD solution was incubated in well for the immobilization, CD became crystalline. The condition did not only concentrate CD on the surface to enhance the probability of linkage formation but also decreased nonspecifically bound tracer by passive adsorption on the surface because the surface was fully covered with CD. However, the optimal CD concentration should be further reexamined in the range of 2.5 – 5.0%.

4.4.3 The effect of benzimidazole tracer concentration on its encapsulation

The results in ***Figure*** 24 indicated that the tracer encapsulation was higher when the tracer concentration increased and the signal difference between adsorbed and immobilized CD surface occurred in amino plate coated with CD while it was not different in the normal one coated with CD. Moreover, for 2.5 μg/ml benzimidazole tracer for the encapsulation, it showed significant difference between adsorbed and immobilized CD of all surfaces coated with various CD concentrations. Especially, the amino plate surface coated with 5% CD, the signal of

DVS/CD surface was about three times higher than to that of CD surface. The signal from CD surface could indicate the total adsorption of CD and that of DVS/CD surface signal could indicate both adsorbed and covalently immobilized CD on microtiter plate surface.

4.4.4 The effect of CD types on benzimidazole tracer encapsulation

Many different chemical moieties can be introduced into CD molecule by the reaction with the hydroxyl group lining the primary and secondary rims of CD; for example hydroxypropyl, methyl, carboxymethyl, and acetyl. The properties of the chemical groups are important to certain physio-chemical properties of the chemically modified CDs (CMCDs) and also have beneficial effect on aqueous solubility and toxicity. So types of β-CD including β-CD, hydroxypropyl-β-CD and methyl-β-CD were investigated for benzimidazole tracer encapsulation. From the result in *Figure* 25 and amino plate coated with β-CD showed the highest signal of both adsorbed and immobilized CD surfaces which could be suggested that β-CD was suitably immobilized by the method using divinylsulfone. This may be because the amount of hydroxyl group in methyl β-CD was lower than that of β-CD. So the probability for reacting with vinyl group of divinylsulfone was lower. However, it was found that hydroxypropyl-β-CD which contained a lot of hydroxyl group surrounding its molecules showed the signal of immobilized CD as low as that of adsorbed surface. Due to the wider inner diameter of hydroxypropyl-β-CD cavity (7.8 A°) is wider than that of β-CD (6.2 A°) (Luong *et al*., 1995). The signal occurred on immobilized and adsorbed surface not only depended on the amount of CD bound on the surface but also depended on the appropriate cavity size for benzimidazole tracer encapsulation.

After the effect of many factors were examined, it was found that the optimal condition for benzimidazole encapsulation was to use amino plate coated with 5% β-CD, 2.5 μg/ml benzimidazole tracer concentration and 0.1 M acetate/citric acid, containing 0.5 M NaCl as washing buffer.

4.5 Capability test of immobilized CD and benzimidazole tracer for detecting carbendazim standard.

The procedure developed for competitive benzimidazole tracer encapsulation had been carefully considered. The pH for carbendazim encapsulation was first determined.

Although in previous experiments, loading buffer of pH 5.0 was normally used, loading buffer of pH 2.5 was also tested in the experiment. Since lower pH tends to increase complex formation between CD and SAB (Ruedekan Saikosin, personal communication). It is consistent with the reports suggested that an ionizable guest, pH plays an important role in complexation (Qi and Rombeger, 1998). Due to the fact that carbendazim containing a single tertiary amine with pKa of 4.0 hence, at loading buffer pH 2.5, the carbendazim may be completely protonated (Gilvgdis and Waters, 1990). The temperature used for carbendazim encapsulated was also considered. Although high temperature increases molecular motion and it generally favors the dissociation of a cyclodextrin complex. There are some studies reported that the stability constant of inclusion complex decreased as temperature was raised from 15°C to 50°C (Gelb *et al*., 1979 and Ishiwata and Kamiya, 1998). So in this study, the temperature for carbendazim encapsulation was maintained at 25 °C because of the above reason and this temperature was also convenient to operate. In addition, The optimal condition for benzimidazole tracer encapsulation and the reduction of non-specifically bound tracer were also considered and examined in preliminary experiments of this study.

According to the previous results, it could be concluded that the method used for CD immobilization on microtiter plate was succeeded. The β-CD was well immobilized on the surface and it was still able to encapsulate guest molecules. In order to apply the use of immobilized CD for the detection of some substances by the competitive encapsulation with benzimidazole tracer, the carbendazim standard was used in this experiment. The approach of the competitive encapsulation was as follows. First, carbendazim standard was loaded into immobilized CD well. After the incubation for 1 hour at room temperature (25°C), unbound molecules were removed by washing with 0.1 M acetate/citric acid buffer, pH 5.0. Next, the benzimidazole tracer was added into the same well and incubated for 1 hour. Then, non-specifically bound tracer was removed by the use of 0.1 M acetate/citric acid buffer containing 0.5 M NaCl as washing buffer and specifically bound tracer activity was determined by the addition of substrate solution containing 0.1 mg/ml TMB and urea hydrogen. Finally, the absorbance at 450 nm was then read after the reaction was stopped by the addition of 2 M sulfuric acid.

Due to the result of this experiment, which investigated the effect of pH in loading buffer on tracer encapsulation, carbendazim was not simutaneously loaded for directly competition with benzimidazole tracer. They were separately loaded because when loading buffer pH 2.5 was used, HRP enzyme of benzimidazole tracer would absolutely lose its activity. Moreover, to enhance carbendazim encapsulated into CD, it was loaded first before loading benzimidazole tracer. As the result in *Figure* 27, it could be concluded that carbendazim was well encapsulated into CD at pH 2.5 in acetate/citric acid buffer and the approach of competitive encapsulation as described was probably applied to detect carbendazim in fruit in the next experiment.

4.6 Capability test of immobilized CD and benzimidazole tracer for carbendazim determination in fruits

Although CDs are capable of encapsulating various kinds of guest with appropriate size and hydrophobicity, the selectivity may be enhanced by the control of the factors influencing complexation (Qi and Romberger, 1998). In previous experiment, we found that carbendazim standard could be detected by competitive benzimidazole tracer encapsulation under specific condition. Therefore, this experiment was designed in order to use the competitive encapsulation system for the determination of carbendazim in fruits. However, sample matrix should be first considered because it may interfere the competitive encapsulation system as false positive. So, it was necessary to use fruit samples, which contained no carbendazim residues as negative control sample in order to determine sample matrix effect. After the sample matrix effect was reduced, the determination of carbendazim in sample would then be performed. The conventional method for the determination of carbendazim residues in food such as HPLC method was necessary to investigate the samples first.

Apple and grape were samples to be used for carbendazim determination. The fruits were selected for study on the basis of information indicating highest usage of benzimidazole fungicide and MLR of carbendazim was established as showen in *Table* 3. Although HPLC method showed that carbendazim residues were not detected in both apple and grape, but there were some interferants in acidic partitioned extract detected near carbendazim peak (*Figure28)*. This result indicated that there were some substances in both apple and grape which were similar to carbendazim. Since carbendazim was determined by reverse-phase LC

system, the interferants should be similar to carbendazim by their polarity and size. The percent recovery of carbendazim in both acidic partitioned sample extracts was very low. On the contrary, there were no interferants detected near carbendazim peak when UV and Fluorescence detectors were used in both basidic partitioned sample extracts.

The percent of recovery by HPLC method was studied in order to control the quality of analytical data obtained from the method. From the results, the recovery of carbendazim in basidic partitioned sample extract was much higher than that in acidic partition. The result was consistent with Gilvydis and Waters (1990) who reported that the recovery from acid partition ranged from about 5 to 25 % while basidic partitioned extract was 60 – 80% (among sample types).

In order to determine the effect of sample matrix on the determination of carbendazim in sample by competitive benzimidazole tracer encapsulation, the sample matrix of grape was used. It was obtained from grape extracted with methanol and partitioned with dichloromethane and then it was forced to dissolve in loading buffer pH 2.5 and 5.0. After it was determined by competitive benzimidazole tracer encapsulation, it showed very clear inhibition curve of benzimidazole tracer in both pH 2.5 and 5.0 (***Figure*** 34). From the result, it was implied that there were some components in grape that could encapsulate into CD cavity and inhibit the encapsulation of benzimidazole tracer like what carbendazim behaved. Moreover, it also indicated that the approach of competitive benzimidazole tracer encapsulation system could be used for the determination of some substances that could encapsulate in CD cavity. However, further study for reducing the interferants in sample matrix was necessary to improve the performance of the competitive encapsulation system.

Food matrix effect is commonly encountered in immunoassay method (Bushway *et al*., 1992). The clean up step is needed not only for the reduction of sample matrix effect but also for concentrating of the specific residue in sample for the determination. Many reports suggested that samples such as fruits and vegetables usually needed the dichloromethane partition for the clean up step to be further determined by both chromatographic (Gilvydis and Waters, 1990 and Hiemstra *et al*., 1995) and ELISA methods (Bushway *et al*., 1992). While other samples such as wine and fruit juices were diluted into 1:10 ratio before determination in order to reduce sample

matrix effect (Bushway *et al*., 1993 and Bushway *et al*., 1995). This was consistent with the result obtained in that the diluted sample showed the reduction in matrix effect. In our experiment, the lowest matrix effect occurred at 0.1 g/ml of grape sample extract.

In the final step of determination of carbendazim in fruits by the competitive benzimidazole tracer encapsulation, spiked sample solution was used. When spiked sample solution was loaded at pH 2.5, it showed that the signal was sharply decreased closely to zero point when carbendazim concentration was in the range of 0.3 –1.0 μg/ml as shown in *Figure* 35. It indicated that carbendazim residue above 0.3 μg/ml could be completely encapsulated in all of CD cavities on microtiter plate surface. Or it could be said that this was the minimum concentration of carbendazim, which completely inhibited the encapsulation of 2.5 μg/ml benzimidazole tracer. Since the sample loaded was 0.1 g/ml spiked with carbendazim 0.3 μg/ml, carbendazim residue was then calculated to be 3 ppm in grape. That was the minimum carbendazim concentration, which could be detected by the complete inhibition of benzimidazole tracer encapsulation. According to the MRL of carbendazim (1999) in berries and other small fruits is 5 ppm as shown in *Table* 3, the approach of competitive benzimidazole tracer encapsulation could be effectively used for the detection of carbendazim at approximately 3 ppm. In further study, the sensitivity of carbendazim determination in grape should be determined by loading carbendazim concentration in the range of 0 – 0.1 μg/ml in 0.1 g/ml grapes extract. The limit of detection is defined as carbendazim concentration in sample extract inhibiting 20 % of benzimidazole tracer encapsulation (Bushway *et al*. 1992).

4.7 Stability test of immobilized CD on microtiter plate for reusing purpose

CDs are considered as stable molecules. It was reported that they were stable toward alkaline, although temperatures were elevated. The thermal stability of CDs was far greater than that of common starch. The melting temperature of CD crystals occurred at around 300 °C. Moreover, CDs containing glucose 6 and 7 units were resistance toward the common amylases but it was degraded by cyclodextrinase. However, strong acid such as hydrochloric acid would hydrolyze the cyclodextrin to yield a mixture of oligosaccharides. The rate-limiting step is the ring opened reaction and the rate of acid hydrolysis increases as functions of both increased temperature and acid concentration (Qi and Kombeger, 1998).

Extreme condition for reusing purpose should be avoided because it may affect the stability of CD and the linkage between amino and hydroxyl groups by divinylsulfone crosslinker. After tracer activity determination, the reaction was stopped by addition of 2 M sulfuric acid. The absorbance at 450 nm was then read, the solution in microtiter plate should be rapidly discarded and washed three times with 300 μl of distilled water in order to prevent CD from the acid hydrolysis. Although CD is stable in alkaline solution, the linkage of amino and hydroxyl groups with divinylsulfone is unstable above pH 8. In the next step, the plate was washed with 1 mM NaOH to remove guest. It should be incubated in short period of time then rapidly washed with water and followed with 0.1 M acetate/citric acid buffer pH 5. After that it was air dried at room temperature, and kept in desiccator. The microtiter plate should be washed with acidic buffer kept in desiccator in order to prevent CD from the degradation by microorganisms with CD hydrolytic enzymes.

4.8 Application potential of the competitive benzimidazole tracer encapsulation into immobilized CD on microtiter plate in comparison with ELISA.

According to the competitive benzimidazole tracer encapsulation approach was modified from the competitive ELISA method, the results could indicate that the approach also be used for the detection of carbendazim residues and other related compounds. In addition, the immobilized CD microtitier plate tended to be reuse while the ELISA plate could not. Although the sensitivity was lower than ELISA methods for benzimidazole residues determination as shown in Table 5, it could be available for the screening purpose and could be developed for the quantitative determination.

CHAPTER V

CONCLUSION

1. Benzimidazole tracer can be constructed by the conjugation of horseradish peroxidase (HRP) and 2-succiamidobenzimidazole (SAB) synthesized by anhydride method from 2-aminobenzimidazole and succinic anhydride. Its activity was 80% of the native HRP.

2. Divinylsulfone is an effective cross-linker for β-CD immobilization on amino polystytrene microtiter plate surface.

3. The immobilized β–CD and its capability of guest encapsulation could be detected by benzimidazole tracer encapsulation.

4. β–CD immobilized on microtiter plate by the use of divinylsulfone cross-linker could be reused by the washing protocol that used 1mM sodium hydroxide solution for removing guest out of CD cavity.

5. The optimal condition for benzimidazole tracer encapsulation into CD was the use of 2.5 μg/ml of benzimidazole tracer for the encapsulation into amino plate coated with 5% CD for 1 hour, at 25 °C and 0.1 M acetate/citric acid buffer containing 0.5 M NaCl, pH 5 as washing buffer.

6. The immobilized β-CD on microtiter plate and benzimidazole tracer could be used for the detection of carbendazim by competitive benzimidazole tracer encapsulation approach.

7. The competitive benzimidazole tracer encapsulation could detect approximately 3 ppm carbendazim residues in grape as the minimum concentration completely inhibiting the benzimidazole tracer encapsulation into immobilized CD on microwell.

8. The competitive encapsulation system could be developed for the quantitative determination of carbendazim or other compounds under specific condition in further study.

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APPENDICES

APPENDIX 1 : Preparation for polyacrylamide gel electrophoresis

Stock reagents:

30% Acrylamide, 0.8% bis-acrylamide, 100 ml

Acrylamide	29.2 g
N, N′ –methylene-bis-acrylamide	0.8 g

Adjusted volume to 100 ml with distilled water

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethame	6.06 g

Adjusted pH to 6.8 with 1M HCl and adjusted volume to 100 ml with distilled water

Solution B (SDS PAGE)

2 M Tris-HCl pH 8.8	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (SDS PAGE)

1 M Tris-HCl pH 6.8	50 ml
10% SDS	4 ml
Distilled water	46 ml

Coomassie Gel Stain, 1 litre

Coomassie Blue R-250	1.0 g
Methanol	450 ml
Distilled water	450 ml
Glacial acetic acid	100 ml

Coomassie Gel Destain, 1 litre

Methanol	100 ml
Glacial acetic acid	100 ml
Distilled water	800 ml

SDS-PAGE

12.5% separating gel

30% acrylamide solution	4.2 ml
Solution B	2.5 ml
Distilled water	3.3 ml
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	10 µl

5.0% stacking gel

30% acrylamide solution	0.67 ml
Solution C	1.0 ml
Distilled water	2.3 ml
10% $(NH_4)_2S_2O_8$	30 µl
TEMED	5 µl

Sample buffer

1 M Tris-HCl pH 6.8	0.6 ml
50% glycerol	5.0 ml
10% SDS	2.0 ml
2-mercaptoethanol	0.5 ml
1% bromophenol blue	1.0 ml
Distilled water	0.9 ml

One part of sample buffer was added to four parts of sample. The mixture was heated 5 minutes in boiling water before loading to the gel.

Electrophoresis buffer, 1 litre

Tris (hydroxymethyl)-aminomethane	3.0 g
Glycine	14.4 g
SDS	1.0 g

Adjusted volume to 1 litre with distilled water

(pH should be approximately 8.3).

APPENDIX 2: Preparation of buffer and reagents for CD immobilization

0.5 M sodium carbonate buffer, pH 11

Na_2CO_3	13.25	g
Distilled water	250	ml
Adjust pH to 11 by the addition of 1 M NaOH		

5% Divinylsulfone in carbonate buffer, pH 11

Divinylsulfone	5	ml
Add 0.5 M sodium carbonate buffer, pH 11 to make	100	ml

5% Cyclodextrin solution in carbonate buffer, pH 11

cyclodextrins	5	g
Add 0.5 M sodium carbonate buffer, pH 11 to make	100	ml

1 M Tris solution

Tris (hydroxymethyl)-aminomethane	30.28	g
Add distilled water to make total volume	250	ml

0.1 M Sodium acetate/citric acid buffer pH 5 (4x)

Citric acid	210.14	g
Sodium acetate	82.00	g
Add distilled water to make total volume	500	ml

APPENDIX 3 : Preparation of buffers for benzimidazole encapsulation

Loading buffer:

0.1 M Sodium acetate/citric acid buffer pH 5 (4x)

Citric acid	210.14 g
Sodium acetate	82.0 g
Add distilled water to make total volume	500 ml

0.1 M Sodium acetate/citric acid buffer pH 3 (2x)

Citric acid	10.33 g
Sodium acetate	0.068 g
Add distilled water to make total volume	250 ml

Washing buffer:

5 mM PBS pH 7, 0.15 M NaCl

NaCl	2.192 g
Na_2HPO_4	0.087 g
NaH_2PO_4	0.088 g
Add distilled water to make total volume	250 ml

5 mM PBS pH 7, 0.5 M NaCl

Nacl	2.922 g
Na_2HPO_4	0.035 g
NaH_2PO_4	0.035 g
Add distilled water to make total volume	100 ml

5 mM PBS pH 7, 0.15 M NaCl and 0.3% Tween

NaCl	0.877 g
Na_2HPO_4	0.035 g
NaH_2PO_4	0.035 g
Tween 20	0.3 ml
Add distilled water to make total volume	100 ml

0.1 M Sodium acetate/citric acid buffer pH 5 (4x)

Citric acid	210.14 g
Sodium acetate	82.0 g
Add distilled water to make total volume	500 ml

0.1 M Sodium acetate/citric acid buffer , 0.5 M NaCl, pH 5

0.1 M Sodium acetate/citric acid buffer pH 5 (4x)	50.0 ml
NaCl	5.88 g
Add distilled water to make total volume	200 ml

0.1 M Sodium acetate/citric acid buffer , 0.3% Tween, pH 5

0.1 M Sodium acetate/citric acid buffer pH 5 (4x)	50.0 ml
Tween 20	0.6 ml
Add distilled water to make total volume	200 ml

Substrate solution

3, 3;, 5, 5; Tetramethylbenzidine (TMB)	1.0 mg
Dimethylsulfoxide (DMSO)	100 µl
Urea hydrogen	1.0 mg
0.1 M Sodium acetate/citric acid buffer pH 5	10 ml

Note: 1.0 mg was first dissolved in DMSO, then diluted with 0.1 M Sodium acetate/citric acid buffer pH 5. It has to mix gently and thoroughly and wait for 10 minutes before adding to urea hydrogen. Moreover, The substrate solution must be fresh preparation before using.

Stop solution:

2 M Sulfuric acid

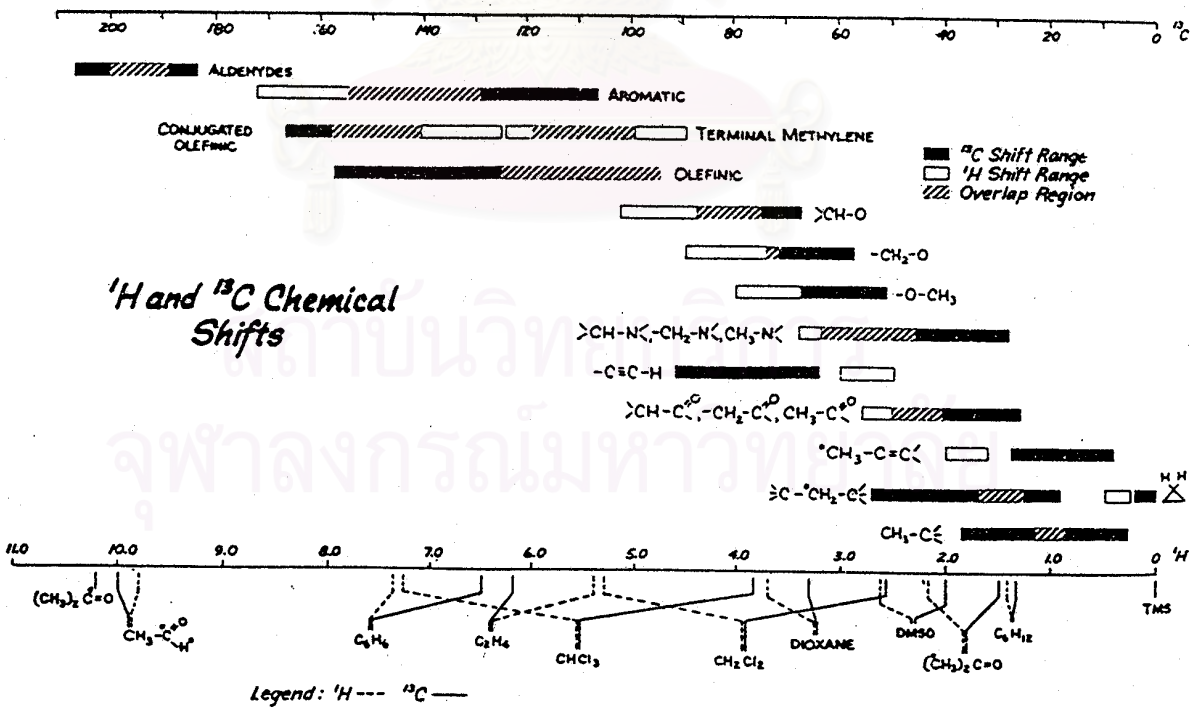
Distilled water	22.3 ml
Conc. Sulfuric acid	2.7 ml
Total volume	25.0 ml

(Note: Add sulfuric acid into distilled water)

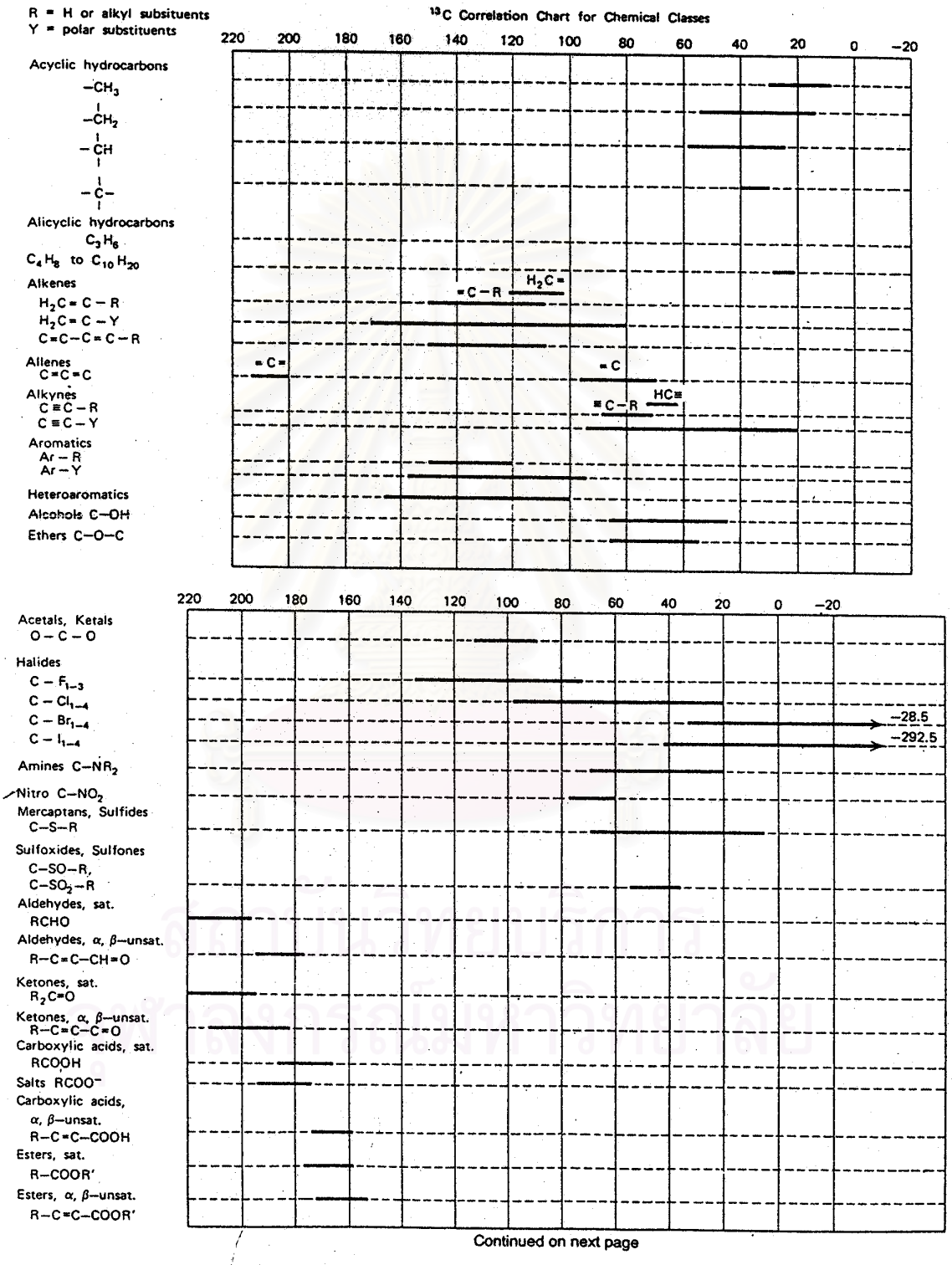
APPENDIX 4 : ^{13}C chemical shift for common deuterated solvents

	Shift	Multi-plicity
Acetic Acid CD_3COOD	178.4, 20.0	1, 7
Acetone $(CD_3)_2CO$	206.0, 29.8	1, 7
Acetonitrile CD_3CN	117.7, 1.3	1, 7
Benzene C_6D_6	128.5	1
(Carbon tetrachloride CCl_4)	96.0	1
Chloroform $CDCl_3$	77.0	3
Dimethyl sulfoxide $(CD_3)_2SO$	39.5	7
Dioxane $C_4D_8O_2$	67.4	1
Methanol CD_3OD	49.0	7
Methylene chloride CD_2Cl_2	53.8	5
Nitromethane CD_3NO_2	57.3	7
Pyridine C_5D_5N	149.9, 135.5, 123.5	3, 3, 3
(Carbon disulfide CS_2)	192.8	1

APPENDIX 5 : Comparision of ^{1}H and ^{13}C chemical shift for common deuterated solvents



APPENDIX 6 : ^{13}C correlation chart for chemical classes



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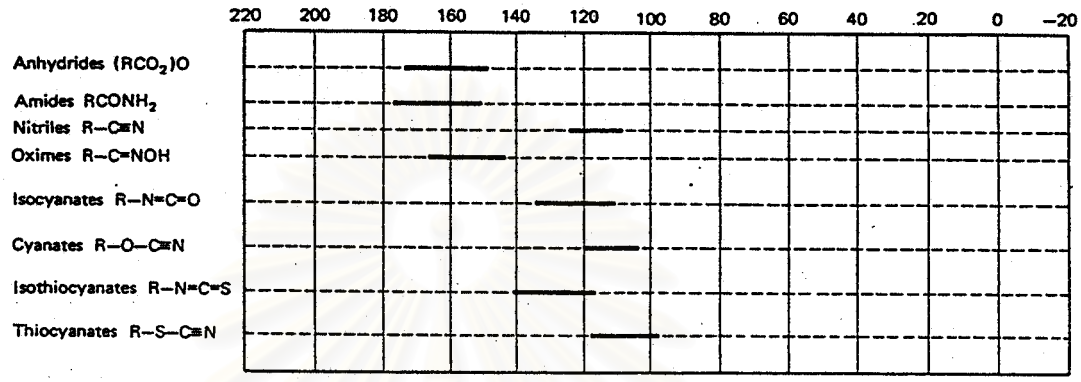
APPENDIX 6 (continued)



BIOGRAPHY

Mrs. Pacharawan Jongmevasana was born on December 10, 1967, at Tak Province. In 1989, she graduated with the Bachelor degree of Science (biology) from Chiang Mai University. After that she had two-year experience in fruit canning manufactory. Since1991, she has been a medical scientist at division of food, department of medical science. She continued to study the master degree in Biotechnology Program at Chulalongkorn University in 1999.