การพัฒนาวิธีตรวจวัดคาร์เบนดาซิมและคาร์บาริลโดยใช้ไซโคลเดกซ์ทรินที่ตรึงบนไมโครไทเทอร์เพลต

นางสาว วิรามศรี ศรีพจนารถ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2546

ISBN 974-17-5165-6

DEVELOPMENT OF CARBENDAZIM AND CARBARYL DETECTION METHOD USING IMMOBILIZED CYCLODEXTRIN ON MICROTITER PLATES

Miss Wiramsri Sriphochanart

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biochemistry

Department of Biochemistry
Faculty of Science
Chulalongkorn University

Academic Year 2003
ISBN 974-17-5165-6

Thesis Title	Development of carbendazim and carbaryl detection method using immobilized cyclodextrin on microtiter plates
By	Miss Wiramsri Sriphochanart
Field of Study	Biochemistry
Thesis Advisor	Manchumas Prousoontorn, Ph.D.
Thesis Co-advisor	Associate Professor Piamsook Pongsawasdi, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Faculty of Science
(Professor Piamsak Menasveta, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Tipaporn Limpaseni, Ph.D.)

……..…… Thesis Advisor
(Manchumas Prousoontorn, Ph.D.)

…….. Thesis Co-advisor
(Associate Professor Piamsook Pongsawasdi, Ph.D.)

…….. Member
(Assistant Professor Suganya Soontaros, Ph.D.)

…….. Member
(Assistant Professor Worawan Bhanthumnavin, Ph.D.)

วิรามศรี ศรีพจนารถ : การพัฒนาวิธีตรวจวัดคาร์เบนดาซิมและคาร์บาริลโดยใช้ไซโคลเดกซ์ทรินที่ตรึงบนไมโครไทเทอร์เพลต. (DEVELOPMENT OF CARBENDAZIM AND CARBARYL DETECTION METHOD USING IMMOBILIZED CYCLODEXTRIN ON MICROTITER PLATES) อ.ที่ปรึกษา : อ. ดร. มัญชุมาส เพราะสุนทร, อ.ที่ปรึกษาร่วม: รศ. ดร. เปี่ยมสุข พงษ์สวัสดิ์; 131 หน้า. ISBN 974-17-5165-6.

งานวิจัยนี้ได้พัฒนาการตรวจวิเคราะห์สารฆ่าราเบนซิมิดาโซลและสารฆ่าแมลงคาร์บาริล โดยวิธีการตรึงบีตาไซโคลเดกซ์ทรินบนโพลิสไตรีนไมโครไทเทอร์เพลท และอาศัยหลักการยับยั้งแบบแข่งขันของวิธี ELISA การทดลองเริ่มจากเคลือบหลุมของไมโครไทเทอร์เพลทด้วย methyl vinyl ether-maleic anhydride copolymer (MMAC) แล้วทำให้เกิดหมู่อะมิโนบนเพลทโดยการเติม 1,6-hexamethylenediamine (HMDA) หลังจากนั้นตรึงบีตาไซโคลเดกซ์ทรินบนผิวอะมิโนเพลท โดยใช้ divinyl sulfone เป็นตัวเชื่อม จากการทดลองพบว่า บีตาไซโคลเดกซ์-ทรินถูกตรึงบนผิวอะมิโนของไมโครไทเทอร์เพลทได้สำเร็จ และยังสามารถจับกับตัวติดตามเบนซิมิดาโซลและตัวติดตามคาร์บาริล ซึ่งเป็นคอนจูเกตระหว่าง 2-succiamidobenzimidazole หรือ 1-(5-carboxypentyl)-3-(1-naphthyl) urea กับ horseradish peroxidase ตามลำดับได้ ขั้นตอนต่อไป ได้นำเพลทที่ตรึงบีตาไซโคลเดกซ์ทรินมาใช้ครั้งแรกในการตรวจวัดสารฆ่าราเบนซิมิดาโซล โดยแข่งขันกับตัวติดตามเบนซิมิดาโซลในการเข้าจับกับไซโคลเดกซ์ทริน เมื่อหาสภาวะที่เหมาะสมสำหรับการตรวจวัด พบว่าความเข้มข้นของตัวติดตามเบนซิมิดาโซลที่ 2.5 μg/ml เหมาะสมสำหรับการแข่งขันเข้าจับกับไซโคลเดกซ์ทริน 4% (w/v) ที่ถูกตรึงบนไมโครไทเทอร์เพลท สารมาตรฐานคาร์เบนดาซิมเข้าจับกับไซโคลเดกซ์ทรินที่บัฟเฟอร์ pH 2.5 และใช้ 0.1M acetate/citric acid buffer pH 5 ที่มี 0.5 M NaCl เป็นบัฟเฟอร์สำหรับล้างการจับแบบไม่จำเพาะ กราฟมาตรฐานของคาร์เบนดาซิมมีค่า IC_{50} และค่าต่ำสุดของการวิเคราะห์ เท่ากับ 1.56 และ 0.2 μg/ml วิธีการแข่งขันกันเข้าจับกับไซโคลเดกซ์ทรินนี้ยังสามารถนำมาประยุกต์ใช้ในการตรวจวัดคาร์บาริล โดยใช้สภาวะของการตรวจวิเคราะห์เช่นเดียวกับการตรวจคาร์เบนดาซิม ยกเว้น pH สำหรับการเข้าจับกับไซโคลเดกซ์ทรินของคาร์บาริลเป็น pH 7.4 และใช้ตัวติดตามคาร์บาริลที่ความเข้มข้น 2.5 μg/ml ค่า IC_{50} ที่ได้จากกราฟมาตรฐานคาร์บาริลคือ 0.4 μg/ml และค่าต่ำสุดของการตรวจวิเคราะห์คาร์บาริลเท่ากับ 0.15 μg/ml งานวิจัยนี้ได้ตรวจวัดตัวอย่างอุ่นที่เติมคาร์เบนดาซิมและคาร์บาริล ผลการทดลองพบว่าเมตริกซ์ (Matrix) ของอุ่นมีผลกระทบต่อการแข่งขันเข้าจับกับไซโคลเดกซ์ทริน ดังนั้น การตรวจวัดควรจะใช้เมตริกซ์ของตัวอย่างในการสร้างกราฟมาตรฐานแทนกราฟมาตรฐานแบบปกติ ผลการทดลองแสดงให้เห็นชัดเจนถึงความยืดหยุ่นของวิธีนี้ที่เหนือกว่าวิธี ELISA ในการตรวจวัดสารที่มีโครงสร้างใกล้เคียงกัน ภายใต้สภาวะที่เหมาะสม

ภาควิชา .....ชีวเคมี..... ลายมือชื่อนิสิต .....

สาขาวิชา.....ชีวเคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา.....2546..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

4372413823 : MAJOR BIOCHEMISTRY
KEY WORD: CYCLODEXTRIN / IMMOBILIZATION / MICROTITER PLATE / BENZIMIDAZOLE / XXX
WIRAMSRI SRIPHOCHANART: DEVELOPMENT OF CARBENDAZIM AND CARBARYL DETECTION METHOD USING IMMOBILIZED CYCLODEXTRIN ON MICROTITER PLATES. THESIS ADVISOR: MANCHUMAS PROUSOONTORN, Ph.D., THESIS COADVISOR: ASSOC. PROF. PIAMSOOK PONGSAWASDI, Ph.D., 131 pp. ISBN 974-17-5165-6.

A method was developed for the immobilization of β-CD on polystyrene microtiter plate for the determination of benzimidazole fungicides and carbaryl insecticide based on competitive inhibition ELISA. The microtiter plate wells were coated with methyl vinyl ether-maleic anhydride copolymer (MMAC). Amino groups were introduced to the MMAC-coated plate by the addition of 1,6-hexamethylenediamine (HMDA). The β-CD was subsequently immobilized by the use of divinyl sulfone as a cross-linking reagent. The result indicated that β-CD was successfully immobilized on the prepared amino plate and was still able to entrap benzimidazole and carbaryl tracers, 2-succiamidobenzimidazole or 1-(5-carboxypentyl)-3-(1-naphthyl) urea conjugated to horseradish peroxidase, respectively. The immobilized β-CD was first used for the determination of benzimidazole fungicide by competitive encapsulation with benzimidazole tracer. The optimum conditions of the assay were determined. The benzimidazole tracer concentration at 2.5 μg/ml was used for the competitive encapsulation into 4% (w/v) β-CD and the loading buffer pH 2.5 was used for carbendazim encapsulation. 0.1M acetate/citric acid buffer, pH 5 containing 0.5 M NaCl was used as washing buffer. The typical standard curve of carbendazim had an IC_{50} value and the detection limit of 1.56 and 0.2 μg/ml, respectively. The competitive encapsulation approach was also applied to the determination of carbaryl. The assay condition was similar to that of carbendazim except for the pH loading buffer used for carbaryl encapsulation by β-CD. The carbaryl standard was incubated at pH 7.4 with immobilized CD-microtiter plate and the carbaryl tracer concentration at 2.5 μg/ml was used for competitive encapsulation. An IC_{50} value obtained from the typical standard curve of carbaryl was 0.4 μg/ml and the limit of detection was 0.15 μg/ml. The method was employed in the detection of carbendazim and carbaryl in grape spiked samples. It was found that competitive encapsulation was affected by sample matrix, thus the standard curves in the presence of matrix were performed and they were recommended to be used in place of the typical standard curve. The overall results clearly demonstrate the flexibility of the method over ELISA for the determination of related compounds under specific conditions.

Department ……Biochemistry……Student's signature……..

Field of study…..Biochemistry……Advisor's signature…….

Academic year……..2003……..Co-advisor's signature ….……..

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my impressive advisor, Dr. Manchumas Prousoontorn. I am very grateful to her great advice, latest information, valuable discussion including indefinite kindness and encouragement. My gratitude is also extended to my co-advisor, Associate Professor Dr. Piamsook Pongsawasdi, for her valuable guidance, suggestion and comment throughout this thesis.

My appreciation is also expressed to Associate Professor Dr. Tipaporn Limpaseni, Assistant Professor Dr. Suganya Soontaros and Assistant Professor Dr. Worawan Bhanthumnavin for their valuable suggestion and comments and also dedicating valuable time for thesis examination. Moreover, I would like to thank Associate Professor Dr. Jariya Boonjawat for the use of Titertek multiskan.

My acknowledgement is also expressed to Assistant Professor Dr. Worawan Bhanthumnavin for the determination and interpretation of 1-(5-carboxypentyl)-3-(1-napthyl) urea NMR spectra, Mr. Theeraphot Tamlongluxkul for mass determination of 1-(5-carboxypentyl)-3-(1-napthyl) urea and Miss Jitpaka Suntudrob for her operations of HPLC for carbendazim and carbaryl determination in fruit. I would like to give a deep thank to my kindness sister, Mrs. Pacharawan Jongmeevasana for her great support, advice and discussion.

Sincere thanks are extended to all staff member and all friends for their friendship, kind assistance and sharing a common fate especially Orn, Yelly, Kung, Ohm, Khem, Chai, Eak, Ed, Aum and Poj.

Finally, the greatest gratitude is expressed to my grandmother, my parents, my brother and my sister for their encouragement, willpower and heartiness support throughout my life.

CONTENTS

		Page
THAI ABSTRACT……...		iv
ENGLISH ABSTRACT…….		v
ACKNOWLEDGEMENTS……...		vi
CONTENTS……...		vii
LIST OF TABLES…….		xii
LIST OF FIGURES……...		xiii
ABBREVIATIONS……		xvii
CHAPTER I INTRODUCTION……		1
1.1	*Cyclodextrins*……..…	1
1.2	*Inclusion complex formation*……..…	1
1.3	*Applications of cyclodextrins*…….	3
1.4	*The immobilization of cyclodextrin*……	8
1.5	*Benzimidazole fungicide*…….	10
1.6	*Carbaryl*…….	11
1.7	*Maximum residue limits (MRLs)*……	11
1.8	*Determinations of carbendazim and carbaryl*……	13
1.9	*The approach for the determination of benzimidazole fungicides and carbaryl by immobilized CD*……	17
1.10	*The objectives of this research* …..…….	19
CHAPTER II MATERIALS AND METHODS……		20
2.1	*Equipments*…….	20
2.2	*Chemical*……	21
2.3	*Buffer and reagent*……..	21
2.4	*Enzyme*…….....	22
2.5	*Synthesis of benzimidazole tracer and carbaryl tracer*……..	22
2.5.1	*Synthesis of 2-succiamidobenzimidazole (SAB)*…….	22
2.5.2	*Synthesis of 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU)*……..	22
2.5.3	*Conjugation of 2-succiamidobenzimidazole (SAB) or 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU) to horseradish peroxidase (HRP)*……	23

Page

2.6 *Characterization of SAB-HRP and CPNU-HRP conjugates*….. 23

2.6.1 *The maximum absorption*……. 23

2.6.2 *The enzyme activity*…….. 23

2.7 *Preparation of amino plate*…….. 24

2.7.1 *Introduction of acid anhydride groups on microtiter plate*…….. 24

2.7.2 *Introduction of amino groups to MMAC-coated plate*… 24

2.7.3 *Introduction of hydrazino groups to MMAC-coated plate*……..... 24

2.7.4 *Determination of amino and hydrazine groups introduced to MMAC-coated plate*……. 24

2.7.5 *Determination of HRP activity*……. 25

2.8 *Immobilization of β-CD on MMAC-HMDA treated plate*…….... 25

2.9 *Capability test of the immobilized β-CD-microtiter plate for benzimidazole tracer encapsulation*…….. 26

2.10 *Optimization for condition of benzimidazole tracer for encapsulating into the immobilized β-CD*……... 26

2.10.1 *Concentration of β-CD*……. 26

2.10.2 *Concentration of benzimidazole tracer (SAB-HRP conjugate)*…….. 27

2.10.3 *Types of washing buffer*……..... 27

2.11 *Capability test of competitive encapsulation with benzimidazole tracer by the immobilized β-CD-microtiter plate for detecting carbendazim standard*.....… 27

2.11.1 *Preparation of benzimidazole tracer*…… 27

2.11.2 *Preparation of carbendazim standard*……. 27

2.11.3 *Construction of carbendazim standard curve by competitive encapsulation with benzimidazole tracer*….. 27

2.12 *Analyses of carbendazim spiked fruit samples by competitive encapsulation with benzimidazole tracer into the immobilized CD-microtiter plate*……..… 28

2.12.1 *HPLC method for determining benzimidazole residues in sample*…… 28

Page

2.12.1.1 *Sample extraction*…….. 28
2.12.1.2 *LC analysis* …… 29
2.12.1.3 *Recovery study of LC method*……..… 29
2.12.2 *Effect of fruit matrices on the determination of carbendazim in sample using the competitive encapsulation with benzimidazole tracer*.....… 30
2.12.3 *Determination of carbendazim spiked fruit using the competitive encapsulation with benzimidazole tracer*…… 30
2.13 *The use of immobilized β-CD on microtiter plate for carbaryl determination*…… 30
2.14 *Concentration of carbaryl tracer (CPNU-HRP conjugate)*…… 30
2.15 *Capability test of competitive encapsulation with carbaryl tracer by the immobilized β-CD-microtiter plate for detecting carbaryl standard*…… 31
2.16 *Analyses of carbaryl spiked fruit samples by competitive encapsulation with carbaryl tracer into the immobilized CD-microtiter plate*…….. 31
2.16.1 *HPLC method for determining carbaryl residues in sample*…….. 31
2.16.1.1 *Sample extraction*……. 31
2.16.1.2 *LC analysis*……... 31
2.16.1.3 *Recovery study of LC method*……... 32
2.16.2 *Effect of fruit matrices on the determination of carbaryl in sample using the competitive encapsulation with carbaryl tracer*…….. 32
2.16.3 *The determination of carbaryl in sample using the competitive encapsulation with carbaryl tracer*…….. 32
CHAPTER III RESULT…… 33
3.1 *Benzimidazole and carbaryl tracers synthesis*…….. 33
3.1.1 *SAB and CPNU synthesis*…….. 35

Page

3.1.2 *Conjugation and purification of benzimidazole and carbaryl tracers (SAB-HRP and CPNU-HRP conjugates)*.. 35

3.2 *Characterization of benzimidazole and carbaryl tracers*……. 41

3.2.1 *The maximum adsorption of benzimidazole and carbaryl tracers*…… 41

3.2.2 *The activity of benzimidazole tracer*…… 44

3.3 *Preparation of amino plate*…….. 44

3.4 *Determination of introduced amino and hydrazino groups to MMAC-coated plates*…… 44

3.5 *Immobilization of cyclodextrin on MMAC-HMDA treated plate*… 49

3.6 *Capability test of immobilized β-CD –microtiter plate for benzimidazole tracer encapsulation*…… 49

3.6.1 *Effect of MMAC concentration*…… 52

3.6.2 *Effect of β-CD concentrations*……. 55

3.6.3 *Effect of benzimidazole tracer concentrations*…… 55

3.6.4 *Effect of washing buffer types*…….. 58

3.7 *Capability test of competitive encapsulation with benzimidazole tracer by immobilized β-CD-microtiter plate for detecting carbendazim standard*……..…….. 60

3.7.1 Construction of carbendazim standard curve by competitive encapsulation with benzimidazole tracer……. 63

3.8 *Analyses of carbendazim spiked fruit samples by competitive encapsulation with benzimidazole tracer into immobilized CD-microtiter plate*…….…… 66

3.8.1 *HPLC determination*…… 66

3.8.2 *Recovery studies of carbendazim in spiked fruit sample by HPLC*……. 69

3.8.3 *Matrix effect on the determination of carbendazim in grape by competitive encapsulation with benzimidazole tracer*…. 69

3.8.4 *Determination of carbendazim in grape by competitive encapsulation with benzimidazole tracer*……... 73

3.9 *Determination of carbaryl using immobilized CD on microtiter plate*….. 76

3.9.1 *Effect of carbaryl tracer concentration on its encapsulation* …….. 80

Page

3.10 *Construction of carbaryl standard curve by competitive Encapsulation with carbaryl tracer*……..…….. 80

3.11 *Analyses of carbaryl spiked fruit samples by competitive encapsulation with carbaryl tracer into immobilized CD-microtiter plate*……. 83

3.11.1 *HPLC determination*……... 83

3.11.2 *Recovery studies of carbaryl in spiked fruit sample by HPLC*……. 86

3.11.3 *Matrix effect on the determination of carbaryl in grape by competitive encapsulation with carbaryl tracer*..... 86

3.11.4 *Determination of carbaryl in grape by competitive encapsulation with carbaryl tracer*…… 86

CHAPTER IV DISCUSSION…… 90

CHAPTER V CONCLUSION……... 113

REFERENCES…….. 114

APPENCICES…… 123

BIOGRAPHY……. 131

LIST OF TABLES

Table **Page**

CHAPTER I

1.1 *Physical properties of α, β, and γ- CD*…… 4

1.2 *Uses of cyclodextrins in various industries*…….. 5

1.3 *Applications of cyclodextrin in analytical uses*…… 7

1.4 *Pesticides maximum residue limits (MRLs)* [a]……... 14

1.5 *Analytical techniques for the determination of pesticide residues*…….... 16

CHAPTER III

3.1 *Specific activities of native HRP and modified HRPs*……... 45

3.2 *Comparison of sensitivity of standard curves for carbendazim*…… 67

3.3 *Comparison of sensitivity of standard curves for carbendazim in the presence of matrix*……..... 78

LIST OF FIGURES

Figure **Page**

CHAPTER I

1.1 *Chemical structure of three kinds of cyclodextrin*……. 2

1.2 *The schematic structure of β-cyclodextrin*……..... 2

1.3 *Schematic representation of CD inclusion complex formation*……. 4

1.4 *Schematic of parent CDs and CD derivatives formation*…….. 6

1.5. *Schematic structure of some supramolecular cyclodextrins*……. 9

1.6 *Schematic structures of benzimidazole fungicides*……. 12

1.7 *Schematic structures of N-methylcarbamate insecticides*…….. 12

1.8 *Amount of carbendazim and carbaryl imported into Thailand between the year 2000-2002*…… 15

1.9 *Illustration of ELISA and immobilized CD principle for the determination of aromatic compounds*……. 18

CHAPTER III

3.1 *Chemical structures of carbendazim and 2-succiamidobenzimidazole*……. 34

3.2 *Chemical structures of carbaryl and 1-(5-carboxypentyl)-3-(1-napthyl) urea*……... 34

3.3 *Absorption spectra of carbendazim and 2-succiamidobenzimidazole*……. 36

3.4 *Absorption spectra of carbaryl and 1-(5-carboxypentyl)-3-(1-napthyl) urea*…… 36

3.5 *Synthesis of 2-succiamidobenzimidazole (SAB) from 2-aminobenzimidazole and succinic anhydride*……. 37

3.6 *Synthesis of 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU) from 1-napthyl isocyanate and 6-aminohexnoic acid*……... 37

3.7 *^{1}H NMR spectra of 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU)*…….. 38

3.8 *^{13}C NMR spectra of 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU)*……. 38

3.9 *MS+ H spectra of 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU)*…… 39

3.10 *Schematic diagram showing the synthesis of peptide bond between HRP and SAB or CPNU by the use of carbodiimide (Bauminger and Wilchek, 1980)*…… 40

3.11 *Purification profile of SAB-HRP conjugate using Sephadex G-25 column*…….. 42

Figure **Page**

3.12 *Purification profile of CPNU-HRP conjugate using Sephadex G-25 column*…….. 42

3.13 *Absorption spectra of horseradish peroxidase, benzimidazole tracer and 2-succiamidobenzimidazole*…… 43

3.14 *Absorption spectra of horseradish peroxidase, carbaryl tracer and 1-(5-carboxypentyl)-3-(1-napthyl) urea*……... 43

3.15 *Introduction of amino and hydrazino groups to MMAC-coated plate*….. 46

3.16 *Schematic diagram illustrating the determination of amino surface using the avidin and biotin system*……. 47

3.17 *Specific and non-specific biotinylation of sulfo-NHS-biotin on a) MMAC-HMDA treated plate and b) MMAC-ADHZ treated plate*…… 48

3.18 *Comparison of the amounts of amino groups on the plate*…… 50

3.19 *Schematic diagram showing the immobilization of cyclodextrin (CD) on amino plate by the use of divinyl sulfone (DVS) as a crosslinker*…… 51

3.20 *The specific and non-specific binding of benzimidazole tracer and HRP onto different types of surfaces*…….. 53

3.21 *Influence of MMAC concentration on immobilization of β-CD to MMAC-HMDA treated plate*……. 54

3.22 *Effect of β-CD concentration on saturating the underlying surface*…….. 56

3.23 *Effect of benzimidazole tracer concentration on encapsulation*……. 57

3.24 *Effect of washing buffer on benzimidazole tracer specifically and non-specifically bound onto different types of surfaces*…….. 59

3.25 *Influence of solvent on carbendazim encapsulation*……... 61

3.26 *Influence of SAB-HRP concentration on carbendazim encapsulation on immobilized CD (MMAC-HMDA-DVS-CD) and adsorbed CD (MMAC-HMDA-CD) surfaces*……. 62

3.27 *Influence of pH on carbendazim encapsulation by immobilized CD (MMAC-HMDA-DVS-CD) and adsorbed CD (MMAC-HMDA-CD) surfaces*……. 64

Figure **Page**

3.28 *Standard curve of carbendazim under optimized competitive inhibition condition by immobilized and adsorbed CD on a) prepared and b) commercially available amino plate*……. 65

3.29 *HPLC chromatograms of carbendazim standard at 0.5 μg/ml obtained by a) UV (at 280 nm) and b) fluorescence detection (at excitation/ emission wavelengths of 280/310)*……. 68

3.30 *HPLC chromatograms of 2 g/ml of grape extract obtained by a) UV (at 280 nm) and b) fluorescence detection (at excitation/emission wavelengths of 280/310)*……. 70

3.31 *HPLC chromatograms of a) 0.5 μg/ml of carbendazim standard and b) 1 ppm of carbendazim spiked grape extract determined by UV detector*……... 71

3.32 *HPLC chromatograms of a) 0.5 μg/ml of carbendazim standard and b) 1 ppm of carbendazim spiked grape extract determined by fluorescence detector*……. 72

3.33 *Influence of grape matrix on competitive encapsulation with benzimidazole tracer into immobilized CD on a) prepared amino plate and b) commercially available amino plate* ……..... 74

3.34 *Influence of DMF on the encapsulation of carbendazim spiked grape extract on a) prepared and b) commercially available amino plates*…… 75

3.35 *Standard curve of carbendazim in the presence of matrix on a) prepared and b) commercially available amino plates*…… 77

3.36 *The specific and non-specific binding of carbaryl tracer and HRP onto different types of surfaces*……... 79

3.37 *Effect of carbaryl tracer concentration on encapsulation*…….. 81

3.38 *Influence of pH on carbaryl encapsulation on immobilized CD (MMAC-HMDA-DVS-CD) and adsorbed CD (MMAC-HMDA-CD) surfaces*…… 82

3.39 *HPLC chromatogram of 1 μg/ml of standard mixture of N-methylcarbamates*……... 84

3.40 *HPLC chromatogram of 1 g/ml grape extracted solution*……... 85

Figure **Page**

3.41 *HPLC chromatogram of 1 ppm of carbaryl spiked grape extracted solution*…….. 87

3.42 *Effect of grape matrix on the competitive inhibition with 2.5 μg/ml carbaryl tracer*……..... 88

3.43 *Standard curve of carbaryl in the presence of matrix by competitive encapsulation with carbaryl tracer* …….. 89

CHAPTER IV

4.1 *Schematic diagram illustrating a) the catalytic cycle of HRP with TMB as chromogenic substrate and b) color development of TMB*……. 95

4.2 *The mechanism of the reaction between sulfo-BNHS and the amino group on the microtiter plate surface*…… 98

4.3 *Schematic chemical and physical configuration of the CovaLink NH surface*……... 99

4.4 *Effect of Tris on immobilization of CD on the well of MMAC-HMDA treated plate*…… 101

ABBREVIATIONS

A	absorbance
CD	cyclodextrin
°C	degree Celsius
DVS	divinyl sulfone
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
μl	microliter
M	molar
ml	milliliter
mM	millimolar
MRLs	maximum residue limits
nm	nanometer
PBS	phosphate buffer saline
SAB	2-succiamidodenzimidazole
TMB	tetramethylbenzidine
w/v	weight by volume
WSC	water soluble carbodiimide
MMAC	methyl vinyl ether-maleic anhydride copolymer
BSA	bovine serum albumin
ADHZ	adipic acid dihydrazide
HMDA	1,6-hexamethylenediamine

CHAPTER I

INTRODUCTION

1.1 Cyclodextrins

Cyclodextrins (CDs) are a class of cyclic oligosaccharides composed of α-(1→4) linked D-glucopyranose units. CDs are produced as a result of intramolecular transglycosylation reaction from degradation of starch by the action of cyclodextrin glycosyltransferase (EC.3.2.1.19) produced from certain microorganisms. The commonly available CDs have six, seven, and eight glucopyranose units (*α*-, *β*-, and *γ*-cyclodextrin, respectively) as shown in ***Figure 1.1*** (Bender, 1986) and their physical properties are shown in ***Table 1.1*** (Szejtli, 1998). CD has a truncated shape, the secondary hydroxyl groups (C2 and C3) are located on the wider edge of the ring and the primary hydroxyl groups (C6) on the narrow edge, and that the apolar C3 and C5 hydrogens and ether-like oxygens are at the inside of the torus-like molecules. This results in a molecule with a hydrophilic outside, which can dissolve in water, and an apolar cavity, which provides a hydrophobic matrix (***Figure 1.2***). As a result, cyclodextrins are able to form inclusion complexes with a wide variety of hydrophobic guest molecules. One or two guest molecules can be entrapped by one, two or three cyclodextrins and offer remarkable effects in properties of guests without the formation of chemical bonds and without changing their structures (Singh *et al.,* 2002). Therefore, the most important characteristic of the CDs is their ability to form inclusion complexes with organic and inorganic molecules**.**

1.2 Inclusion complex formation

In liquid and solid phases, organic and inorganic molecules of appropriate size can be incorporated into the cyclodextrin cavity to form inclusion complexes. The incorporated compounds and cyclodextrins are referred to as guests and hosts, respectively. The driving force of the formation of these complexations involve several types of intramolecular interaction such as (1) Van der Waals interaction, (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 molecule encapsulation. The selectivity and sensitivity of the complexation are strongly dependent on the analyte (inclusate) geometry and functional

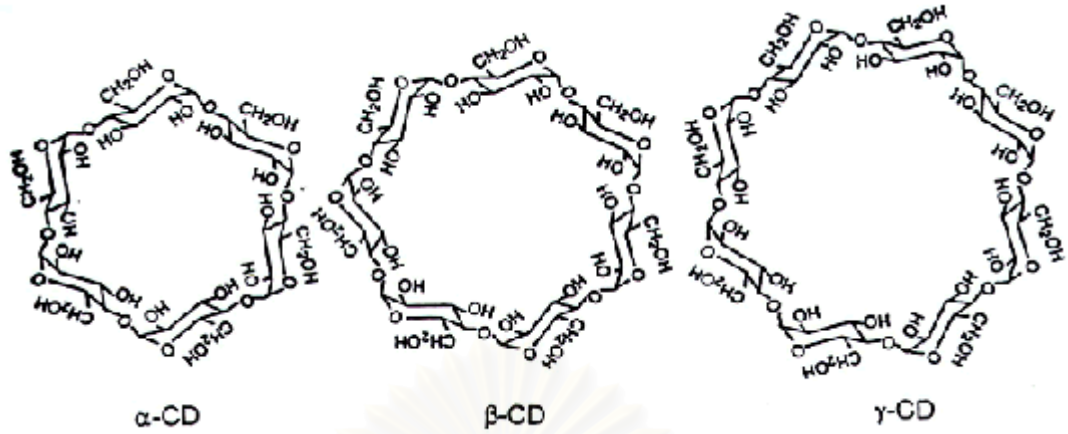


Figure 1.1. Chemical structure of three kinds of cyclodextrin (Szejtli, 1990).

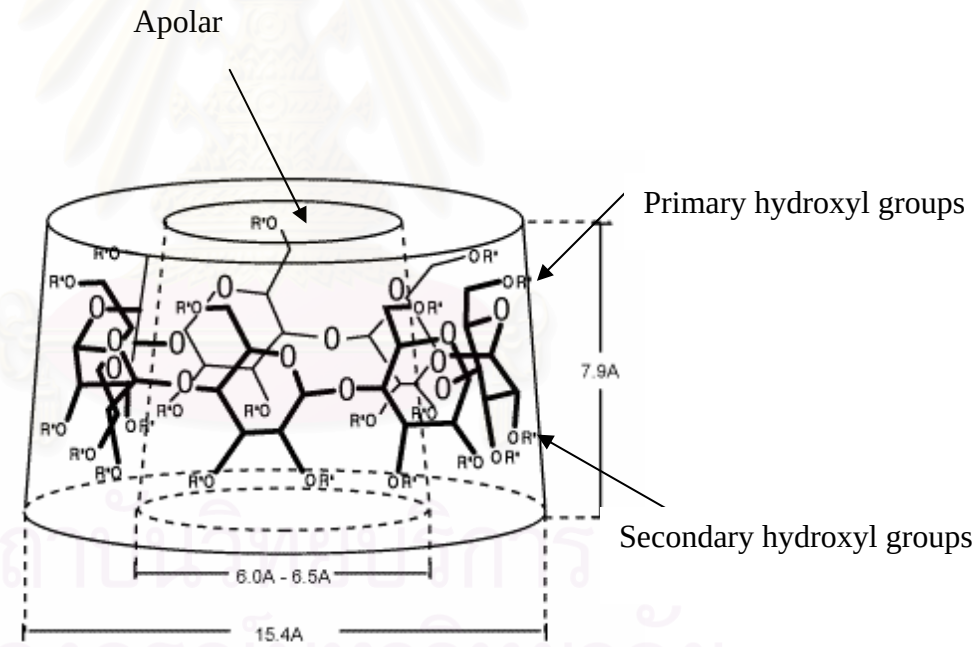


Figure 1.2. The schematic structure of β-cyclodextrin (Szejtli, 1998).

group orientation (Szetjli, 1998). α-CD, which has the smallest cavity among cyclodextrins, best accommodates a benzene nucleus while β-CD best accommodates a naphthalene nucleus. Usually, a single guest molecule is encapsulated into the cavity of a single cyclodextrin molecule to form a 1:1 inclusion complex. However, two small guest molecules are often incorporated into a single cyclodextrin cavity, resulting in the formation of a 1:2 host-guest inclusion complex. In some cases, one guest molecule, which is bulky or long, is encapsulated by two cyclodextrin molecules, leading to the formation of a 2:1 host-guest inclusion complex (Yang *et al*., 2003).

Figure 1.3 shows the complexation process. The dissolved cyclodextrin is the "host" molecule, and the "driving force" of the complex formation is the substitution of the high-enthalpy water molecules by an appropriate "guest" molecule.

1.3 Applications of cyclodextrins

CDs are widely used in many fields, such as foods, cosmetics, toiletries, environmental, agricultural industries, and pharmaceuticals. With their characteristic molecular structures, CDs are capable of forming inclusion complexes with various organic compounds by incorporating them into the CD cavities. Since these CDs change the physical and chemical properties of incorporated guest compounds, the following general applications of CDs are suggested by many investigators (Singh *et al.*, 2002). The uses of CDs in various industries are summarized in ***Table 1.2***.

Chemical or enzymatic modifications of CDs (***Figure 1.4***) can alter their physical properties. Due to the availability of multiple reactive hydroxyl groups, the functionality of CDs is greatly increased. CDs are modified through substituting various functional compounds on the primary and/or secondary face of the molecule. Through modifications, the applications of cyclodextrins are expanded. The modified CDs have shown to improve solubility, stability against light or oxygen and help control the chemical activity of guest molecules (Singh *et al.*, 2002).

Recently, the application of CDs in analytical chemistry in both chromatographic and non-chromatographic uses has remarkably increased. For the application in chromatographic area (***Table 1.3***), CDs, native and modified ones, are used as chemically bonded or adsorbed ligands in stationary phase or in mobile phase in HPLC, GC, capillary electrophoresis and MECC for chiral and enantiomer separations (Singh *et al.*, 2002).

In the field of non-chromatography, CDs are used as sensors, detectors and indicators. Dansylglycine-modified cyclodextrin (DnsC4-β-CD) immobilized on the

Table 1.1. Physical properties of α, β, and γ- CD (Szejtli, 1998)

	α	β	γ
Glucopyranose units	6	7	8
Molecular weight	972	1135	1297
Solubility in water at 25 ^{o}C	14.2	1.85	23.2
Internal cavity diameter (^{o}A)	5.3	6.5	8.3
External diameter (^{o}A)	14.6	15.4	17.5
Height of torus (^{o}A)	7.9	7.9	7.9
Melting range (^{o}C)	255-260	255-265	240-245
Water molecules in cavity	6	11	17

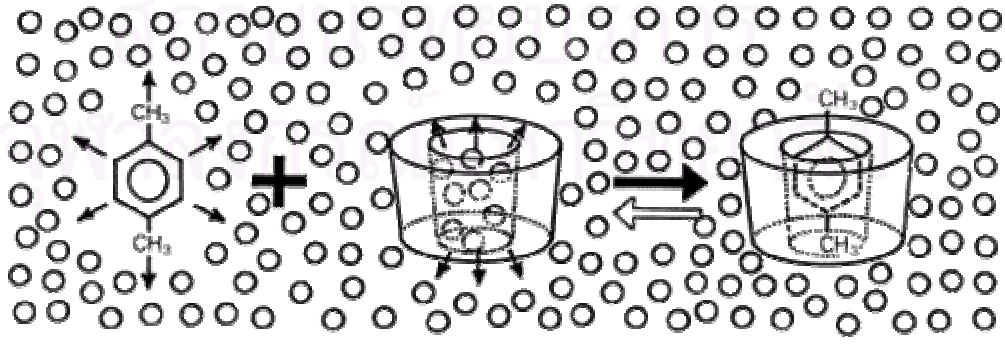
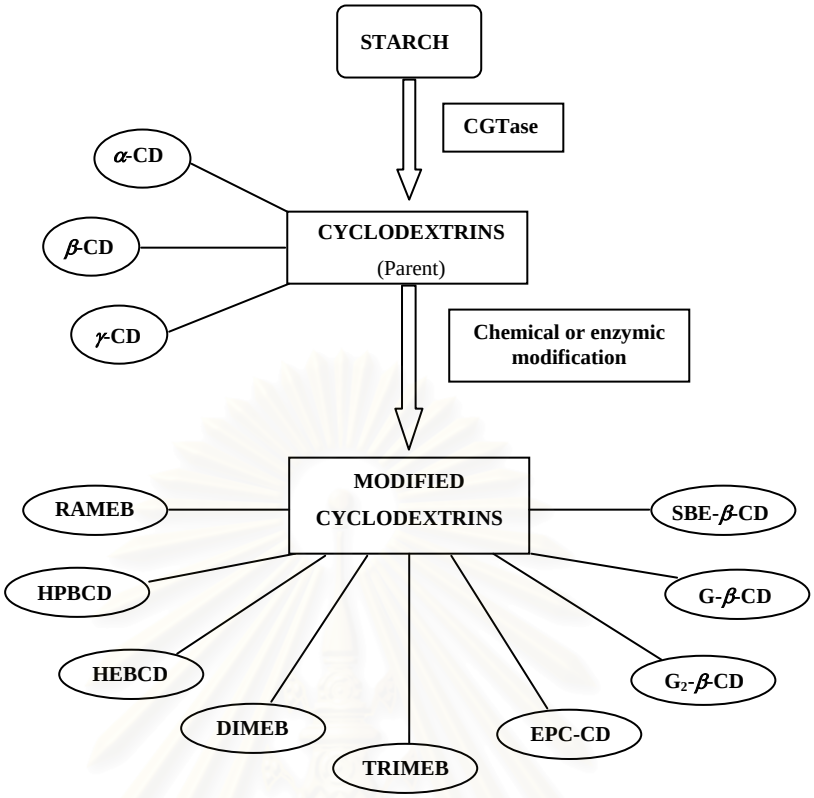


Figure 1.3. Schematic representation of CD inclusion complex formation. *p-Xylene is the guest molecule; the small circles represent the water molecules (Szejtli, 1998).*

Table 1.2. Uses of cyclodextrins in various industries

Application fields	Uses	Type of CD	References
Pharmaceutical	• Increase in bioavailability (by increased solubility and stability) of nimuselide • Protein and peptide delivery such as growth hormone, interleukin-2,asparteme, albumin and MABs	β-CD, 2HP-β-CD Modified CDs	Vavia and Adhage,1999 Uekama *et al.*, 1998
Environmental	• Entrapment of iodine in nuclear waste management • Increase the solubility of the hydrocarbon for biodegradation and bioremediation	Normal CDs, Methylated CDs β-CD	Szente *et al*., 1999 Bardi *et al.*, 2000
Food and flavors	• Remove cholesterol from animal products such as egg, dairy products • Control bitterness in tannins, plant and fungal extracts, skim milk hydrolysates and overcooked tea and coffee and remove these bitter components from citrus fruit juices	β-CD Cross-linked CD polymer	Hedges, 1998 Hedges, 1998
Cosmetics	• Stabilization, odor control, process improvement upon conversion of a liquid ingredient to a solid form, flavor protection and flavor delivery in lipsticks and water solubility and enhanced thermal stability of oils	β-CD	Buschmann and Schollmeyer, 2002
Agricultural	• Delay germination of seed • Increase of water solubility of benzimidazole-type fungicides	β-CD α–CD, β-CD	Szetjli, 1998 Lezcano *et al.*, 2002



- **RAMEB**: randomly methylated beta-CD
- **HPBCD**: hydroxypropyl-beta-CD
- **HEBCD**: hydroxyethyl-beta-CD
- **DIMEB**: heptakis(2,6-dimethyl)-beta-CD
- **TRIMEB**: heptakis(2,3,6-trimethyl)-beta-CD
- **EPC-CD**: crosslinked with epichlorhydrin
- **G_2-β-CD**: 6-O-maltosyl-beta-CD
- **G-β-CD**: 6-O-glycosyl-beta-CD
- **SBE-β-CD**: sulfobutylether-beta-CD

Figure 1.4. Schematic of parent CDs and CD derivatives formation *(modified from Singh et al., 2002).*

Table 1.3. Applications of cyclodextrin in analytical uses

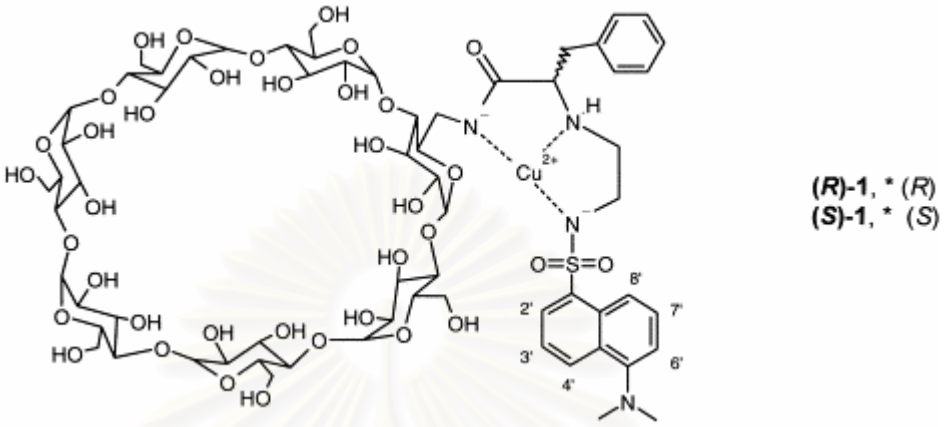
Chromatographic uses	Examples	references
High Performance Liquid Chromatography (HPLC)	• *β*-CDs polymers were immobilized onto silica particles as stationary phases to form inclusion complexes between polyethylene glycols (PEGs) bearing hydrophobic ends. • A novel chiral stationary phase derived from heptakis (6-azido-dexy-2,3-di-*O*-phenylcabamoylated)-*β*-CD immobilized onto amino-functionalzed silica gel via multiple urea linkages.	David *et al*., 2001 Chen *et al*., 2002
Micella Electrokinetic Capillary Chromatography (MECC)	• A borax buffer containing sodium deoxycholate modified with methyl-*β*-CD was used for separation and detection of phospholipids in MECC system. • *β*-CD added to the buffer system was used as a chiral selector and separation of DL-amino acids.	Zhang *et al*., 2000 Ma *et al*., 2002
Gas-liquid chromatography	• Chiral recognition ability of *α*-CD with regard to some monoterpenoids	Asztemborska *et al*., 2003
Capillary gas chromatography	• Enantiomer separation of mandelates and their analogs, which are important intermediates in asymmetric synthetic and pharmaceutical chemistry, was investigated by capillary gas chromatography using different cyclodextrin derivative chiral stationary phases (CD CSPs)	Nie *et al*., 2001

cellulose membrane decreases its fluorescence intensity with increasing concentration of guest molecules, indicating that the host changes the location of the dansyl group from inside to outside the cyclodextrin cavity upon guest accommodation (Tanabe *et al.*, 2001). *β*-cyclodextrin polymer is used as the immobilization matrix for peroxidase and mediator in the fabrication of a sensor for hydrogen peroxide determination (Zhu *et al*., 2000).

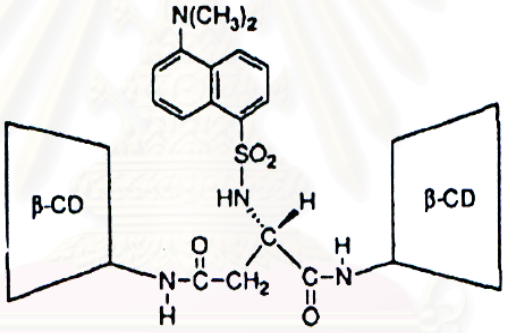
Moreover, cyclodextrin chemistry is one of the most important fields of supramolecular chemistry. CD supramolecules are CDs modified with chromophore, fluorophore, photoactive metal complexes or other easily detecting functional groups in order to be used as sensing molecules. ***Figure 1.5a*** displays two modified cyclodextrins 6-deoxy-6-*N*-[N^{α}-(N^{2}-dansylaminoethyl)-*R*-(or *S*)-phenylalaninamide]-*β*-cyclodextrin, containing a metal binding site and dansyl fluorophore. Both CDs were shown to form copper (II) complexes with fluorescence quenching. Addition of D- or L-amino acid to the copper (II) complexes induced a "switch on" of the fluorescence which was enantioselective for Pro, Phe and Trp (Pagliari *et al.*, 2000). Dansyl modified *β*-CD dimer (***Figure 1.5b***) is the fluorescent CD that can bind a large molecule by co-operation of two CD units. Dansyl modified *β*-CD dimer responded to the presence of steroids with an increase or decrease of the fluorescence intensity depending on the substitution pattern of the steroid (Ueno, 1996). Furthermore, a new supramolecular sensitizer for mercury (II) ion based on an alkylated *β*-cyclodextrin/meso-tetraphenylporphyrin (TPP) inclusion complex has been proposed (Yang *et al*., 2002). The formation of supramolecular complex causes a remarkable increase of the porphyrin metallation rate following the fluorescence quenching of TPP at its maximum emission wavelength. As can be seen from the applications of CDs to be used as molecular sensing molecules mentioned above, therefore CDs are quite attractive to be developed in the field of biosensor.

1.4 The immobilization of cyclodextrin

In analytical chemistry, various methods have been reported for the immobilization of CDs on many supports such as silica, cellulose membrane and gold surfaces. In the chromatographic field, most of the attention is paid to the use of the immobilized CDs as the stationary phase. Hattori *et al.* (1986) reported that 6-deoxyamino-*β*-cyclodextrin has been immobilized through its epoxy glyceride group on hydrophilic gel beads to be used as high-performance liquid chromatographic adsorbent. In addition, the immobilization of mono- (6-azido-6-deoxy)-perfunctionalised cyclodextrins onto the surface of aminized



a.



b.

Figure 1.5. Schematic structure of some supramolecular cyclodextrins.

a) copper(II) complexes of dansylated cyclodextrins ***(R)-1*** *and* ***(S)-1***

b) Dansyl modified β-CD dimer

silica gel was achieved by the Staudinger reaction and was applicable as chiral stationary phase for enantioseparation (Zhang *et al.*, 1999).

For the non-chromatographic use of CDs, heptakis (6-methylthio-6-deoxy)-mono-2-(*O*–methylanthraquinyl)-*β*-cyclodextrin has been immobilized on gold electrode surfaces through a chemisorption (Stine *et al*., 1996) and this was used to exhibit favorable electron transfer rates on electrochemical analysis. Moreover, dansylglycine-modified cyclodextrin (DNS C4-*β*-CD) was shown to be immobilized on oxidized cellulose membrane by reductive amination using $NaBH_3CN$ as a chemosensor for detecting molecules such as cyclohexanol, borneol, deoxycholic acid and hydrodeoxycholic acid (Tanabe *et al*., 2001). Recently, *β*-CD was immobilized onto chitosan beads by cross-linking with 1, 6-hexamethylene diisocyanate (HMDI) reagent and used for cholesterol adsorption from egg yolk (Chiu *et al*., 2004).

1.5 Benzimidazole fungicide

Benzimidazole fungicides are a group of systemic agrochemicals with fungitoxin activity, which include carbendazim, benomyl, thiabendazole and thiophanate-methyl (***Figure 1.6***). They are widely used for the control of diseases in fruit trees, crops, vegetables, and cereals. In addition, benzimidazole fungicides can be used as a preservative in textile, papermaking, leathers and paint industry (Thomas *et al*., 1996). They are formulated as an aqueous dispersion, aqueous suspension, flowable water, dispersible granules and wettable powder. Benomyl (methyl (1-(butylcarbamoyl)-1*H*-benzimidazole-2-yl) carbamate) is one of the most widespread used systemic fungicides. However, its distribution in the environment is not clearly understood because benomyl is unstable in solution and decomposes rapidly to carbendazim (methyl-2-benzimidazolecarbamate, MBC) by loss of its butylcarbamoyl side chain (Bushway *et al*., 1990). Thiophanate-methyl is a benzimidazole precursor. It is also 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. Since carbendazim can be presented simultaneously in plant material, lasting several weeks, it may become accessible to organisms feeding on leaf litter. Soil and sediments may contain residues of carbendazim for up to 3 years and the residues constituted an important risk for human health owing to their chronic toxicity (Blasco *et al*., 2002).

Although benzimidazole fungicides are used with great success in agricultural applications which have led to a continued increase in their use, their carcinogenic activity is of great concern. Therefore, the monitoring of these fungicide residues is necessary for proper assessment of exposure to fungicides through food.

1.6 Carbaryl

N-methylcarbamates such as carbaryl, carbofuran, methiocarb and aldicarb (***Figure 1.7***) are broad-spectrum pesticides. Carbaryl (1-naphthyl *N*-methylcarbamate) is one of the most widely used insecticide in agriculture because of its effectiveness against numerous insect pests and for control of pests on fruits, vegetables, cotton and many other crops, as well as, on poultry and domestic animals (Demirbas, 1998). Carbaryl is a neurotoxic carbamate insecticide which inhibits the action of acetylcholinesterase (AchE) that is an essential component of insect, fish, bird and mammal nervous systems. This enzyme controls the chemical reaction that transforms acetylcholine into choline after acetylcholine has been used to transmit nerve impulses across the functions between nerves (Gupta, 1994). Carbamates do not only inhibit insect acetylcholinesterase but also strongly interfere with neural transmission in other organisms, including humans (Mickova *et al.*, 2003). They have high toxicity to humans through the oral and inhalation routes of exposure. Since they are regarded as a potential hazard for the environment and human health, continuous assessment and monitoring these insecticides in processed food and in particular fruit products are required.

1.7 Maximum residue limits (MRLs)

Maximum residue limit (MRL) is the legal level of pesticide that can be present in foodstuff. MRLs have been set by government agencies in order to regulate the use of agrochemicals that affect the health of the consumers from harmful levels of foods. Many 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. The example of pesticide MRLs in crops accepted by the World Food Agriculture Organization, the World Health Organization and Codex are

---

***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.

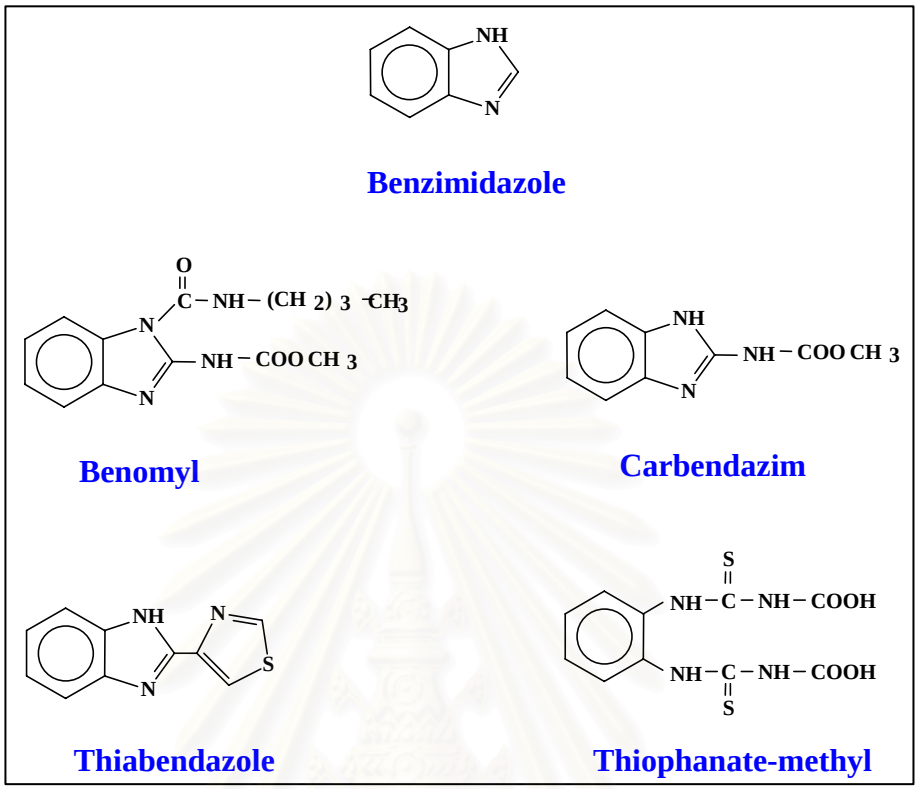


Figure 1.6. Schematic structures of benzimidazole fungicides.

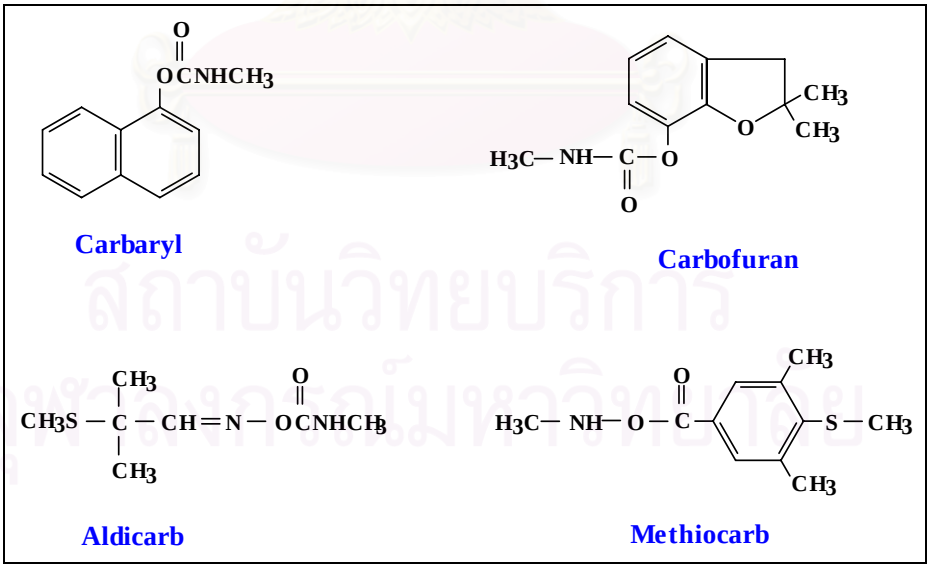


Figure 1.7. Schematic structures of N-methylcarbamate insecticides.

shown in ***Table 1.4***. Noticeably, most fruits and vegetables in the table show pesticides MRLs in the range of 0.1-10 mg/kg. Therefore, the limit of detection and quantification for the pesticide determination should correspond to MRLs. Although quantities of carbendazim and carbaryl imported into Thailand have increased between the year 2000-2002 as shown in ***Figure 1.8***, there are no specific carbendazim MRLs and carbaryl MRLs in any crops.

1.8 Determinations of carbendazim and carbaryl

Because of their acute toxicity and widespread use, monitoring of these residues has become crucial in pesticide control and health care. Traditional analytical methods for trace determination of residues such as benzimidazole fungicides (e.g. carbendazim, thiabendazole, benomyl) and *N*-methylcarbamates insecticides (e.g. carbaryl, aldicarb, carbofuran) in agriculture products are chromatographic techniques.

For benzimidazole fungicides, the use of liquid chromatography especially, high performance liquid chromatography (HPLC) with detector for instance ultraviolet (UV) (Muccio *et al*., 1999) and diode array (DAD) (Thomas *et al*., 1996) has been reported. *N*-methylcarbamates have been determined by the use of HPLC with fluorescence spectrometry (FS) (Nunes *et al*., 1999) and mass spectrometry (MS) (Fernandez *et al.*, 2001). Delgado *et al*. (2001) studied the analysis of carbamates by gas chromatography (GC) with flame ionization and nitrogen-phosphorus detection.

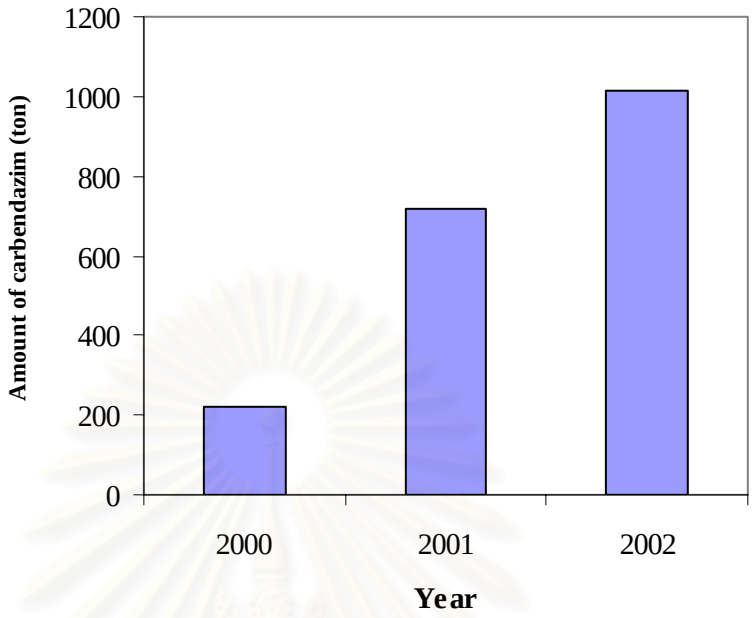
Although these methods are sensitive and well established, they require complex and expensive instruments that have to be managed by highly qualified personnel. For the past sixteen years, enzyme-linked immunosorbent assay (ELISA) methods have been developed for a significant number of pesticides (Newsome and Collins, 1987; Brandon *et al*., 1993; Bushway *et al*., 1994; Abad *et al*., 1999; Nunes *et al*., 1999; Moreno *et al*., 2001; Szekacs *et al*., 2003). ELISA is an analytical method based on the interaction of an analyte and its specific antibody with high affinity and specificity. It is simple, cost-effective, field-portable and does not require sophisticated instrument and is able to accurately and precisely analyze a large number of samples simultaneously. ***Table 1.5*** shows the chronological technique developments for the determination of benzimidazole fungicides and *N*-methylcarbamate insecticides.

Table 1.4. Pesticides maximum residue limits (MRLs) [a]

Pesticide	Food	MRL (mg/kg)	Pesticide	Food	MRL (mg/kg)
Benomyl	Food grains	0.5	Carbendazim	Food grains	0.5
	Vegetables	0.5		Vegetables	0.5
	Mango	0.2		Mango	2.0
	Banana (whole)	1.0		Banana (whole)	1.0
	Fruit	5.0		Fruits	5.0
	Cotton seeds	0.1		Cotton seeds	0.1
	Groundnut	0.1		Groundnut	0.1
	Sugar beet	0.1		Sugar beet	0.1
	Dry fruit	0.1		Dry fruits	0.1
	Eggs	0.1		Eggs	0.1
	Meat & poultry	0.1		Meat & poultry	0.1
	Milk & milk	0.1		Milk & milk	0.1
	products	5.0		products	5.0
	Citrus fruit	1.0		Orange	2.0
	Apricot	2.0		Tomato	5.0
	Grapes	1.0		Grapes	10.0
	Peaches			Citrus fruits	5.0
				Strawberries	3.0
Carbaryl	Food grains	0.5		Avocado	5.0
	Leafy vegetables	10.0		Lettuce (head)	0.5
	Potatoes	0.2		Cucumbers	2.0
	Cotton seed (whole)	1.0		Celery	10.0
	Apples	3.0		Peaches	2.0
	Grapes	5.0		Melons	0.05
	Apricots	10.0		Rape seed	0.2
	Peaches	3.0		Soya been, dry	5.0
	Plums	3.0		Pumpkins	3.0
	Citrus fruits	7.0			
	Cherries	5.0	Cabofuran	Food grains	0.1
	Blueberries	7.0		Milled food	0.03
	Blackberries	10.0		grains	0.1
	Kiwifruit	10.0		Fruits &	0.1
	Strawberries	7.0		vegetables	0.1
	Mushrooms	5.0		Oil seeds	0.1
	Cereal grains	5.0		Sugarcane	0.05
				Potato	0.5
				Soybean, dry	0.2

[a] From: Codex (2 September, 1999)

a)



b)

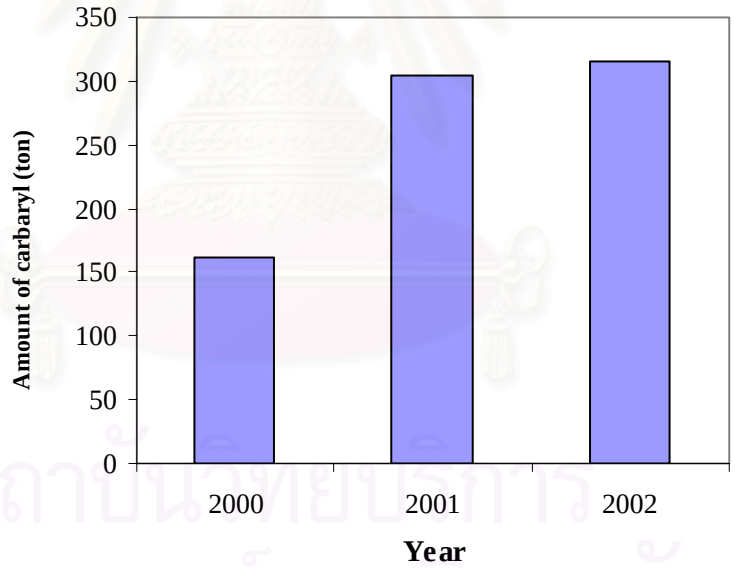


Figure 1.8. Amount of carbendazim and carbaryl imported into Thailand between the year 2000-2002: *a) carbendazim and b) carbaryl reported by Agricultural Regulatory Division, Department of Agriculture.*

Table 1.5. Analytical techniques for the determination of pesticide residues

Analytical technique	Type of pesticide residues	LOD or LOQ
Chromatography		
• Reversed-phase HPLC using an ion-pairing mobile phase with UV and fluorescence detector	Carbendazim, thiabendazole and methyl thiophanate in fruit product (Sannino, 1995)	LOD = 0.01 ppm for carbendazim and methyl thiophanate LOD = 0.001 ppm for thiabendazole
• LC with MS	imidacloprid, carbendazim methiocarb and hexythiazox in peaches and nectarines (Blasco *et al*., 2002)	LOQ = 0.02 ppm
• HPLC with diode-array detector	Carbendazim in water (Thomas *et al*., 1996)	LOD = 0.075 ppm
• HPLC with electrospray mass spectrometric	Carbofuran, carbaryl and methiocarb in baby food (Mickova *et al*., 2003)	LOD = 0.001 ppm
• GC with flame ionization	Carbaryl, carbofuran and propham in potato (Delgado *et al*., 2001)	LOD= 0.21ppm for carbaryl LOD= 0.11ppm for propham LOD=0.14ppm for carbofuran
ELISA		
• Competitive ELISA	Carbaryl in apple and grape juices (Abad and Montoya, 1995)	LOD = 0.065 ppb
• Competitive ELISA	Carbofuran, carbaryl and methiocarb in baby food (Mickova *et al*., 2003)	LOD = 0.3 ppb for carbofuran LOD = 0.04 ppb for carbaryl LOD = 0.02 ppb for methiocarb
• Indirect ELISA (monoclonal antibodies)	Thiabendazole in potatoes and apple (Brandon *et al*., 1993)	LOD = 0.2 ppm
• Competitive ELISA (Benzimidazole and thiabendazole kit)	Benzimidazole and thiabendazole residues in bovine liver (Brandon *et al.*, 1998)	LOD = 0.7 ppb for benzimidazole LOD = 0.2 ppb For thiabendazole

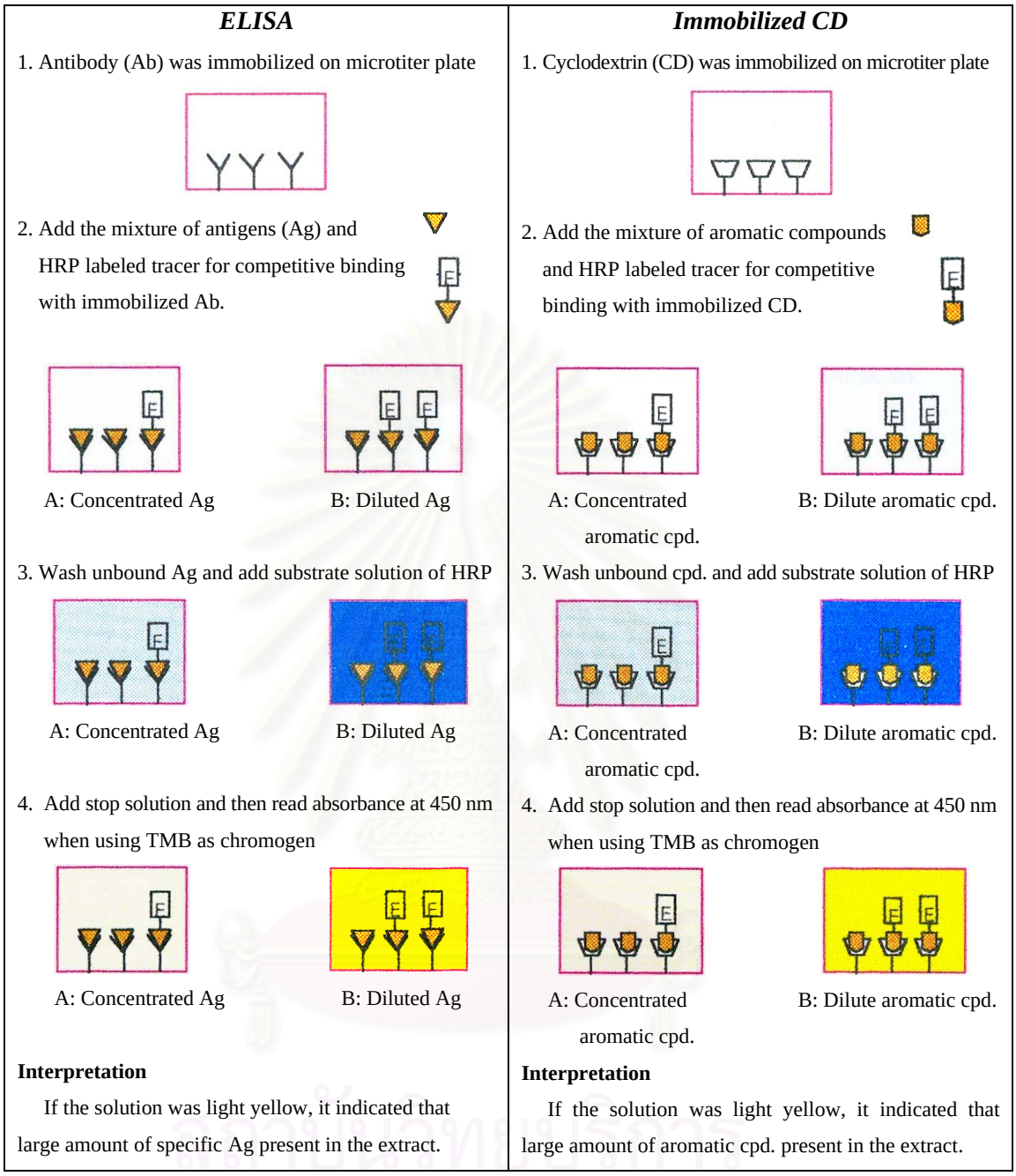
LOD = Limits of detection

LOQ = Limits of quantity

1.9 The approach for the determination of benzimidazole fungicides and carbaryl by immobilized CD

Both of chromatographic and ELISA methods have some disadvantages for the determination of benzimidazole fungicides and *N*-methylcarbamates insecticides. In chromatographic techniques, although sensitive and well established, the methods are not very well suited for the analysis of a large number of samples. They are time consuming because of sample preparation and the need for clean-up step.

While ELISAs are simple, have high specificity and sensitivity, require short analysis time and can be used in field adaptable assay but antibodies immobilized on a microtiter plate cannot be reused. Because they are denatured by solution used to stop enzyme activity. Moreover ELISA kits (commercially available ELISAs) are rather expensive because they have to be imported. In this sense, the application of immobilized CDs as sensing molecules instead of antibodies is very interesting. There have been reports that described the encapsulation of benzimidazole and carbaryl by β-CD (Lezcano *et al*., 2002; Barbato *et al*., 2000; Saikosin *et al*., 2002). Jongmeevasana (2000) reported the use of immobilized β-CD on amino microtiter plate for the determination of benzimidazole fungicides. The method was developed based on competitive-inhibition enzyme immunoassay (Kaufman and Clower, 1991) as shown in ***Figure 1.9***. The figure displays steps involved in the determination of aromatic compounds by immobilized CD which is modified from ELISA principle. First, CD was immobilized on microtiter plate surface as antibody-like. Secondly, pesticide and pesticide-tracer solutions were added into the wells for competitive encapsulation. Then unbound molecules were washed and pesticide-tracers encapsulated into CDs cavities were examined by enzyme substrate reaction. Finally, stop solution was added to the reaction and the absorbance was then read. The amount of carbendazim and carbaryl could be quantified by using carbendazim and carbaryl calibration curve, respectively. However, the commercially available amino microtiter plate is very expensive. The present work thus focuses on the development of the immobilized CD on polystyrene microtiter plate for the detection of pesticide residues by coating the plate with methyl vinyl ether-maleic anhydride copolymer (MMAC). Amino groups were introduced with either 1, 6-hexamethylenediamine (HMDA) or adipic acid dihydrazide (ADHZ) (Satoh *et al.*, 1998).



Note: the presence of Ag or aromatic cpd. in extract reduces the amount of the tracer bound. Thus, the color intensity was decreased whenever the amount of Ag or aromatic cpd. increase.

Figure 1.9. Illustration of ELISA and immobilized CD principle for the determination of aromatic compounds: *Major steps consist 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 (Jongmeevasana, 2000).*

Even though the specificity and affinity between CD and guest molecule are lower than those of antigen and antibody, for screening purpose, it is possible to use the immobilized CD for the determination of carbendazim and other aromatic compounds such as carbaryl under optimal conditions.

1.10 The objectives of this research are:

1. To test the capability of encapsulation with benzimidazole tracer or carbaryl tracer by the β-CD immobilized on prepared amino microtiter plate
2. To compare the capability of β-CD immobilized on prepared and commercially available amino microtiter plate for the determination of carbendazim by competitive encapsulation with benzimidazole tracer
3. To apply the method for the determination of carbaryl by competitive carbaryl tracer encapsulation using CD immobilized on prepared amino microtiter plate

CHAPTER II

MATERIALS and METHODS

2.1 Equipments

Analytical balance: - Sartorius BP 310S, Scientific Promotion Co., Ltd., Germany
- Mettler AB 204-S, Mettler-Toledo, Switzerland

Autopipette: - Pipetman, Gilson, France
- HT, High tech lab, Poland
- HT multichannel pipette, High tech lab, Poland
- Finnpipette digital multichannel pipette, Labsystem Inc., Finland

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

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

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

ELISA microtiter plate: CovaLink NH, Nunc, Denmark

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

FT-NMR: JEOL JNM-A500 NMR spectrometer, Tokyo, Japan

Homogenizer: Ultra-turrax T 25, IKA-Labortechnik, Germany

HPLC: - Thermo Separation Products, U.S.A.
- Spectra-Physic, Spectra system P4000, Pickering Laboratories PCK 5100 postcolumn recaction module, U.S.A.

: Analytical column: Ultracarb 5 C8 4.60 ×150 mm, Phenomenex, U.S.A.
: Zobax 5 C8 4.60 × 250 mm, Phenomenex, U.S.A.

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

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

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

MS: API-4000 LC/MS/MS triple quadrupole system, Applied Biosystems, U.S.A.

pH meter: Mettler MP 220 pH Meter, Mettler-Toledo, Switzerland

Rotary vacuum evaporator: Rotavapor R-200, Buchi, Switzerland

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

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

2.2 Chemical

Acetronitrile**,** HPLC grade, LAB-SCAN, Ireland

2-Aminobenzimidazole, Fluka, Switzerland

ε-Amino-*n*-caproic acid (6-amino-*n*-hexanoic acid), Sigma, U.S.A.

Adipic acid dihydrazide, Sigma, U.S.A.

Biotin 3-sulfo-*N*-hydroxysuccinimide ester, Sigma, U.S.A.

Carbaryl, Chem Service, U.S.A.

Carbendazim, Chem Service, U.S.A.

β-Cyclodextrin, Sigma, U.S.A.

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

Divinylsulfone, Aldrich, Germany

1, 6-Hexane-diamine (HMDA), Sigma, U.S.A.

1-Naphthyl isocyanate, Aldrich, Germany

Poly (methyl vinyl ether-*alt*-maleic anhydride) (MMAC), Aldrich, Germany

Succinic anhydride, Fluka, Switzerland

3, 3', 5, 5'-Tetramethylbenzidine, Sigma, U.S.A.

Tetrahydrofuran, LAB-SCAN, Ireland

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

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

2.3 Buffer and reagent

Loading buffer for encapsulation:

- 10 mM phosphate buffer saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, pH 7.4
- 0.1 M Sodium acetate/ citric acid buffer, pH 2.5 and 5

Washing buffer for the encapsulation:

- 0.1 M Sodium acetate/ citric acid buffer, pH 5
- 0.1 M sodium acetate/ citric acid buffer containing 0.5 M NaCl, pH 5
- 50 mM Tris buffer, pH 7.5 (TB)
- 10 mM Tris-buffer saline, pH 7.5 (TBS)

CD coating reagent:

- 0.5 M Sodium carbonate buffer, pH 11
- 1 M Tris solution

2.4 Enzyme

Avidin-peroxidase labeled, Sigma, U.S.A.

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

2.5 Synthesis of benzimidazole tracer and carbaryl tracer

The general procedure for the synthesis of both tracers is described for benzimidazole, although the similar procedure was also carried out for carbaryl.

Benzimidazole and carbaryl tracers were horseradish peroxidase (HRP) conjugated to either 2-succiamidobenzimidazole (SAB) or 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU), respectively. They were first prepared to be used in the determination of benzimidazole fungicides or carbaryl based on competitive-inhibition enzyme immunoassay method. Due to the fact that SAB and CPNU could be encapsulated into CD owing to their hydrophobicity. The occurred inclusion complexes could be investigated through enzyme-substrate reaction.

2.5.1 Synthesis of 2-succiamidobenzimidazole (SAB)

The synthesis of SAB was modified from the method previously reported (Newsome and Shields, 1981). 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 recrystallized with methanol. The white solid was air-dried. The product was identified and confirmed by fourier transform nuclear magnetic resonance spectroscopy (FT-NMR) and mass spectrometry (MS)

2.5.2 Synthesis of 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU)

CPNU was synthesized by the method previously described by Marco and his co-workers (1993). A solution of 1-napthyl isocyanate (1.7 g, 0.001 mol) and 6-aminohexanoic acid (1.3 g, 0.01 mol) in tetrahydrofuran (THF) (100 ml) was stirred at room temperature overnight. The white solid was isolated by filtration. Subsequently, the solvent was evaporated and the pure compound was recrystallized from acetronitrile. The white solid was then air-dried. The product was identified and confirmed by fourier transform nuclear magnetic resonance spectroscopy (FT-NMR) and mass spectrometry (MS).

2.5.3 Conjugation of 2-succiamidobenzimidazole (SAB) or 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU) to horseradish peroxidase (HRP)

After SAB and CPNU were prepared, the benzimidazole and carbaryl tracers consisting of SAB or CPNU coupled to horseradish peroxidase (HRP) were then synthesized according to Jongmeevasana (2000) with some modifications.

For SAB-HRP conjugate, 1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (56.4 mg) in distilled water (0.5 ml) was added to a stirred solution of HRP (5 mg) in 1.0 ml of 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 pH was adjusted to 7 with dilute HCl. Gentle stirring was continued for 20 hours at room temperature (Newsome and Shields, 1981). The conjugate was then concentrated by Centricon with membrane MW cut-off of 10,000 dalton. After that the solution was applied to a Sephadex-G25 (Hitrap^{TM} desalting column), pre-equilibrated and eluted with 5 mM PBS to separate the excess SAB and conjugated protein. Finally, aliquots of the conjugate solution were stored at –20 °C.

For CPNU-HRP conjugate, the method used was carried out in the same manner as described above except that 1 mg of CPNU dissolved in 100 μl DMF and diluted in 1 ml PBS was used instead of 2.3 mg SAB dissolved in 1 ml NaOH.

2.6 Characterization of SAB-HRP and CPNU-HRP conjugates

2.6.1 The maximum absorption

Native HRP, benzimidazole and carbaryl tracers (modified HRPs) were dissolved in 5 mM PBS to have an appropriate absorbance reading at 405 nm. The maximum absorption was scanned in the range of 250-450 nm.

2.6.2 The enzyme activity

The method for peroxidase activity determination, according to Bos *et al.* (1981) was used to examine activity of native HRP, benzimidazole and carbaryl tracers (modified HRPs). The native and modified HRP solutions of 100 μl were added to 400 μl of a freshly prepared substrate mixture containing 0.1 mg/ml of 3, 3', 5, 5' – tetramethylbenzidine (TMB) and 0.1 mg/ml of urea hydrogen peroxide (H_2O_2) in 0.1 M sodium acetate / citric acid buffer, pH 5 (see appendix in detail). The increase in the absorbance at 655 nm was measured spectrophotometrically in for 0-180 seconds. One unit of enzyme activity was defined as the amount of enzyme which oxidizes 1 μmol of

TMB per minute under the experimental conditions. The specific activities of modified HRPs were compared to those of the native one.

2.7 Preparation of amino plate

The procedures were carried out according to the method previously reported (Satoh *et al.*, 1998). The amino microtiter plates were prepared using methyl vinyl ether-maleic anhydride copolymer (MMAC) and 1, 6 hexamethylenediamine (HMDA) or adipic acid dihydrazide (ADHZ).The amino group of HMDA or hydrazino group of ADHZ would react with anhydride group of MMAC, resulting in plates having amino or hydrazino groups, respectively.

2.7.1 Introduction of acid anhydride groups on microtiter plate

Two hundred microlitres of 1.25 mg/ml of methyl vinyl ether-maleic anhydride copolymer (MMAC) dissolved in dimethyl sulfoxide was added to each well of the microtiter plate and allowed to stand at room temperature (25 °C) for 30 min. After that the solution was removed and the amino groups were introduced as described in 2.7.2.

2.7.2 Introduction of amino groups to MMAC-coated plate

Two hundred microlitres of 10 mg/ml of 1, 6 hexamethylenediamine (HMDA) dissolved in water were added to the wells coated with MMAC and allowed to stand at room temperature (25 °C) for 2 hours. The solution in the wells was then removed and the wells were thoroughly washed twice with water.

2.7.3 Introduction of hydrazino groups to MMAC-coated plate

The wells coated with MMAC were prepared and a solution of 200 μl of adipic acid dihydrazide (ADHZ) in water (10 mg/ml) was added, and allowed to stand for 2.5 hours. The solution in the wells was removed and the wells were washed twice with water.

2.7.4 Determination of amino and hydrazino groups introduced to MMAC-coated plate

The biotinylation was used to detect the amino group introduced to the plate. *N*-hydroxysulfosuccinimide ester of biotin (sulfo-BNHS) which was added to the wells would bind to the amino groups on the plate. Avidin- peroxidase (AV-HRP)

conjugate which was subsequently added would then bind specifically to the biotinylated surface. The enzyme activity of peroxidase was then determined spectrophotometrically at 450 nm using TMB and H_2O_2 (section 2.7.5) (Satoh *et al.*, 1998).

A 50 μl aliquot of a serial dilution of sulfo-BNHS in 50 mM citrate-phosphate buffer, pH 5 (CPB) was added to each well of the amino plate or commercially available amino plate and allowed to stand at room temperature (25 °C) for 1 hour. After incubation, 200 μl of 50 mM Tris buffer, pH 7.5 (TB) was added and incubated for 15 min to maintain the water solubility of MMAC-coated plate. The wells were then washed with 10 mM Tris-buffered saline, pH 7.5 (TBS) three times, and 200 μl of 3% (w/v) BSA/TBS was added to the wells and incubated for 2 hours to block any non-specific binding. After wells were washed with 250 μl of TBS three times, 50 μl of diluted AV-HRP conjugated with TBS at final dilution of 1:1600 was added to each well and allowed to stand at room temperature (25 °C) for 1 hour. After incubation with HRP conjugates, the wells were washed with 0.1 M sodium acetate / citric acid buffer, pH 5 containing 0.5 M NaCl (washing buffer) three times, followed by three washes with 0.1 M sodium acetate / citric acid buffer, pH 5 (assay buffer) before subjected to HRP activity assay.

2.7.5 Determination of HRP activity

A solution of 100 μl of chromogenic substrate solution consisting of 0.1 mg/ml TMB and 0.1 mg/ml urea H_2O_2 was added and incubated at room temperature (25 °C) for 4 min. The color development was stopped by the addition of 50 μl of 2 M H_2SO_4 and the absorbance was then read at 450 nm.

2.8 Immobilization of β-CD on MMAC-HMDA treated plate

A bifunctional cross-linking reagent, divinyl sulfone was used for the immobilization of the β-CD on HMDA-coated. The procedure for the immobilization of β-CD was modified from the method for carbohydrate immobilization on amino polystyrene microtiter plate surface (Hatakeyama *et al.*, 1996).

A solution of 300 μl of 5% (v/v) divinyl sulfone in 0.5 M carbonate buffer, pH 11, was added into each well and incubated at room temperature for 1 hour. The well were then washed 3 times with 300 μl of 0.5 M carbonate buffer, pH 11, followed by incubation with 300 μl of 4% (w/v) β-CD in 0.5 M carbonate buffer, pH 11 for 18 hours at room temperature. The wells were thoroughly washed 4 times with 300 μl of distilled

water and incubated for 15 minutes to remove excess β-CD. Another 300 μl of distilled water heated to 40 °C was added and incubated for 15 minutes, 4 times, to ensure that the excess β-CD was removed. After that any remaining vinyl groups were blocked with 1M Tris in each well for 1 hour. Then wells were washed twice with 300 μl of distilled water and another 3 times with 300 μl of 0.1 M sodium acetate/citric acid buffer, pH 5. The immobilized β-CD microtiter plate was air dried and kept in desiccator at room temperature before used.

2.9 Capability test of the immobilized β-CD-microtiter plate for benzimidazole tracer encapsulation

To examine the immobilized β-CD on microtiter plate surface, benzimidazole tracer was used as guest molecule for encapsulation. The procedure was as follows.

Fifty microlitres of various benzimidazole concentrations (0-20 μg/ml) in 0.1 M sodium acetate/ citric acid buffer, pH 5, were added into the β-CD immobilized plates and incubated for 1 hour at room temperature. The solution was removed and wells were washed 3 times with 300 μl of 0.1 M sodium acetate/ citric acid buffer, pH 5, containing 0.5 M NaCl (washing buffer) and followed by 3 washes with 0.1 M sodium acetate/ citric acid buffer, pH 5 (assay buffer). Then HRP activity was determined as described under section 2.7.5.

2.10 Optimization for condition of benzimidazole tracer for encapsulating into the immobilized β-CD

Various factors such as concentration of β-CD, benzimidazole tracer and types of washing buffer used were determined to find the optimum condition for the encapsulation of benzimidazole tracer into immobilized β-CD. The procedure was carried out in the same way described under section 2.9 except that each parameter was varied.

2.10.1 Concentration of β-CD

Fifty microlitres of β-CD concentrations at 1%, 2.5%, 4% and 5% (w/v) dissolved in 0.5 M carbonate buffer, pH 11, were used in the immobilized process for benzimidazole tracer encapsulation.

2.10.2 Concentration of benzimidazole tracer (SAB-HRP conjugate)

To determine the optimal concentration of tracer used for encapsulation into immobilized β-CD, 50 μl of 0.3, 0.7, 1.3, 2.5, 5.0, 10 and 20 μg/ml of benzimidazole tracer were added into each well.

2.10.3 Types of washing buffer

In this experiment, different types of washing buffer were prepared and were used to wash the nonspecifically bound benzimidazole tracer. Six types of buffer, 0.1 M sodium acetate/ citric acid buffer pH 5, 0.1 M sodium acetate/ citric acid buffer pH 5 containing 0.5 M NaCl, 0.1 M sodium acetate/ citric acid buffer pH 5 containing 0.05% tween 20, 50 mM Tris buffer pH 7.5 (TB), 10 mM Tris buffer saline (TBS) containing 0.5 M NaCl and 0.5 M carbonate buffer pH 10 were used.

2.11 Capability test of competitive encapsulation with benzimidazole tracer by the immobilized β-CD-microtiter plate for detecting carbendazim standard

The competitive encapsulation between carbendazim standard and benzimidazole tracer based on a competitive inhibition ELISA was investigated. Different carbendazim and benzimidazole tracer concentrations and the pH used to dilute carbendazim standard were analyzed. The procedure was performed as follows.

2.11.1 Preparation of benzimidazole tracer

An aliquot of benzimidazole tracer solution was diluted in 0.1 M sodium acetate/ citric acid, pH 5.0 to get 1.25, 2.5 and 5.0 μg/ml working solution.

2.11.2 Preparation of carbendazim standard

The carbendazim stock standard solution was prepared by dissolving 2 mg of carbendazim standard in 10 ml of dimethylformamide (DMF) (200 μg/ml). This solution was then used to prepare working standard solution in the 0.01-50 μg/ml range by 1:2 serial dilution in 0.1 M sodium acetate/ citric acid buffer, pH 2.5 and 5.

2.11.3 Constraction of carbendazim standard curve by competitive encapsulation with benzimidazole tracer

The immobilization of β-CD on the prepared amino microtiter plates were performed as described under section 2.7 and 2.8. Then the immobilized CD plates received 50 μl of working carbendazim standard solution and incubated for 1 hour at

room temperature. The wells were then washed twice with 250 μl of 0.1 M sodium acetate/ citric acid buffer, pH 5.0 to remove unbound carbendazim. After that 50μl of benzimidazole tracer at concentrations of 1.25, 2.5 and 5.0 μg/ml was added into the wells and the mixture was incubated for 1 hour at room temperature. After the plates were washed 3 times with 300 μl of 0.1 M sodium acetate/ citric acid buffer, pH 5.0, containing 0.5 M NaCl (washing buffer), followed by 3 washes with 0.1 M sodium acetate/ citric acid buffer, pH 5.0 (assay buffer), the enzyme activity was determined as described in section 2.7.5.

2.12 Analyses of carbendazim spiked fruit samples by competitive encapsulation with benzimidazole tracer into the immobilized CD-microtiter plate

The use of immobilized CD and benzimidazole tracer to detect carbendazim spike in fruit consisted of three parts. First, fruit sample was confirmed by HPLC analysis that it did not contain residues of carbendazim to be used for recovery studies (see section 2.12.1.3) and matrix interference analysis (see section 2.12.2). In the second part, the effect of fruit matrices which may interfere with the competitive determination system was checked and in the last step, fruit sample spiked with carbendazim was determined by competitive benzimidazole tracer encapsulation into immobilized CD-microtiter plate.

2.12.1 HPLC method for determining benzimidazole residues in sample

Sample extraction and LC determination were modified from Givydis and Waters (1990) as follows.

2.12.1.1 Sample extraction

In this study, grape was used as a sample. Inedible portions such as seeds were removed and the edible portion was homogenized with blender. Ten grams of grapes were homogenized with 30 ml of methanol for 2 minutes with an Ultra-turrax T25 apparatus from IKA-Labortechnik. The mixture was then filtered through whatman no. 1 and rinsed with 20 ml methanol. The pH of the filtrate was adjusted to 7.5-8.0 using sodium hydroxide (5 M and 1M) and 1 M hydrochloric acid, as necessary (strong alkaline condition during adjustment should be avoided) and transferred to a separatory funnel. The carbendazim was extracted twice with dichloromethane. Ten milliliters of saturated sodium chloride solution and 50 ml of dichloromethane were subsequently added. The mixture was shaken for 1 minute. Then the organic layer (dichloromethane

layer) was separated and dehydrated with anhydrous Na_2SO_4. For the aqueous layer, it was re-extracted by adding 3 g of NaCl and 50 ml dichloromethane. The organic layer was again separated and dehydrated with anhydrous Na_2SO_4. The dry organic layers were mixed and evaporated just to dryness using vacuum rotary evaporator with 30 °C water bath. The residues were then redissolved in 5 ml of methanol, hence, the concentration of sample-extracted solution was 2 g/ml. The solution was filtered through 0.45 μm membrane before subjected to LC system.

2.12.1.2 LC analysis

Analysis of carbendazim was performed on a reverse phase Ultracarb 5 C8 (150x4.6 mm) column coupled to fluorescence detector in tandem with UV detector. The wavelength of UV detector was set at 280 nm. Excitation and emission wavelengths of fluorescence detector were set at 280 and 310 nm, respectively. The mobile phase consisted of 40% of acetonitrile and 60% of 0.01 M potassium hydrogen phosphate was used and a flow rate was kept at 0.5 ml/min. The system was equilibrated for at least 30 minutes before use and the sample injection volume was 20 μl. Chromatographic response (peak retention times, heights, and/or areas) of standard and sample solutions was determined and the amount of the residues was calculated. If further dilutions were necessary, methanol was used as diluent. If the system was not in use for a short period of time, the column was rinsed with 70% acetonitrile for at least 1.5 hour to prevent salt deposition and 50% of methanol in water was used when the system was not in use for a long time.

2.12.1.3 Recovery study of LC method

Once confirmed by HPLC analysis that sample did not contain carbendazim residues, 10 g of prepared grape were spiked with carbendazim solution at 1 ppm in methanol. Carbendazim solution at 1 ppm was prepared from the 200 μg/ml stock solution. After the sample was spiked, carbendazim was extracted and determined as described under 2.12.1.1 and 2.12.1.2, respectively. This was done to check whether the extraction method was appropriate.

The recovery was calculated by the equation (PAM, 1991)

$$\text{Recovery (\%)} = \frac{\text{Amount of carbendazim determined}}{\text{Amount of carbendazim spiked}} \text{ x100}$$

2.12.2 Effect of fruit matrices on the determination of carbendazim in sample using the competitive encapsulation with benzimidazole tracer

The effect of grape matrix was evaluated prior to the application of the method to detect carbendazim. There were other unidentified compounds in the food matrix apart from carbendazim that could be encapsulated into CD cavity when they were incubated under the same condition as carbendazim standard. Therefore, the fruit sample without carbendazim residues (as confirmed by HPLC) was extracted as mentioned in 2.12.1.1 and was used to check the matrix effects. Two milliliters of the extract were pipetted into 15 ml graduated tube and then 2 ml of 0.1 M sodium acetate/ citric acid buffer, pH 2.5 or 5.0 (loading buffer) was added. The bottom layer (methanol layer) was evaporated by passing nitrogen gas until the bottom layer disappeared. The upper layer (2 g/ml sample-extracted solution) was diluted in loading buffer to get the final concentration of 0.05, 0.1, 0.25 and 0.5 g/ml. They were used for the encapsulation in replace of carbendazim standard described in protocol 2.11.3.

2.12.3 Determination of carbendazim spiked fruit using the competitive encapsulation with benzimidazole tracer

The concentration of grape extract in 2.12.2 which showed the lowest interference by food matrix was spiked with carbendazim standard at 0-50 μg/ml. The spiked solutions were then loaded into immobilized CD wells in order to determine carbendazim residues by the competitive encapsulation as described for carbendazim standard determination (protocol 2.11.3).

2.13 The use of immobilized β-CD on microtiter plate for carbaryl determination

After the optimized condition for the benzimidazole tracer encapsulation by immobilized CD was achieved, the method was applied for the determination of carbaryl in fruit sample. First, carbaryl tracer was synthesized as mentioned in section 2.5.3, then it was checked whether it could be encapsulated by immobilized CD. The main difference was the pH loading buffer used between carbendazim and carbaryl for the encapsulation into β-CD. The method was performed in the same manner as for the encapsulation of benzimidazole tracer as described previously in section 2.9.

2.14 Concentration of carbaryl tracer (CPNU-HRP conjugate)

Although the optimized condition for the carbendazim was used, carbaryl tracer concentration still had to be determined to get an appropriate concentration for the

detection of carbaryl. A solution of 50 μl carbaryl tracer at concentrations of 0.3, 0.7, 1.3, 2.5, 5.0, 10 and 20 μg/ml were used for encapsulation into immobilized β-CD to find the optimal tracer concentration.

2.15 Capability test of competitive encapsulation with carbaryl tracer by the immobilized β-CD-microtiter plate for detecting carbaryl standard

The procedure was performed in the same manner as described in 2.11. The carbaryl stock solution was also dissolved in DMF but it was diluted with two types of buffer, 0.1 M sodium acetate/ citric acid buffer, pH 5.0 and 10 mM phosphate buffer saline containing 137 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS) to prepare for the working solutions. They were used for the investigation of pH loading buffer for carbaryl encapsulation into immobilized CD. The carbaryl tracer at the concentration of 2.5 μg/ml was used to study the competitive encapsulation between carbaryl standard and carbaryl tracer.

2.16 Analyses of carbaryl spiked fruit samples by competitive encapsulation with carbaryl tracer into the immobilized CD-microtiter plate

The analysis of carbaryl spiked fruit sample consisted of 3 steps as described for the determination of carbendazim in section 2.12.3. First, grape sample was checked by HPLC that it did not have any carbaryl residues. Next, the grape matrix effect was then investigated. Finally, grape extracted solution spiked with carbaryl was determined by competitive encapsulation with carbaryl tracer into the CD immobilized on microtiter plate.

2.16.1 HPLC method for determining carbaryl residues in sample

The determination of carbaryl residues in sample also consisted of sample extraction and LC analysis. Carbaryl was analyzed by HPLC according to the method described by Parfitt (2000).

2.16.1.1 Sample extraction

The procedure for the extraction of carbaryl from grape was carried out in the same way as described for carbendazim (section 2.12.1.1) except that there was no need to adjust the pH of the filtrate containing carbaryl to 7.5-8.0.

2.16.1.2 LC analysis

The analytical column was a reverse phase Zobax 5 μm C-8 (4.6×250 mm) column with fluorescence detector. Excitation and emission wavelengths of

fluorescence detector were 340 nm and 435 nm, respectively. The mobile phase was acetronitrile:water and was set a flow rate at 1.2 ml/min. The initial composition was 12% acetonitrile held from 0 to 5 minutes, followed by a linear gradient to 70% acetonitrile from 5 to 10 minutes to equilibrate the column. Ten microlitres of sample solution were injected into LC system and carbaryl was eluted with 70% acetronitrile from 10 to 35 minutes. Chromatographic response (peak retention times, heights, and/or areas) of standard and sample solutions was determined and the amount of the carbaryl residue was calculated. If further dilutions were necessary, methanol was used as diluent. Before stopping, the system was re-equilibrated at initial conditions (12% acetonitrile) from 35 to 45 minutes.

2.16.1.3 Recovery study of LC method

The percentage recovery of carbaryl residues was calculated as described in 2.12.1.3.

2.16.2 Effect of fruit matrices on the determination of carbaryl in sample using the competitive encapsulation with carbaryl tracer

The grape matrix was checked whether they contained other substances in the sample apart from carbaryl that could be encapsulated into CD cavity when they were incubated in the same condition as carbaryl standard. After that the extracted sample solution with no carbaryl at concentration of 2 g/ml was then diluted in the range of 0-1 g/ml with either 0.1 M sodium acetate/ citric acid buffer, pH 5.0 or 10 mM phosphate buffer saline containing 137 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS) to study for the pH loading buffer. The solutions were then added onto the immobilized CD plate for the investigation of an appropriate concentration of fruit matrix. The method was performed as in section 2.12.2.

2.16.3 Determination of carbaryl spiked fruit using the competitive encapsulation with carbaryl tracer

The appropriate concentration of grape extract which showed the lowest interference was spiked with carbaryl standard at 0-50 μg/ml in PBS, pH 7.4. The solutions were then loaded into immobilized CD wells in order to determine carbaryl residues by the competitive encapsulation method similar to that previously described for carbendazim standard determination (protocol 2.11.3).

CHAPTER III

RESULT

The primary objective of this study was to develop a sensitive method for the determination of benzimidazole fungicides using immobilized CD on microtiter plate surfaces. The results were then compared to the method that was previously reported by Jongmeevasana (2000). In this previous report, CD was immobilized on commercially available amino plates for the determination of benzimidazole fungicides. However, this kind of plates is very expensive. The present work describes the preparation of amino microtiter plate and hopefully, the plates prepared should be more sensitive due to larger amounts of amino groups introduced. In addition, the method was extended to the determination of carbaryl residues and fruit samples spiked with carbendazim or carbaryl were used to demonstrate the applicability of the method.

The principle procedures of this approach were 1) to synthesize benzimidazole and carbaryl tracers, 2) to prepare amino plates, 3) to immobilize β-CD on prepared amino plates, 4) to examine whether the immobilized β–CD could encapsulate benzimidazole or carbaryl tracers, 5) to optimize benzimidazole tracer encapsulation and 6) to detect carbendazim standard and carbendazim spiked fruit sample using the β-CD immobilized on prepared and commercially available amino plates. Finally, the immobilized CD on prepared amino microtiter plate was further applied for the determination of carbaryl.

3.1 Benzimidazole and carbaryl tracers synthesis

Benzimidazole tracer was synthesized to be used for the determination of benzimidazole fungicide based on competitive-inhibition enzyme immunoassay method. This method was also adapted for the determination of carbaryl. Therefore, it was necessary to also synthesize carbaryl tracer for the competitive-inhibition with carbaryl.

Benzimidazole and carbaryl tracers were horseradish peroxidase (HRP) conjugated to either 2-succiamidobenzimidazole (SAB) or 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU), respectively. On account of the carbendazim and carbaryl structures, they did not have reactive functional groups for the conjugation. Therefore, SAB and CPNU which had carboxyl groups in the molecules were constructed. Structures of carbendazim and SAB are shown in ***Figure 3.1*** and those of carbaryl and CPNU are shown in ***Figure 3.2***. SAB and CPNU were then conjugated to HRP. There

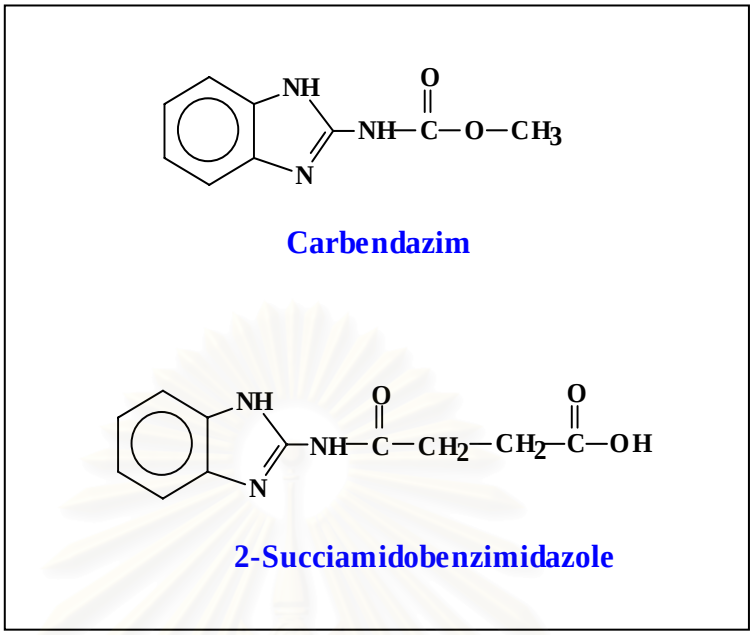


Figure 3.1. Chemical structures of carbendazim and 2-succiamidobenzimidazole.

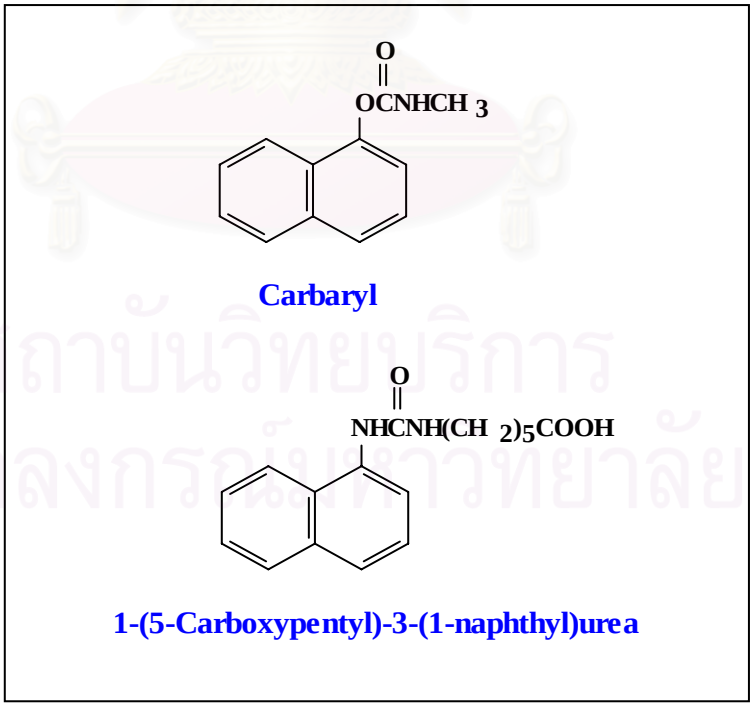


Figure 3.2. Chemical structures of carbaryl and 1-(5-carboxypentyl)-3-(1-naphthyl) urea.

have been reports regarding carbendazim and carbaryl can be encapsulated into CD cavity (Barbato *et al*., 2000; Lezcano *et al*., 2002; Saikosin *et al*., 2002). Hence, SAB and CPNU which have similar structures observed by similar absorption spectrum (***Figure 3.3 and 3.4***) to carbendazim and carbaryl, respectively should also be encapsulated and could be used to investigate the amount of immobilized CD on microtiter plate surface. Inclusion complexes occurred between either benzimidazole tracer and CD or carbaryl tracer and CD could be detected by HRP activity assay.

3.1.1 SAB and CPNU synthesis

SAB was synthesized from 2-aminobenzimidazole and succinic anhydride (***Figure 3.5***) according to Newsome and Shields (1981). SAB, which was used in this study, was received from Jongmeevasana (2000).

CPNU was synthesized from 1-naphthyl isocyanate (1.7 g, 0.01 mol) and 6-aminohexanoic acid (1.3 g, 0.01 mol) as shown in ***Figure 3.6.*** The method was previously described by Marco and his co-workers (1993). The white solid product (0.719 g, 54.8% yield) obtained and its functional groups were analysed by ^{1}H and ^{13}C NMR spectroscopy (Scientific and Technological Research Equipment Center, CU). The ^{1}H NMR spectrum in ***Figure 3.7*** showed that CPNU molecule consisted of –CH in aromatic ring, –NH group and –COOH group. The ^{13}C NMR spectrum in ***Figure 3.8*** similarly showed –CH in aromatic ring, $-CH_2$ and –COOH groups. This corresponded to the structure of CPNU shown in ***Figure 3.2*** and thus, confirmed that the product obtained was CPNU. MS+H spectroscopy was used for the determination of its molecular mass (National Science and Technology Development Agency). ***Figure 3.9*** shows that the molecular mass of CPNU was 301 daltons and this also corresponded to its structure and previous report (Marco *et al*., 1993).

3.1.2 Conjugation and purification of benzimidazole and carbaryl tracers (SAB-HRP and CPNU-HRP conjugates)

SAB and CPNU were coupled to HRP as benzimidazole and carbaryl tracers, respectively by 1-(3-(dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. Carbodiimide was used for the synthesis of peptide bond between carboxyl group of SAB or CPNU and amino group of HRP (***Figure 3.10***). Following the conjugation, the solution was concentrated by Centricon (membrane MW cut-off of 10,000 Da) before subjected to Sephadex G-25 gel filtration in order to remove excess SAB or CPNU. The

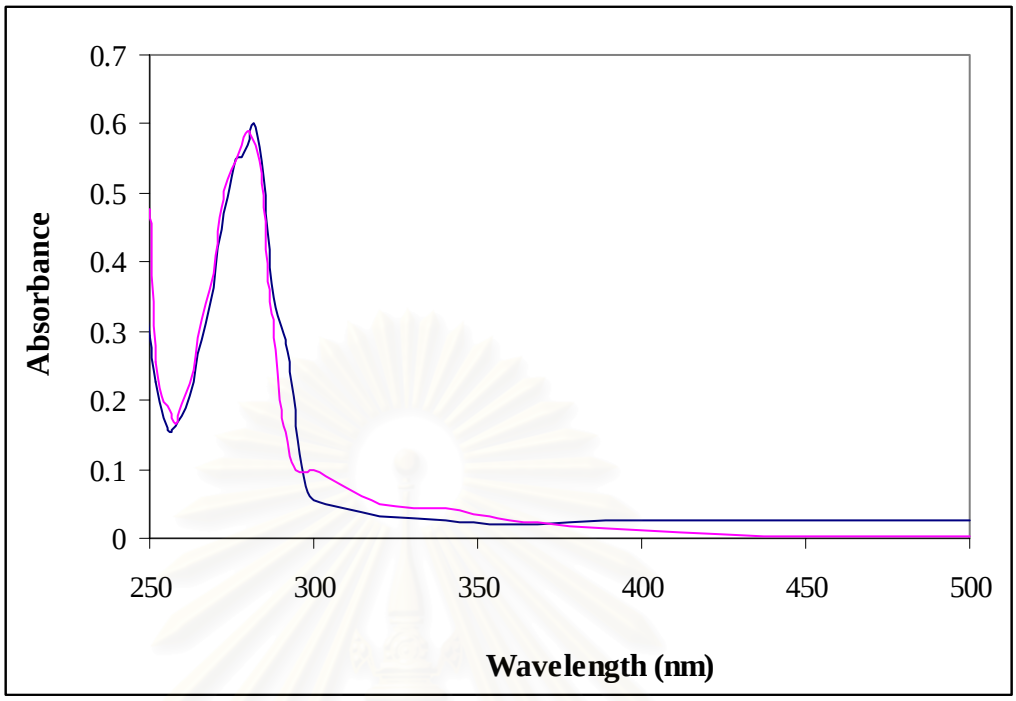


Figure 3.3. Absorption spectra of carbendazim (——) and 2-succiamidobenzimidazole (——).

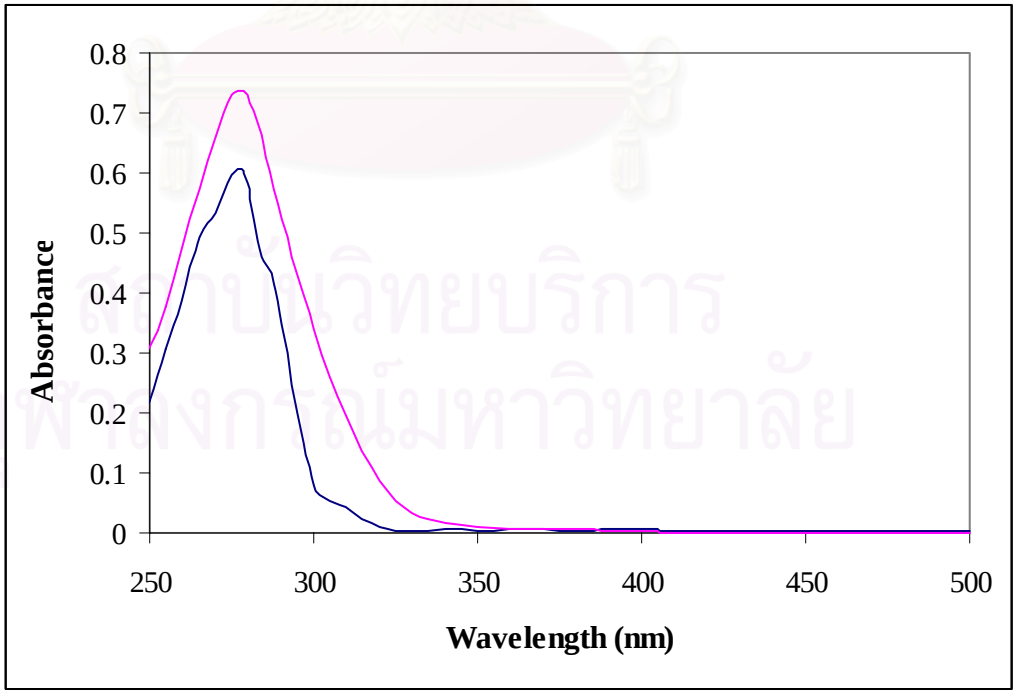


Figure 3.4. Absorption spectra of carbaryl (——) and 1-(5-carboxypentyl)-3-(1-naphthyl) urea (——).

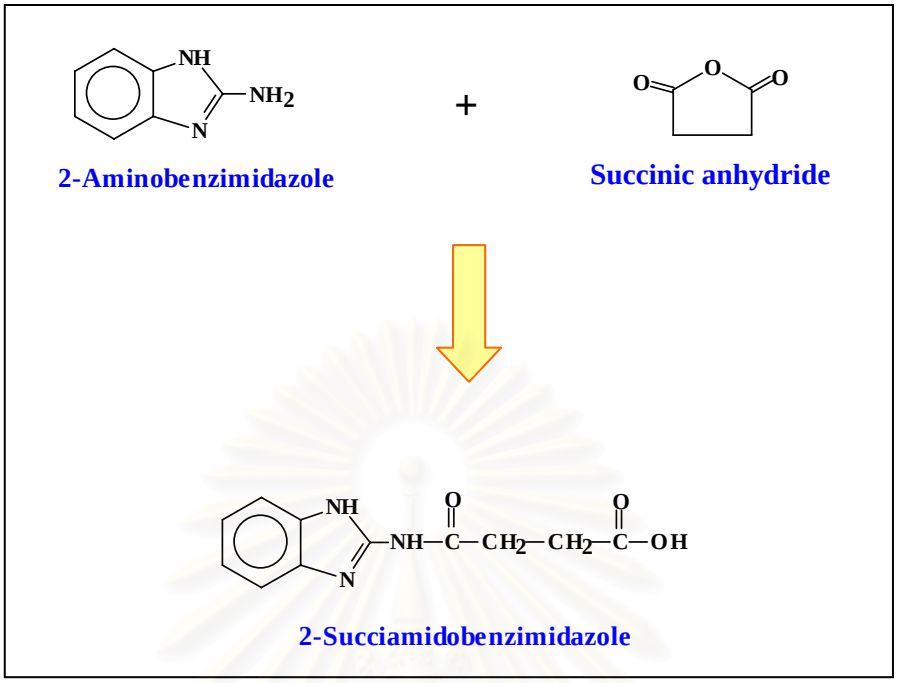


Figure 3.5. Synthesis of 2-succiamidobenzimidazole (SAB) from 2-aminobenzimidazole and succinic anhydride.

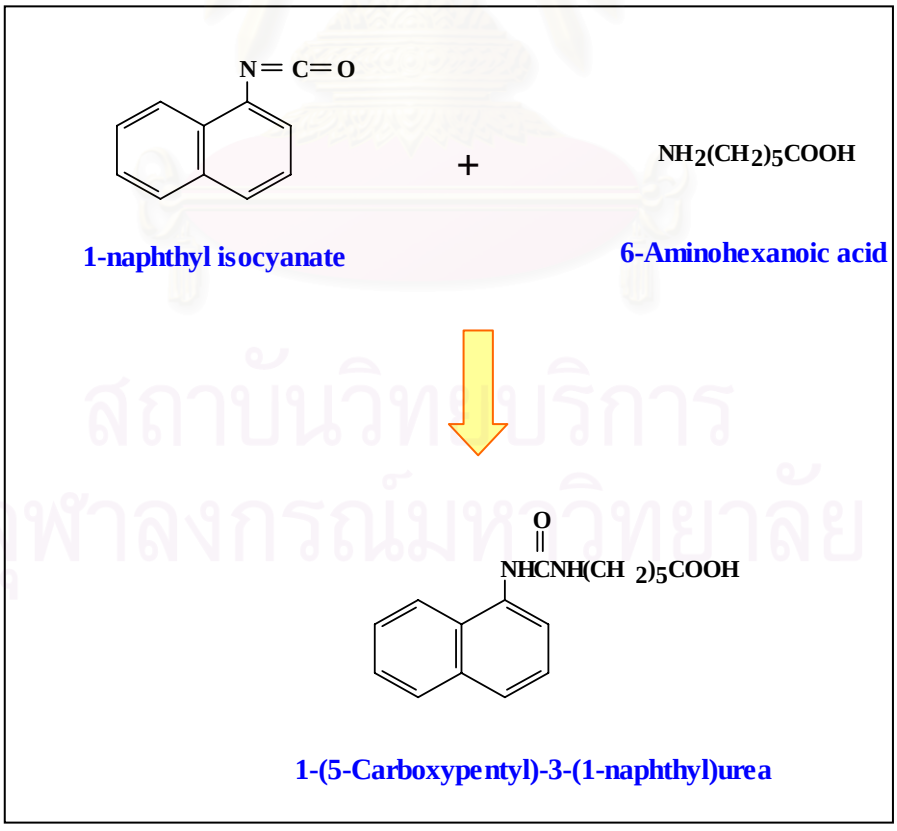


Figure 3.6. Synthesis of 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU) from 1-napthyl isocyanate and 6-aminohexanoic acid.

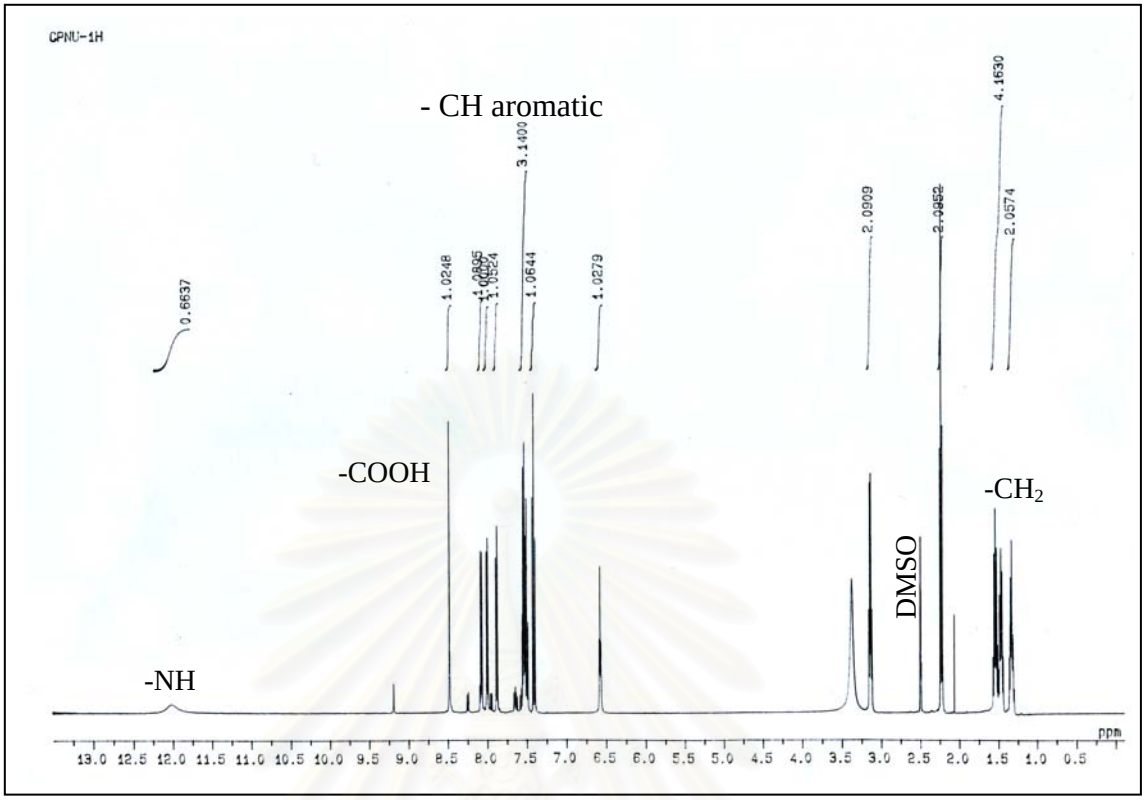


Figure 3.7. ^{1}H NMR spectra of 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU): *CPNU was dissolved in DMSO for the determination.*

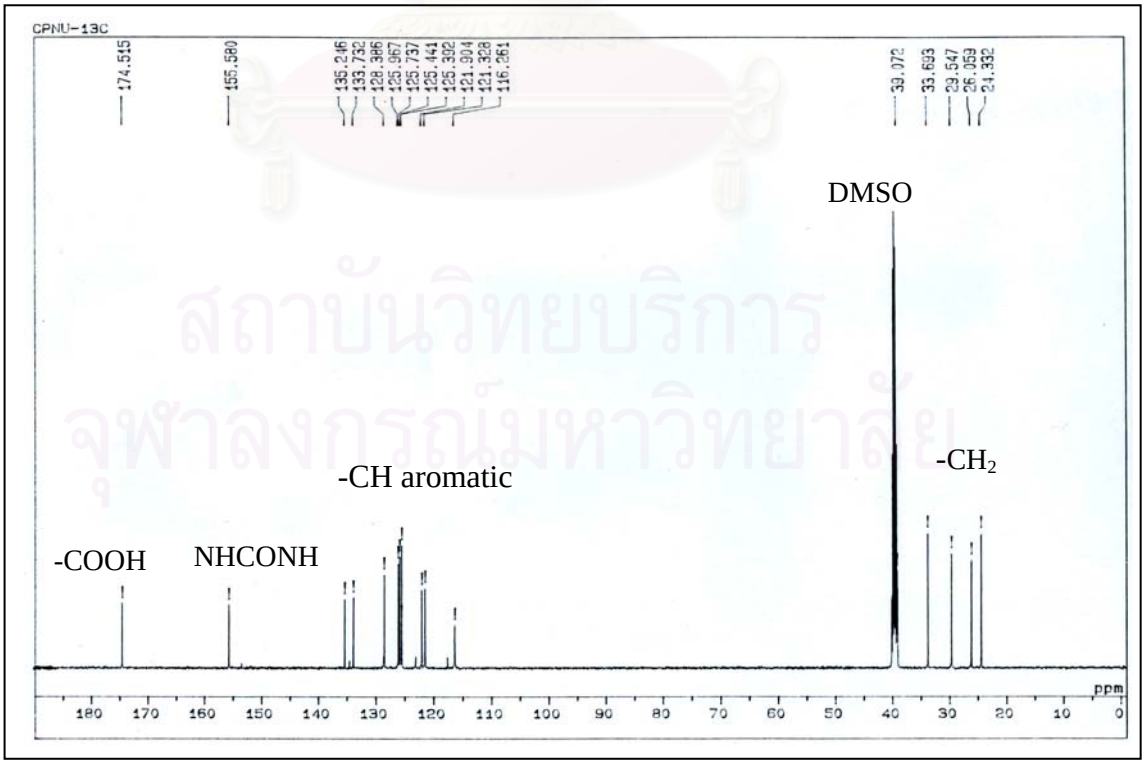
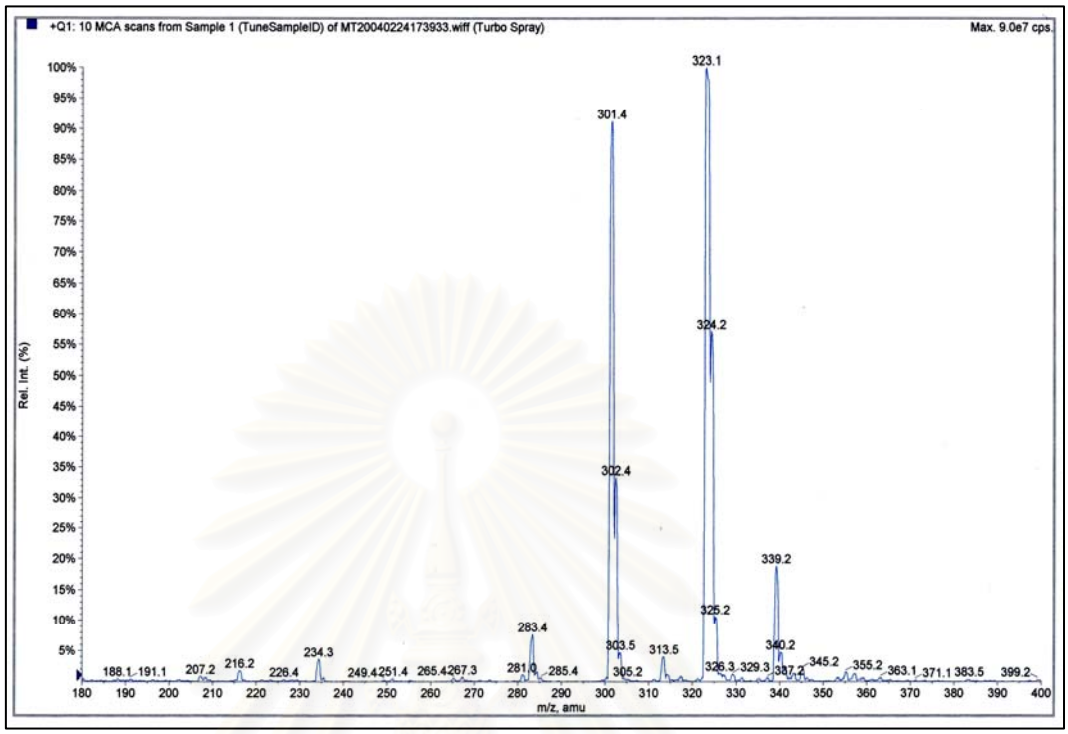


Figure 3.8. ^{13}C NMR spectra of 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU): *CPNU was dissolved in DMSO for the determination.*

a)



b)

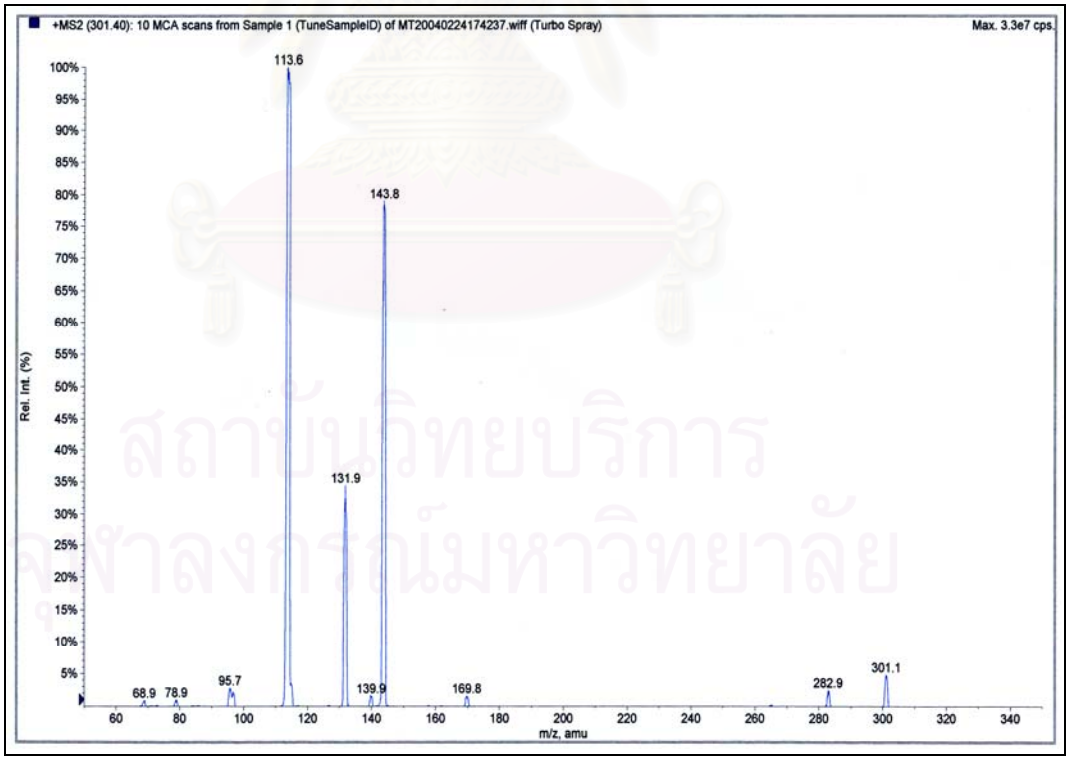


Figure 3.9. MS+ H spectra of 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU): *CPNU was dissolved in acetronitrile for the determination: a) Total Ion Chromatogram (TIC) of synthesized product, b) Mass spectrum of peak 301.4 obtained from a LC/MS/MS analysis.*

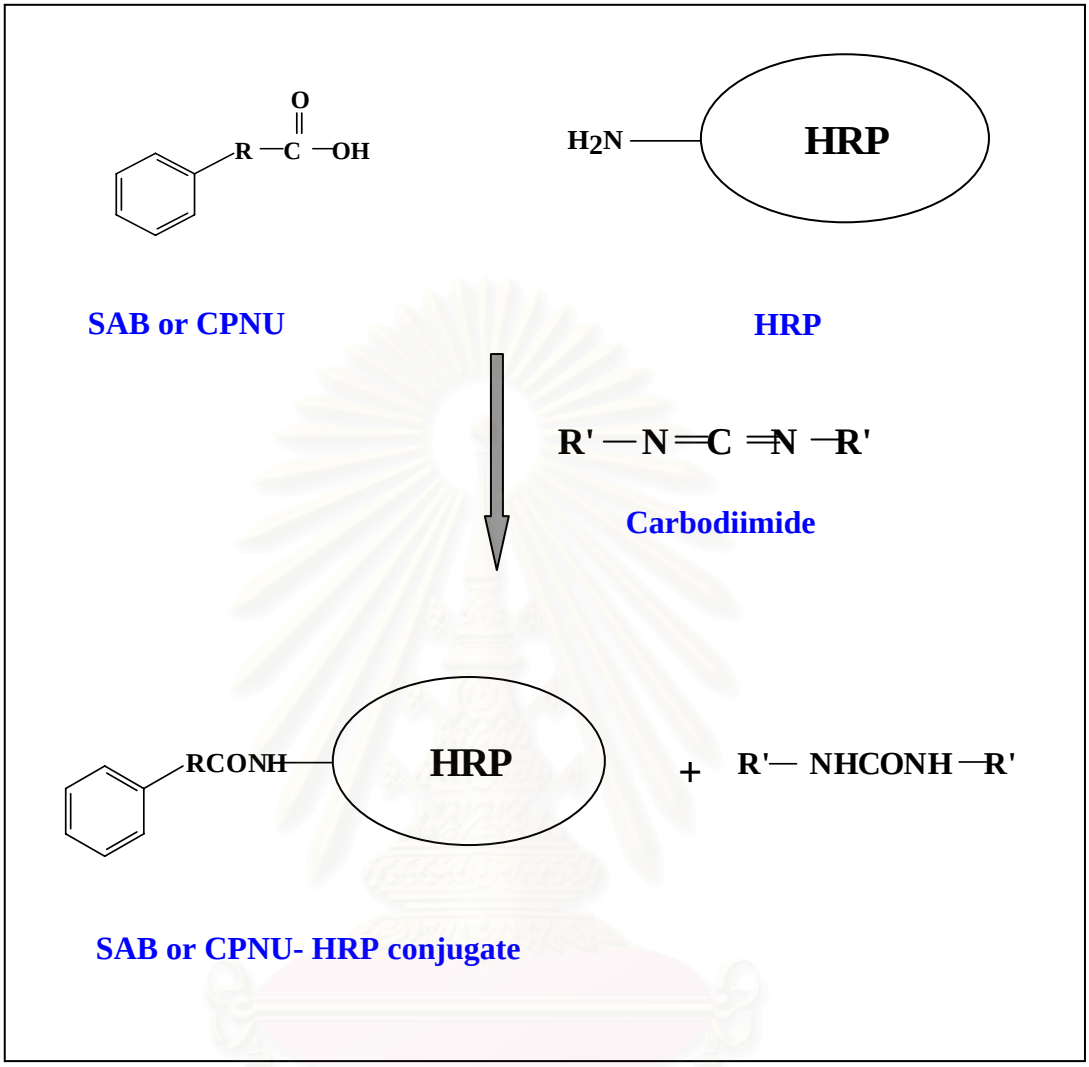
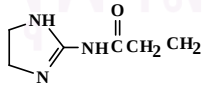
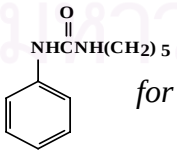


Figure 3.10. Schematic diagram showing the synthesis of peptide bond between HRP and SAB or CPNU by the use of carbodiimide (Bauminger and Wilchek, 1980). *HRP = horseradish peroxidase; SAB = 2-succiamidobenzimidazole; CPNU = 1-(5-carboxypentyl)-3-(1-napthyl) urea.*

R =  *for SAB or R =*  *for CPNU and R' is aliphatic.*

resulting purification profile of SAB-HRP conjugate is shown in ***Figure 3.11***. A single peak of HRP (A_{405}) was obtained after elution with 5 mM phosphate buffer saline (PBS) containing 0.15 M NaCl, pH 7. Fractions 4 to 6 were then pooled.

Figure 3.12 shows the purification profile of CPNU-HRP conjugate using the same column as described above and fractions number 3 to 5 which contained HRP were pooled. Another A_{280} peak without HRP conjugated was found suggesting that an excess of CPNU was separated from the CPNU-HRP conjugate. Some properties of both benzimidazole and carbaryl tracers were then examined and compared to those of native HRP.

3.2 Characterization of benzimidazole and carbaryl tracers

Benzimidazole and carbaryl tracers (modified HRPs) were examined for their biochemical properties such as their maximum absorption and recovered activity. These were then compared with the native HRP.

3.2.1 The maximum absorption of benzimidazole and carbaryl tracers

The absorption spectra of HRP, SAB, CPNU and the modified HRPs were compared. They were prepared in 5 mM PBS and analyzed over the wavelength range of 250-450 nm. The absorption spectra of native HRP, SAB-HRP conjugate and SAB are shown in ***Figure 3.13***. The maximum absorption peak of SAB was at 282 nm and two peaks at 280 and 405 nm were found for the native HRP. For SAB-HRP conjugate, two maximum absorption peaks were observed at 282 and 405 nm. Under the same concentration of native and modified HRP (defined by the absorbance at 405 nm), the absorbance at 280 nm of SAB-HRP was much higher than that of the native one and slightly shifted towards 282 nm. This confirmed that SAB was coupled to HRP. Thus, significantly enhanced the maximum absorption at 280 nm. In the case of carbaryl tracer (***Figure 3.14***), HRP also had two maximum absorption peaks and CPNU exhibited maximum absorption peak at 277 nm. When CPNU-HRP conjugate was examined, the maximum absorption was observed at 405 nm with a broad spectrum at around 280 nm. In comparison with the spectrum of native HRP, there was only slight difference between CPNU-HRP conjugate and the native one. When CPNU-HRP counjugate was further used as carbaryl tracer for the determination of carbaryl by competitive encapsulation, this can also prove that CPNU was conjugated to HRP if HRP activity was observed after encapsulation by the immobilized CD.

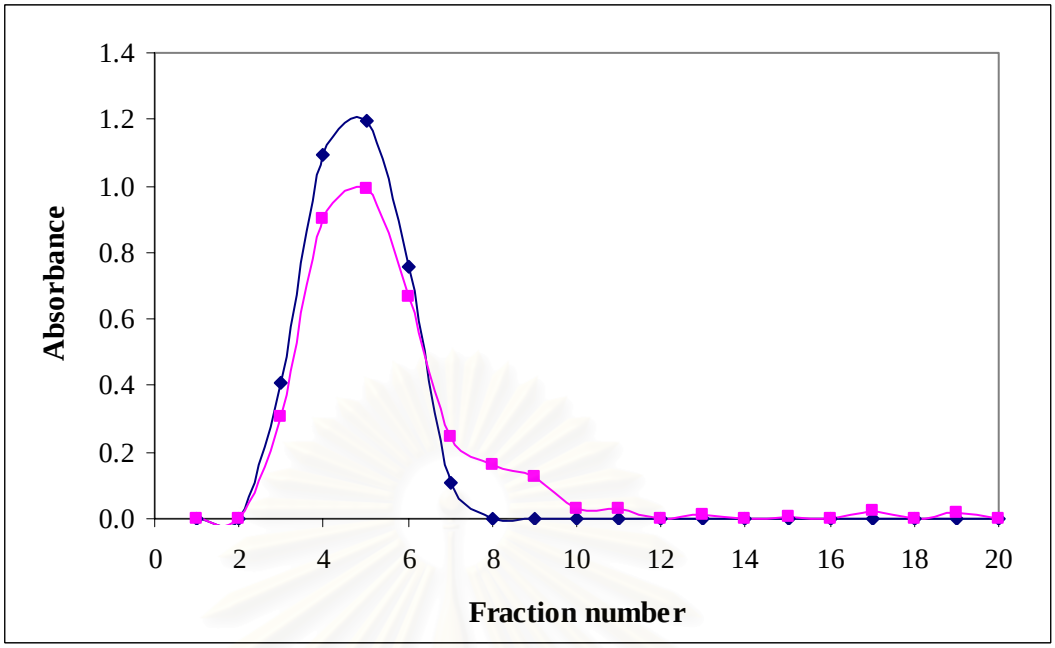


Figure 3.11. Purification profile of SAB-HRP conjugate using Sephadex G-25 column. *The profile shows the absorbance at 405 nm (◆) and 280 nm (■). A 0.5 ml fraction was collected from gel filtration column and purified conjugate was eluted with 5 mM phosphate buffer containing 0.15 M NaCl, pH 7.*

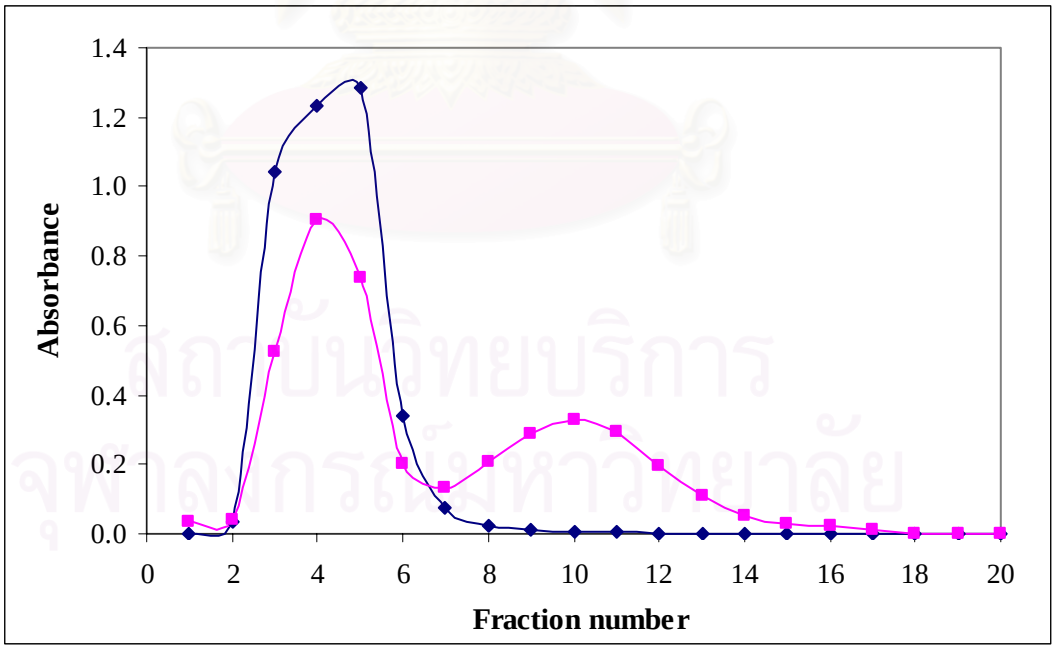


Figure 3.12. Purification profile of CPNU-HRP conjugate using Sephadex G-25 column. *The profile shows the absorbance at 405 nm (◆) and 280 nm (■). A 0.5 ml fraction was collected from gel filtration column and purified conjugate was eluted with 5 mM phosphate buffer containing 0.15 M NaCl, pH 7.*

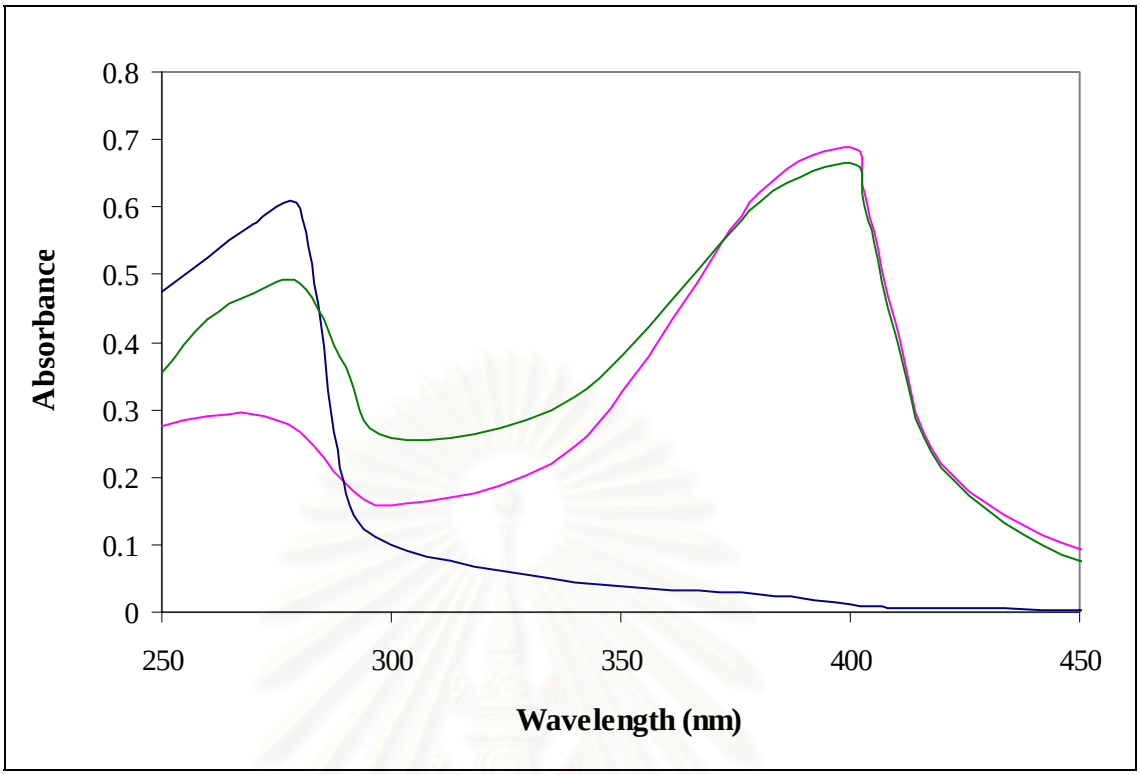


Figure 3.13. Absorption spectra of horseradish peroxidase (——), benzimidazole tracer (——) and 2-succiamidobenzimidazole (——).

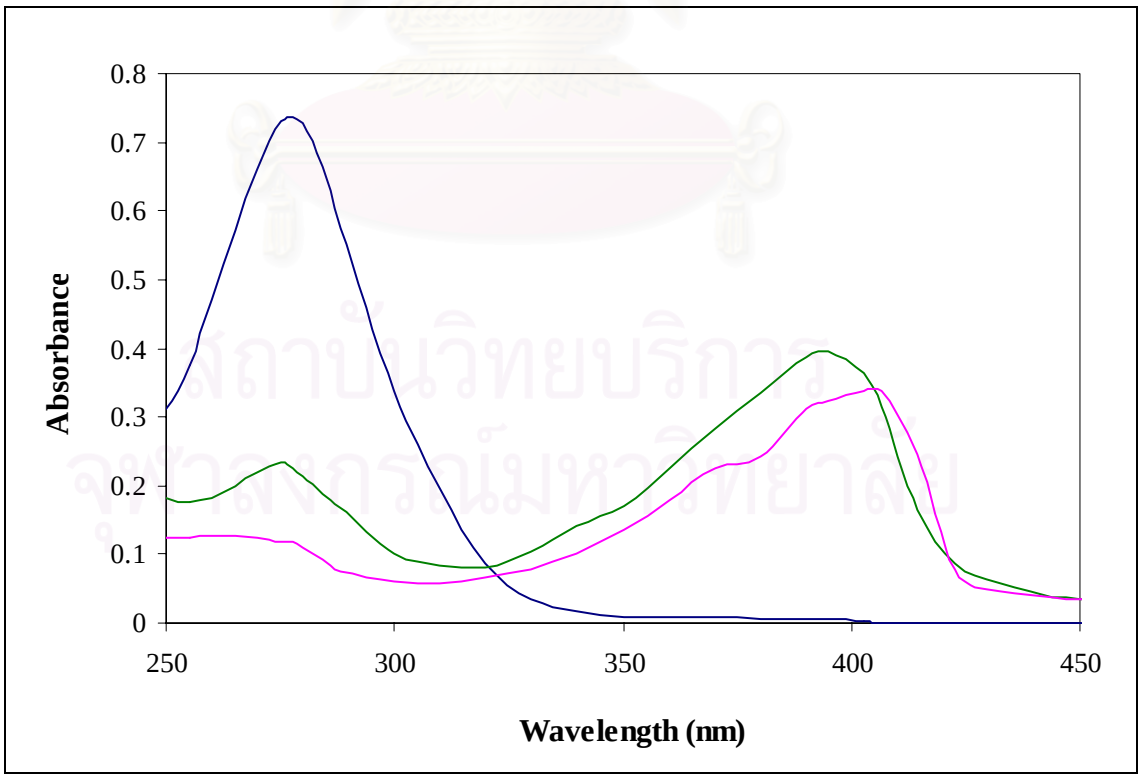


Figure 3.14. Absorption spectra of horseradish peroxidase (——), carbaryl tracer (——) and 1-(5-carboxypentyl)-3-(1-napthyl) urea (——).

3.2.2 The activity of benzimidazole tracer

The specific activities of native HRP, SAB-HRP and CPNU-HRP conjugates were examined spectrophotometrically using substrate solution containing TMB and H_2O_2 in 0.1 M sodium acetate/ citric acid, buffer pH 5. As presented in ***Table 3.1***, the specific activities of HRP, SAB-HRP and CPNU-HRP were calculated to be 2707, 2224 and 2464 unit/mg, respectively. The relative activity of SAB-HRP was 82% whereas CPNU-HRP was 90% compared with the native HRP.

3.3 Preparation of amino plate

As mentioned previously that the commercially available amino plate is very expensive. Therefore, the purpose of this experiment was to prepare the amino plate using methyl vinyl ether-maleic anhydride copolymer (MMAC) for the immobilization of β-CD. Satoh *et al*. (1998) have reported the successful use of MMAC for the immobilization of small biomolecules such as peptides and linear oligosaccharides.

First, the polystyrene microtiter plates were coated with MMAC. MMAC is a water-insoluble polymer containing active acid anhydride groups which can react with the amino groups of ligands to form stable covalent amide bonds (Isosaki *et al.*, 1992 and Satoh *et al.*, 1998). Amino groups or hydrazino groups were then introduced to the MMAC-coated plates with the addition of either 1, 6 hexamethylenediamine (HMDA) or adipic acid dihydrazide (ADHZ), respectively (***Figure 3.15***).

3.4 Determination of introduced amino and hydrazino groups to MMAC-coated plates

A derivative of biotin, the sulfo N-hydroxysuccinimide ester (sulfo-BNHS), was used in order to determine the amino and hydrazino groups on MMAC-coated plates. The addition of sulfo-BNHS resulted in the biotinylation to both plates and the amount of biotin bound was assessed by the addition of avidin-peroxidase conjugate (AV-HRP) as shown in ***Figure 3.16***. To confirm the results were a consequence of surface biotinylation through MMAC and HMDA (or ADHZ), two control experiments were performed. The first control experiment was carried out to determine whether there was non-specific adsorption of HMDA or ADHZ onto the plate. HMDA or ADHZ was added to the plate with no MMAC, and after incubation, sulfo-BNHS and AV-HRP solution were added, respectively. In this control experiment, the non-specific adsorption of HMDA (***Figure 3.17a***) and ADHZ (***Figure 3.17b***) was found to be 32 and 30 % of the specifically bound value. In the second control experiment, sulfo-BNHS and AV-HRP solutions were incubated with the plate with no MMAC and HMDA or ADHZ to see if there was any non-specific binding of AV-HRP to the plate. After the HRP activity was

Table 3.1. Specific activities of native HRP and modified HRPs

Samples	Specific activity (Unit/mg)	Relative activity (%)
HRP	2707	100
SAB-HRP	2224	82
CPNU-HRP	2464	90

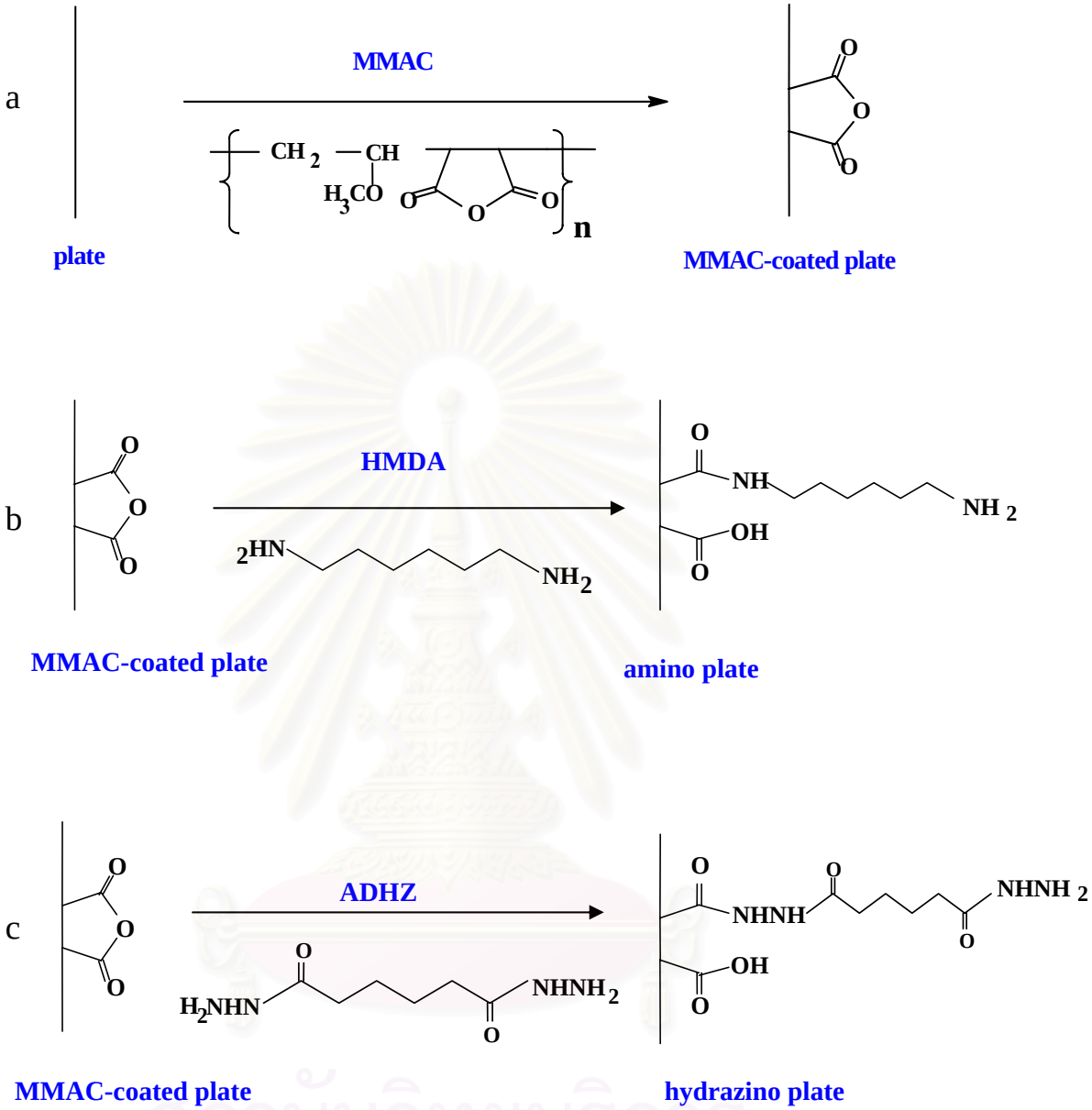
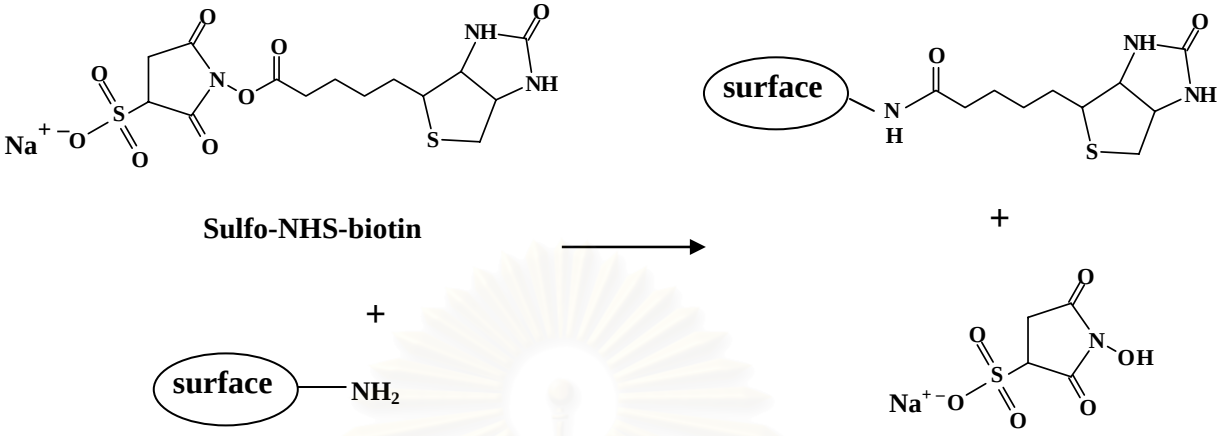
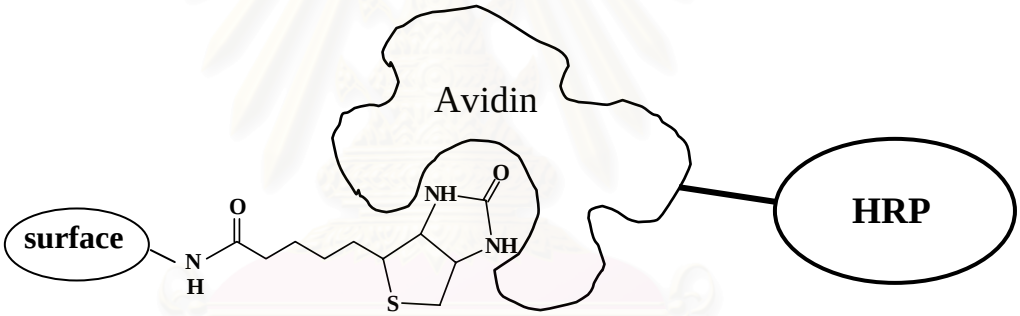


Figure 3.15. Introduction of amino and hydrazino groups to MMAC-coated plate. *Acid anhydride groups of MMAC were coated on the well (a) and they were coupled by amino groups of HMDA (b) or hydrazino group of ADHZ (c).*



a.



b.

Figure 3.16. Schematic diagram illustrating the determination of amino surface using the avidin and biotin system*. a) Sulfo-NHS-biotin binds to the amino group on the plate surface. b) Avidin-HRP conjugate then binds specifically to the biotinylated surface.*

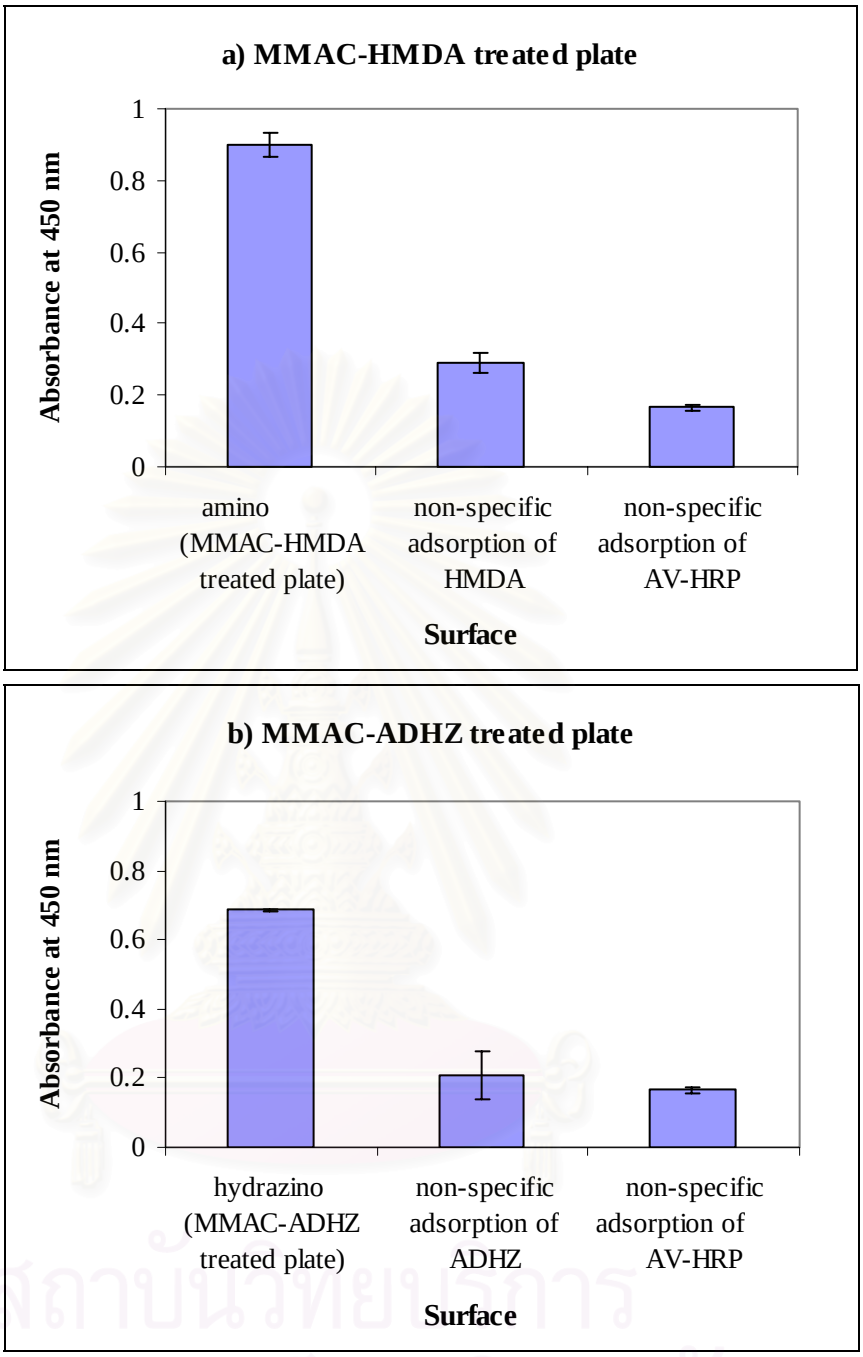


Figure 3.17. Specific and non-specific biotinylation of sulfo-NHS-biotin on a) MMAC-HMDA treated plate and b) MMAC-ADHZ treated plate. *1.25 mg/ml of MMAC, 10 mg/ml of HMDA and 10 mg/ml of ADHZ were coated to the plate in the case of biotinylation through MMAC and HMDA or ADHZ. For control experiments, some solutions were omitted. The concentration of AV-HRP at 0.625 μg/ml and 1 mg/ml of sulfo-NHS-biotin were used. Bars: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

determined, it was found that the non-specific adsorption of AV-HRP to a suitably "blocked" surface was only 18 and 24 % of the specifically bound AV-HRP on MMAC-HMDA and MMAC-ADHZ treated plates, respectively. Hence, it was concluded that the biotinylation was achieved specifically through MMAC and HMDA or ADHZ bridge.

Next, the amounts of amino groups introduced to the plate in this study were compared with those of the commercially available plate. From ***Figure 3.18***, it can be seen that sulfo-BNHS added was biotinylated in the dose-dependent manner until it reached saturation and the amounts of amino groups introduced to both HMDA and ADHZ-treated plates were much larger than those of commercially available amino plates. At saturation for biotinylation, the signal from HMDA-treated plates was slightly higher than that of ADHZ-treated plates. Therefore, HMDA was chosen for the preparation of amino plates.

3.5 Immobilization of cyclodextrin on MMAC-HMDA treated plate

After the polystyrene plates were coated with MMAC, amino groups were introduced by HMDA. The β-CDs were then covalently immobilized on MMAC-HMDA treated plate using divinyl sulfone as a crosslinker. Divinyl sulfone (DVS) is a bifunctional cross-linking reagent, which can react with both amino and hydroxyl groups. Thus, DVS could be used to cross-link the amino groups on the plate surfaces and hydroxyl groups of β-CDs. The use of divinyl sulfone for carbohydrate immobilization on amino polystyrene plate surface was previously described by Hatakeyama *et al.* (1996). ***Figure 3.19*** shows the strategy for CD immobilization on amino microtiter plate.

3.6 Capability test of immobilized β-CD –microtiter plate for benzimidazole tracer encapsulation

After the immobilization, the amount of immobilized β-CD was determined by the colour development of SAB-HRP conjugate used as benzimidazole tracer. In addition, the ability of β-CD for guest encapsulation can be assessed. In the initial experiment, 2% (w/v) of β-CD and 0.40 μg/ml of SAB-HRP conjugate were used. To prove that β-CD was covalently immobilized through MMAC-HMDA-DVS bridge, various control experiments were performed. This was done by the addition of SAB-HRP on different types of surfaces: 1) amino (MMAC-HMDA), 2) DVS (MMAC-HMDA-DVS),

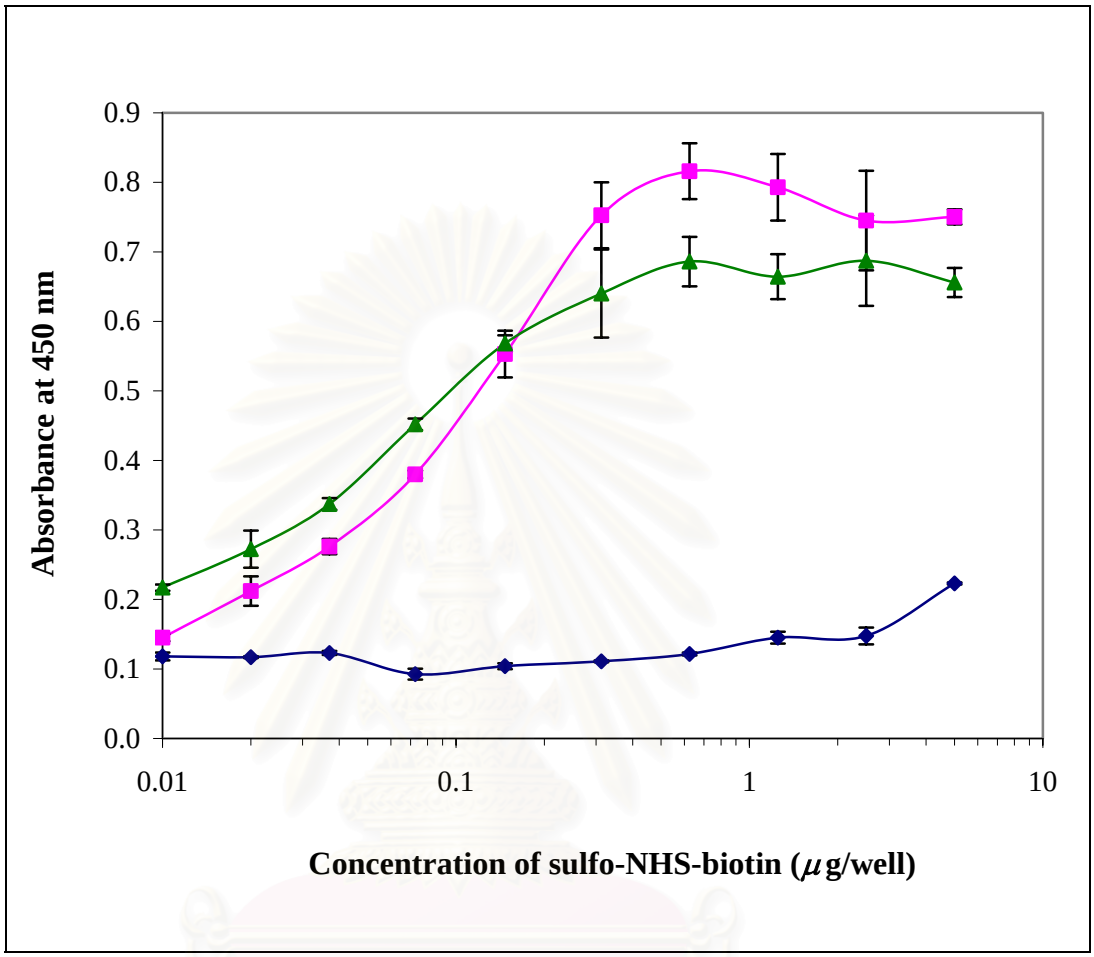


Figure 3.18. Comparison of the amounts of amino groups on the plate.

The amounts of amino groups on amino plates prepared with HMDA (■) or ADHZ (▲) and commercially available amino plate (◆) were determined with sulfo-NHS-biotin. Points: means of triplicate wells. Error bars represent the standard deviation of triplicate wells.

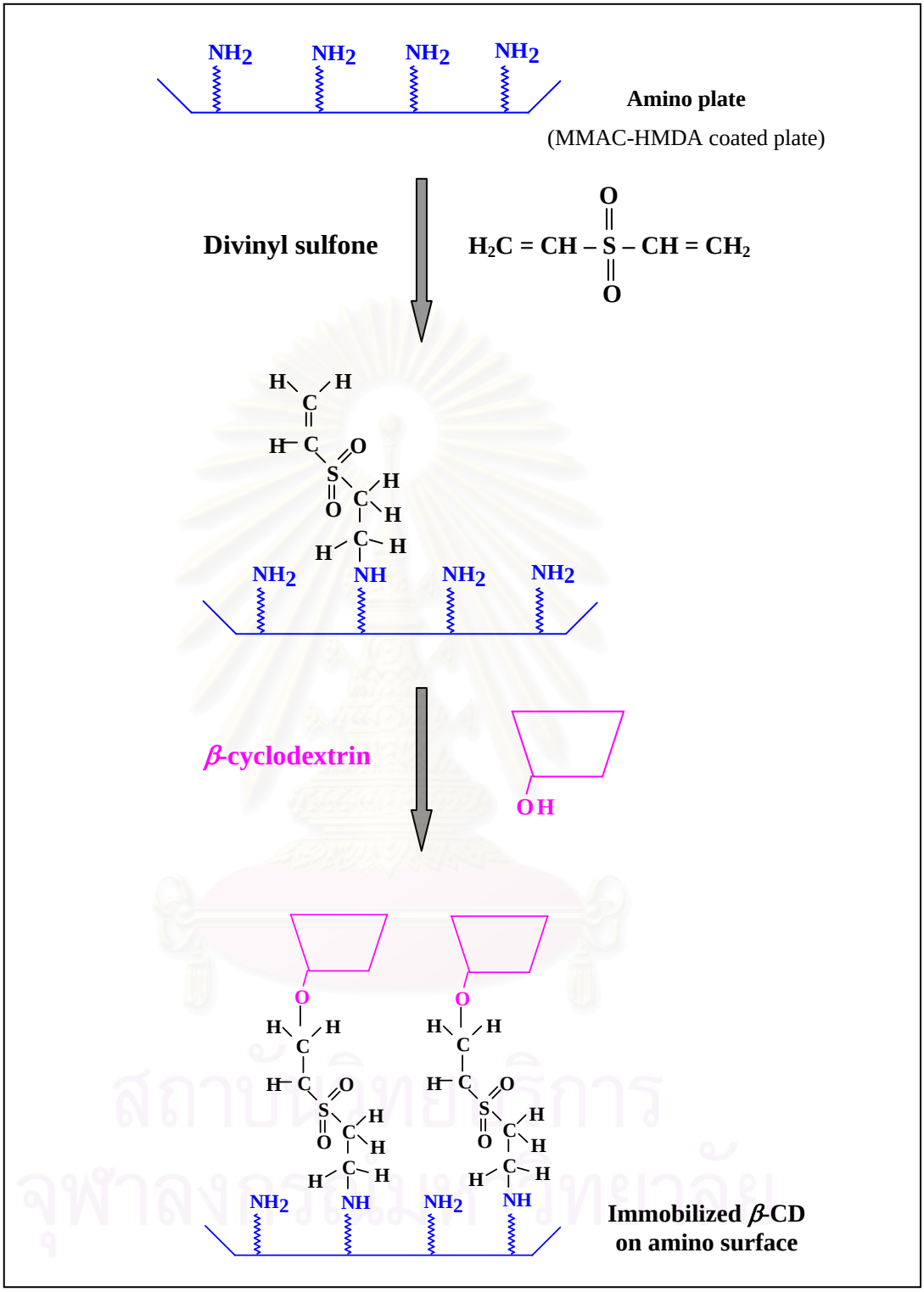


Figure 3.19. Schematic diagram showing the immobilization of cyclodextrin (CD) on amino plate by the use of divinyl sulfone (DVS) as a crosslinker.

3) adsorbed CD (MMAC-HMDA-β-CD) and 4) immobilized CD (MMAC-HMDA-DVS-β-CD). The specific binding of SAB-HRP conjugate by CD encapsulation was studied on the adsorbed and immobilized CD surfaces. While the amino and DVS surfaces were used to investigate non-specific binding of SAB-HRP conjugate. In another set of experiments, native HRP at the same concentration was used instead of SAB-HRP to see if the immobilized HRP activity observed was due to SAB-HRP encapsulation or just HRP adsorption. After HRP and SAB-HRP conjugate were incubated with various types of surfaces and washed with 0.1 M sodium acetate/ citric acid buffer, pH 5, containing 0.5 M NaCl, the HRP activity was then determined. ***Figure 3.20*** shows the amount of HRP activity bound to each surface. To compare binding behavior between native HRP and SAB-HRP conjugate, it was found that the activities of the native HRP bound to all surfaces were significantly lower than SAB-HRP conjugate activities. This suggested that HRP showed little adsorption on surfaces and could not encapsulate into CD cavity. For SAB-HRP conjugate, although considerably greater amounts were bound when compared to native HRP, the signals were comparable to those found when CD was simply adsorbed in the wells and high non-specific adsorption of SAB-HRP was also observed on DVS and amino surfaces. The variation in immobilized SAB-HRP activity in each experiment was also found to be high witnessed by the large error bars. This could be due to the low concentration of SAB-HRP conjugate used for encapsulation or the low concentration of β-CD used for immobilization. To maximize the specific binding of SAB-HRP and minimize its non-specific adsorption, concentration of MMAC, β-CD and benzimidazole tracer and type of washing buffer were optimized.

3.6.1 Effect of MMAC concentration

To investigate the influence of MMAC concentration on the immobilization of β-CD, the plates were coated with different concentration of MMAC varying from 0.25 to 5 mg/ml in DMSO. The amino groups were introduced to the MMAC coated plates by 10 mg/ml of HMDA and 2% (w/v) of β-CDs were then immobilized on the amino plates through DVS. Benzimidazole tracer at the concentration of 10 μg/ml was used to give concentration 0.5 μg/well (50 μl) for encapsulation by immobilized CD. Three control experiments were carried out in the same way as described in section 3.6. It can be seen in ***Figure 3.21*** that 0.5 and 1.25 mg/ml of MMAC showed the highest signals of SAB-HRP conjugate on immobilized CD SAB-HRP on

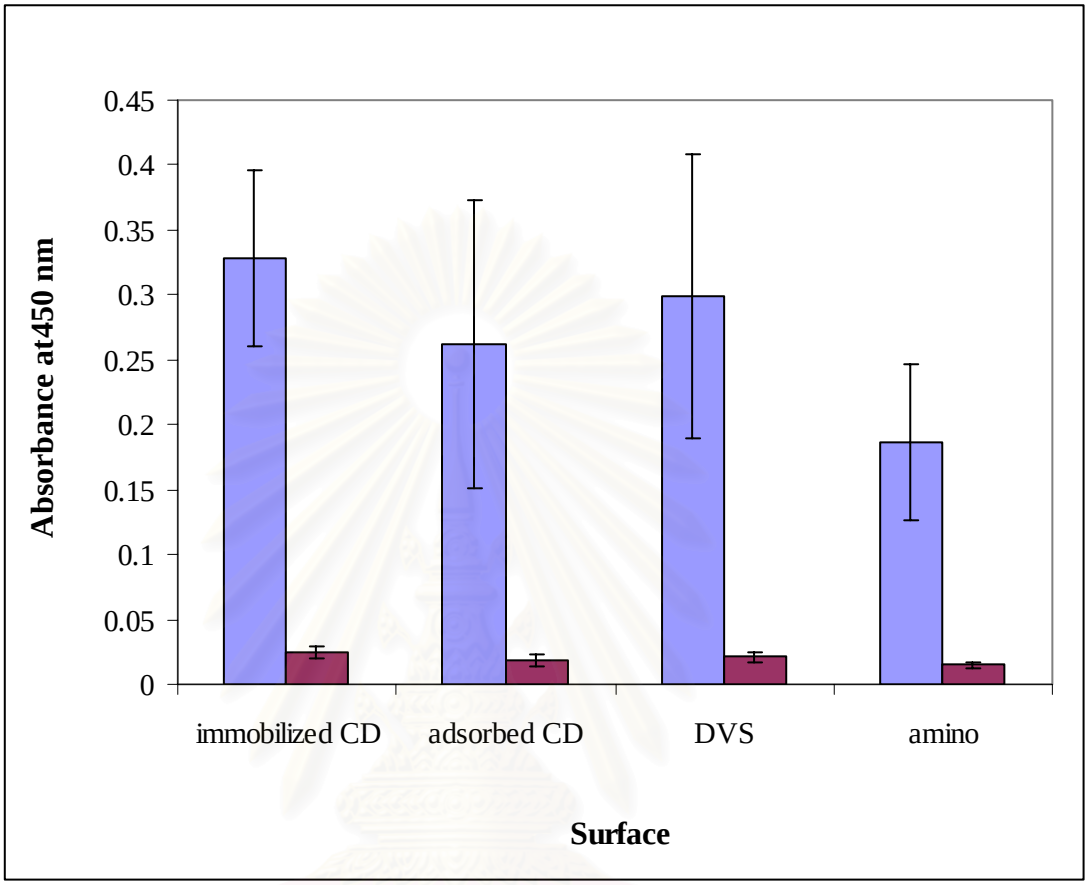


Figure 3.20. The specific and non-specific binding of benzimidazole tracer and HRP onto different types of surfaces*. 1.25 mg/ml of MMAC was coated and 10 mg/ml of HMDA was added to the plate. 2% β-CD was immobilized on HMDA-treated plate. 0.40 μg/ml of benzimidazole tracer (■) and HRP (■) were used for binding. The immobilized CD, adsorbed CD, DVS and amino plate surfaces referred to as MMAC-HMDA-DVS-CD, MMAC-HMDA-CD, MMAC-HMDA-DVS and MMAC-HMDA surfaces, respectively. Bars: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

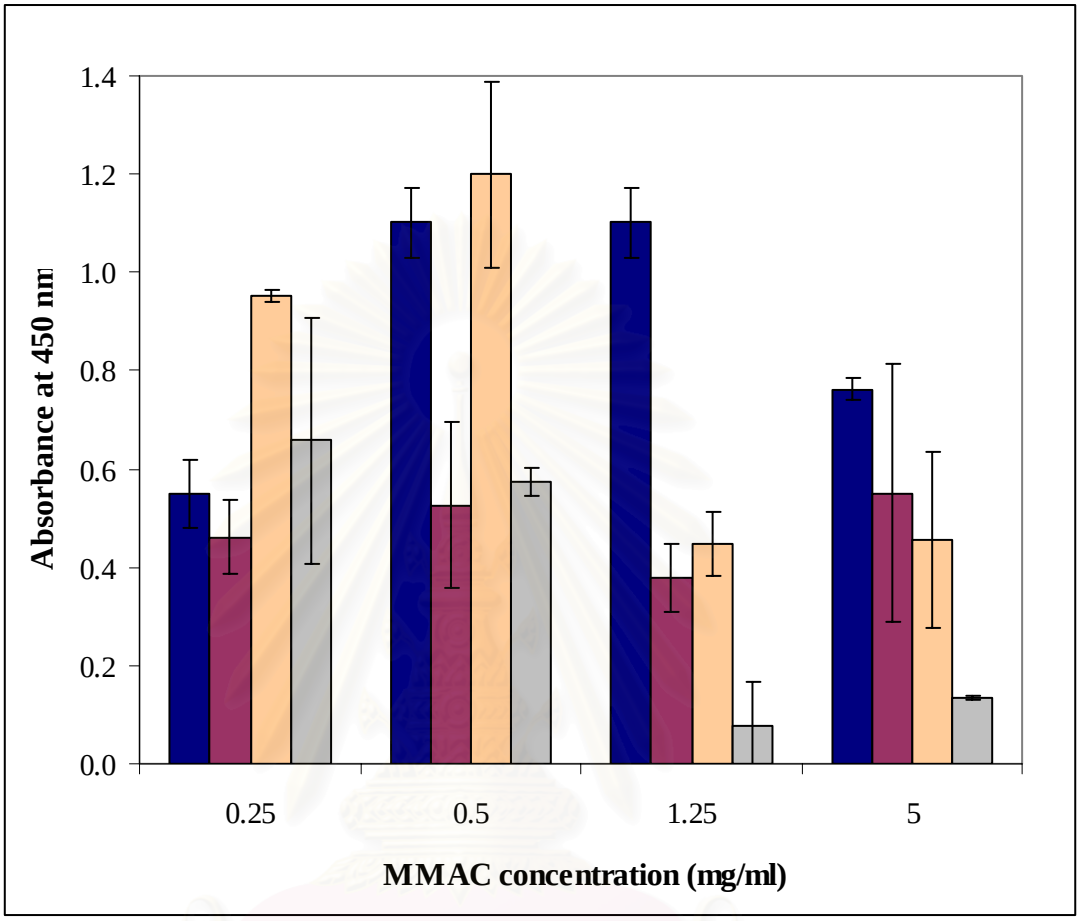


Figure 3.21. Influence of MMAC concentration on immobilization of β-CD to MMAC-HMDA treated plate. *Various concentrations of MMAC (0.25, 0.5, 1.25, 5 mg/ml) were coated and 10 mg/ml of HMDA was added to the plate. 2% β-CD was immobilized on HMDA-coated plate. 10 μg/ml benzimidaole tracer (SAB-HRP conjugate) was used for encapsulation. The immobilized CD (■), adsorbed CD (■), DVS (■) and amino plate (■) surfaces referred to as MMAC-HMDA-DVS-CD, MMAC-HMDA-CD, MMAC-HMDA-DVS and MMAC-HMDA surfaces, respectively. Bars: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

adsorbed CD was found to be higher as well. At 0.25 mg/ml of MMAC used, the signals of SAB-HRP conjugate immobilized on CD surface through MMAC-HMDA-DVS were lower than the non-specific adsorption of SAB-HRP. Moreover, when 5 mg/ml of MMAC was used, the SAB-HRP activity bound to immobilized CD and adsorbed CD surfaces were comparable. Hence, suitable MMAC concentration for the immobilization of β-CD was 1.25 mg/ml because it showed the highest difference between specific and non-specific immobilization of SAB-HRP.

3.6.2 Effect of β-CD concentrations

After the optimal concentration of MMAC was achieved, another parameter that needed to be optimized was the amount of β-CD used for immobilization to obtain enough sites for the interaction with SAB-HRP to yield coloured product. The plate was coated with 1.25 mg/ml of MMAC, followed by the addition of HMDA (10 mg/ml) to introduce amino groups. The amino plate was then immobilized with different concentration of β-CD through DVS, ranging from 1 to 5 % (w/v) in 0.5 M carbonate buffer, pH 11. The amount of immobilized or adsorbed CD was determined by 10 μg/ml of benzimidazole tracer. The control experiments were performed as described in section 3.6. As shown in ***Figure 3.22***, the plate covalently immobilized with 4% β-CD was found to give the highest coverage of SAB-HRP and the difference in HRP activities between specifically and non-specifically bound molecules was also found to be the highest. Therefore, 4% (w/v) of β-CD concentration was selected for the immobilization.

3.6.3 Effect of benzimidazole tracer concentrations

Further optimization for benzimidazole tracer encapsulation was performed by varying concentrations of benzimidazole tracer. The appropriate concentration of tracer was determined in order to cover all the guest binding sites on CD. However, too high tracer concentration may lead to high non-specific adsorption on the surface. The effect of benzimidazole tracer concentrations on its encapsulation is shown in ***Figure 3.23***. The control experiments were carried out as previously described in section 3.6. The benzimidazole tracer concentrations applied to the plates were varied from 0.3 to 20 μg/ml. As the amount of benzimidazole tracer added to the immobilized CD surface increased, the activity of HRP conjugate increased and reached saturation when 5 μg/ml of benzimidazole tracer was used. For other surfaces, adsorbed CD, DVS

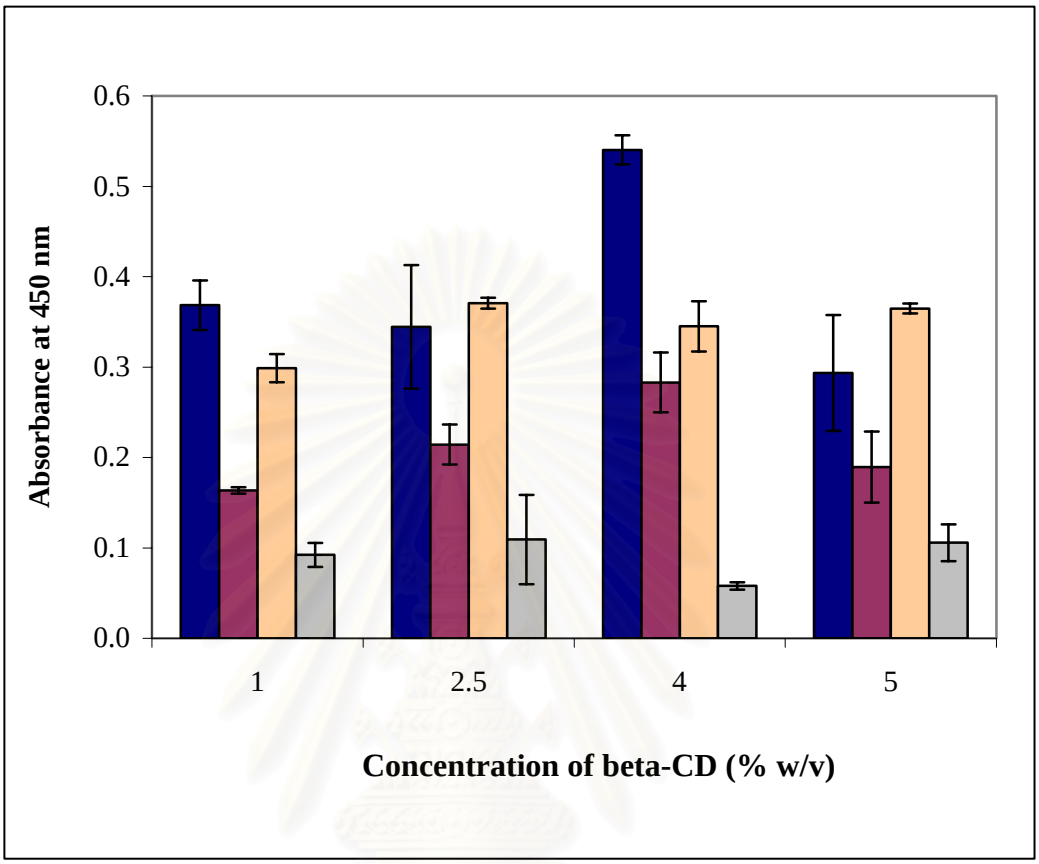


Figure 3.22. Effect of β-CD concentration on saturating the underlying surface. *Various concentrations of β-CD (1, 2.5, 4 and 5% w/v) were immobilized. 10 μg/ml benzimidaole tracer (SAB-HRP conjugate) was used for encapsulation. The immobilized CD (■), adsorbed CD (■), DVS (■) and amino plate (■) surfaces referred to as MMAC-HMDA-DVS-CD, MMAC-HMDA-CD, MMAC-HMDA-DVS and MMAC-HMDA surfaces, respectively. Bars: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

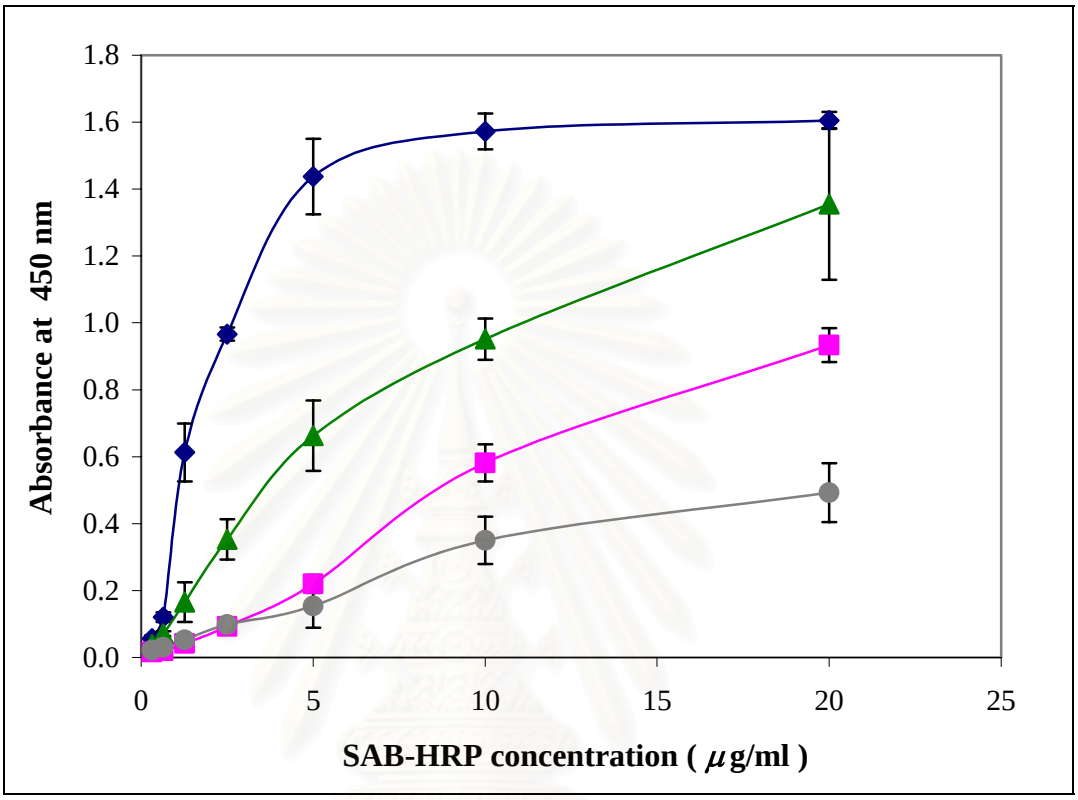


Figure 3.23. Effect of benzimidazole tracer concentration on encapsulation. *4% (w/v) β-CD was immobilized on MMAC-HMDA coated plate: 0.3-20 μg/ml benzimidazole tracer (SAB-HRP conjugate) was used for encapsulation. The immobilized CD (◆), adsorbed CD (■), DVS (▲) and amino plate (●) surfaces referred to as MMAC-HMDA-DVS-CD, MMAC-HMDA-CD, MMAC-HMDA-DVS and MMAC-HMDA surfaces, respectively. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

and amino surfaces, the signals displayed the same patterns but they were lower than those found in immobilized CD surfaces. In addition, benzimidazole tracer at the concentration of 5 μg/ml showed high contrast between specific and non-specific binding onto the underlying surfaces. This suggested that β-CD was successfully covalently immobilized on MMAC-HMDA treated plate through DVS and was still able to encapsulate SAB-HRP. Therefore, the concentration of benzimidazole tracer at 5 μg/ml was then chosen for the next experiment.

3.6.4 Effect of washing buffer types

From the previous experiment, some non-specific binding of SAB-HRP on the surfaces was observed. This could be due to either hydrophobic or electrostatic interaction between the two. Different types of washing buffer were then determined to reduce this non-specific interaction. It has been reported that high salt and high pH could disrupt the electrostatic interaction and the use of detergent e.g. Tween could disrupt the hydrophobic interaction. Many types of washing buffer such as 0.1 M sodium acetate/ citric acid buffer, pH 5, 0.1 M sodium acetate/ citric acid buffer, pH 5, containing 0.5 M NaCl, 0.1 M sodium acetate/ citric acid buffer, pH 5, containing 0.05% tween 20, 50 mM Tris buffer pH 7.5 (TB), 10 mM Tris buffer saline (TBS) containing 0.5 M NaCl and 0.5 M carbonate buffer pH 10 were used. The result is collected in ***Figure 3.24***. Three control experiments were also carried out as described in section 3.6. The ***Figure 3.24*** clearly shows that the interaction between SAB-HRP and DVS or amino plate can be disrupted by high salt concentration at acidic pH when 0.1 M sodium acetate/ citric acid buffer, pH 5 was used. When Tween and high pH buffer were used, not only non-specifically adsorbed SAB-HRP was removed but the SAB-HRP did not seem to be encapsulated into CD cavity.

As a result, 0.1 M sodium acetate/ citric acid buffer, pH 5, containing 0.5 M NaCl was selected to wash the non-specifically adsorbed SAB-HRP.

From ***Figure 3.21-3.24***, the signal of HRP conjugate obtained from immobilized CD was significantly higher than that of adsorbed CD surface. Hence, it was necessary to covalently immobilize CD on amino plate. The optimal conditions for covalent immobilization of β-CD were summarized as follows. Microtiter plate was coated with 200 μl of MMAC at the concentration of 1.25 mg/ml in DMSO and amino groups were then introduced by 200 μl HMDA (10 mg/ml in water). The amino plates were activated with 5% (v/v) of DVS and 4% (w/v) of β-CD was added. The concentration of benzimidazole tracer at 5 μg/ml was used for encapsulation and finally, 0.1 M sodium acetate/ citric acid buffer, pH 5, containing 0.5 M NaCl was used as washing buffer.

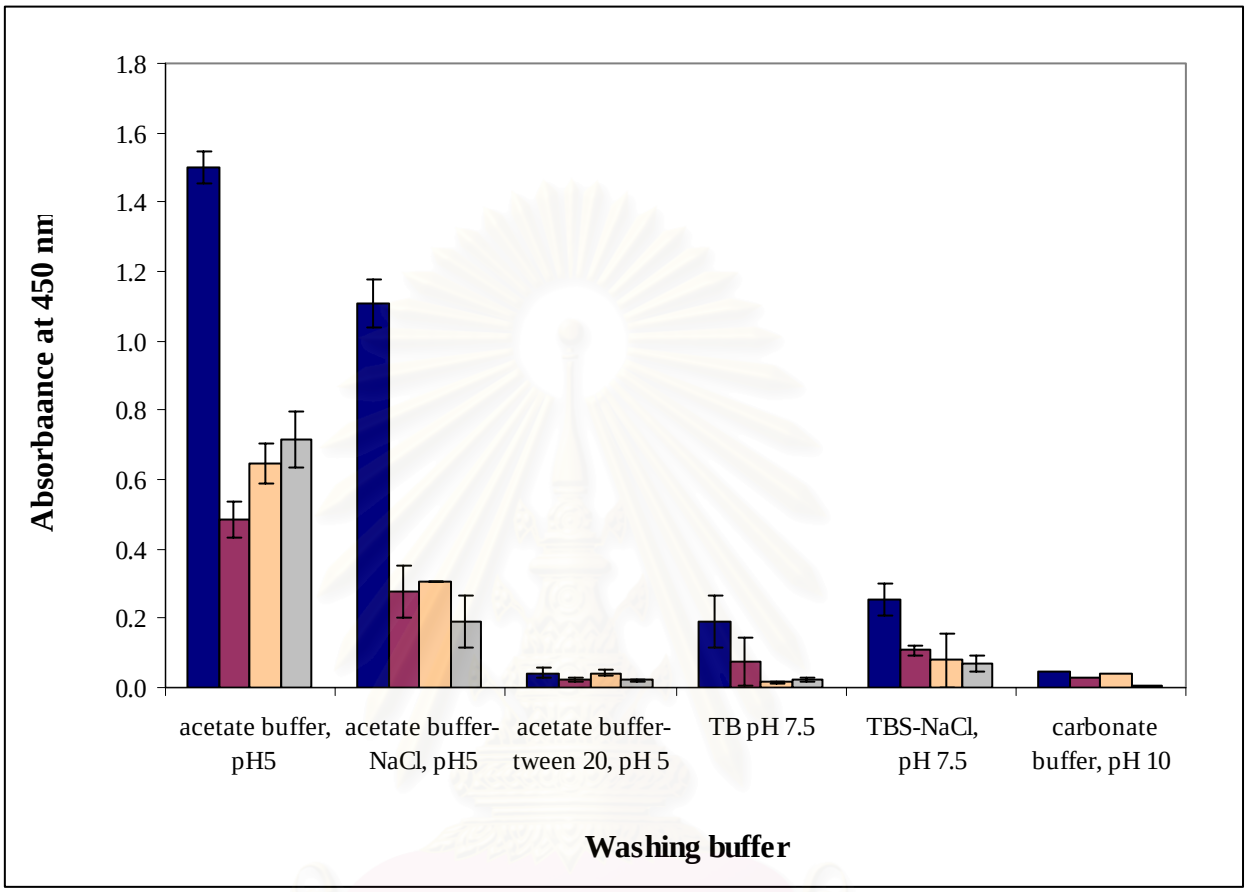


Figure 3.24. Effect of washing buffer on benzimidazole tracer specifically and non-specifically bound onto different types of surfaces. *4% β-CD was coated on amino surface. 5 μg/ml benzimidazole tracer was used for encapsulation and washed with different types of washing buffers. The immobilized CD (■), adsorbed CD (■), DVS (■) and amino (■) surfaces referred to as MMAC-HMDA-DVS-CD, MMAC-HMDA-CD, MMAC-HMDA-DVS and MMAC-HMDA surfaces, respectively. Bars: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

3.7 Capability test of competitive encapsulation with benzimidazole tracer by immobilized β-CD-microtiter plate for detecting carbendazim standard

To assess the potential of the immobilized CD for the determination of carbendazim by competitive benzimidazole tracer encapsulation, carbendazim standard was used to construct the standard curve. Several parameters involved in the competitive benzimidazole tracer encapsulation including solvent used to dissolve carbendazim standard, benzimidazole tracer concentration and pH used for carbendazim encapsulation were examined. Recent study has indicated that solvent has an effect on the encapsulation of carbendazim (Ni *et al.*, 2002), thus, the solvent used to dissolve it was determined. The carbendazim stock standard solutions were prepared by dissolving 2 mg of carbendazim standards in 10 ml of methanol or *N*, *N*-dimethylformamide (DMF) (200 μg/ml) and they were subsequently diluted in 0.1 M sodium acetate/ citric acid buffer, pH 5 to make the working solution in the range of 0 to 100 μg/ml. Effect of solvents on the sensitivity and reactivity between CD and carbendazim is shown in ***Figure 3.25***. For DMF, a typical curve of carbendazim was obtained. As it corresponded to competitive assays, the signal was inversely proportional to the analyte concentration. On the contrary, when methanol was used to dissolve carbendazim, carbendazim did not seem to be encapsulated by immobilized CD and a significant decrease in the reactivity was observed. Thus, a typical curve was not obtained. Therefore, DMF was used to dissolve carbendazim as stock standard solution. To investigate the optimal benzimidazole tracer concentration for competitive inhibition with standard carbendazim, the tracer concentration of 1.25, 2.5 and 5 μg/ml were used. The result is shown in ***Figure 3.26***. At all concentrations used, HRP conjugate activity immobilized or adsorbed on CD surface was again found to be inversely proportional to carbendazim concentration. Nevertheless, the signal obtained from adsorbed CD surface was lower than that from the immobilized CD surface. Considering the immobilized CD surface, it was found that the amount of HRP activity bound were comparable between 2.5 and 5 μg/ml of SAB-HRP conjugate used but at 1.25 μg/ml of SAB-HRP, the amount of HRP activity bound was much less. This could be explained by the fact that the tracer concentration at 2.5 and 5 μg/ml already saturated the underlying surface whereas tracer at 1.25 μg/ml did not reach saturation yet. Hence, in the presence of carbendazim standard, the benzimidazole tracer concentration of 2.5 μg/ml was used for the determination of carbendazim by competitive encapsulation.

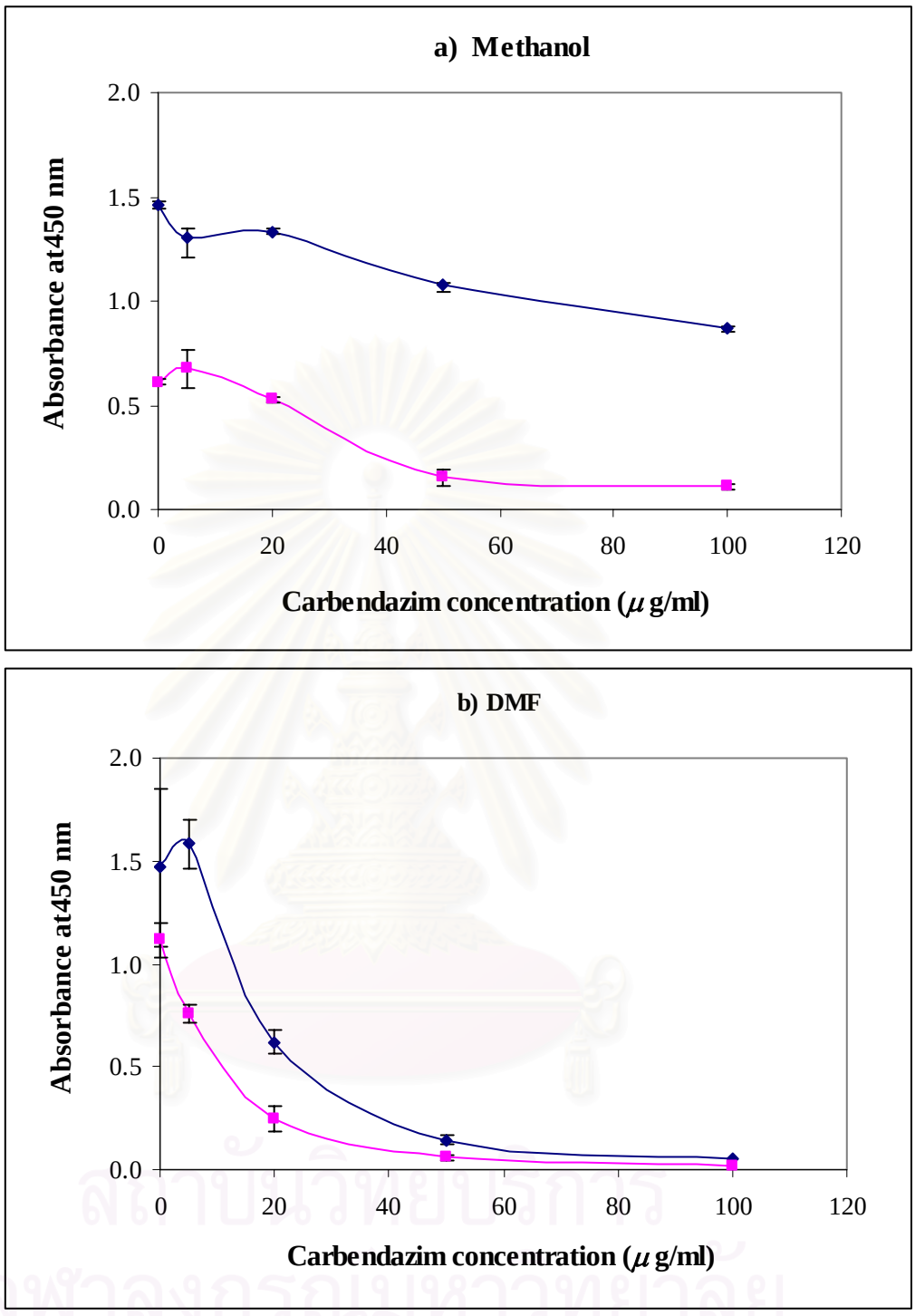


Figure 3.25. Influence of solvent on carbendazim encapsulation: *stock standard solution of carbendazim (200 μg/ml) was dissolved in a) methanol and b) DMF, then diluted in 0.1 M sodium acetate/ citric acid buffer, pH 5 to prepare working solution. The immobilized CD (◆) and adsorbed CD (■) surfaces referred to MMAC-HMDA-DVS-CD and MMAC-HMDA-CD, respectively. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

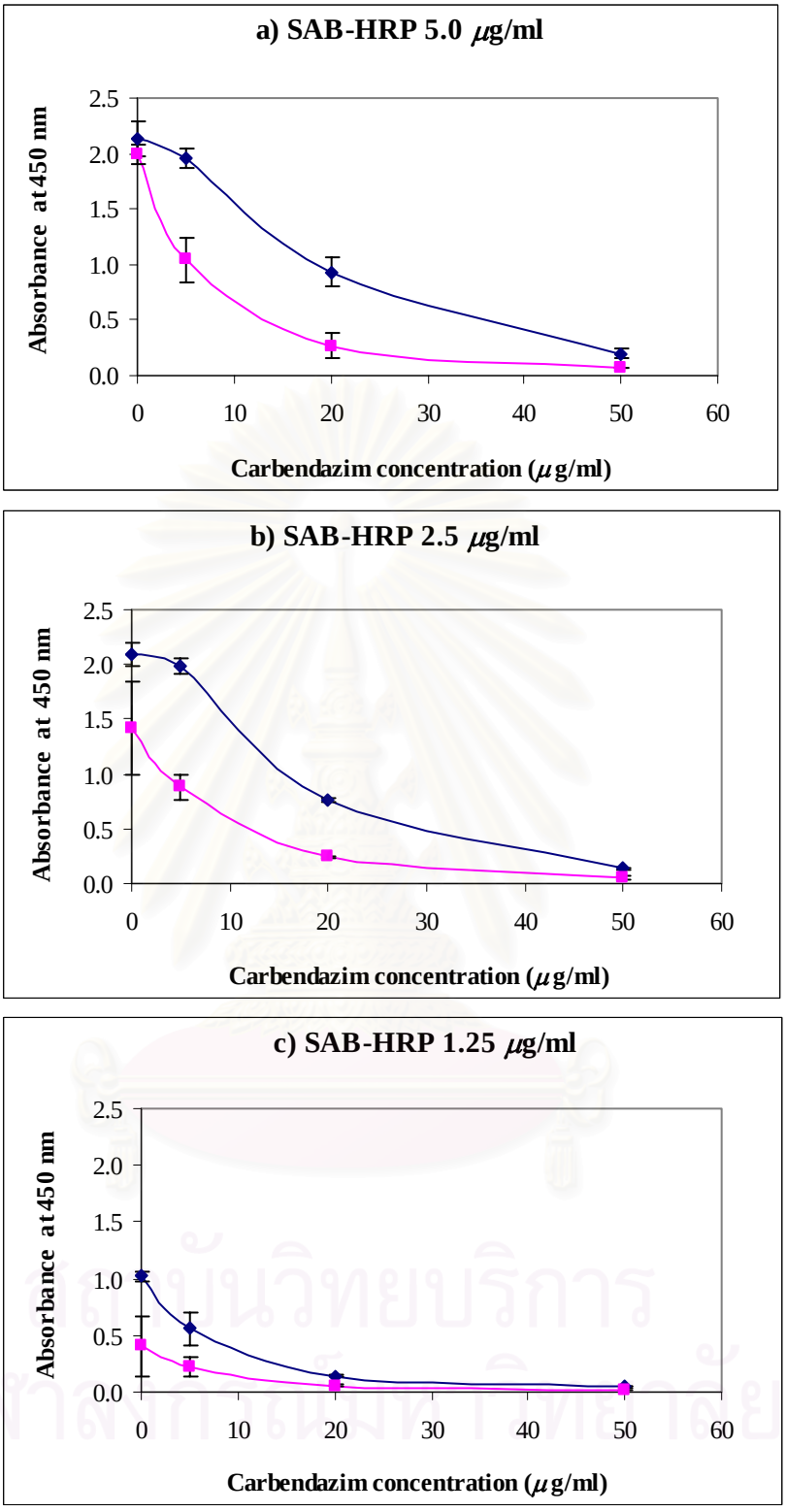


Figure 3.26. Influence of SAB-HRP concentration on carbendazim encapsulation on immobilized CD (MMAC-HMDA-DVS-CD) (◆) and adsorbed CD(MMAC-HMDA-CD) (■) surfaces: *a) 5.0 μg/ml, b) 2.5 μg/ml and c) 1.25 μg/ml SAB-HRP were used for competitive encapsulation. Carbendazim standard was dissolved in DMF and diluted in acetate/citric acid buffer pH 5.0. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

The pH dependence of the carbendazim standard encapsulation was then evaluated. The best pH condition was selected based on the sensitivity of the assay, expressed as carbendazim concentration that reduced the assay signal to 50% of the maximum value (IC_{50}). Ni *et al*. (2002) have reported that at pH 2, carbendazim could be encapsulated by β-CD and the loading buffer at pH 5 was used to check whether carbendazim could be encapsulated at the same pH as benzimidazole tracer or not. ***Figure 3.27*** shows the competitive curve of carbendazim standard using 0.1 M sodium acetate/ citric acid buffer pH 2.5 and 5 as loading buffer. For immobilized CD surface, the signal obtained when carbendazim was loaded at pH 2.5 was more rapidly decreased than that obtained when loading buffer at pH 5.0 was used. In addition, the IC_{50} value of carbendazim loaded at pH 2.5 was 10 μg/ml but when loading buffer at pH 5 was used, the IC_{50} value increased. This suggested that the use of immobilized CD for carbendazim encapsulation at pH 2.5 was more sensitive than that at pH 5. Therefore, 0.1 M sodium acetate/ citric acid buffer pH 2.5 was used as loading buffer for carbendazim standard encapsulation.

3.7.1 Construction of carbendazim standard curve by competitive encapsulation with benzimidazole tracer

After all the parameters involved in this competitive inhibition experiment were optimized, calibration curve was again established using the standard dilution series of carbendazim starting from 0.01 to 50 μg/ml. The stock standard solution of carbendazim was dissolved in DMF and subsequently diluted in acetate/citric acid buffer pH 2.5 to make working solution. The benzimidazole tracer concentration of 2.5 μg/ml was used for competitive encapsulation. Standard curve for carbendazim in the optimum condition is shown in ***Figure 3.28a***. A typical standard curve for carbendazim was obtained when SAB-HRP was encapsulated into immobilized CD surface. In contrast, very little HRP activity was found on the adsorbed CD surface and a typical standard curve was not observed either. In order to properly calculated for the working range of the assay, IC_{50} and the limit of detection, the obtained standard curve was normalized by expressing the absorbance as the percentage of the maximum response (B/B_0 x 100) (see Appendix 3). The working range of the assay for standards, defined by concentrations giving 20-80 % inhibition of the maximum signal (or the central section of the standard curve with a nearly linear response), was 0.4-12.5 μg/ml. The IC_{50} value was 1.56 μg/ml and the limit of detection, calculated as the carbendazim concentration that reduced maximum

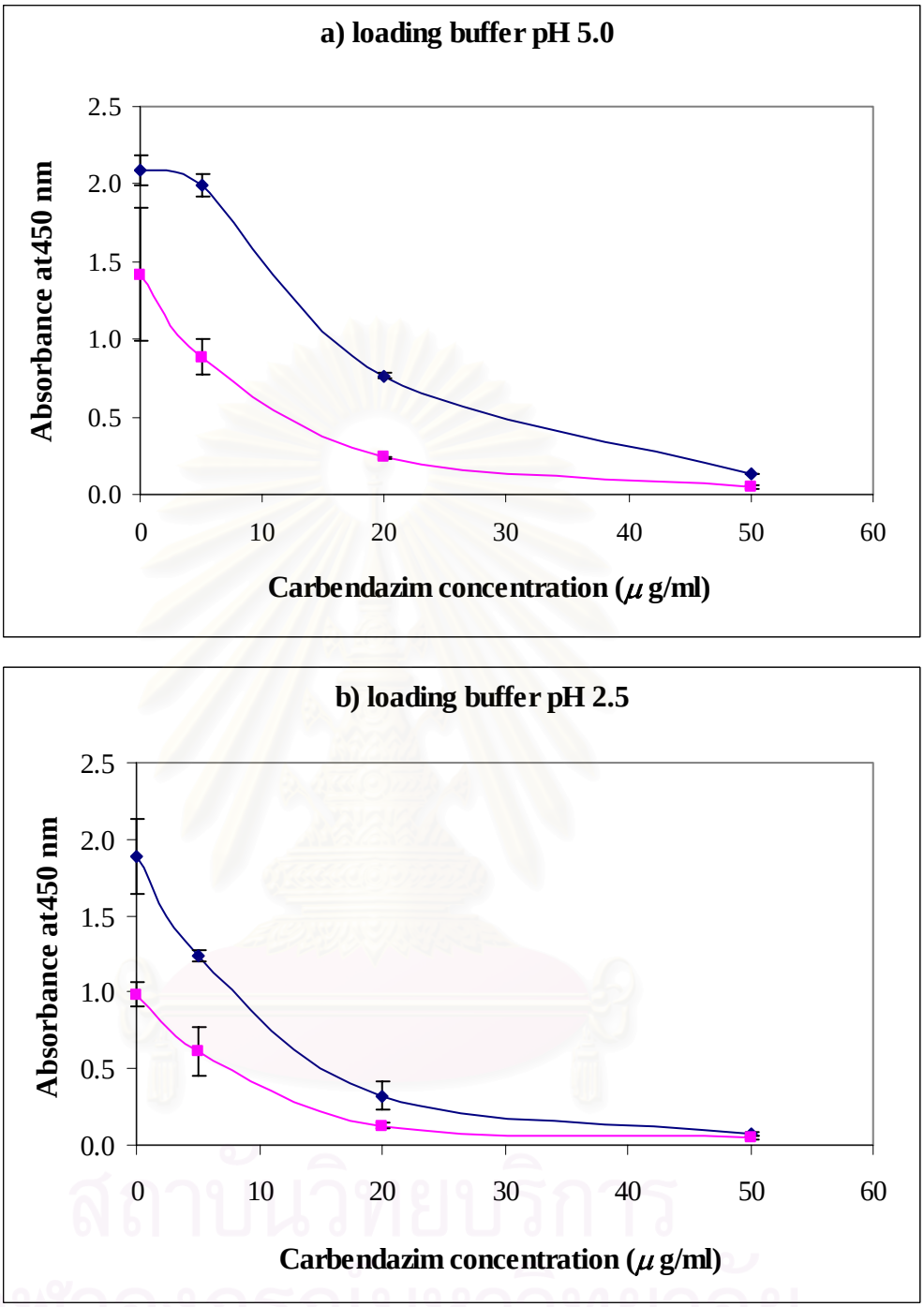


Figure 3.27. Influence of pH on carbendazim encapsulation by immobilized CD (MMAC-HMDA-DVS-CD) (◆) and adsorbed CD (MMAC-HMDA-CD) (■) surfaces: *Carbendazim was dissolved in DMF and diluted in acetate/citric acid buffer a) pH 5.0 and b) pH 2.5. SAB-HRP at 2.5 μg/ml was used for competitive encapsulation. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

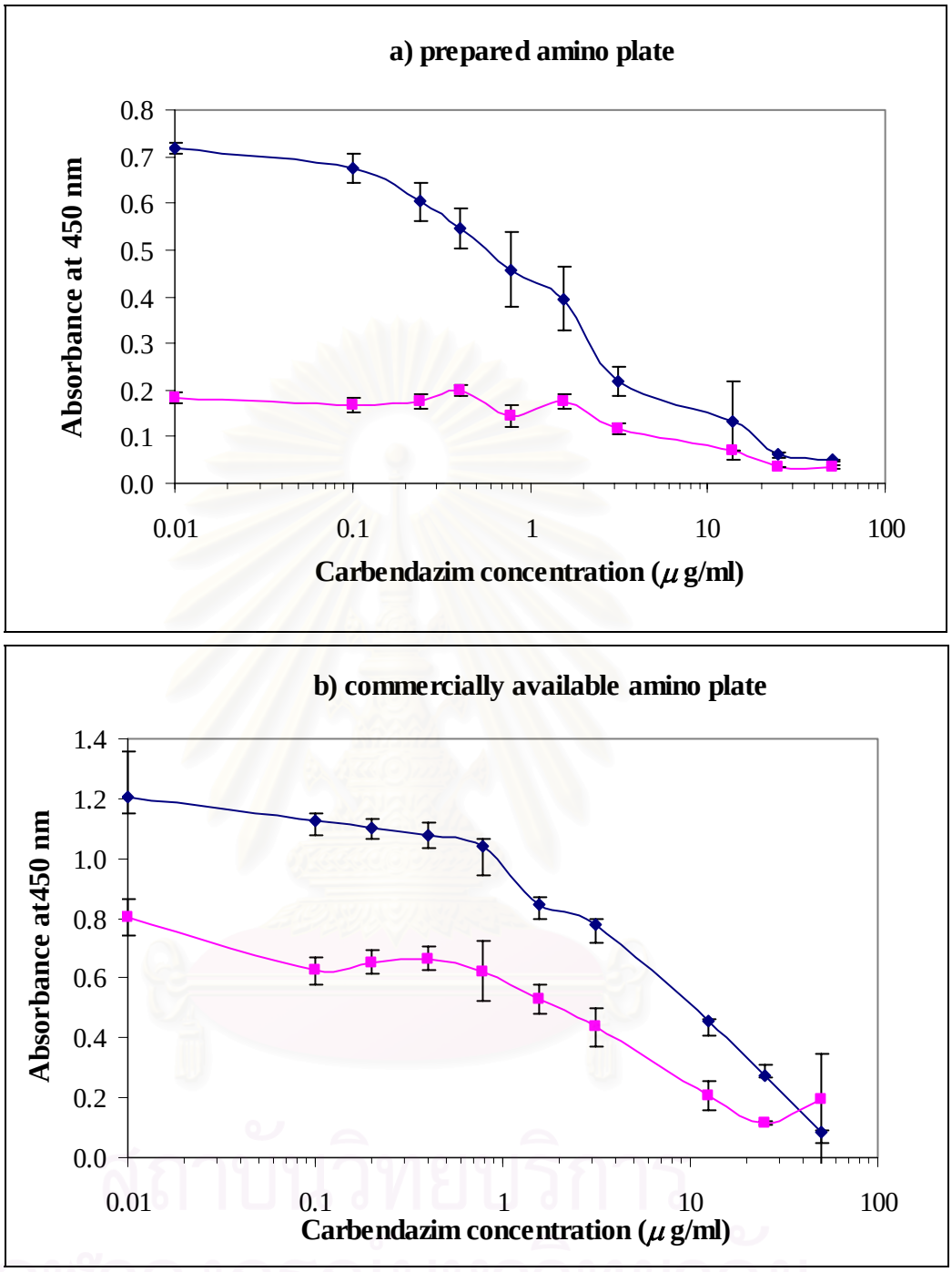


Figure 3.28. Standard curve of carbendazim under optimized competitive inhibition condition by immobilized (MMAC-HMDA-DVS-CD) (◆) and adsorbed CD (MMAC-HMDA-CD) (■) on a) prepared and b) commercially available amino plate. *2.5 μg/ml of benzimidazole tracer was used for competitive encapsulation by 4% β-CD. Carbendazim standard was dissolved in DMF and diluted in acetate/ citric acid buffer, pH 2.5. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

absorbance by 10 %, was 0.2 μg/ml. In comparison with the use of commercially available amino plate, 4 % (w/v) of β-CD was immobilized on commercially available amino plate through DVS. The standard dilution series of carbendazim ranging from 0.01 to 50 μg/ml were used for encapsulation under optimized condition. The result in ***Figure 3.28b*** showed that the typical standard curve was also observed on immobilized CD surface but relatively high amount of SAB-HRP activity was found on the adsorbed CD surface. The working range, IC_{50} and the limit of detection were summarized in ***Table 3.2***. It can clearly be seen that the immobilized CD on prepared amino plate was more sensitive for the determination of carbendazim than the commercially available one.

3.8 Analyses of carbendazim spiked fruit samples by competitive encapsulation with benzimidazole tracer into immobilized CD-microtiter plate

Following the analysis of carbendazim standard using benzimidazole tracer encapsulation by immobilized β-CD, attempts were made to analyze carbendazim residues in fortified fruit sample. Grapes were chosen as model matrices to evaluate the possibility of this method for the analysis of carbendazim in fruit. Thus, a negative control was set where samples were confirmed by HPLC that they did not contain carbendazim residues. The carbendazim-free samples were then used for recovery studies and for the determination of matrix interference. The matrix dilution that provided accurate and precise determination of carbendazim was then undertaken. Finally, carbendazim standard was spiked into the grape extract to find the limit of detection (LOD) of this method.

3.8.1 HPLC determination

Grape was checked whether it contained carbendazim residues by HPLC with UV and fluorescence detectors. Detection of carbendazim by UV detector was carried out at 280 nm which was the maximum adsorption of carbendazim and for the detection of carbendazim by fluorescence detector, it was operated at 280 and 310 nm as wavelengths for excitation and emission, respectively (Gilvydis and Walters, 1990). First, carbendazim standard was injected into HPLC column to determine its retention time (R_t). The peak at 6.353 minutes was observed when UV detector was used (***Figure 3.29a***) and R_t at 6.413 minutes was seen when determined by fluorescence (***Figure 3.29b***). Sample extraction was then performed in the same way as described for the extraction of carbendazim (section 2.12.1.1). Briefly, grape was extracted with methanol and

Table 3.2. Comparison of sensitivity of standard curves for carbendazim

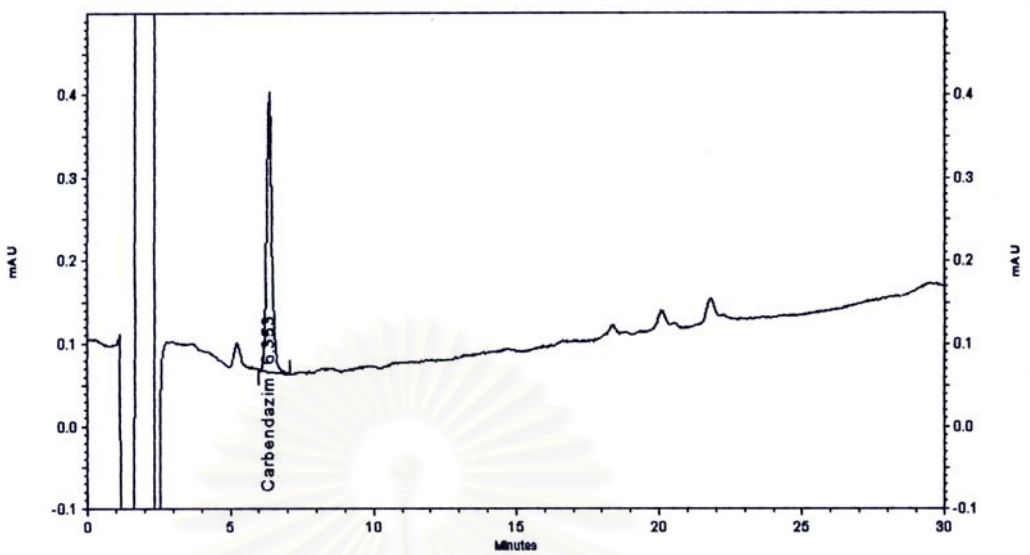
Type of amino plate	Parameters		
	IC_{50} (μg/ml)	LOQ (μg/ml)	LOD (μg/ml)
Prepared	1.56	0.4-12.5	0.2
Commercially available	7	1-25	0.8

IC_{50} : the inhibition concentration at 50%

LOQ : the lower and upper limit of quantification

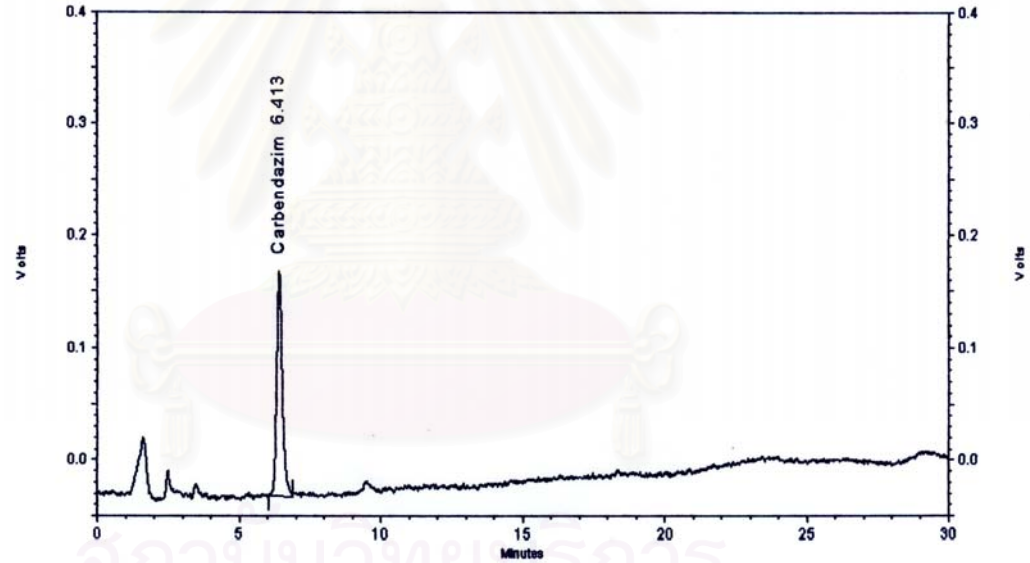
LOD : the limit of detection

a)



UV Results

b)



Fluorescence Results

Figure 3.29. HPLC chromatograms of carbendazim standard at 0.5 μg/ml obtained by a) UV (at 280 nm) and b) fluorescence detection (at excitation/emission wavelengths of 280/310). *An Ultracarb 5 C8 reverse phase column (4.6x150 mm) was used and eluted with 40% of acetonitrile and 60 % of 0.01 M potassium hydrogen phosphate at a flow rate of 0.5 ml/min.*

partitioned into dichloromethane after alkalization of the extract. After extraction, samples were analyzed by HPLC and it was found that no carbendazim residues were detected (***Figure 3.30a*** and ***b***). The samples were then used for recovery studies and matrix effect studies.

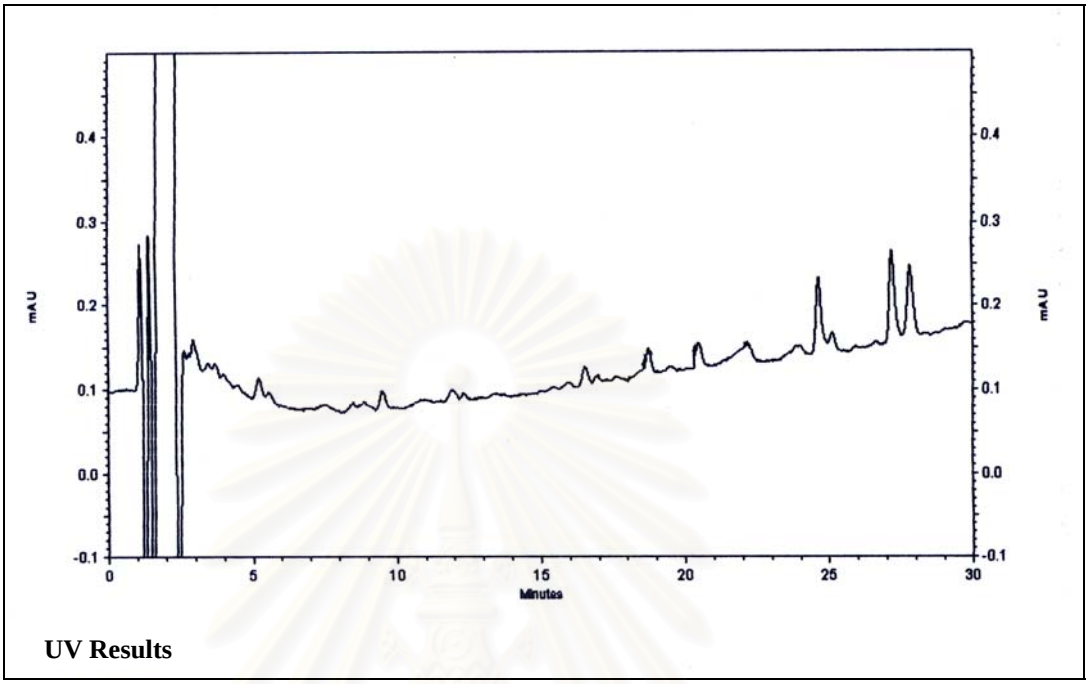
3.8.2 Recovery studies of carbendazim in spiked fruit sample by HPLC

The recovery studies were essential to evaluate the extraction efficiency from grape fruit sample. Although the HPLC used for carbendazim determination is the standard method recommended in Pesticide Analytical Method (PAM. Volumn I, 1991), the accuracy of the method should be assessed in term of the percent recovery by analysis of known added amounts of carbendazim. Recovery test was performed by fortifying grape which was found negative with 1 ppm of carbendazim standard solution and extracted for the determination of carbendazim residues as described in section 2.12.1.1. Finally, the amount of carbendazim in spiked fruit sample was analyzed by HPLC. It was found that carbendazim peaks in spiked fruit sample were eluted at 6.373 minutes and 6.447 minutes in UV and fluorescence chromatograms, respectively by comparing the retention time with that of standard carbendazim (***Figure 3.31-3.32***). Besides, less sample background was seen in fluorescence chromatogram (***Figure 3.31b and 3.32b***). The recovery of carbendazim was calculated on the basis of its peak area in comparison with that of the standard carbendazim. It was found that the recovery determined using UV detector was 79% and 90% for the fluorescence detector. From these results, it was possible to use the above extraction method for the determination of carbendazim in grape.

3.8.3 Matrix effect on the determination of carbendazim in grape by competitive encapsulation with benzimidazole tracer

Sample matrix effects are quite common in analysis, so it is advisable to determine the influence of the matrix of interest (grape) on the competitive benzimidazole tracer encapsulation prior to the application of the method to sample containing analyte. Accordingly, it was tested by incubating a variable proportion of grape matrix with immobilized β-CD, followed by the competitive inhibition with benzimidazole tracer. The experiment was undertaken in order to determine the matrix dilution that provided accuracy and precision on the system under specific conditions and the comparison between immobilized β-CD on prepared and commercially available amino plate was studied.

a)



b)

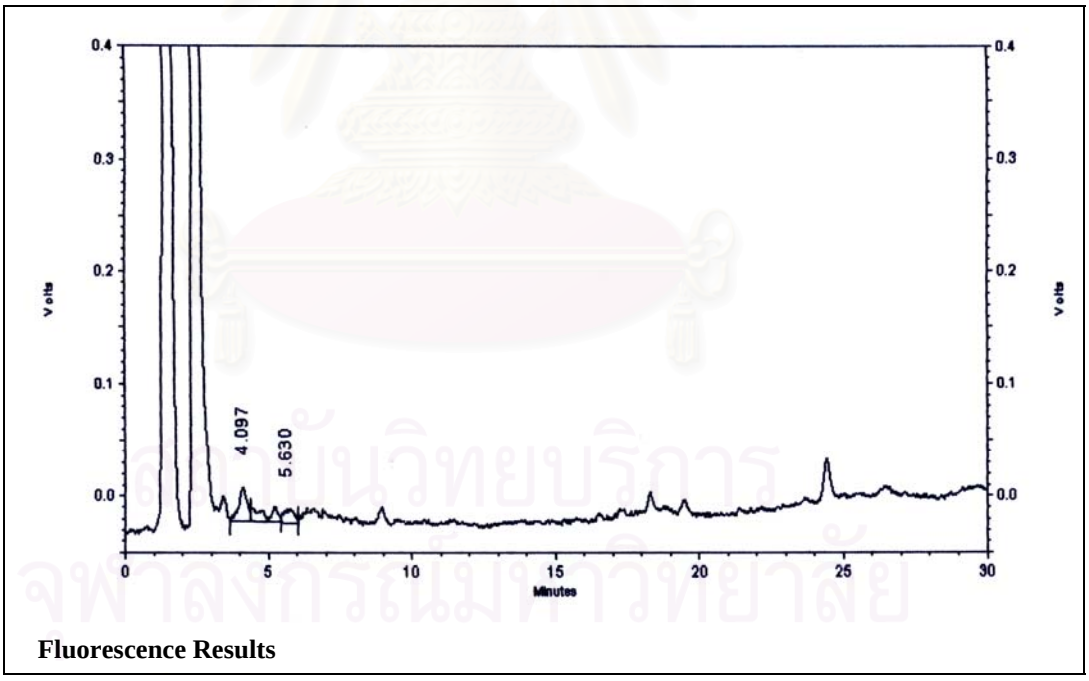
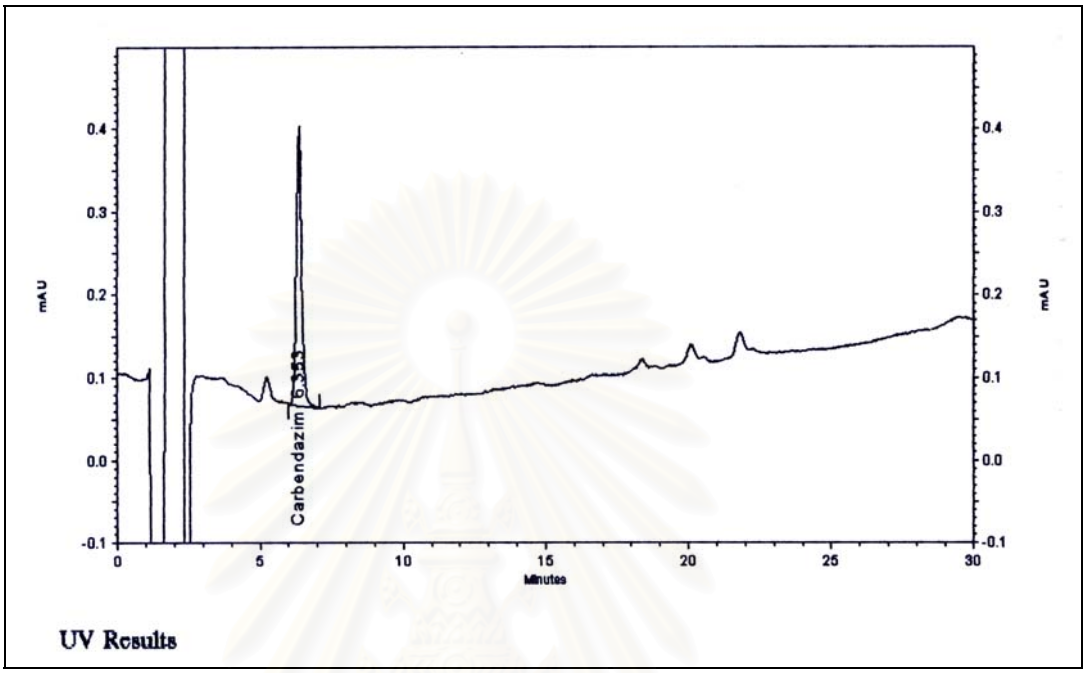


Figure 3.30. HPLC chromatograms of 2 g/ml of grape extract obtained by a) UV (at 280 nm) and b) fluorescence detection (at excitation/emission wavelengths of 280/310). *An Ultracarb 5 C8 reverse phase column (4.6x150 mm) was used and eluted with 40% of acetonitrile and 60 % of 0.01 M potassium hydrogen phosphate at a flow rate of 0.5 ml/min.*

a)



b)

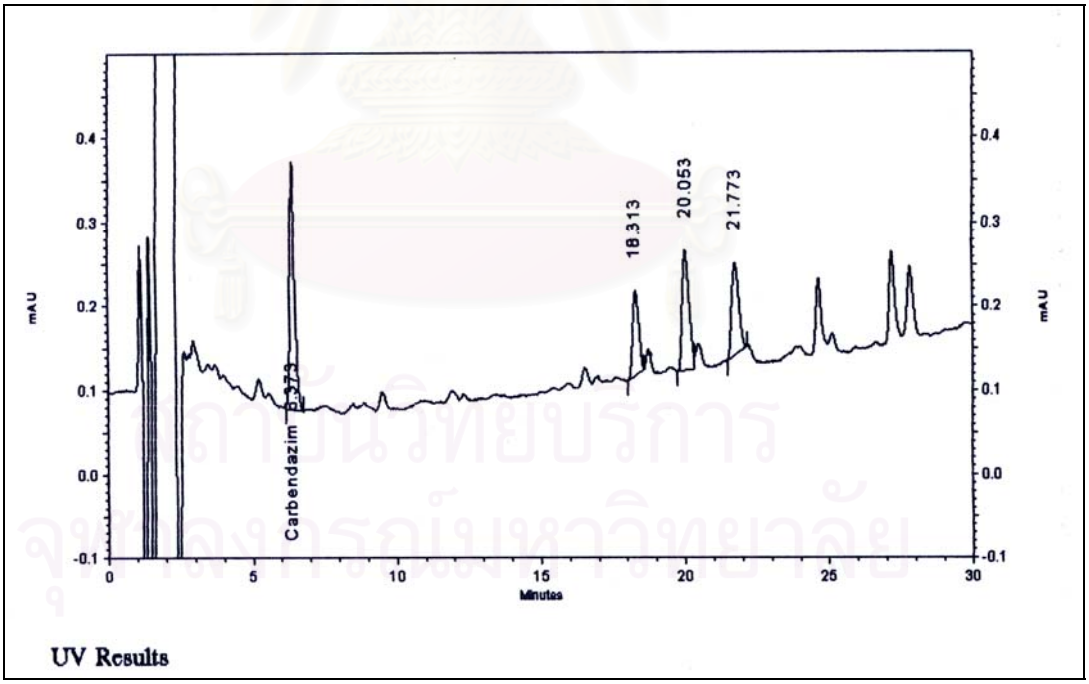
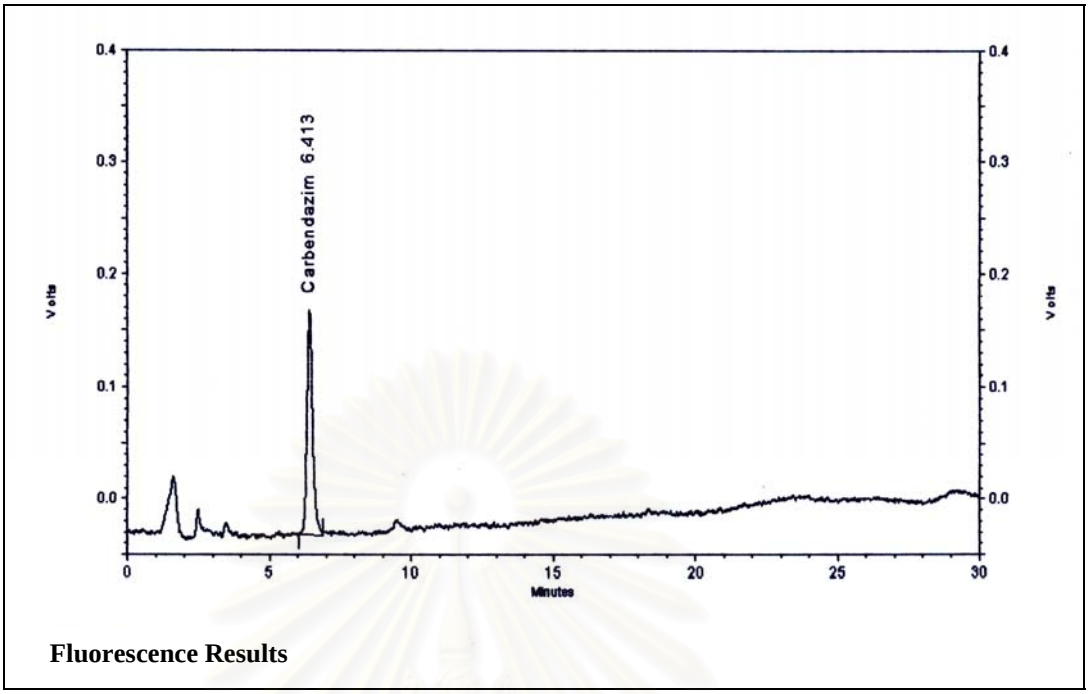


Figure 3.31. HPLC chromatograms of a) 0.5 μg/ml of carbendazim standard and b) 1 ppm of carbendazim spiked grape extract determined by UV detector. *An Ultracarb 5 C8 reverse phase column (4.6x150 mm) was used and eluted with 40% of acetonitrile and 60 % of 0.01 M potassium hydrogen phosphate at a flow rate of 0.5 ml/min. UV detector was measured at 280 nm.*

a)



b)

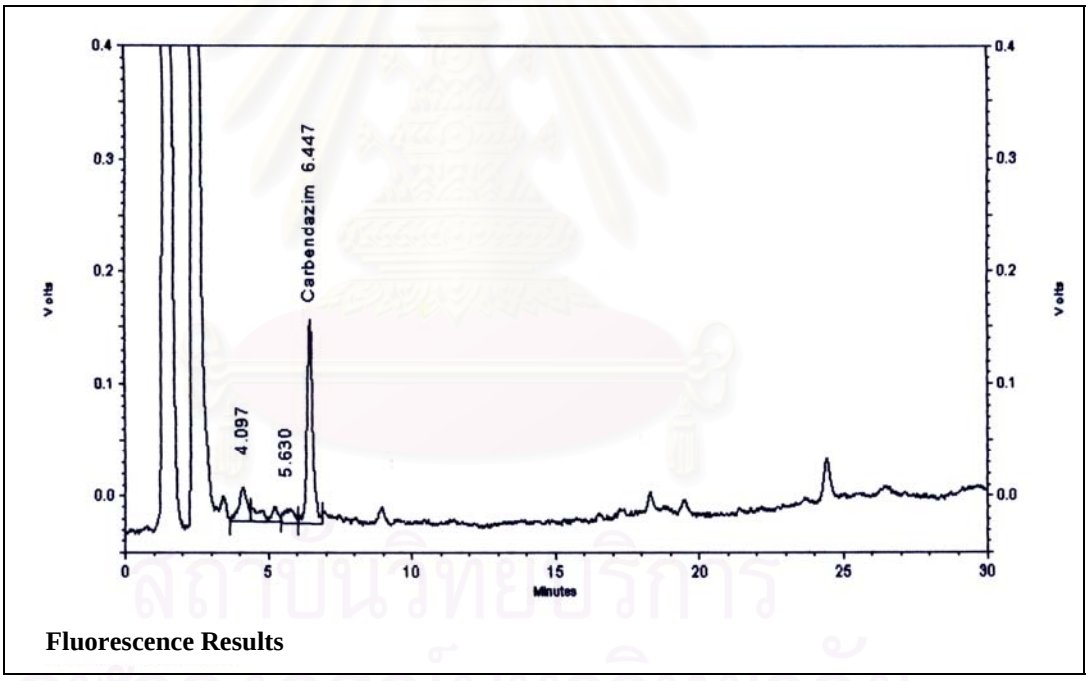


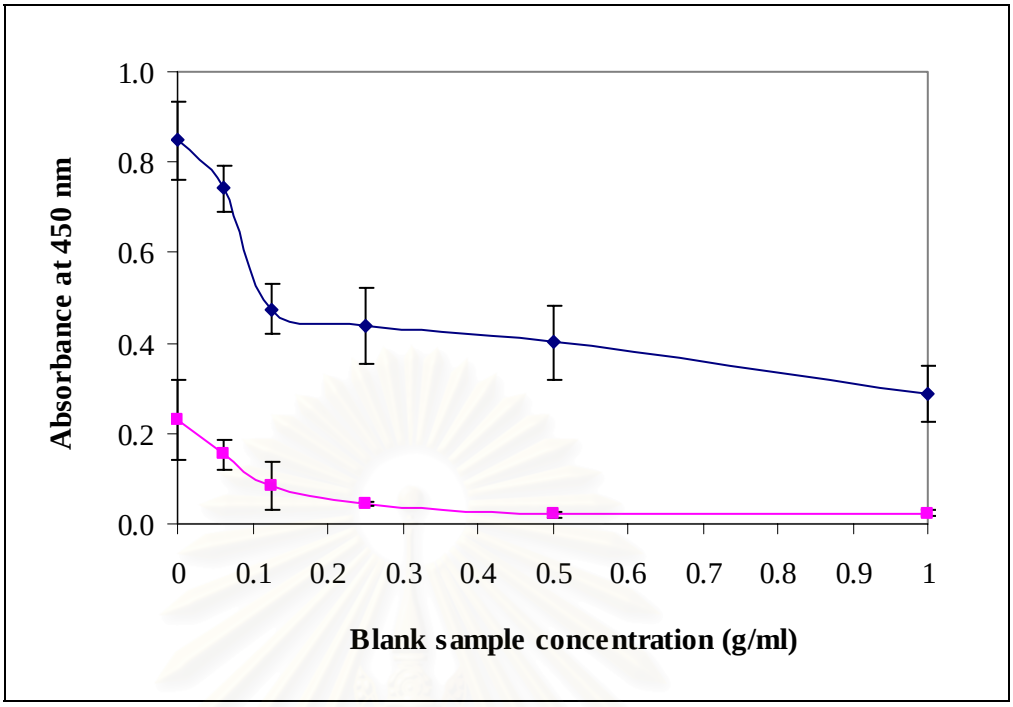
Figure 3.32. HPLC chromatograms of a) 0.5 μg/ml of carbendazim standard and b) 1 ppm of carbendazim spiked grape extract determined by fluorescence detector. *An Ultracarb 5 C8 reverse phase column (4.6x150 mm) was used and eluted with 40% of acetonitrile and 60 % of 0.01 M potassium hydrogen phosphate at a flow rate of 0.5 ml/min. Excitation and emission wavelengths of fluorescence detector were set at 280 and 310 nm, respectively.*

The concentration of grape extract at 2 g/ml prepared according to section 2.12.2 was diluted with loading buffer pH 2.5 to get final concentrations of 0.05, 0.1, 0.25, 0.5 and 1 g/ml. These grape extracts were used for the competitive encapsulation with 2.5 μg/ml benzimidazole tracer by immobilized CD. Another set of experiment was performed where β-CD was simply adsorbed on both amino plates. In both cases of β-CD immobilized on prepared and commercially available amino plates, it can be seen that the sample matrix could inhibit the encapsulation of benzimidazole tracer and the observed effects depended on the fruit proportion (***Figure 3.33***). At low concentration of grape matrix (0.05g/ml), the maximum absorbance was slightly reduced. The presence of the matrix higher than that strongly reduced the maximum absorbance which could lead to the underestimation of carbendazim in grape sample. Thus, grape matrix should be diluted to get a concentration of 0.05 g/ml in an assay to be accurately analyzed by this method.

3.8.4 Determination of carbendazim in grape by competitive encapsulation with benzimidazole tracer

The concentration of grape extract at 0.05 g/ml which showed the lowest interference was spiked with carbendazim standard ranging from 0-2.5 μg/ml to get the concentration of carbendazim residues at 0-50 μg/ml in 1 g/ml grape extract. The use of β-CD immobilized on prepared and commercially available amino plates for the determination of carbendazim was compared. It was found that the signal of HRP conjugate bound to immobilized CD on prepared amino plate decreased until the carbendazim standard concentration increased to 0.04 μg/ml. When the carbendazim concentration was higher than that, the signal was found to fluctuate and deviate from a typical standard curve as shown in ***Figure 3.34a***. For commercially available amino plate, the signal of HRP conjugate bound to immobilized CD was also fluctuated and again a typical curve was not obtained (***Figure 3.34b***). The HRP conjugate activity bound to adsorbed CD surfaces was found to be significantly lower. Additionally, solvent employed to dissolve carbendazim may affect the characteristics of the assay. This was observed when the proportion of DMF was high (at high concentration of carbendazim used). Therefore, stock standard solution of carbendazim containing low concentration of DMF was prepared by dissolving 1 mg of carbendazim in 0.5 ml DMF

a)



b)

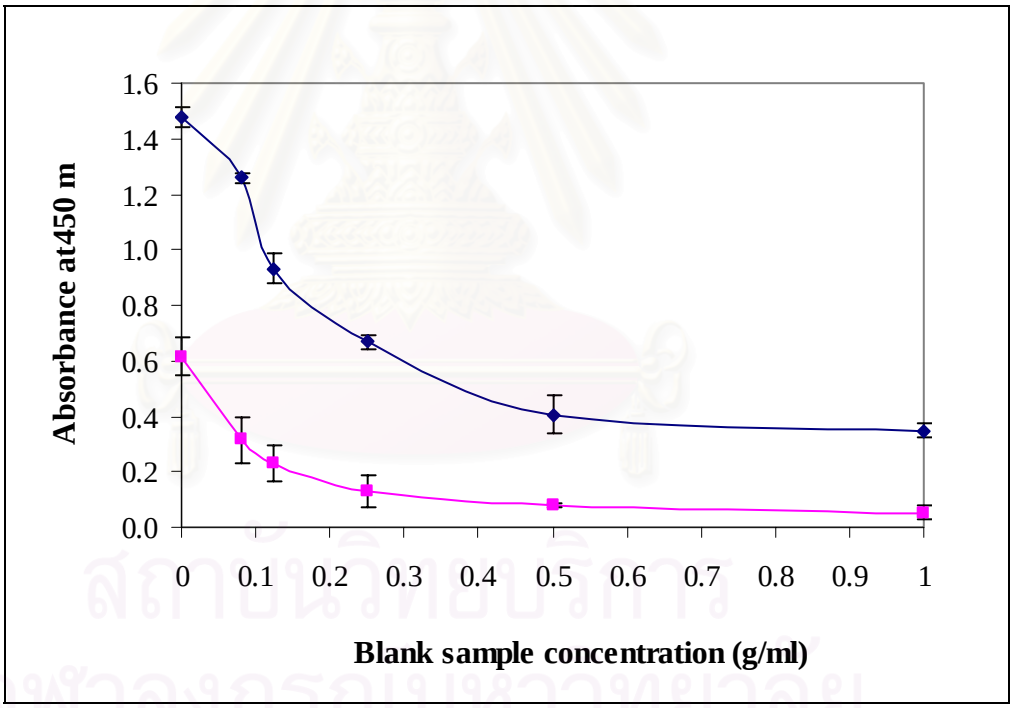


Figure 3.33. Influence of grape matrix on competitive encapsulation with benzimidazole tracer into immobilized CD on a) prepared amino plate and b) commercially available amino plate. *The immobilized CD (◆) and adsorbed CD (■) surfaces were investigated. 2.5 µg/ml benzimidazole tracer was used. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

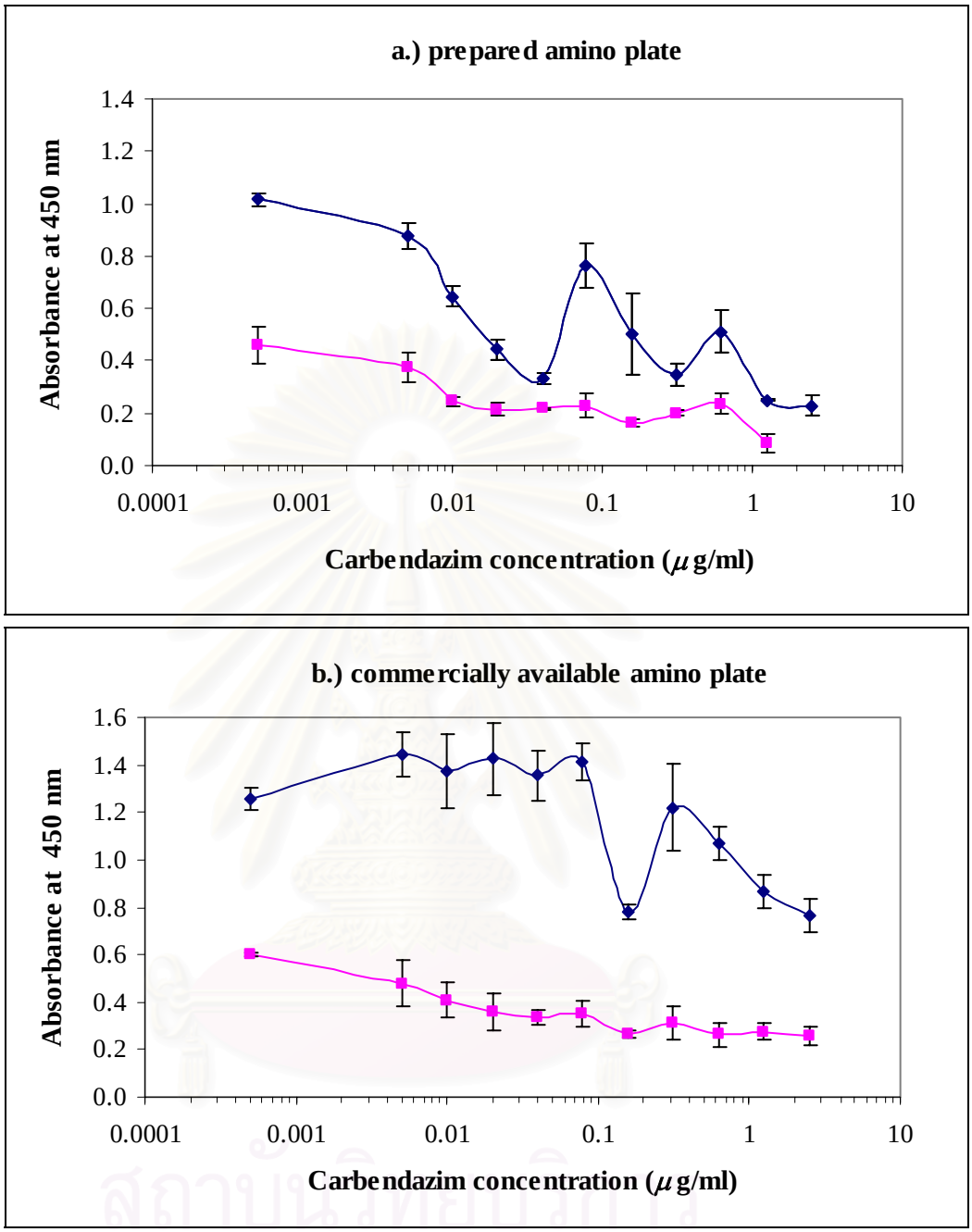


Figure 3.34. Influence of DMF on the encapsulation of carbendazim spiked grape extract on a) prepared and b) commercially available amino plates. *2 mg of carbendazim standard was dissolved in 10 ml DMF and spiked into 0.05 g/ml grape extract and loaded at pH 2.5. Benzimidazole tracer at the concentration of 2.5 μg/ml was used for competitive encapsulation. The immobilized CD (◆) and adsorbed CD (■) surfaces referred to as MMAC-HMDA-DVS-CD and MMAC-HMDA-CD, respectively. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

and 0.1 M acetate/ citric acid buffer pH 2.5 (loading buffer) was added to make 10 ml in volumetric flask. Grape extract at concentration of 0.05 g/ml was spiked with carbendazim standard ranging from 0-2.5 μg/ml and the inhibition curve on prepared amino plate was re-determined (***Figure 3.35a***). In the case of commercially available amino plate, carbendazim standard concentrations ranging from 0 to10 μg/ml were used (***Figure 3.35b***). Typical competitive inhibition curve was seen in both of amino plates. The IC_{50}, the operative working range and the limit of detection of the assay are summarized in ***Table 3.3***. It can be concluded that the analysis of carbendazim by CD immobilized on prepared amino microtiter plate was more significantly sensitive than that immobilized on commercially available plate.

3.9 Determination of carbaryl using immobilized CD on microtiter plate

Considering the results obtained previously, the immobilized β-CD on prepared amino microtiter plate was able to successfully detect carbendazim residues in spiked fruit sample. To illustrate the applicability of the method, it was extended to the determination of carbaryl by competitive carbaryl tracer encapsulation. The complexation between carbaryl and β-CD was recently reported (Barbato *et al*., 2000; Saikosin *et al*., 2002). The preparation of the amino microtiter plate and the optimal competitive inhibition system for example, the concentration of β-CD and tracer used were similar to the determination of carbendazim except for the pH loading buffer used for carbaryl encapsulation by β-CD.

First, the carbaryl tracer (CPNU-HRP conjugate) was synthesized and characterized as described in section 3.1 and 3.2, respectively. The encapsulation study of CPNU-HRP conjugate on adsorbed CD (MMAC-HMDA-CD) and immobilized CD (MMAC-HMDA-DVS-CD) was subsequently determined and compared with the native HRP. Moreover, the amino (MMAC-HMDA) and DVS (MMAC-HMDA-DVS) surfaces were used to investigate non-specific binding of both enzymes. After 4% of β-CD was immobilized on prepared amino plate, 5 μg/ml of CPNU-HRP conjugate and native HRP were applied onto various types of surfaces prior to HRP activity determination. The result is shown in ***Figure 3.36***. It was found that CPNU-HRP conjugate activities bound to all surfaces were significantly higher than those of HRP and the signal of specific binding on immobilized CD surface was the highest. This suggested that CPNU conjugated to HRP was encapsulated by immobilized CD and thus, could be used as tracer for the determination of carbaryl by competitive carbaryl tracer encapsulation.

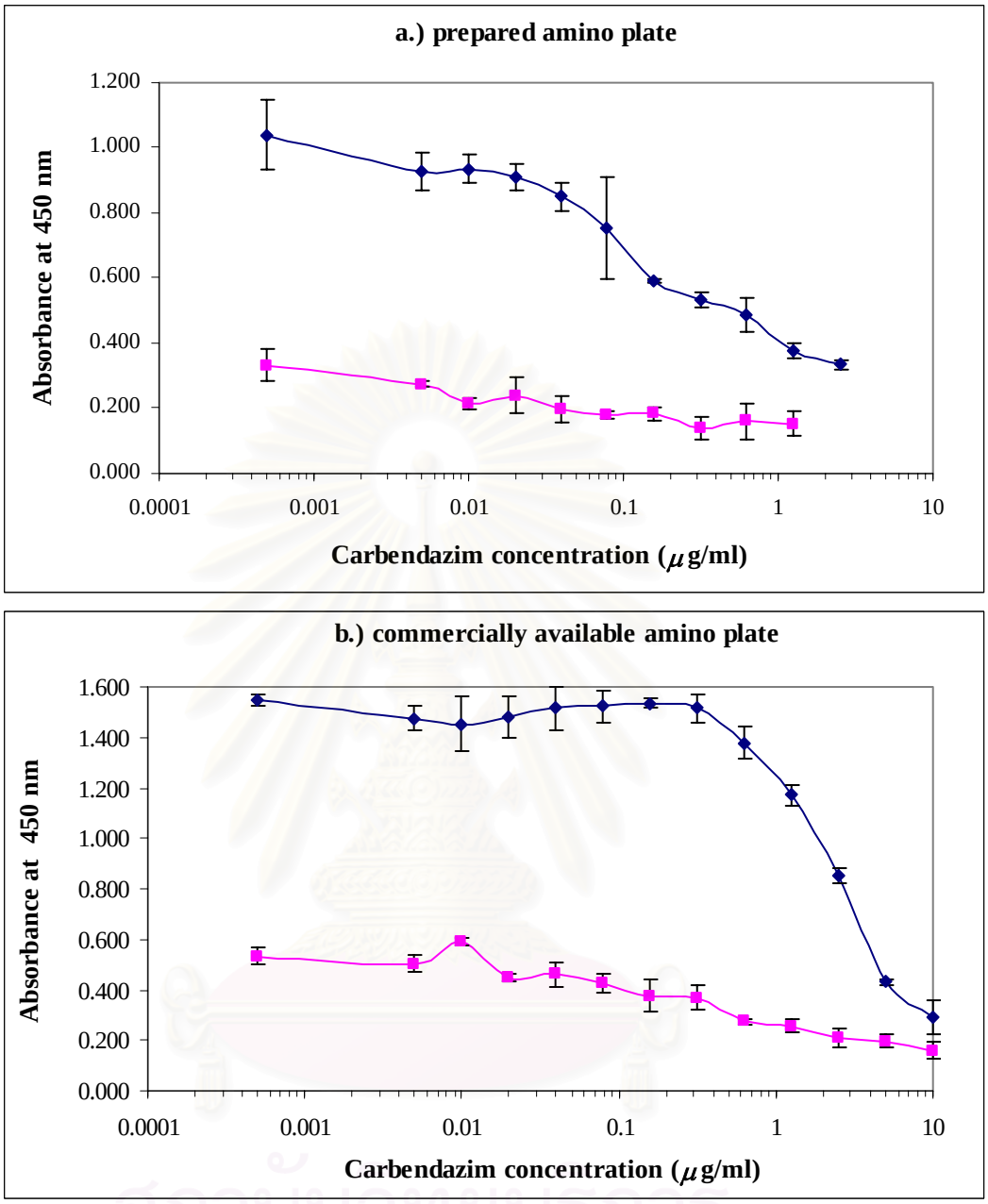


Figure 3.35. Standard curve of carbendazim in the presence of matrix on a) prepared and b) commercially available amino plates. *1 mg of carbendazim standard was dissolved in 0.5 ml DMF and loading buffer pH 2.5 was added to make 10 ml in volumetric flask and 0.05 g/ml grape extract was then spiked. Benzimidazole tracer at the concentration of 2.5 μg/ml was used for competitive encapsulation. The immobilized CD (◆) and adsorbed CD (■) surfaces referred to as MMAC-HMDA-DVS-CD and MMAC-HMDA-CD, respectively. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

Table 3.3. Comparison of sensitivity of standard curve of carbendazim in the presence of matrix

Type of amino plate	Parameters		
	IC_{50} (μg/ml)	LOQ (μg/ml)	LOD (μg/ml)
Prepared	0.1	0.04-0.7	0.02
Commercially available	2	0.7-5	0.5

IC_{50} : the inhibition concentration at 50%

LOQ : the lower and upper limit of quantification

LOD : the limit of detection

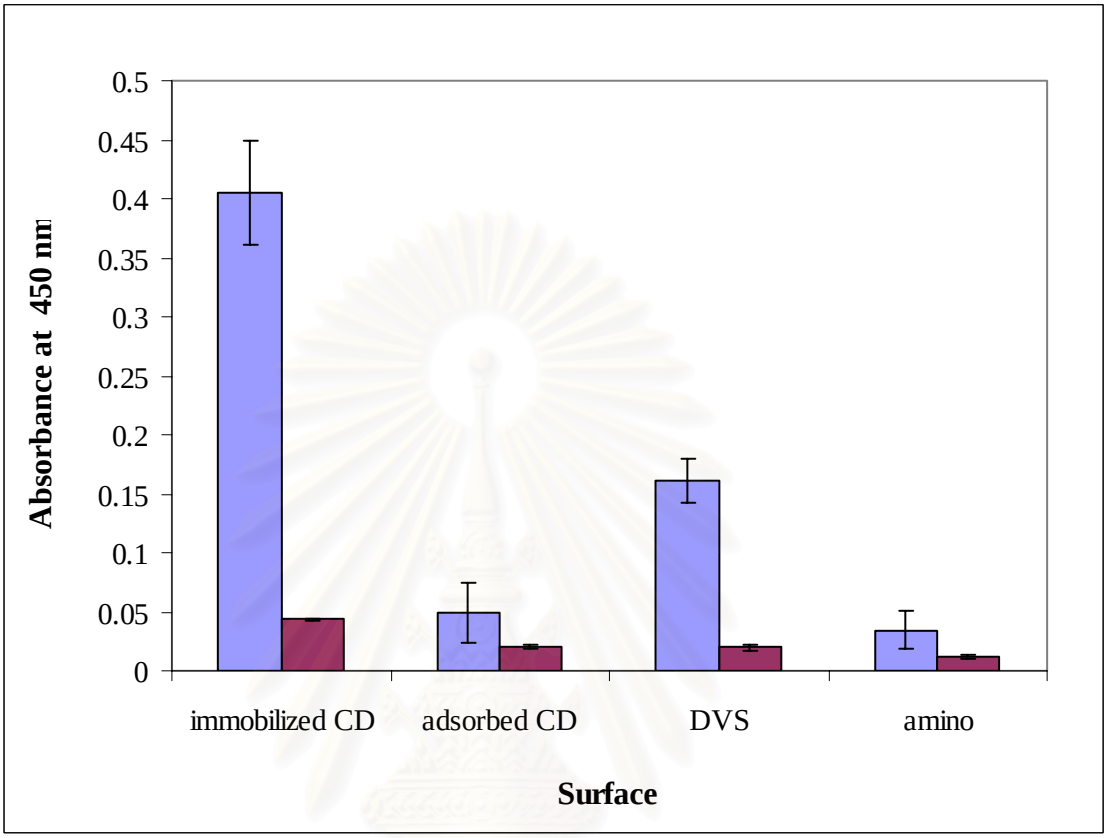


Figure 3.36. The specific and non-specific binding of carbaryl tracer and HRP onto different types of surfaces*. 4% β-CD was immobilized on MMAC-HMDA treated plate. 5 μg/ml of carbaryl tracer (■) and HRP (■) were used for binding. The immobilized CD, adsorbed CD, DVS and amino plate surfaces referred to as MMAC-HMDA-DVS-CD, MMAC-HMDA-CD, MMAC-HMDA-DVS and MMAC-HMDA surfaces, respectively. Bars: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

3.9.1 Effect of carbaryl tracer concentration on its encapsulation

Even though the optimal benzimidazole tracer concentration for its encapsulation was observed at 5 μg/ml (section 3.6.3), the appropriate concentration of carbaryl tracer was still necessary to be investigated. This should exhibit the difference in CPNU-HRP conjugate activity bound on immobilized CD and other surfaces (adsorbed CD, DVS, and amino surfaces). Several concentrations of the carbaryl tracer were incubated with various types of surfaces in order to find the optimal tracer concentration for the encapsulation. As can be seen in ***Figure 3.37***, the saturation curve of carbaryl tracer was observed when the amount of tracer added increased to the immobilized CD surface. For other surfaces, the activities of HRP conjugate showed the same patterns but they are lower than those found in immobilized CD surface. The minimum carbaryl tracer concentration which covered all the guest binding sites on CD was 5 μg/ml. This result illustrated that the saturation concentration of carbaryl tracer was similar to that of benzimidazole tracer (see ***Figure 3.23***). Thus, it was decided to skip the experiment varying tracer concentration and use the carbaryl tracer concentration at 2.5 μg/ml for the determination of carbaryl standard and carbaryl spiked fruit which was the same as in the carbendazim determination experiment (section 3.7).

3.10 Construction of carbaryl standard curve by competitive encapsulation with carbaryl tracer

The effect of pH was first evaluated based on the IC_{50} value of competitive standard curve. Two types of buffer: 0.1 M sodium acetate/ citric acid buffer, pH 5 and 10 mM sodium phosphate buffer pH 7.4 containing 137 mM NaCl and 2.7 mM KCl (PBS) were used to find a suitable pH for carbaryl encapsulation. Pacioni and Veglia (2003) have reported that at pH 7, carbaryl was shown to be encapsulated by β-CD and the loading buffer at pH 5 was assessed to see if carbaryl could be encapsulated at the same pH as carbaryl tracer. Another set of experiment was carried out where CD was simply adsorbed onto the amino plate.The carbaryl stock standard solutions were prepared by dissolving 1 mg of carbaryl standard in 0.5 ml of DMF and 9.5 ml of loading buffer pH 5 or pH 7.4. They were subsequently diluted with loading buffer pH 5 or pH 7.4 in the range of 0 to 100 μg/ml. The carbaryl tracer concentration of 2.5 μg/ml was used for competitive encapsulation. From ***Figure 3.38a and b,*** competitive inhibition curves were obtained in both pH and the signal of adsorbed CD surface was

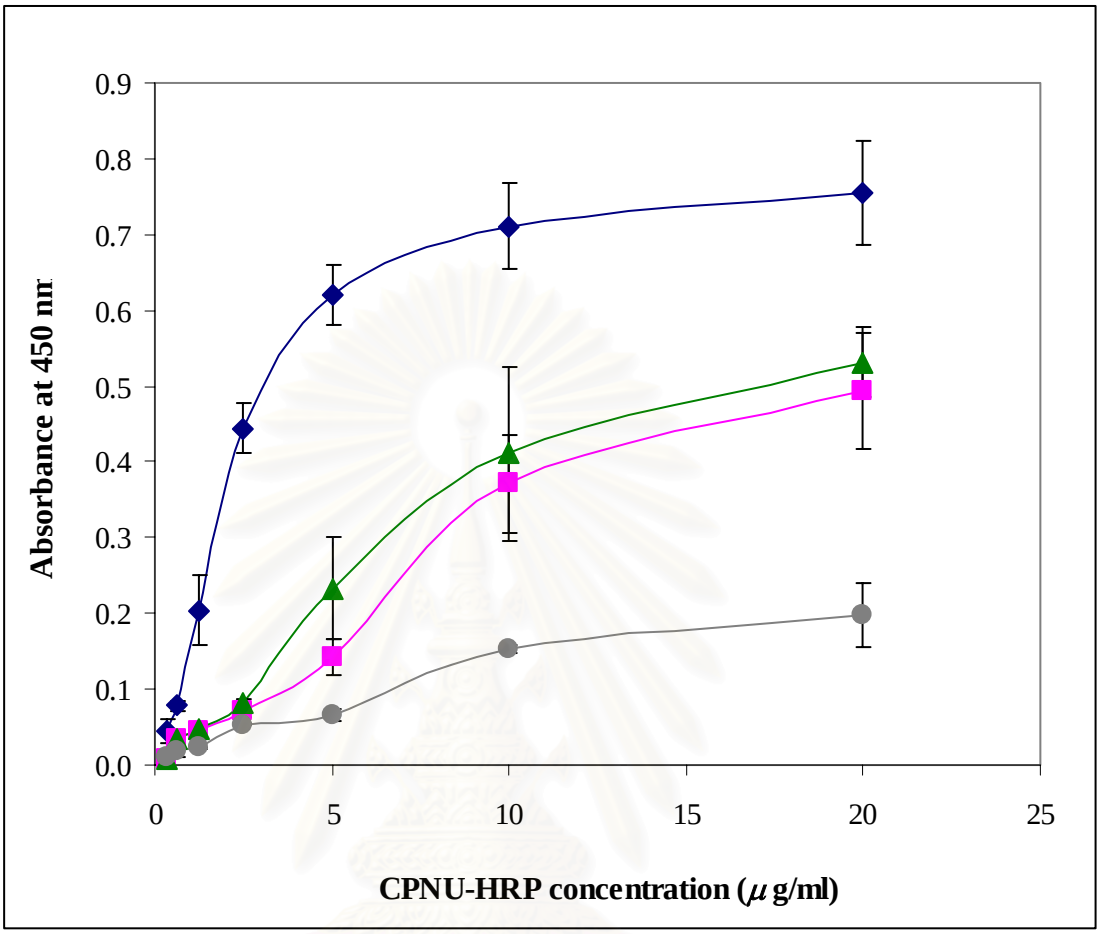


Figure 3.37. Effect of carbaryl tracer concentration on encapsulation. *4% (w/v) β-CD was immobilized on MMAC-HMDA treated plate: 0.3-20 μg/ml of carbaryl tracer (CPNU-HRP conjugate) was used for encapsulation. The immobilized CD (◆), adsorbed CD (■), DVS (▲) and amino plate (●) surfaces referred to as MMAC-HMDA-DVS-CD, MMAC-HMDA-CD, MMAC-HMDA-DVS and MMAC-HMDA surfaces, respectively. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

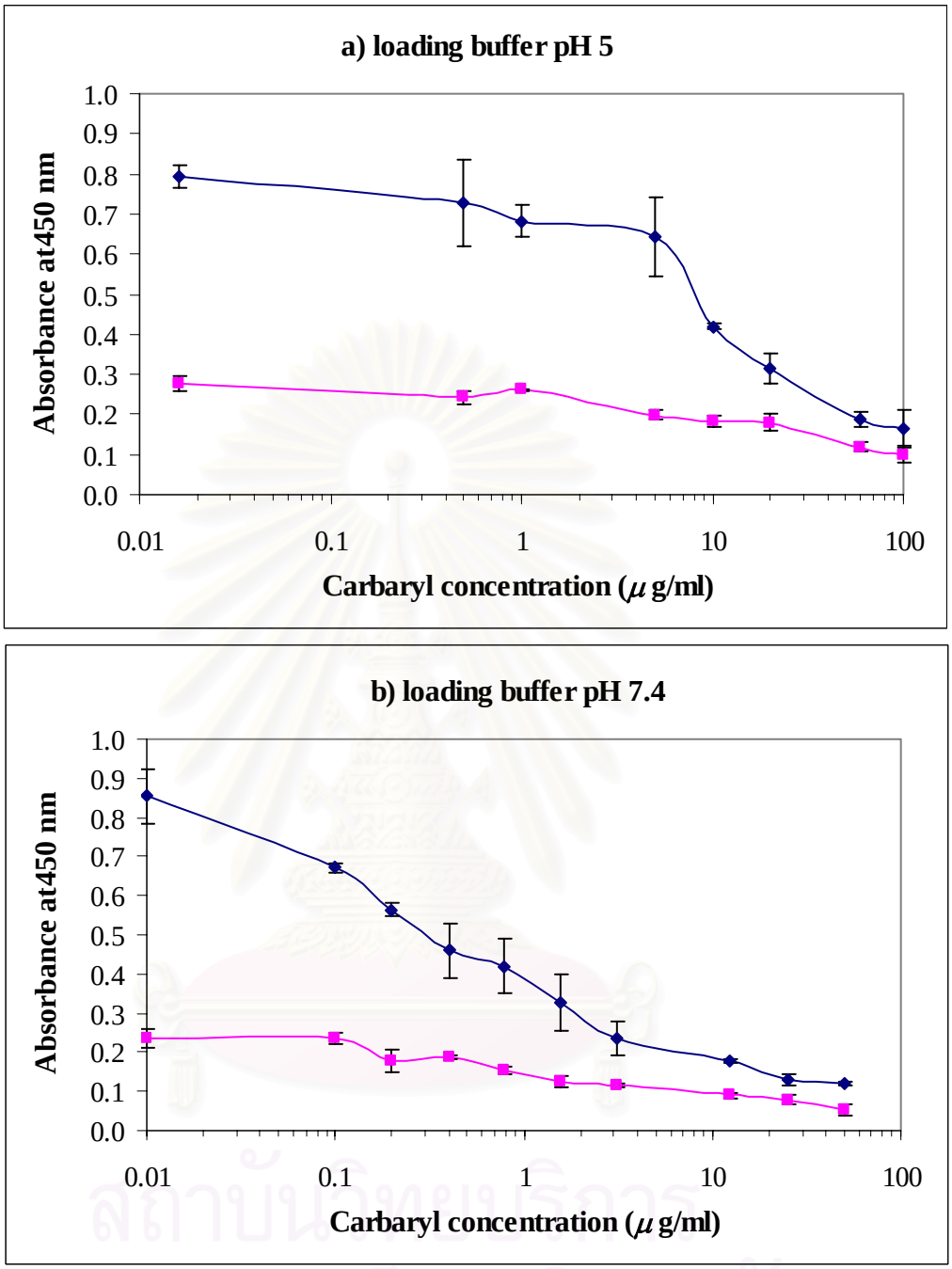


Figure 3.38. Influence of pH on carbaryl encapsulation on immobilized CD (MMAC-HMDA-DVS-CD) (◆) and adsorbed CD (MMAC-HMDA-CD) (■) surfaces: *carbaryl was dissolved in DMF and diluted in a) acetate/citric acid buffer pH 5 and b) PBS pH 7.4. CPNU-HRP at 2.5 μg/ml was used for competitive encapsulation. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

lower than that of the immobilized CD surface. The assay for carbaryl when it was loaded at pH 7.4 was more sensitive than that loaded at pH 5, which was observed by a rapid decrease in signal and the working range covered lower concentration values. Moreover, IC_{50} value of carbaryl loaded at pH 7.4 was 0.4 μg/ml and when loading buffer at pH 5 was used, the IC_{50} value increased (10 μg/ml). Therefore, 10 mM sodium phosphate buffer pH 7.4 containing 137 mM NaCl and 2.7 mM KCl (PBS) was further used as loading buffer for carbaryl standard encapsulation.

A typical standard curve of carbaryl loaded with pH 7.4 displays the working range of 0.2-3 μg/ml and the limit of detection was 0.15 μg/ml.

3.11 Analyses of carbaryl spiked fruit samples by competitive encapsulation with carbaryl tracer into immobilized CD-microtiter plate

The rationale and the procedure for the determination of carbaryl spiked fruit sample using immobilized CD were similar to the determination of carbendazim spiked fruit sample. The method consisted of three parts. First, fruit sample was determined whether it contained carbaryl or not by HPLC. Fruit samples that did not contain carbaryl were set as a negative control. They were used for recovery studies and to determine the matrix effects. In the second experiment, if the matrix interference occurred, the matrix dilution had to be evaluated to provide accurate and precise determination of carbaryl. Finally, the limit of detection (LOD) of carbaryl spiked fruit sample assessed by this method was determined.

3.11.1 HPLC determination

Grape was also chosen as a model sample and was checked for carbaryl by HPLC with fluorescence detector. The excitation and emission wavelength were set at 340 nm and 435 nm, respectively (Parfitt, 2000). First, a standard mixture of N-methylcarbamate pesticides was injected into the HPLC column to determine for the retention time (R_t) of carbaryl. Chromatogram in ***Figure 3.39*** presents R_t of N-methylcarbamate pesticides and the peak of carbaryl was eluted at R_t of 20.51 minutes. Sample extraction was then performed in the same way as described for the extraction of carbaryl (section 2.16.1.1). Grape was extracted with methanol and partitioned into dichloromethane. After extraction, samples were analyzed by HPLC and it was found that no carbaryl residues were detected (***Figure 3.40***). The samples were then used for recovery studies and matrix effect studies.

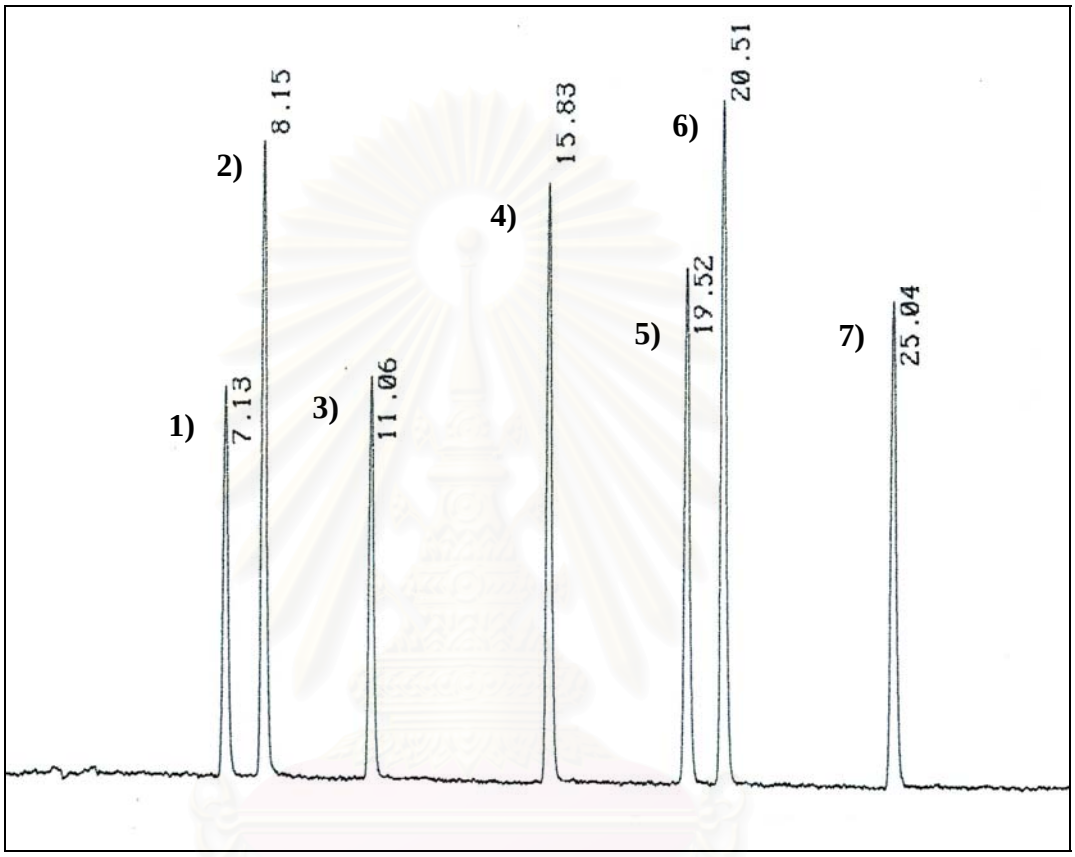


Figure 3.39. HPLC chromatogram of 1 μg/ml of standard mixture of N-methylcarbamates. *A Zobax 5 C8 reverse phase column (4.6x250 mm) was used and eluted with 70% of acetonitrile at a flow rate of 1.2 ml/min with fluorescence detector. Excitation and emission wavelengths of fluorescence detector were set at 340 nm and 435 nm, respectively. Peak identification: 1) oxamyl, 2) methomyl, 3) 3-OH carbofuran, 4) aldicarb, 5) carbofuran, 6) carbaryl and 7) methiocarb.*

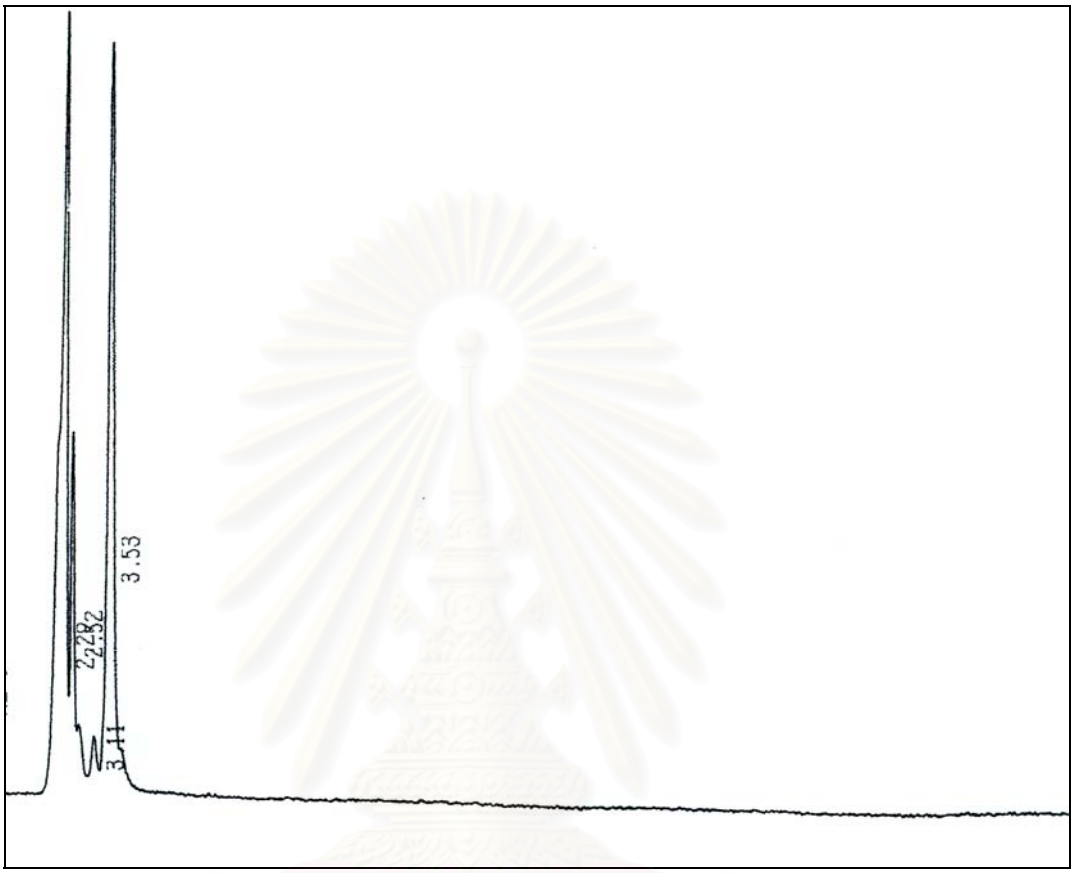


Figure 3.40. HPLC chromatogram of 1 g/ml grape extracted solution. *A Zobax 5 C8 reverse phase column (4.6x250 mm) was used and eluted with 70% of acetonitrile at a flow rate of 1.2 ml/min with fluorescence detector. Excitation and emission wavelengths of fluorescence detector were 340 nm and 435 nm, respectively.*

3.11.2 Recovery studies of carbaryl in spiked fruit sample by HPLC

In this study, the accuracy of the HPLC and the efficiency of the extraction method were determined in term of the percent recovery. The analysis of percent recovery was performed by fortifying known amounts of carbaryl in fruit sample that contained no carbaryl. It was subsequently extracted and determined for recovered carbaryl by HPLC. From fluorescence spectra shown in ***Figure 3.40***, there were no carbaryl residues in grape extract. Grape was then spiked with carbaryl standard at 1 ppm and extracted for the determination of carbaryl residues. It was found that carbaryl was eluted at 20.50 minutes (***Figure 3.41***) and the percent recovery determined by fluorescence detector was 84.1% which was calculated on the basis of its peak area compared to that of the standard carbaryl (***Figure 3.39***). This result indicated that the carbaryl extraction method could be used for the determination of carbaryl in grape.

3.11.3 Matrix effect on the determination of carbaryl in grape by competitive encapsulation with carbaryl tracer

It was necessary to assess the effect of sample matrix on the system of competitive carbaryl tracer encapsulation under specific conditions. The concentration of grape extract at 2 mg/ml was diluted with PBS, pH 7.4 to get the final concentration of 0.05, 0.1, 0.25, 0.5 and 1 g/ml. They were used for the competitive encapsulation with 2.5 μg/ml carbaryl tracer. As shown in ***Figure 3.42***, it was found that 0.05 g/ml of grape extract showed the lowest interference to the assay method. At higher concentration, the sample matrix could inhibit the encapsulation of carbaryl tracer. Therefore, grape extract had to be diluted to get a concentration of 0.05 g/ml prior to the determination of carbaryl in grape.

3.11.4 Determination of carbaryl in grape by competitive encapsulation with carbaryl tracer

In the previous experiment, the concentration of grape extract at 0.05 g/ml was found to be sufficient since it showed the lowest interference. Consequently, it was spiked with carbaryl standard ranging from 0-2.5 μg/ml to get the level of carbaryl residues at 0-50 μg/ml in 1 g/ml grape extract. The carbaryl tracer concentration of 2.5 μg/ml was used for competitive encapsulation. A typical inhibition curve of carbaryl is shown in ***Figure 3.43***. As it corresponded to competitive assays, the signal was inversely proportional to the analyte concentration. The working range or the central section of the curve with a nearly linear response was 0.02-0.35 μg/ml. In addition, the IC_{50} value which is the concentration of carbaryl that can inhibit half encapsulation of carbaryl tracer was 0.06 μg/ml and the limit of detection was 0.015 μg/ml.

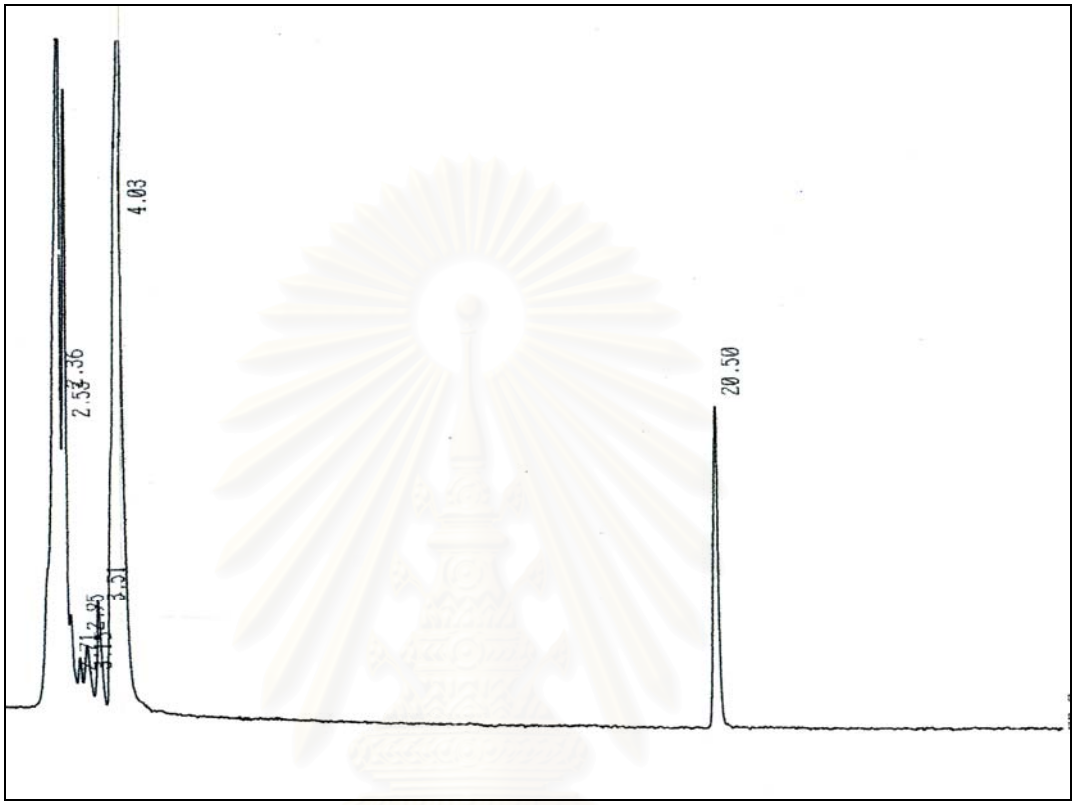


Figure 3.41. HPLC chromatogram of 1 ppm of carbaryl spiked grape extracted solution. *A Zobax 5 C8 reverse phase column (4.6x250 mm) was used and eluted with 70% of acetonitrile at a flow rate of 1.2 ml/min with fluorescence detector. Excitation and emission wavelengths of fluorescence detector were 340 nm and 435 nm, respectively.*

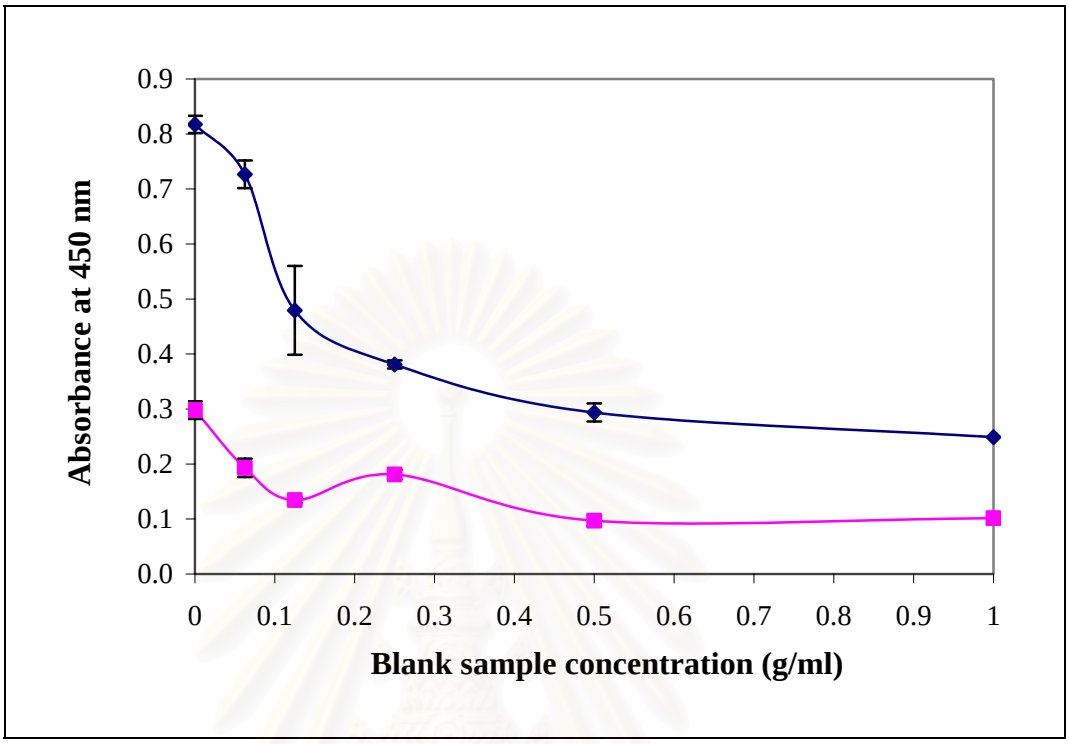


Figure 3.42. Effect of grape matrix on the competitive inhibition with 2.5 μg/ml carbaryl tracer. *Grape extracted solution was loaded at pH 7.4. The immobilized CD (◆) and adsorbed CD (■) surfaces referred to MMAC-HMDA-DVS-CD and MMAC-HMDA-CD, respectively. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

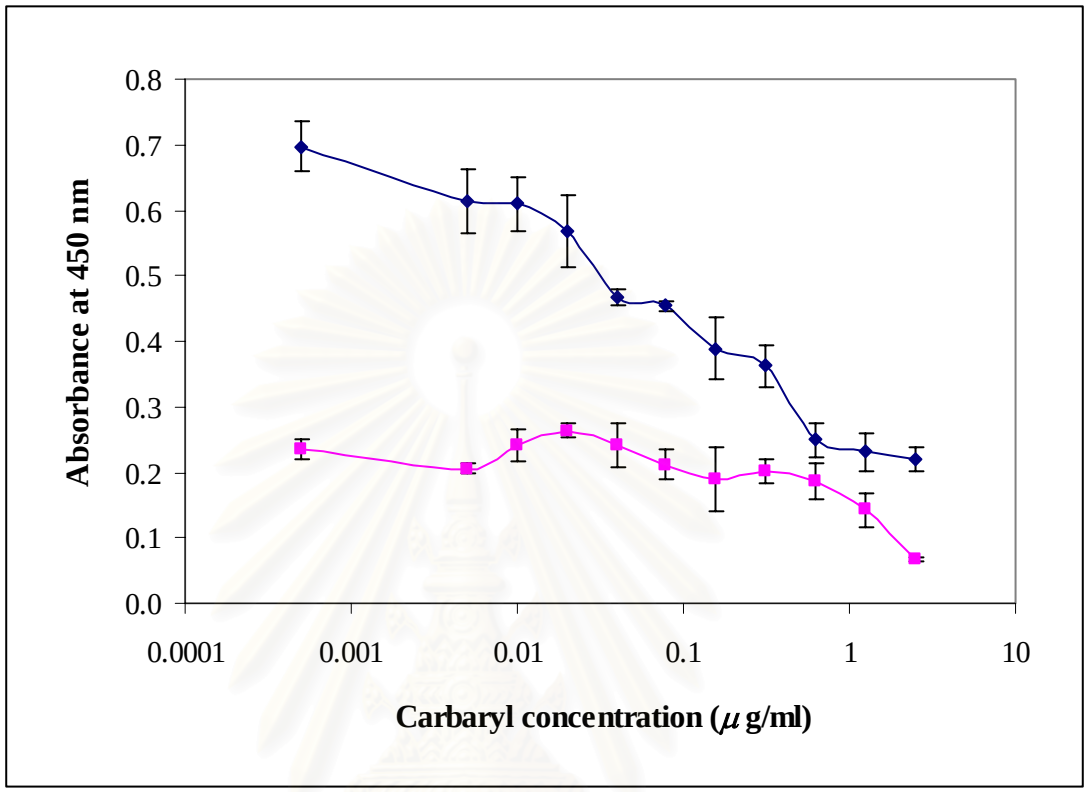


Figure 3.43. Standard curve of carbaryl in the presence of matrix by competitive encapsulation with carbaryl tracer. *1 mg of carbaryl standard was dissolved in 0.5 ml DMF and loading buffer pH 7.4 was added to make 10 ml in volumetric flask. 0.05 g/ml grape extract was then spiked with 0-2.5 μg/ml of carbaryl standard and loaded at pH 7.4. Carbaryl tracer at concentration of 2.5 μg/ml was used for competitive encapsulation. The immobilized CD (◆) and adsorbed CD (■) surfaces referred to MMAC-HMDA-DVS-CD and MMAC-HMDA-CD, respectively. Points: means of triplicate wells. The error bars represent the standard deviation of triplicate wells.*

CHAPTER IV

DISCUSSION

In Thailand, benzimidazole fungicides and carbaryl are still widely used as broad spectrum fungicides and insecticide, respectively for their high activity against many major agricultural pests (www.doa.go.th/toxic/menu.html). Owing to their extensive use and thus contamination in soil, water and food has become a serious problem. Several methods have then been developed for their determination, both in environmental and food samples, including high-performance liquid chromatography (HPLC) (Massey *et al.*, 1995; Bernal *et al.*, 1997; Muccio *et al.*, 1999; Gou *et al.*, 2000; Sandahl *et al.*, 2000; Orejuela and Silva, 2003) and enzyme-linked immunosorbent assay (ELISA) (Newsome and Collins, 1987; Bushway, 1996; Nunes *et al*., 1998; Abad *et al.*, 2001; Brandon *et al*., 2002; Mickova *et al*., 2003). Developing of new analytical methods for their determination is of great interest in many laboratories. Recently, molecular host-guest systems have attracted much attention due to the fact that guest molecules can be targeted by forming inclusion complex with the hosts. Cyclodextrins (CDs) which are cyclic oligosaccharides have the capacity to bind a variety of guest molecules inside the apolar cavity. Many potential applications require the immobilization of CDs, for example, the use of immobilized CDs as sensors for the determination of tetracyclines (Gong and Zhang, 1997; Yang *et al*., 2000), warfarin (Badia and Diaz-Garcia, 1999), steroids (Kummer *et al*., 1996) and terpenes (Clarot *et al.*, 2000). Very few studies have been reported on molecular recognition for analytical purposes of pesticides with CDs.

Jongmeevasana (2000) was first reported the use of β-CD immobilized on commercially available amino plates for the determination of benzimidazole fungicides. The determination method was based on competitive inhibition ELISA. Nevertheless, this kind of plate was very expensive. Following this line, the present work described the preparation of amino plate by the use of methyl vinyl ether-maleic anhydride copolymer (MMAC) and 1, 6-hexamethylenediamine (HMDA) and β-CD was subsequently immobilized. The determination of benzimidazole fungicides was then investigated and the result was compared to the previous method. In addition, the complexation of carbaryl molecule with β-CD has been reported (Barbato *et al*., 2000; Saikosin *et al*., 2002). Hence, the immobilized β-CD on prepared amino microtiter plate was also applied for the detection of carbaryl residues.

4.1 Benzimidazole and carbaryl tracers synthesis

The use of *β*-CD immobilized on microtiter plates for the determination of benzimidazole fungicides and carbaryl was based on competitive-inhibition enzyme immunoassay. Thus, benzimidazole and carbaryl tracers were constructed. 2-Succiamidobenzimidazole (SAB) and 1-(5-carboxypentyl)-3-(1-napthyl) urea (CPNU) conjugated to horseradish peroxidase (HRP) were used as benzimidazole and carbaryl tracers, respectively for competitive encapsulation into immobilized *β*-CD-microtiter plate.

In ELISA technique for the determination of benzimidazole fungicides and carbaryl, SAB (Newsome and Shields, 1981) and CPNU (Marco *et al*., 1997) were haptens that coupled to human albumin or bovine serum albumin (BSA) as the immunogen and HRP as tracers. In this method, CD was used instead of antibody and SAB and CPNU conjugated to HRP as tracers were used to compete with carbendazim and carbaryl, respectively into immobilized CD cavity. Owing to the carbendazim and carbaryl structures, they lack reactive functional groups for the conjugation (***Figure 3.1*** and ***3.2***). Therefore, carboxyl groups were introduced into the molecules by chemical procedures. Carbendazim (Lezcano *et al*., 2002) and carbaryl (Barbato *et al*., 2000 and Saikosin *et al*., 2000) have been shown to be encapsulated by *β*-CD, so SAB and CPNU which have the similar structures and thus, similar properties (***Figure 3.3*** and ***3.4***) to carbendazim and carbaryl should also be able to encapsulate into CD cavities. The assay of HRP conjugate activity was investigated for the amount of the encapsulated tracers.

4.1.1 SAB and CPNU synthesis

Newsome and Shield (1998) described that SAB was synthesized by the reaction of acid anhydride. 2- aminobenzimidazole formed amide bond with succinic anhydride to give SAB as a product. The product obtained was identified as SAB by NMR spectroscopy and the molecular mass was 233 as reported by Jongmeevasana (2000).

CPNU was synthesized from 1-naphthyl isocyanate and 6-aminohexanoic acid via amide formation. After construction of CPNU, its chemical structure was analyzed by ^{13}C and ^{1}H NMR spectroscopy. NMR spectra showed that it consisted of –CH in aromatic ring, $-CH_2$, -COOH and –NH which corresponded to the functional groups of CPNU. Additionally, the molecular mass of the product was found to be 300 (M plus H as 301). This value was in good agreement with that calculated from its chemical structure and also coincided with the previous report (Marco *et al*., 1993).

4.1.2 Conjugation and purification of benzimidazole and carbaryl tracers (SAB-HRP and CPNU-HRP conjugates)

SAB and CPNU were covalently attached to HRP as benzimidazole and carbaryl tracers using 1-(-3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (a water-soluble carbodiimide, WSC). Carbodiimides are important tools for the preparation of conjugates used in a lot of immunotechnologies and for protein studies. Bauminger and Wilchek (1980) introduced the use of carbodiimides as cross linkers in the preparation of immunizing conjugates. Peptide bond was formed between amino groups of proteins and carboxyl groups of hapten molecules (***Figure 3.10***). ε-Amino group of lysyl residues of protein carrier are the amino groups most involved in the reaction (Bauminger and Wilchek, 1980). The urea formed during the reaction can be removed by dialysis or by gel filtration when used to couple haptens to high molecular weight carriers. The preparative procedure is simple and easy to perform. Therefore, SAB and CPNU were conjugated to HRP by WSC. First, HRP was activated by WSC, carboxyl groups of SAB and CPNU were subsequently coupled to the activated HRP possibly through the amine in the side-chain of lysine. After concentrated with Centricon (membrane MW cut-off 10,000 Da), the HRP conjugates were separated from excess SAB or CPNU (observed by another A_{280} peak eluted after protein peak) (***Figure 3.11*** and ***3.12***) and urea (by product) by Sephadex G-25 gel filtration prior to characterization of the conjugates. Gel filtration chromatography separates protein according to size. The column matrix is a cross-linked polymer with pores of selected size (Nelson and Cox, 2000). HRP (large molecule) migrated faster than SAB or CPNU (small molecules) because it was too large to enter the pores in the beads and hence took a more direct route through the column. The smaller molecules entered the pores and were slowed by the more labyrinthine path they took through the column.

4.2 Characterization of benzimidazole and carbaryl tracers

After preparation of the SAB-HRP and CPNU-HRP conjugates, the biochemical properties including maximum absorption and activity of benzimidazole and carbaryl tracers were investigated. In order to prove that they were HRP conjugates, those properties were compared with those of the native HRP.

4.2.1 Maximum absorption of benzimidazole and carbaryl tracers

The spectrophotometric property of SAB, CPNU, native HRP and modified HRPs (benzimidazole and carbaryl tracers) was determined. Absorption

maximum wavelengths of the native HRP were observed at 280 and 405 nm due to amino acid with aromatic side chains and heme groups in HRP molecule (Molina *et al*., 2003). SAB and CPNU which have aromatic rings in their molecules showed the peaks at 282 and 277 nm, respectively. When SAB-HRP conjugate was scanned, two maximum absorption peaks were observed at 282 and 405 nm. Owing to SAB molecules introduced to benzimidazole tracer, the absorbance at 280 nm of the HRP conjugate significantly increased in comparison with the native HRP (***Figure 3.13***). This confirmed that SAB was linked to HRP. For carbaryl tracer, the maximum absorption peaks were observed at 280 nm and nearly at 405 nm. Although same concentration of native and CPNU-HRP was used (defined by the absorbance at 405 nm), the peak of CPNU-HRP conjugate at 280 nm was slightly higher than that of native HRP. Thus, another way to confirm that CPNU was conjugated to HRP was to check whether carbaryl tracer could be encapsulated by immobilized CD-microtiter plate or not when it was used in competitive encapsulation to determine carbaryl. It was found that CPNU-HRP activity was observed on the plate, indicating that CPNU was successfully coupled to the protein (***Figure 3.36***).

4.2.2 The activity of benzimidazole and carbaryl tracers

The specific activity of modified HRPs was determined before being used as tracers. Generally, the activity of HRP was partially lost during the chemical modification because of the extreme condition used and thus, resulted in its conformational changes.

HRP is an important heme-containing enzyme that has been used in chemiluminescent assay and enzyme immunoassay as enzyme tracer (Hashimoto *et al*., 1998; Szulowski *et al*., 1999; Totsune *et al*., 1999; Ximenes *et al*., 2001; Metelitza *et al*., 2004). It consists of 308 amino acid residues and the molecular weight has been reported to be approximately 40,000 Dalton (Welinder, 1976). HRP catalyzes the transfer of two electrons from a substrate (DH) to hydrogen peroxide, generating H_2O and an oxidized donor (colored end product, D) as shown below.

$$H_2O_2 + 2DH \xrightarrow{\text{Peroxideses}} 2H_2O + D$$

The basis of colorimetric assays has been used for the determination of HRP activity. A number of chromogenic substrates for HRP are available such as o-phenylenediamine (OPD), 3, 3'-diaminobenzidine (DAB) and 2, 2'-azino-di(3-

ethylbenzthiazoline)-6-sulphonic acid (ABTS) (Engvall, 1980; Graham and Karnovsky, 1996; Watanabe *et al*., 2002). However, those compounds were found to be mutagenic and possibly carcinogenic (Ames *et al*., 1975; Garner, 1975; Voogd *et al*., 1980). A developed compound, 3,3',5,5'-tetramethylbenzidine (TMB), was shown to be a non-carcinogenic chromogen with high intensity of color development for the end point determination (Bos *et al*., 1981; Cattaneo and Luong, 1994; Madersbacher and Berger, 2000; Metelitza *et al*., 2004). The catalytic cycle of HRP with TMB and color development of TMB are illustrated in ***Figure 4.1***. 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 ($TMB{\cdot}TMB^{2+}$), it turns to be blue species with the absorption coefficient of 35,800 $M^{-1}cm^{-1}$ at 650 nm. While the second product (TMB^{2+}), observed after the addition of H_2SO_4, is the yellow one with the absorption coefficient of 67,300 $M^{-1}cm^{-1}$ at 450 nm. The color development of HRP in this study used TMB as a chromogenic substrate due to its high intensity and safety. The relative activities of SAB-HRP and CPNU-HRP conjugates were 82% and 90%, respectively to that of the native HRP. It could be concluded that the conjugation between lysine residues of HRP and carboxyl groups of SAB and CPNU via carbodiimide method hardly disturbed HRP activity. This was because lysine residues normally located in the exposed region of most proteins and they are usually not involved in the enzyme activity. Hence, the activity of HRP still remained.

4.3 Preparation of amino plate

An objective of this experiment was to prepare the amino plate from polystyrene microtiter plate using MMAC and HMDA. The prepared amino plate was then used for the immobilization of β-CD.

Conventional ELISA procedures were usually carried out by immobilizing antigen or antibody on a polystyrene microtiter plate through adsorption which suffer from several shortcomings. These include: 1) inconsistent ELISA values in different wells and plates; 2) long incubation times; 3) non-reproducible results due to detachment of biomolecules during washing; and 4) lower sensitivity. On the other hand, covalent binding is more sensitive, minimizes nonspecific binding and eliminates those problems (Isosaki *et al*., 1992; Nahar *et al*., 2001; Bora *et al*., 2002). Immobilization of proteins on microplate wells by simple adsorption is convenient, but it can be inefficient, especially if proteins are hydrophilic or small in size (Suzuki *et al*., 1997).

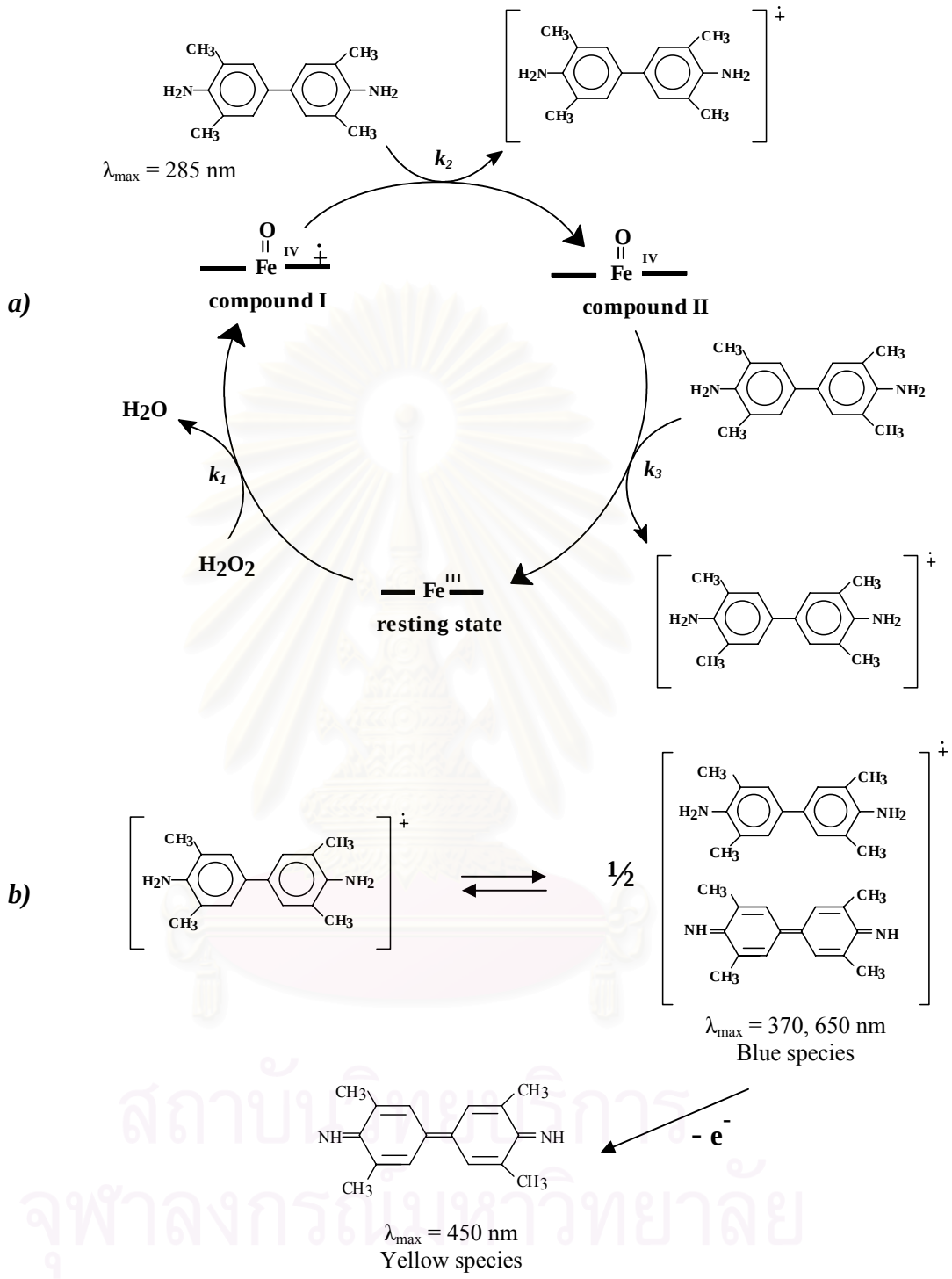


Figure 4.1. Schematic diagram illustrating a) the catalytic cycle of HRP with TMB as chromogenic substrate and b) color development of TMB. *The rate constants k_1, k_2 and k_3 represent the rate of compound I formation, rate of compound I reduction and rate of compound II reduction, respectively (modified from Veitch, 2004 and Cattaneo and Luong, 1994). Compound I-III are HRP with different reduction states of Fe.*

If the substances are coupled to the well via covalent bonds, the immobilization should be stable and efficient. Hence, several improved covalent immobilization methods have been reported, e.g., Ukeda *et al*. (1996) has reported that glucose oxidase and peroxidase were immobilized on amino microtiter plate using glutaraldehyde as cross-linker. Suzuki *et al*. (1997) reported that polyvinylbenzyl lactonoylamide (PVLA) was strongly adsorbed to the hydrophobic well surface, and its lactonamide part could be oxidized with periodate to generate aldehyde groups. Proteins were then immobilized covalently to the aldehyde groups by reductive amination under mild conditions. Morvover, Hatakeyama *et al*. (1996) reported that carbohydrates were chemically immobilized on amino microtiter plate by a bifunctional cross-linking reagent, divinyl sulfone. MMAC has been successfully used for the immobilization of protein ligands on microtiter plates (Isosaki *et al*., 1992). MMAC is a water-insoluble polymer with active acid anhydride groups that react with amino groups of ligands to form stable covalent amide bonds. Moreover, due to its high hydrophobicity, it seems to bind to the surface of the wells by hydrophobic interactions and this binding is expected to be stable in aqueous solutions (Isosaki *et al*., 1992). However, no good method for the direct and efficient immobilization of small peptides or oligosaccharides has been observed. Until 1998, Satoh *et al*. described a newly developed MMAC method to immobilize small peptides and small linear oligosaccharides by improving the method with addition of 1, 6-hexamethylenediamine (HMDA) and adipic acid dihydrazine (ADHZ).

According to the developed MMAC method, the preparation of amino plate was carried out as follows. ELISA plates were first coated with MMAC and an excess of active anhydride groups was introduced. They were subsequently reacted with either amino groups of HMDA or hydrazino groups of ADHZ. Finally, the wells of microtiter plates were obtained with either amino or hydrazino groups having long spacer arms as shown in ***Figure 3.15***.

Since carbendazim and carbaryl were conjugated to HRP via its lysine residues, the orientation of the immobilized enzyme would be in random fashion. This may prevent substrates from reaching its active site if an enzyme active site was held towards the matrix. However, the use of a spacer arm introduced to the plate was expected to reduce this steric hindrance, and thus, effective protein immobilization was obtained.

4.4 Determination of amino and hydrazino groups introduced to MMAC-coated plates

After the amino microtiter plates were prepared, the amounts of amino and hydrazino groups introduced to MMAC-coated plates were determined by the use of avidin-biotin system.

Biotin (vitamin H, coenzyme R; F.W. = 244.3) is a water soluble vitamin that binds with high affinity ($k_a = 10^{15}$ M^{-1}) to avidin (M_r = 66,000), a tetrameric, basic glycoprotein, abundant in raw egg white. The interaction between avidin and biotin is the non-covalent interaction. Biotin can be attached to a variety of proteins, nucleic acid, lipid and lectins, often without altering their properties. Similarly, avidin (or streptavidin, its nonglycosylated prokaryotic equivalent) can be joined to reporter enzymes such as HRP and alkaline phosphatase whose activity can be used to quantitate avidin-biotin-target complexes. Derivatives of biotin, biotinyl-*N*-hydroxysuccinimide ester (BNHS), biotinyl-p-nitrophenyl ester (BNP) and sulfo-*N*-hydroxysuccinimide biotin ester (sulfo-BNHS), are used to biotinylation of proteins, peptides and other molecules via amino group (Wilchek and Bayer, 1990).

For the examination of amino and hydrazino groups on microtiter plate surfaces, the sulfo-BNHS and avidin-HRP conjugate (AV-HRP) were used as biotinylated probe and reporter, respectively. The reaction involves the nucleophilic attack between the unprotonated amino groups on surfaces and carbonyl groups of sulfo-BNHS ester. This results in a stable amide bond and sulfo-NHS groups are subsequently left from the biotinylated surfaces as leaving groups (***Figure 4.2***). AV-HRP added would then bind specifically to the biotinylated surfaces prior to determination of HRP activity. The amount of amino groups on MMAC-HMDA treated plate was comparable to that of hydrazine groups on MMAC-ADHZ treated plate and much higher than that of the commercially available amino plate (***Figure 3.18***). Similar result was reported by Satoh *et al*. (1998) that the amount of amino groups on HMDA treated plates was much larger than that of commercially available amino plates. This could be due to large amount of amino groups was introduced. In addition, the amino groups on prepared amino plate were primary amine ($-NH_2$) while those on commercially available amino plate were secondary amine ($-NHCH_3$) (***Figure 4.3***). It can be concluded that the amino plate was successfully prepared by the use of developed MMAC method and HMDA was chosen for the introduction of amino group to MMAC-coated plate. This was because the amount of amino groups on HMDA-treated plate was slightly higher than that of ADHZ-treated plate at saturation (***Figure 3.18***).

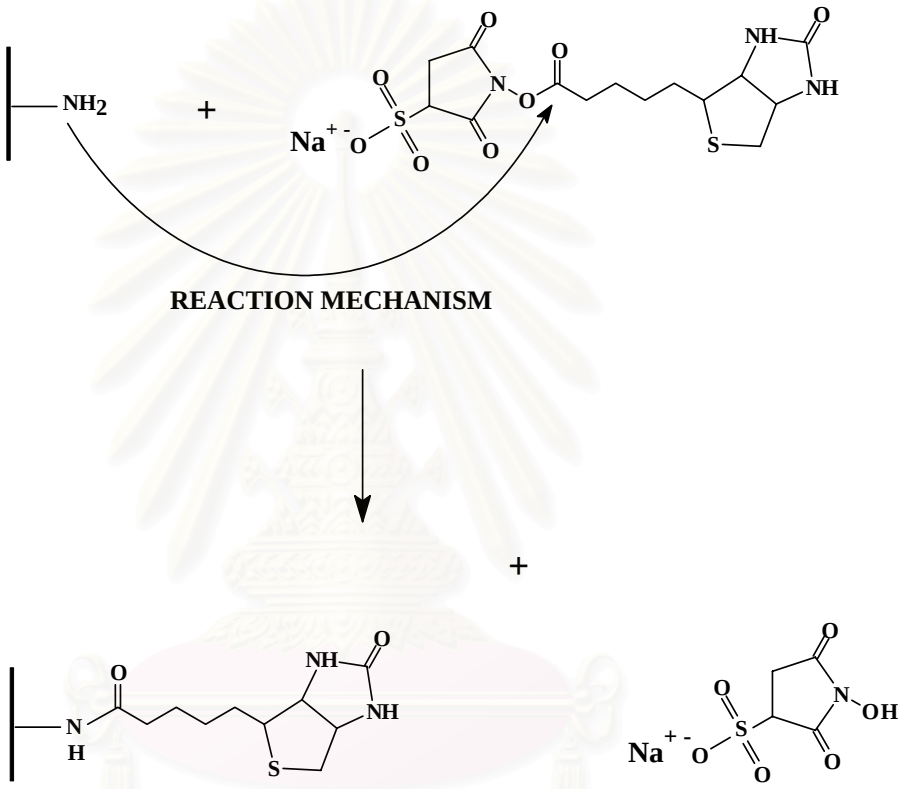


Figure 4.2. The mechanism of the reaction between sulfo-BNHS and the amino group on the microtiter plate surface.

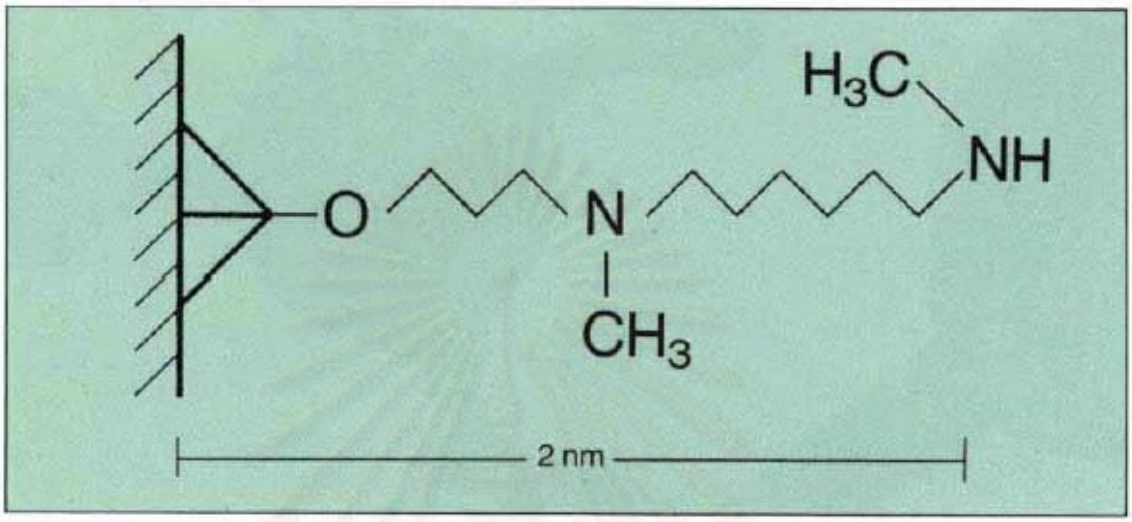


Figure 4.3. Schematic chemical and physical configuration of the CovaLink NH surface (http://www.nuncbrand.com/page. asp?ID=553&lang=GB).

4.5 Immobilization of cyclodextrin on MMAC-HMDA coated plate

There are many studies described the methods for the immobilization of carbohydrates and oligosaccharides on microtiter plates. Satoh *et al*. (1999) reported that the formyl groups of reducing end of oligosaccharides reacted with hydrazino groups of ADHZ on MMAC-ADHZ treated plate to form Schiff bases. These bases were then reduced to stable alkylamine bonds by reductive amination using $NaBH_3CN$. In addition, chemical immobilization of the carbohydrates on amino type microtiter plates using a bifunctional cross-linking reagent, divinyl sulfone (DVS) was carried out by Hatakayama *et al*., (1996). For CD, Jongmeevasana, (2000) has reported that CD could also be immobilized on CovaLink NH, a microwell plate with secondary amino groups bound to its surface by DVS. Nevertheless, this commercially available amino plate is very expensive. Therefore, we attempted to use prepared amino plate (MMAC –HMDA treated plate) as a solid phase for β-CD immobilization and DVS was used as a bifunctional cross-linking reagent. The DVS coupling method is well suited for the attachment of hydroxyl groups and amino groups (Porath, 1974). According to primary and secondary hydroxyl groups on the rims of CD cavity, CD can be covalently immobilized to the amino plate with the use of DVS (***Figure 3.19***).

First, amino groups on MMAC-HMDA treated plate were activated with DVS for 1 hour. In the subsequent step, the DVS activated surface would couple to hydroxyl group of CD. After incubation for 18 hours at room temperature (25 °C), excess CD was removed by washing with water. Finally, the addition of 1M Tris was used to block the remaining vinyl groups. Moreover, Tris was found to be an appropriate buffer for protein immobilization because it maintained the water insolubility of MMAC coated surfaces (Isosaki *et al*., 1992). When acid anhydride groups of MMAC reacted with HMDA, they produced amino groups and carboxyl groups to the plate. The water solubility of HMDA-MMAC treated plate increased when the reaction was carried out in phosphate or citrate buffer. On the contrary, when the reaction was performed in Tris, the amino group of Tris could form amide group with the remaining carboxyl group of MMAC. Thus, the HMDA-MMAC treated surface maintained its water insolubility (***Figure 4.4***).

The orientation of immobilized CD could not be controlled. Both of primary and secondary hydroxyl groups could be covalently coupled to the surfaces. If the immobilized secondary hydroxyl groups occur, the narrow rim would expose. In this situation, it would be difficult for the guest molecule to be encapsulated into CD cavity.

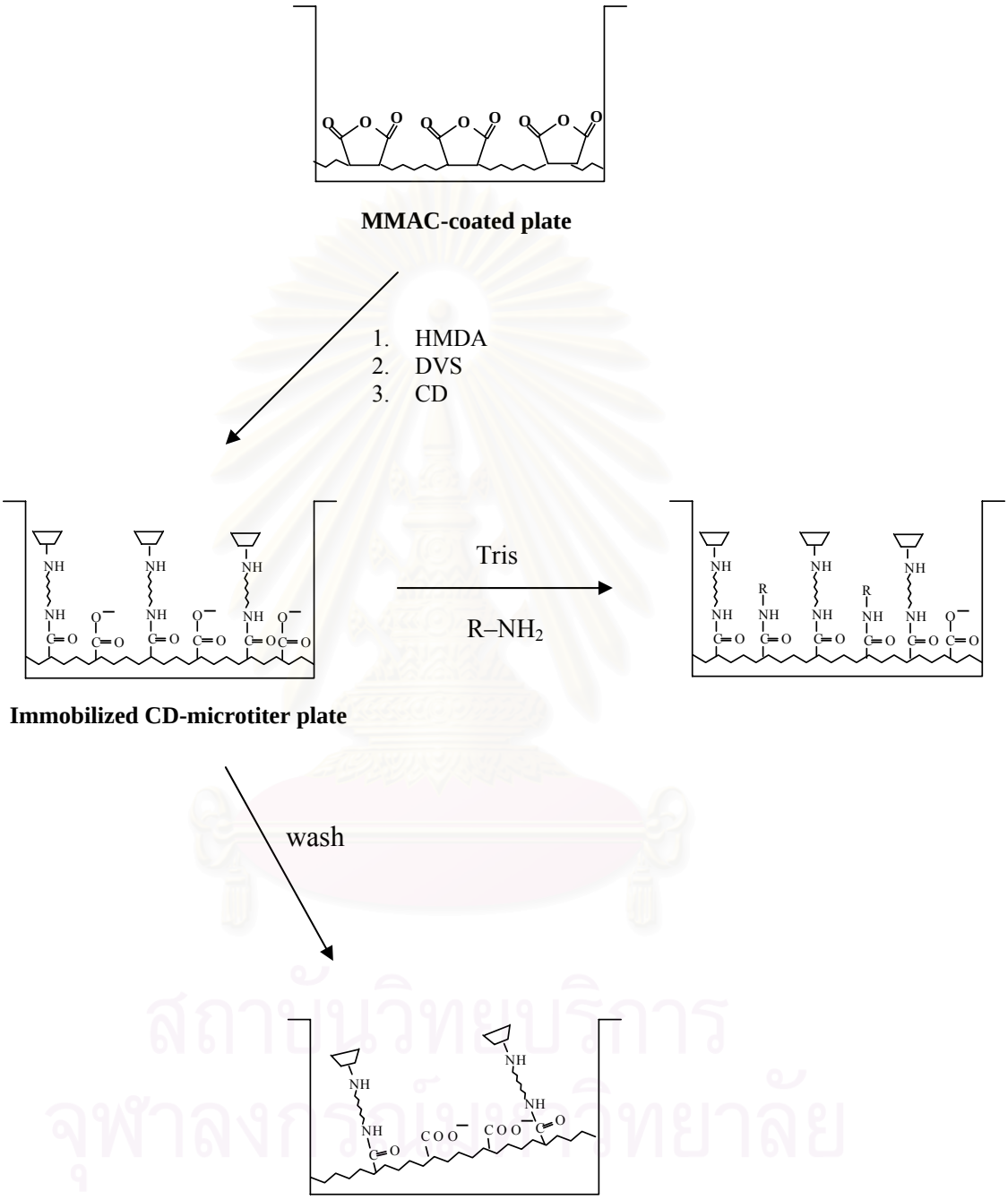


Figure 4.4. Effect of Tris on immobilization of CD on the well of MMAC-HMDA treated plate.

However, Szejtli (1998) reported that C-2-OH group of one glucopyranoside unit can form a hydrogen bond with the C-3-OH group of the adjacent glucopyranose unit. Thus, a complete secondary belt is formed by these H bonds, resulting in a rather rigid structure of β-CD and the chance of the CD immobilization via its primary hydroxyl is higher than secondary hydroxyl groups.

4.6 Capability test of immobilized β-CD –microtiter plate for benzimidazole tracer encapsulation

After the immobilization of β-CD, the benzimidazole tracer (SAB-HRP conjugate) was used for encapsulation in order to assess the ability of immobilized CD for guest encapsulation and to determine the amount of immobilized CD on surfaces. To confirm that CD was covalently immobilized on MMAC-HMDA treated plate through DVS, four control surfaces were performed: 1) amino (MMAC-HMDA), 2) DVS (MMAC-HMDA-DVS), 3) adsorbed CD (MMAC-HMDA-CD) and 4) immobilized CD (MMAC-HMDA-DVS-CD). The benzimidazole tracer encapsulated into β-CD immobilized or adsorbed on surfaces was assessed for their specific binding. While the amino and DVS surfaces were used to investigate the non-specific adsorption of benzimidazole tracer. The binding behavior between SAB-HRP conjugate and native HRP on all surfaces was compared to confirm that the HRP activity observed was due to SAB-HRP encapsulation and not just HRP adsorption. The result showed that the SAB-HRP conjugate activities bound to all surfaces were significantly higher than the native HRP activities (***Figure 3.20***). This suggested that native HRP could not encapsulate into CD cavity and showed little adsorption on surfaces. Considering the signal of SAB-HRP conjugate, it was found that CD was bound to amino surface by both adsorption and immobilization through DVS and high non-specific adsorption of SAB-HRP conjugate was observed. Besides, the high variation of SAB-HRP activity on all surfaces was obtained (observed by the large error bars). This could be due to the fact that the concentration of SAB-HRP conjugate (0.40 μg/ml) used for encapsulation was too low and may not reach saturation yet. Hence, it would not cover all the guest binding sites on the immobilized CD. And as a result, the specific and non-specific binding could not be distinguished. Another factor that may have caused this was the low concentration of β-CD (2% w/v) used for immobilization to cover all the microtiter well. Hence, the further experiment was designed to find the condition to reduce this non-specific interaction with the surface.

4.6.1 Effect of MMAC concentration

According to Satoh *et al*. (1998) and Isosaki *et al*. (1992), MMAC with M_r = 41,000 was used to coat the well of microtiter plate. In this study, we used MMAC with M_r = 216,000 which was higher than previous reports. Therefore, the appropriate concentration used to coat the plate was examined. Benzimidazole tracer concentration was increased to 10 μg/ml which gave 0.5 μg/well (50 μl). The result in ***Figure 3.21*** showed that the highest signals of SAB-HRP conjugate were observed on immobilized CD surfaces when 0.5 and 1.25 mg/ml of MMAC were used. At MMAC concentration of 5 mg/ml, the signal on immobilized CD surface was lower. This could be explained by the fact that MMAC at high concentration did not seem to adsorb on microtiter plate as a monolayer. As a result, CD could not be immobilized on this surface efficiently and SAB-HRP conjugate would not be able to cover all guest binding sites on the immobilized CD. On the contary, at too low concentration of MMAC, SAB-HRP conjugate was found to give relatively high non-specific adsorption. However, at 1.25 mg/ml, the highest different signal between specific and non-specific binding of SAB-HRP conjugate was observed. Hence, 1.25 mg/ml of MMAC was used to coat the polystyrene microtiter plate prior to the introduction of the amino group with HMDA.

4.6.2 Effect of β-CD concentration

β-CD concentration at 1%, 2.5 %, 4% and 5% (w/v) was immobilized on MMAC-HMDA treated plate through DVS in order to examine the suitable concentration. For the immobilized CD surface, the signal of SAB-HRP obtained at 4%β-CD was found to be the highest and the difference in HRP activities between specifically and non-specifically bound molecules was also found to be the highest (***Figure 3.22***). However, it also showed that the signal of SAB-HRP conjugate non-specifically bound to DVS (MMAC-HMDA-DVS) was significantly higher than that simply adsorbed onto amino surfaces (MMAC-HMDA). This indicated that SAB-HRP conjugate could be immobilized on DVS surface via its amino groups. When concentration of β-CD was high, the coverage of SAB-HRP was expected to increase. Nevertheless, this phenomenon did not occur when the plate was immobilized with 5% of β-CD. When 5% of β-CD solution was incubated with the microtiter well for a long time, CD became crystallized. Therefore, all of CD molecules may not be able to be immobilized on the surfaces. Thus, the SAB-HRP conjugate activity bound on 5% of immobilized β-CD was lower than that of 4% of immobilized β-CD. The β-CD

concentration at 4% was then chosen for the immobilization on MMAC-HMDA treated plate.

4.6.3 Effect of benzimidazole tracer concentration

The optimal benzimidazole tracer concentration was determined and the result in ***Figure 3.23*** presented a dose-dependent manner at benzimidazole tracer concentration between 0 and 5 μg/ml. The SAB-HRP conjugate activity bound to immobilized CD surface was the highest. It could be concluded that β-CD was successfully immobilized on the MMAC-HMDA treated plate by the use of DVS as a cross-linker. However, high concentration of benzimidazole tracer used for encapsulation into immobilized CD could increase the non-specifically bound tracer on other surfaces (amino, DVS and adsorbed CD surfaces). Benzimidazole tracer concentration at 5 μg/ml was the concentration that covered the entire guest binding sites on immobilized CD and it also showed high contrast between specific and non-specific binding. Thus, 5 μg/ml of benzimidazole tracer was selected for the encapsulation in the next experiment.

4.6.4 Effect of washing buffer

The results obtained from previous experiments indicated that SAB-HRP conjugate could absorb on DVS and amino surfaces. Besides, CD was not only immobilized on MMAC-HMDA-DVS surface but also adsorbed on amino surface too. The different type of washing buffer was then investigated to reduce the non-specific interaction of SAB-HRP conjugate and the underlying surface. In ELISA, false positive reactions can sometimes occur. These false positive reactions are considered to be mainly dependent on non-specific bindings such as electrostatic and hydrophobic interaction of other proteins or biomolecules to non-occupied spaces on the surface of ELISA plate (Miyakava *et al*., 2001). Both salt and pH predominantly affect the dissociation of non-specifically bound (electrostatic interaction) protein (Sidorova and Rau, 2001). The result in ***Figure 3.24*** showed that 0.1 M sodium acetate/ citric acid buffer, pH 5 with high salt concentration (0.5 M NaCl) could disrupt the interaction between SAB-HRP and DVS or amino plate in contrast with the use of the same buffer without salt. This suggested that a change in ionic strength could affect the electrostatic interaction between SAB-HRP and the surfaces. When Tween was used, the SAB-HRP activity observed was very low on all surfaces. Tween is a detergent and has been used

to eliminate the hydrophobic interaction (Giovannoli *et al*., 2003), thus it can also disrupt the adsorption of MMAC on microtiter plate. When, the surfaces were washed with high pH buffer, both specific and non-specific binding of SAB-HRP were also removed. According to Ni *et al.* (2002), carbendazim was encapsulated into β-CD cavity under acidic solution. Hence, SAB which had similar structure to carbendazim could not be encapsulated at high pH buffer. As a result, 0.1 M sodium acetate/ citric acid buffer, pH 5, containing 0.5 M NaCl was used as washing buffer.

4.7 Capability test of competitive encapsulation with benzimidazole tracer into immobilized β-CD-microtiter plate for detecting carbendazim standard

The previous results concluded that β-CD was successfully immobilized on prepared amino plate and its ability to form inclusion complex still remained. Hence, the feasibility of the β-CD immobilized on microtiter plate was tested with carbendazim standard by using competitive encapsulation with benzimidazole tracer. First, carbendazim standard was loaded onto 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. Next, the benzimidazole tracer was added and incubated for 1 hour. Non-specifically bound tracer was then 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 color development of HRP. Several factors which affected the competitive encapsulation with benzimidazole tracer into β-CD for detecting carbendazim standard were studied. The major influence on the formation of the inclusion complex between β-CD and carbendazim was the solvent used to dissolve carbendazim. It has been reported that solvent has an effect on the encapsulation and the inclusion complex can be accomplished in co-solvent system (Singh *et al.*, 2002; Valle (Process Biochemistry, article in press)). Because carbendazim did not dissolve in aqueous solution, methanol and DMF were then assessed for the preparation of carbendazim stock standard solution. Methyl 2-benzimidazole carbarmate (MBC) (Bushway *et al*., 1993) and thiabendazole (Abad *et al*., 2001) were dissolved in methanol and DMF, respectively before they were determined by ELISA. After carbendazim was dissolved in methanol and DMF to make stock standard solution, it was subsequently diluted with loading buffer prior to encapsulation by immobilized CD-microtiter plate. It was found that in the presence of DMF, a typical competitive inhibition curve was obtained, suggesting that carbendazim in DMF could be

encapsulated into CD cavity better than that in methanol. This could be explained by the fact that carbendazim was held within the cavity of CD by hydrophobic interaction. Thus, carbendazim in DMF which was non-polar solvent was more entrapped by CD than in methanol which was polar solvent. In addition, Valle (Process Biochemistry, article in press) reported that the more soluble the CD in the solvent, the more molecules become available for complexation. For the solubility in organic solvent, β-CD was insoluble in methanol. In comparison with DMF, the solubility of β-CD was 32 g/100ml in 100 % DMF and 3 g/100 ml in 50% DMF at 25 ° C (Ensuiko). Therefore, the formation of inclusion complex in DMF was better than in methanol. Another factor that had an effect on the competitive encapsulation was the concentration of benzimidazole tracer. The result in ***Figure 3.26*** showed that benzimidazole tracer concentration at 2.5 μg/ml was suitable for the competitive encapsulation. This was lower than the concentration that covered the entire guest binding sites on the immobilized CD (***Figure 3.23***). This was due to the decrease in guest binding site when carbendazim was first encapsulated into CD. Another parameter that had to be optimized was the pH used to load carbendazim. According to Ni *et al*. (2002), at pH below 4.5 (the pk_a of its basic guanadinium group), the carbendazim is protonated (ionized form). This formation of a resonance stabilized cation of carbendizim is responsible for the complex formation by β-CD. Thus, the loading buffer at pH 2.5 was investigated for cardendazim encapsulation. The loading buffer at pH 5 was also determined to see if carbendazim could be encapsulated at the same pH as benzimidazole tracer or not. The pH used for benzimidazole tracer encapsulation had to be set at 5 to keep HRP activity. The result in ***Figure 3.27*** indicated that at pH 2.5, inclusion complex between carbendazim and β-CD increased. This corresponded to the previous study (Ni *et al*., 2002) and is consistent with Jongmevasana (2000) who reported that carbendazim could be encapsulated by β-CD at pH 2.5.

4.7.1 Construction of carbendazim standard curve by competitive encapsution with benzimidazole tracer

In an ELISA format with a known amount of antibodies linked to a solid support and a fixed concentration of enzyme conjugates, the photometric determination of the enzyme activity by absorption is related to the analyte concentration via a dose-response curve. Such calibration curves are constructed with standard antigen concentrations. When the response is plotted vs. the logarithm of the anatyle concentration, the curve

has a sigmoidal shape with a relatively linear portion around the point of half inhibition (IC_{50}). The working range of the calibration curve is defined by the lower and upper limits which can be exploited for the determination of analytes of interest (Hennion and Barcelo, 1998).

The optimal condition for the carbendazim determination using competitive encapsulation with benzimidazole tracer was as follows. The stock standard solution of carbendazim was dissolved in DMF and subsequently diluted in acetate/citric acid buffer pH 2.5 to make working solution. The benzimidazole tracer concentration of 2.5 μg/ml was used for competitive encapsulation. The comparison study between β-CD immobilized on a prepared and commercially available amino plate was investigated. The result in ***Table 3.2*** showed that the IC_{50} value, the working range and LOD obtained from prepared amino plate were lower than those obtained from commercially available amino plate. This suggested that the competitive encapsulation assay performed on the prepared amino plate was more sensitive. This could be due to the large amount of NH_2 group introduced to the polystyrene microtiter plate. As a result, the amount of CD immobilized to this plate was higher. In addition, the prepared amino plate had an amino spacer group between CD and the surface, the immobilized CD should not be affected by steric hindrance. Hence, it was easy for the guest molecule and enzyme tracer to encapsulate into CD cavity.

In comparison with other ELISA for the determination of carbendazim, Bushway *et al*., (1993) used antibody to carbendazim was employed and the LOD was 0.05 μg/ml. The LOD obtained from ELISA kit (RapidAssay test kit) was 0.1 ng/ml (Meulenberg *et al.*, 1995). In this study, the LOD obtained from the prepared amino plate was 0.2 μg/ml. Although the competitive encapsulation with benzimidazole tracer in this work was found to be less sensitive, the LOD obtained was still lower than the MRL set for carbendazim (1999).

4.8 Analyses of carbendazim spiked fruit samples by competitive encapsulation with benzimidazole tracer into immobilized CD- microtiter plate.

An ELISA has potential advantage over HPLC in that it requires no clean-up procedures. Nevertheless, the antigen-antibody interactions can be affected by a variety of compounds originating from various samples and this phenomenon is called matrix effect (Nunes *et al*., 1998; Mareno *et al*., 2001; Watanabe *et al*., 2002; Giovannoli *et al*., 2003). This experiment was designed in order to use the competitive encapsulation

system for the determination of carbendazim in fruit based on competitive ELISA. Similar to ELISA, the matrix of sample extract may interfere with the competitive encapsulation because CD is able to form inclusion complexes with a wide range of guest molecules. Therefore, matrix effect should be first assessed to see if it gives false positive results before the application of the assay to samples containing the analytes. For this reason, it was necessary to check whether the fruit sample contained carbendazim residues or not by conventional method, HPLC.

Grape was selected as fruit sample due to the basis of information indicating highest usage of benzimidazole fungicide and MRLs of carbendazim was established as shown in ***Table 1.4***. The HPLC chromatogram in ***Figure 3.30*** indicated that the grape sample used did not contain carbendazim residues. The carbendazim-free sample was then used to determine for the influence of the matrix on the performance of the competitive encapsulation with benzimidazole tracer. Grape extracted with methanol and partitioned into dichloromethane was forced to dissolve in the loading buffer pH 2.5 prior to the determination. Both of prepared and commercially available amino plates displayed the competitive inhibition curve (***Figure 3.33***). Although, there was no carbendazim, it was found that some compounds in grape could be encapsulated into CD cavity and inhibited the benzimidazole tracer encapsulation. There have been many reports indicating that sample dilution was a feasible way to minimize matrix effect, thus allowing the direct analysis of grape sample (Bushway and Savage, 1990; Abad and Montoya, 1995; Abad *et al*., 2001; Botchkareva *et al*., 2003). In this experiment, grape extract diluted 20-fold (0.05 g/ml) showed the lowest matrix effect in both prepared and commercially available amino plates.

After an appropriate concentration of grape matrix was determined, the matrix effect was investigated again with real sample spiked carbendazim by constructing a dose-response curve as in the preparation of carbendazim standard curve. A shift to the right or to the left, whilst keeping parallelism, corresponds to an increase or a loss in the sensitivity and may indicate the need of performing measurements by preparing the standard curves with the matrix (Hennion and Barcelo, 1998). From ***Figure 3.34***, typical curve was not observed in both of those plates. It could be due to the high amount of DMF employed to dissolve carbendazim which could modify the curve pattern. Thus, it may be necessary to construct a dose-response curve with the addition of the same amount of organic solvent to the matrix sample (Hennion and Barcelo, 1998). Therefore, stock standard solution of carbendazim containing low concentration

of DMF was prepared by dissolving 1 mg of carbendazim in 0.5 ml DMF and 0.1 M acetate/ citric acid buffer pH 2.5 (loading buffer) was added to make 10 ml. The result in ***Figure 3.35*** showed the typical standard curve. However, interfering compounds were not completely eliminated since the inhibition curve in ***Figure 3.35*** was found to be different from the standard curve of carbendazim in ***Figure 3.28***. In other words, the operative working range of the inhibition curve (0.04-0.7 μg/ml and 0.7-0.5 μg/ml obtained from prepared and commercially available amino plates, respectively) was lower than the typical standard curve of carbendazim (0.4-12.5 μg/ml and 1-25 μg/ml obtained from prepared and commercially available amino plates, respectively). This indicated that although grape extract was diluted, the matrix effect still interfered with our system. Therefore, to determine carbendazim in grape sample, the addition of carbendazim to grape extract shown in ***Figure 3.35*** should be used to construct the standard curve. From ***Table 3.3***, it can be concluded that the determination of carbendazim in grape by using β-CD immobilized on prepared amino plate was more sensitive than the commercially available amino plate. In addition, the MRLs for carbendazim (1999) in grape and other fruits range from 1.0 to 10 ppm as shown in ***Table 1.4***. The LOD obtained from prepared amino plate was 0.02 μg/ml in 0.05 g/ml grape extract and it was calculated to be 0.4 ppm. Therefore, the approach of competitive encapsulation with benzimidazole tracer should have the capability to determine carbendazim in fruit sample.

4.8.1 Recovery studies of carbendazim in spiked fruit sample by HPLC

The percent recovery was also studied in order to evalute the extraction efficiency from fruit sample. Grape which had no carbendazim residue was fortified with 1 ppm of carbendazim. The procedure for extraction of carbendazim was then carried out, followed by the determination of carbendazim by HPLC. It was found that the percent recovery obtained using UV detector was 79% and 90% when fluorescence detector was used. These values corresponded to those reported by Gilvydis and Walter (1990). Hence, the extraction method described here was appropriate to extract carbendazim from grape.

4.9 Determination of carbaryl using immobilized CD on microtiter plate

Interestingly, Barbato *et al*. (2000) and Saikosin *et al*. (2002) reported that carbaryl could be encapsulated by β-CD. Hence, the determination of carbaryl by competitive carbaryl tracer encapsulation was focused for the applicability of this method. As in the determination of carbendazim, the carbaryl tracer (CPNU-HRP conjugate) was first synthesized and characterized. Subsequently, carbaryl tracer encapsulation into β-CD immobilized on prepared amino plate was investigated. The result in ***Figure 3.36*** and ***3.37*** indicated that CPNU was confirmed to be coupled to HRP by carbodiimide method because it was encapsulated into β-CD immobilized on microtiter plate. The minimum carbaryl tracer concentration which covered all the guest binding sites on CD was 5 μg/ml which was the same as benzimidazole tracer. When the determination of carbendazim standard by competitive encapsulation with benzimidazol tracer was examined, the optimal benzimidazole tracer concentration was found to be 2.5 μg/ml. Thus, the experiment varying the concentration of carbaryl tracer was skipped and the concentration of carbaryl tracer at 2.5 μg/ml was then used for competitive encapsulation for the determination of carbaryl.

4.10 Construction of carbaryl standard curve by competitive encapsution with carbaryl tracer

In this experiment, the procedure was performed in the same way as in the competitive encapsulation with benzimidazole tracer except for the pH of the loading buffer used. Barbato *et al*. (2000) and Saikosin *et al*. (2002) reported that carbaryl could form inclusion complex with β-CD at neutral pH. Hence, 10 mM sodium phosphate buffer pH 7.4 containing 137 mM NaCl and 2.7 mM KCl (PBS) was used. The loading buffer at pH 5 was also assessed to see if carbaryl could be encapsulated at the same pH as carbaryl tracer. Another type of loading buffer (0.1 M sodium acetate/ citric acid buffer, pH 5) was then used to find a suitable pH for carbaryl encapsulation. Carbaryl stock standard solution was dissolved in 0.5 ml of DMF and diluted in loading buffer pH 5 and 7.4 before being loaded to the plate. The competitive inhibition curves were observed at both pH and the IC_{50} value of carbaryl loaded at pH 7.4 was lower than that of carbaryl loaded pH 7. This suggested that at pH 7.4, carbaryl could favorably form inclusion complex than at pH 5. Therefore, 10 mM sodium phosphate buffer pH 7.4 containing 137 mM NaCl and 2.7 mM KCl (PBS) was used as loading buffer for carbaryl standard encapsulation.

The typical standard curve of carbaryl was observed as shown in ***Figure 3.38b*** and the LOD was 0.15 μg/ml. To compare the sensitivity with other ELISA, the LOD reported by Abad and Montoya (1995) which used antibody to carbaryl was 0.065 ng/ml and the LOD obtained from ELISA kit (RapidAssay test kit) was 0.25 ng/ml (Meulenberg *et al.*, 1995). Again, the competitive encapsulation with carbaryl tracer for the determination of carbaryl was found to be less sensitive. However, our developed method can still be used because the LOD value was lower than the MRL set for carbaryl.

4.11 Analyses of carbaryl spiked fruit samples by competitive encapsulation with carbaryl tracer into immobilized CD-microtiter plate

Similar to the carbendazim determination, the matrix effect on the competitive encapsulation with carbaryl tracer was first examined. HPLC method was used to confirm that the sample had no carbaryl residues. From HPLC chromatogram in ***Figure 3.40***, it was found that grape did not contain carbaryl residue. To verify the matrix effect, the grape extract with methanol and partitioned into dichloromethane was forced into loading buffer pH 7.4. The result showed that the matrix had an effect on the response of the competitive encapsulation with carbaryl tracer and the lowest interference was at 0.05 g/ml of grape extract (***Figure 3.42***). Grape extract at 0.05 g/ml was then spiked with carbaryl to construct a standard curve. The typical standard was observed as shown in ***Figure 3.43***. Nevertheless, sample matrix effects still interfered with the assay, observed by the difference between the standard curve in the presence of matrix in ***Figure 3.43*** and the standard curve of carbaryl in ***Figure 3.38b***. Thus, the addition of standard carbaryl to grape extract shown in ***Figure 3.43*** should be used to construct the standard curve for the determination of carbaryl in grape sample. The LOD was 0.015 μg/ml in 0.05 g/ml grape extract and it was calculated to be 0.3 ppm. The MRL for carbaryl in grape (1999), is 5 ppm (***Table 1.4***). Thus, this method was able to determine carbaryl in fruit sample.

4.11.1 Recovery studies of carbendazim in spiked fruit sample by HPLC

The recovery of carbaryl was also studied by fortifying grape with 1 ppm carbaryl in order to evaluate the extraction efficiency. It was found that the percent recovery was 90%. This suggested that the extraction method used was suitable for the determination of carbaryl in grape.

From the result obtained, it can be concluded that the β-CD immobilized on prepared amino microtiter plate was feasible for the determination of the pesticide residues by competitive encapsulation with tracer. In comparison to other ELISA methods, our method was less sensitive. This was explained by the higher affinity between antigen and antibody than that of β-CD and carbendazim or carbaryl. However, the immobilized β-CD can detect both carbendazim and carbaryl by changing pH for encapsulation. Whereas in ELISA technique, antibodies must be raised by the haptens which had similar structures to each pesticide. Therefore, the use of immobilized CD is interesting and is a suitable method for determining carbendazim and carbaryl in fruit samples, and it can be anticipated that the competitive encapsulation with tracer could also be applied to the determination of other pesticide residues or other compounds which have similar structures for screening purpose.

CHAPTER V

CONCLUSION

1. Amino plate was successfully prepared by coating the polystyrene microtiter plate with 1.25 mg/ml MMAC and 10 mg/ml HMDA was added to introduce the amino group.
2. β-CD was covalently immobilized onto the prepared amino microtiter plate using a cross-linking agent, divinyl sulfone.
3. The β-CD-immobilized microtiter plate could encapsulate benzimidazole and carbaryl tracers.
4. The optimal condition for carbendazim determination was: 0.1 M acetic/citric acid buffer, pH 2.5 as loading buffer, bezimidazole tracer concentration at 2.5 μg/ml, and 0.1 M acetic/citric acid buffer, pH 5 containing 0.5 M NaCl as washing buffer.
5. Under optimal condition, the construction of carbendazim standard curve by competitive encapsulation on prepared amino plate was compared with that on commercially available plate. The IC_{50} value (1.56 μg/ml), the working range (0.4-12.5 μg/ml) and the limit of detection (0.2 μg/ml) obtained from the prepared amino plate were lower than those from commercially available amino plate (IC_{50} = 7 μg/ml, working range = 1-25 μg/ml and LOD = 0.8 μg/ml).
6. The analysis of carbendazim spiked grape sample was interfered by the matrix of grape extract. Thus, the standard curve of carbendazim in the presence of matrix was prepared. The IC_{50} value, the working range and the detection limit obtained from prepared amino plate were 0.1, 0.04-0.7 and 0.02 μg/ml, respectively. Whereas the IC_{50} value, the working range and the detection limit obtained from commercially available amino plate were 2, 0.7-5 and 0.5 μg/ml, respectively.
7. For carbaryl determination, the optimum condition of the assay was similar to that of carbendazim except that 10 mM sodium phosphate buffer pH 7.4 containing 137 mM NaCl and 2.7 mM KCl (PBS) was used as the loading buffer.
8. The IC_{50} value, the working range and the limit of detection of the carbaryl standard curve were 0.4, 0.2-3 and 0.15 μg/ml, respectively.
9. For the standard carbaryl curve in the presence of the matrix, the IC_{50} value, the working range and the detection limit were 0.06, 0.02-0.35 and 0.015 μg/ml, respectively.

REFERENCES

Abad, A., Manclus, J. J., Moreno, M. J. and Montoya, A. 2001. Determination of thiabendazole in fruit juices by a new monoclonal enzyme immunoassay. *J. AOAC. Int.* **84**: 156-61.

Abad, A. and Montoya, A. 1995. Application of a monoclonal antibody-based ELISA to the determination of carbaryl in apple and grape juices. *Anal. Chim. Acta* **311**: 365-370.

Abad, A., Moreno, M. J., Pelegri, R., Martinez, M. I., Saez, A., Gamon, M. and Montoya, A. 1999. Determination of carbaryl, carbofuran and methiocarb in cucumbers and strawberries by monoclonal enzyme immunoassays and high-performance liquid chromatography with fluorescence detection: An analytical comparison. *Journal of Chromatography A* **833**: 3-12.

Ames, B. N., Karman, H.O. and Yamasaki, E., 1975. Hairdye are mutagenic: Identification of avariety of mutagenic ingredients. *Proc. Natl.* Acad. Sci. USA, **72**, 2423-2427.

Asztemborska, M., Sybilska, D., Nowakowski, R., and Perez, G. 2003. Chiral recognition ability of [alpha]-cyclodextrin with regard to some monoterpenoids under gas-liquid chromatographic conditions. *Journal of Chromatography A* **1010**: 233-242.

Badia, R. and Diaz-Garcia, M. E. 1999. Cyclodextrin-Based Optosensor for the Determination of Warfarin in Waters. *J. Agric. Food Chem.* **47**: 4256 -4260.

Barbato, F., Rotonda, M. I. L., Miro, A., Morrica,P. and Quaglia, F. 2000. Inclusion complexation of carbaryl and beta-cyclodextrin in solution and in the solid state. *Journal of Iclusion Phenomena and Macrocyclic Chemistry* **38**:423-433.

Bardi, L., Mattei, A., Steffan, S. and Marzona, M. 2000. Hydrocarbon degradation by a soil microbial population with beta-cyclodextrin as surfactant to enhance bioavailability. *Emzyme Microb. Technol*. **27**: 709-713.

Bauminger, S. and Wilchek, M., 1980. *Method in enzymology*: the used of carbodiimides in the preparation of immunizing conjugates. **70**: 151-159. New York; Academic Press.

Bernal, J. L., Nozal, M. J. del, Jimenez, J. J. and Atienza, J. 1997. High-performance liquid chromatographic determination of benomyl and carbendazim residues in apiarian samples*1. *Journal of Chromatography A* **787**: 129-136.

Blasco, C., Fernandez, M., Pico, Y., Font, G. and Manes, J. 2002. Simultaneous determination of imidacloprid, carbendazim, methiocarb and hexythiazox in

peaches and nectarines by liquid chromatography-mass spectrometry. *Anal. Chim. Acta* **461**: 109-116.

Bora, U., Chugh, L. and Nahar, P. 2002. Covalent immobilization of proteins onto photoactivated polystyrene microtiter plates for enzyme-linked immunosorbent assay procedures. *Journal of Immunological Methods* **268**: 171-177.

Bos, E. S., Doelen, A. A., Rooy, N., Shuurs, A. H. W. M. 1981. 3, 3', 5, 5'-Tereamethylbenzidine as and Ames test negative chromogen for horseradish peroxidase in enzyme immunoassay. *J. Immunoassay* **2**: 187-204.

Botchkareva, A. E., Eremin, S. A., Montoya, A., Manclus, J. J., Mickova, B., Rauch, P., Fini, F. and Girotti, S. 2003. Development of chemiluminescent ELISAs to DDT and its metabolites in food and environmental samples. *J. Immunol. Method.* **283**: 45-57.

Brandon, D. L., Binder, R. G., Wilson, R. E. and Montague, W. C. 1993. Analysis of thiabendazole in potatoes and apple by ELISA using monoclonal antibodies. *J. Agric. Food Chem*. **41**: 996-999.

Brandon, D. L., Holland, K. P., Dreas, J. S. and Henry, C. 1998. Rapid screening for benzimidazole residues in bovine liver. *J. Agric. Food Chem*. **46**:3653-3656.

Buschmann, H. J. and Schollmeyer, E. 2002. Application of cyclodextrins in cosmetic products: a review. *J. Cosmet. Sci.* **53**: 185-191.

Bushway, R. J. 1996. Complementation of direct-injection high-performance liquid chromatography and enzyme-linked immunosorbent assay for the analysis of thiabendazole in fruit juices and concentrates. *J Chromatogr A* **754**: 431-5.

Bushway, R. J., Paradis, L. R. and Perkins, L. B. 1993. Determination of methyl-2-benzimidazolecarbamate in wine by competitive inhibition enzyme immunoassay. *J. AOAC. Int.* **76**: 851-856.

Bushway, R. J., Savage, S. A. and Ferguson, B. S. 1990. Determination of methyl 2-benzimidazolecarbamate in fruit juices by immunoassay. *Food Chem.* **35**: 51-58.

Bushway, R. J., Young, B. E. S., Paradis, L. R., Perkins, L. B., Martin, S. K. and Brown, M. P. 1994. Determination of methyl-2-benzimidazolecarbamate in bulk fruit concentrates by competitive-inhibition enzyme immunoassay. *J. AOAC Int.* **77**: 1237-1243.

Cattaneo M. V. and Luong J. H. T. 1994. A Stable Water-Soluble Tetramethylbenzidine-2-Hydroxypropyl-[beta]-cyclodextrin Inclusion Complex and Its Applications in Enzyme Assays. *Anal. Biochem.* **223**: 313-320.

Chen, L., Zhang, L. F., Ching, C. B. and Ng, S. C. 2002. Synthesis and chromatographic properties of a novel chiral stationary phase derived from heptakis(6-azido-6-deoxy-

2,3-di-O-phenylcarbamoylated)-[beta]-cyclodextrin immobilized onto amino-functionalized silica gel via multiple urea linkages. *J. Chromatogr. A*. **950**: 65-74.

Clarot, I., Cledat, D., Battu, S. and Cardot, P. J. P. 2000. Chromatographic study of terpene derivatives on porous graphitic carbon stationary phase with [beta]-cyclodextrin as mobile phase modifier. *Journal of Chromatography A* **903**: 67-76

David, C., Millot, M. C. and Sebille, B. 2001. High-performance liquid chromatographic study of the interactions between immobilized [beta]-cyclodextrin polymers and hydrophobically end-capped polyethylene glycols. *Journal of Chromatography B: Biomedical Sciences and Applications* **753**: 93-99.

Delgado S., M. J., Rubio Barroso, S., Toledano Fernandez-Tostado, G. and Polo-Diez, L. M. 2001. Stability studies of carbamate pesticides and analysis by gas chromatography with flame ionization and nitrogen-phosphorus detection*1. *J. Chromatogr. A* **921**: 287-296.

Demirbas, A. 1998. Spectrophotometric determination of carbaryl pesticide and its hydrolysis product in soil and strawberry samples. *The Science of The Total Environment* **220**: 235-241.

Ensuido Sugar Refining. *Isoeleat® branched cyclodextrin*. Japan: Ensuido Sugar Refining Co., LTD.

Fernandez, M., Rodriguez, R., Pico, Y. and Manes, J. 2001. Liquid chromatographic-mass spectrometric determination of post-harvest fungicides in citrus fruits. *J. Chromatogr. A* **912**: 301-310.

Garner, R. C., Walpole, A. L. and Ando, T. 1975. Test of some benzidine analogues for microsomal activation to bacterial mutagens. *Cancer Letters* **1**: 39-42.

Gilvydis, D. M. and Walter, S. M. 1990. Ion-pairing liquid chromatographic determination of benzimidazole fungicides in foods. J. Assoc. Off. Anal. Chem. **73**: 753-761.

Giovannoli, C., Giraudi, G., Baggiani, C., Tozzi, C., Anfossi, L. and Dolci, M. 2003. Determination of the insecticide fenoxycarb in apple leaf samples by an enzyme-linked immunosorbent assay. *Anal. Chim. Acta* **478**: 271-280.

Gou, Y., Eisert, R.and Pawliszyn, J. 2000. Automated in-tube solid-phase microextraction-high-performance liquid chromatography for carbamate pesticide analysis. *Journal of Chromatography A* **873**: 137-147.

Gong, Z. and Zhang, Z.1997. Determination of tetracyclines with a modified [beta]-cyclodextrin based fluorosensor. *Analytica Chimica Acta* **351**: 205-210.

Gupta, R. C. 1994. Carbofuran toxicity. *Journal of Toxicology and Environmental Health* **43**: 383-418.

Hashimoto, Y., Ikeda, I., Ikeda, M., Takahashi, Y., Hosaka, M., Uchida, H., Kono, N., Fukui, H., Makino, T. and Honjo, M. 1998. Construction of a specific and sensitive sandwich enzyme immunoassay for 20 kDa human growth hormone. *Journal of Immunological Methods* **221**: 77-85.

Hatakeyama, T., Murakami, K., Miyamoto, Y. and Yamasaki, N. 1996. An Assay for Lectin Activity Using Microtiter Plate with Chemically Immobilized Carbohydrates. *Anal. Biochem.* **237**: 188-192.

Hattori, K., Takahashi, K. and Mikami, M. 1986. Novel high-performance liquid chromatographic adsorbents prepared by immobilization of modified cyclodextrins. *Journal of Chromatography* **355**: 383-391.

Hedges, A. R. 1998. Industrial Applications of Cyclodextrins. *Chem. Rev.* 98: 2035-2044.

Hegedus, G., Belai, I. and Szekacs, A. 2000. Development of an enzyme-linked immunosorbent assay (ELISA) for the herbicide trifluralin. *Anal. Chim. Acta* **421**: 121-133.

Hennion, M. C. and Barcelo, D. 1998. Strengths and limitations of immunoassays for effective and efficient use for pesticide analysis in water samples: A review. *Anal. Chim. Acta* **362**: 3-34.

http://www.doa.go.th/toxic/menu.html. [online]

http://www.nuncbrand.com/page. asp?ID=553&lang=GB. [online]

Irwin, P. L., Brouillette, J. N. and Hicks, K. B. 1995. Cyclomaltoheptaose ([beta]-cyclodextrin) and hydroxyethyl-substituted [beta]-cyclodextrin inclusion complex formation with chlorogenic acid: solvent effects on inclusion complex stability. *Carbohydrate Research* **269**: 201-216.

Isosaki, K., Seno, N., Matsumoto, I., Koyama, T. and Moriguchi, S. 1992. Immobilization of protein ligands with methyl vinyl ether-maleic anhydride copolymer. *J. Chromatogr.* **597**: 123-128.

Jongmeevasana, P. 2000. Immobilization of cyclodextrin for the determination of benzimidazole fungicides. Master' s Thesis, Faculty of Science, Chulalongkorn University.

Kaufman, B. M. and Clower, M., Jr. 1991. Immunoassay of pesticides. *J. Assoc. Off. Anal. Chem.* **74**: 239-247.

Kummer, M., Palme, H.-J. and Werner, G. 1996. Resolution of enantiomeric steriod by high-performance liquid chromatography on chiral stationary phases. *Journal of Chromatography A* **749**: 61-68.

Lezcano M., W. A. S., Novo, M., Rodriguez-Nunez, E. and Tato, J. V. 2002. Complexation of Several Benzimidazole-Type Fungicides with α - and β - Cyclodextrins. *J. Agric. Food Chem.* **50**: 108 -112.

Ma, H. M., Wang, Z. H. and Su, M. H. 2002. New triazine spectroscopic reagent for the separation of DL- amino acids by micellar electrokinetic chromatography. J. Chromatogr. A **955**: 125-131.

Madersbacher, S. and Berger, P. 2000. Antibodies and Immunoassays. *Methods* **21**: 41-50.

Marco, M. P., Shirley, J. G., Cheng, H. M., Liang, Z. Y., Bruce, D. and Marco, H. P. 1993. Development of an enzyme-linked immunosorbent assay for carbaryl. *J. Agric. Food Chem.* **41**.

Massey, K. A., Van Engelen, D. L. and Warner, I. M. 1995. Determination of carbaryl as its primary metabolite, 1-naphthol, by reversed-phase high-performance liquid chromatography with fluorometric detection. *Talanta* **42**: 1457-1463.

Mickova, B., Zrostlikova, J., Hajslova, J., Rauch, P., Moreno, M. J., Abad, A. and Montoya, A. 2003. Correlation study of enzyme-linked immunosorbent assay and high-performance liquid chromatography/tandem mass spectrometry for the determination of N-methylcarbamate insecticides in baby food. *Anal. Chim. Acta* **495**: 123-132.

Miyakawa, H., Kawaguchi, N., Kikuchi, K., Kitazawa, E., Kawashima, Y., Yajima, R. and Itoh, Y.2001. False positive reaction in ELISA for IgM class anti-M2 antibody and its prevention. *Hepatology Research* **20**: 279-287.

Metelitza, D. I., Karasyova, E. I., Grintsevich E. E. and Thorneley, R. N. F.2004. Peroxidase-catalyzed co-oxidation of 3,3',5,5'-tetramethylbenzidine in the presence of substituted phenols and their polydisulfides. *Journal of Inorganic Biochemistry* **98**: 1-9.

Meulenberg, P. E., Mulder, W. H. and Stoks, P. G. 1995. Immunoassays for pesticides. Environmental Science & Technology **29**: 553-561.

Molina , D. L., Heering , H. A., Smulevich , G., Tudela , J., Thorneley , R. N. F., Canovas , F. G. and Lopez, J. N. R. 2003. Purification and characterization of a new cationic peroxidase from fresh flowers of *Cynara scolymus* L. *Journal of Inorganic Biochemistry* 94: 243–254.

Muccio, D.A., Girolimetti, S., Attard Barbini, D., Pelosi, P., Generali, T., Vergori, L., De Merulis, G., Leonelli, A. and Stefanelli, P. 1999. Selective clean-up applicable to aqueous acetone extracts for the determination of carbendazim and thiabendazole in fruits and vegetables by high-performance liquid chromatography with UV detection. *J. Chromatogr. A* **833**: 61-65.

Moreno, M. J., Abad, A., Pelegri, R., Martinez, M. I., Saez, A., Gamon, M. and Montoya, A. 2001. Validation of a Monoclonal Enzyme Immunoassay for the

Determination of Carbofuran in Fruits and Vegetables. *J. Agric. Food Chem.*, **49**: 1713 -1719.

Nahar, P., Wali, N. M. and Gandhi, R. P.2001. Light-Induced Activation of an Inert Surface for Covalent Immobilization of a Protein Ligand. *Analytical Biochemistry* **294**: 148-153.

Nelson, D. L. and Cox, M. M. 2000. *Lehninger Principles of Biochemistry*: Amino acids, peptides and proteins. 3rd ed. New York: Worth Publishers. 115-158.

Newsome, W. H. and Collins, P. G. 1987. Enzyme-linked immunosorbent assay of benomyl and thiabendazole in some foods. *J. Assoc. Off. Anal. Chem.* **70**: 1025-1027.

Newsome, W. H. and Shields, J. B. 1981. a radioimmunoassay for benomyl and methyl 2-benzimidazolecarbamate on food crops. *J. Agric. Food Chem.* **29**: 220-222.

Ni, N., Sanghvi, T. and Yalkowsky, S. H. 2002. Solubilization and preformulation of carbendazim. *International Journal of Pharmaceutics* **244**: 99-104.

Nie, M. Y.,Zhou, L. M., Wang, Q. H. and Zhu D. Q. 2001. Enantiomer separation of mandelates and their analogs on cyclodextrin derivative chiral stationary phases by capillary GC. *Anal. Sci*. **17**: 1183-1187.

Nunes, G. S., Marco, M. P., Farre, M. and Barcelo, D. 1999. Direct application of an enzyme-linked immunosorbent assay method for carbaryl determination in fruits and vegetables. Comparison with a liquid chromatography-postcolumn reaction fluorescence detection method. *Anal. Chim. Acta* **387**: 245-253.

Nunes, G. S., Marco, M. P., Ribeiro, M. L. and Barcelo, D. 1998. Validation of an immunoassay method for the determination of traces of carbaryl in vegetable and fruit extracts by liquid chromatography with photodiode array and mass spectrometric detection. *J. Chromatogr. A* **823**: 109-120.

Orejuela, E. and Silva, M. 2003. Monitoring some phenoxyl-type N-methylcarbamate pesticide residues in fruit juices using high-performance liquid chromatography with peroxyoxalate-chemiluminescence detection. *J. Chromatogr. A* **1007**: 197-201.

Pagliari, S., Corradini, R., Galaverna, G., Sforza, S., Dossena, A. and Marchelli, R. 2000. Enantioselective sensing of amino acids by copper(II) complexes of phenylalanine-based fluorescent [beta]-cyclodextrins. *Tetrahedron Letters* **41**: 3691-3695.

Parfitt, C. H. Chapter editor. 10 Pesticide and Industrial Chemical Residues in Horwitz W. editor. *AOAC official Method 985.23 N-methylcarbamate Insecticide and Metabolite Residues*. AOAC International: Gaitheosburh 41-44.

Porath, J. 1974. *Method in enzymology*: General methods and coupling procedures. **34**: 13-30. New York: Academic Pess.

Saikosin, R., Limpaseni, T. and Pongsawasdi, P. 2002. Formation of inclusion complexes between cyclodextrin and carbaryl and characteriazation of the complexes. *Journal of Inclusion Phenomena and Macrocyclic Chemistry* **44**: 191-196.

Sandahl, M., Mathiasson, L. and Jonsson, J. A. 2000. Determination of thiophanate-methyl and its metabolites at trace level in spiked natural water using the supported liquid membrane extraction and the microporous membrane liquid-iquid extraction techniques combined on-line with high-performance liquid chromatography. *Journal of Chromatography A* **893**: 123-131.

Sannino, A. 1995. Investigation into contamination of processed fruit products by carbendazim, methyl thiophanate and thiabendazole. *Food Chem.* **52**: 57-61.

Satoh, A., Kojima, K., Koyama, T., Ogawa, H. and Matsumoto, I. 1998. Immobilization of saccharides and peptides on 96-well microtiter plates coated with methyl vinyl ether-maleic anhydride copolymer. *Anal. Biochem.* **260**: 96-102.

Sidorova, N. Y. and Rau, D. C. 2001. Linkage of EcoRI dissociation from its specific DNA recognition site to water activity, salt concentration, and pH: separating their roles in specific and non-specific binding1. *Journal of Molecular Biology* **310**: 801-816.

Singh, M., Sharma, R. and Banerjee, U. C. 2002. Biotechnological applications of cyclodextrins. *Biotechnology Advances* **20**: 341-359.

Stine, K. J., Andrauskas, D. M., Khan, A. R., Forgo, P. and D'Souza, V. T. 1996. Electrochemical study of self-assembled monolayers of a [beta]-cyclodextrin methyl sulfide covalently linked to anthraquinone. *Journal of Electroanalytical Chemistry* **465**: 209-218.

Suzuki, N., Quesenberry, M. S., Wang, J. K., Lee, R. T., Kobayashi, K. and Lee, Y. C. 1997. Efficient Immobilization of Proteins by Modification of Plate Surface with Polystyrene Derivatives*1. *Analytical Biochemistry* **247**: 412-416.

Szejtli, J. 1998. Introduction and General Overview of Cyclodextrin Chemistry. *Chem. Rev.* **98**: 1743-1754.

Szejtli, J. 1990. The cyclodextrins and their applications in biotechnology. *Carbohydrate Polymers* **12**: 375-392.

Szekacs, A., Le, H. T. M., Szurdoki, F. and Hammock, B. D. 2003. Optimization and validation of an enzyme immunoassay for the insect growth regulator fenoxycarb. *Anal. Chim. Acta* **487**: 15-29.

Szente, L., Fenyvesi, E. and Szejtli, J. 1999. Entrapment of iodine with cyclodextrins: Potential application of cyclodextrins in nuclear waste management. *Environ. Sci. Technol.* **33**: 4495-4498.

Szulowski, K., Iwaniak, W., Pilaszek, J., Truszczynski, M.and Chrobocinska, M.1999. The ELISA for the examination of hare sera for anti-Brucella antibodies. *Comparative Immunology, Microbiology and Infectious Diseases* **22**: 33-40.

Tanabe, T., Touma, K., Hamasaki, K. and Ueno, A. 2001a. Fluorescent cyclodextrin immobilized on a cellulose membrane as a chemosensor system for detecting molecules. *Anal. Chem.* **73**: 1877-1880.

Tanabe, T., Touma, K., Hamasaki, K. and Ueno, A. 2001b. Immobilized fluorescent cyclodextrin on a cellulose membrane as a chemosensor for molecule detection. *Anal. Chem.* **73**: 3126-3130.

Thomas, D. H., Lopez-Avila, V., Betowski, L. D. and Van Emon, J. 1996. Determination of carbendazim in water by high-performance immunoaffinity chromatography on-line with high-performance liquid chromatography with diode-array or mass spectrometric detection. *J. Chromatogr. A* **724**: 207-217.

Totsune, H., Ohno, C., Kambayashi, Y., Nakano, M., Ushijima, Y., Tero-Kubota, S. and Ikegami, Y. 1999. Characteristics of Chemiluminescence Observed in the Horseradish Peroxidase-Hydrogen Peroxide-Tyrosine System. *Archives of Biochemistry and Biophysics* **369**: 233-242.

Uekama, K., Hirayama, F. and Irie, T. 1998. Cyclodextrin drug carrier systems. *Chem. Rev.* **98**: 2045-2076.

Ueno, A. 1996. Review: fluorescent cyclodextrins for molecule sensing. *Supramolecular Science* **3**: 31-36.

Ukeda, H. F. Y. Ohira, M. and Sawamura, M. 1996. Immobilized Enzyme-Based Microtiter Plate Assay for Glucose in Foods. *J. Agric. Food Chem.* **44**: 3858-3863.

U.S. Food and Druge Administration. 1994. *Pesticide FDA Pesticide Analytical Manual volume I*: Section 105 and 405.

Vavia, P. R. and Adhage, N. A. 1999. Inclusion complexation of nimesulide with beta-cyclodextrins. Drug Dev. Ind. Pharm. **25**: 543-545.

Veitch, N. C. 2004. Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry* **65**: 249-259.

Valle, D. E. M. M. Cyclodextrins and their uses: a review. *Process Biochemistry* **In Press, Corrected Proof**.

Voogd, C. E., Stel, J. J. and Jacobs, J. J. J. A. A. 1980. On the mutagenic action of some enzyme immunoassay substrates. *J. Immunol. Method* **36**: 55-61.

Watanabe, E., Kubo, H. and Nakazawa, H. 2002. Group-specific enzyme-linked immunosorbent assay for fenitrooxon and 3-methyl-4-nitrophenol in water samples based on a polyclonal antibody. *Anal. Chim. Acta* **460**: 99-110.

Welinder, K. G. 1976. Covalent structure of the glycoprotein horseradish preoxidase (EC 1.11.1.7). *FEBSLETTERS* **72**: 19-23.

Wilchek, M. and Bayer, E. A. 1990. *Method in enzymology*: Introduction to avidin-biotin technology. 184: 5-13. New York: Academic Pess.

Ximenes, V. F., Campa, A. and Catalani, L. H. 2001. The Oxidation of Indole Derivatives Catalyzed by Horseradish Peroxidase Is Highly Chemiluminescent*1. *Archives of Biochemistry and Biophysics* **387**: 173-179.

Yang, R., Li, K., Wang, K., Liu, F., Li, N. and Zhao, F. 2003. Rayleigh light scattering study on the supramolecular interactions of [beta]-cyclodextrin derivatives with tetrakis(4-methoxylphenyl)porphyrin. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **59**: 153-161.

Yang, R., Li, K. a., Wang, K., Liu, F., Li, N. and Zhao, F. 2002. Cyclodextrin-porphyrin supramolecular sensitizer for mercury(II) ion. *Anal. Chim. Acta* **469**: 285-293.

Yang, R. H., Wang, K. M., Xiao, D., Yang, X. H. and Zhang, L. D. 2000. A selective sensing membrane for the determination of tetracycline with heptakis (2,6-di-O-isobutyl)-[beta]-cyclodextrin as the substrate. *Microchemical Journal* **64**: 213-220.

Zhang, L., Krylov, S. N., Hu, S. and Dovichi, N. J. 2000. Methyl-[beta]-cyclodextrin modified micellar electrokinetic capillary chromatography with laser-induced fluorescence for separation and detection of phospholipids. *Journal of Chromatography A* **894**: 129-134.

Zhang, L. F., Wong, Y. C., Lei, C., Chi, B. C. and Ng, S. C. 1999. A Facile Immobilization Approach for Perfunctionalised Cyclodextrin onto Silica via the Staudinger Reaction. *Tetrahedron Letters* **40**: 1815-1818.

Zhu, M., Han, S. and Yuan, Z. 2000. [beta]-Cyclodextrin polymer as the immobilization matrix for peroxidase and mediator in the fabrication of a sensor for hydrogen peroxide. *Journal of Electroanalytical Chemistry* **480**: 255-261.

APPENDICES

APPENDIX 1: Preparation of buffer and reagents for CD immobilization

0.5 M sodium carbonate buffer, pH 11

Na_2CO_3	10.6 g
$NaHCO_3$	2.1 g
Add distilled water to make total volume	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

4% Cyclodextrin solution in carbonate buffer, pH 11

Cyclodextrins	4 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	1.05 g
Sodium acetate	1.64 g
Add distilled water to make total volume	250 ml

APPENDIX 2: Preparation of buffer for carbendazim and carbaryl encapsulation

Loading buffer:

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

Citric acid	8.4 g
Sodium acetate	13.12 g
Add distilled water to make total volume	500 ml

5 mM PBS pH 7.4, 0.137 M NaCI, 2.7 mM KCl

NaCI	2.192 g
Na_2HPO_4	0.087 g
NaH_2PO_4	0.088 g
KCl	0.004 g
Add distilled water to make total volume	200 ml

Washing buffer:

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

Citric acid	1.05 g
Sodium acetate	1.64 g
Add distilled water to make total volume	250 ml

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

0.1 M Sodium acetate/citric acid buffer pH 5 (4x)	50.0 ml
NaCI	5.88 g
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 gentle 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	25.0 ml

(Note: Add sulfuric acid into distilled water)

APPENDIX 3: Normalization of standard curves

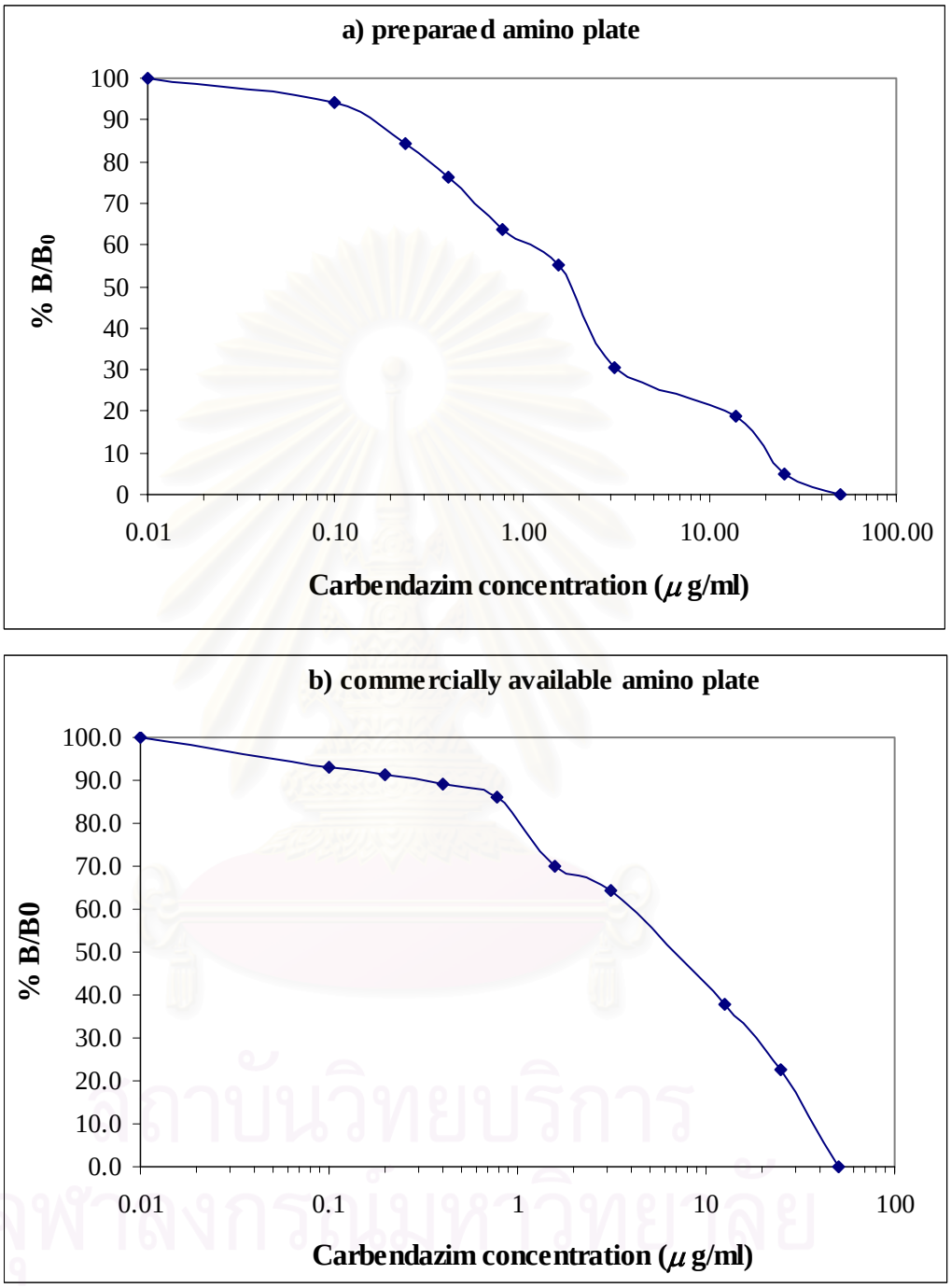


Figure 1. Normalized standard curve of carbendazim by competitive encapsulation with benzimidazole tracer into immobilized CD on a) prepared and b) commercially available amino plate.

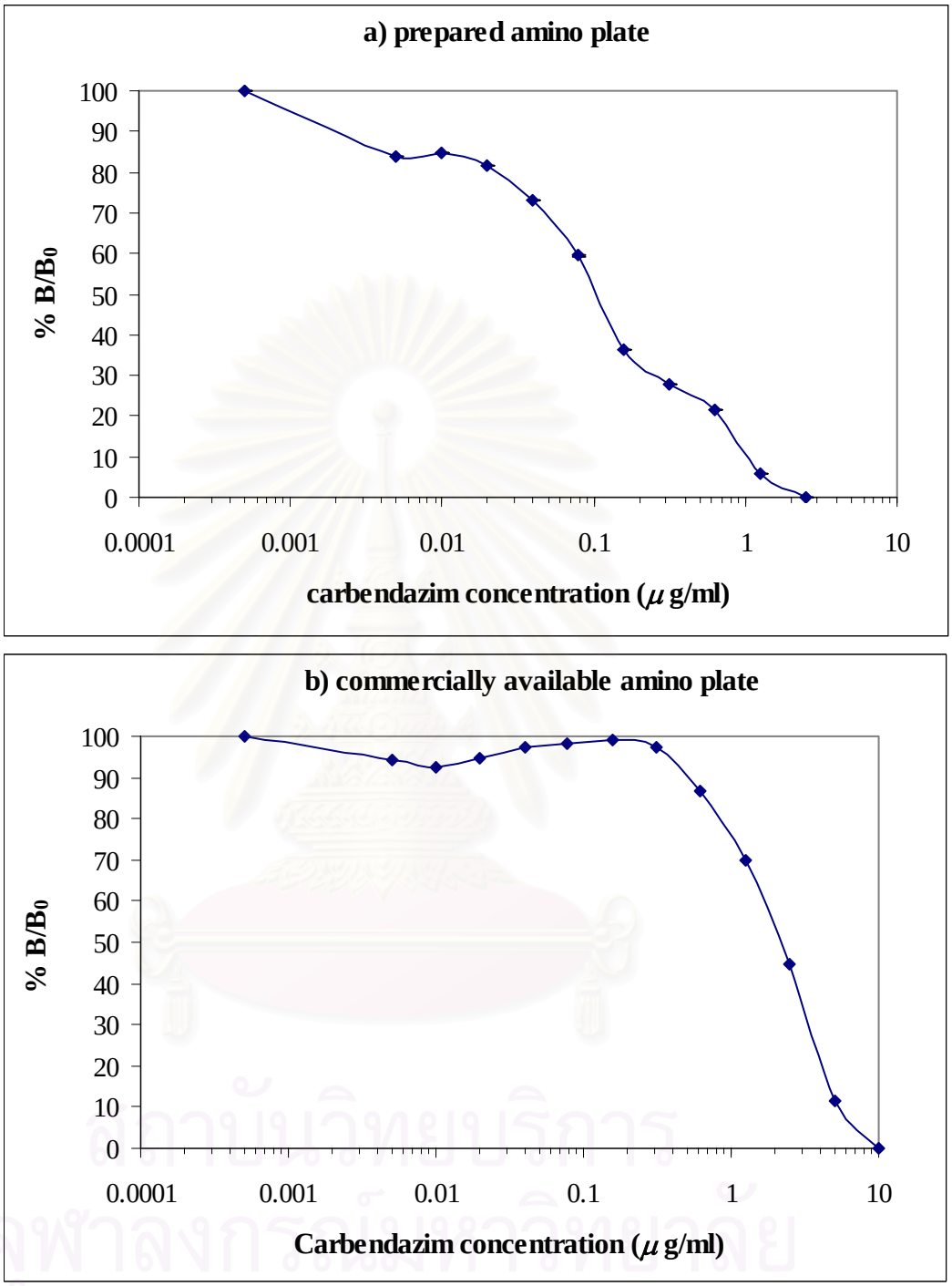


Figure 2. Normalized standard curve of carbendazim in the presence of matix on a) prepared and b) commercially available amino plates.

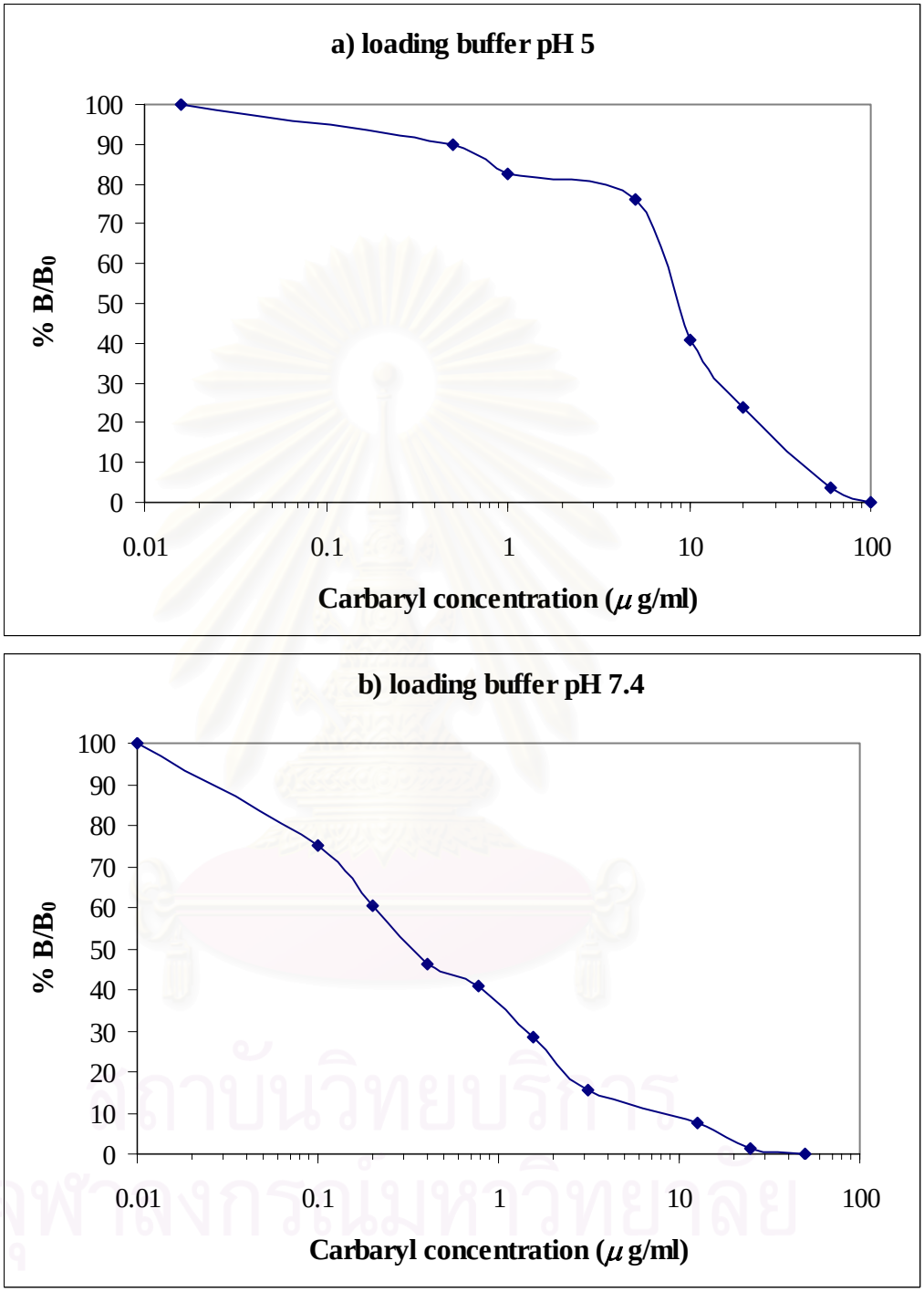


Figure 3. Normalized standard curve of carbendazim by competitive encapsulation with carbaryl tracer into immobilized CD on prepared amino plate.

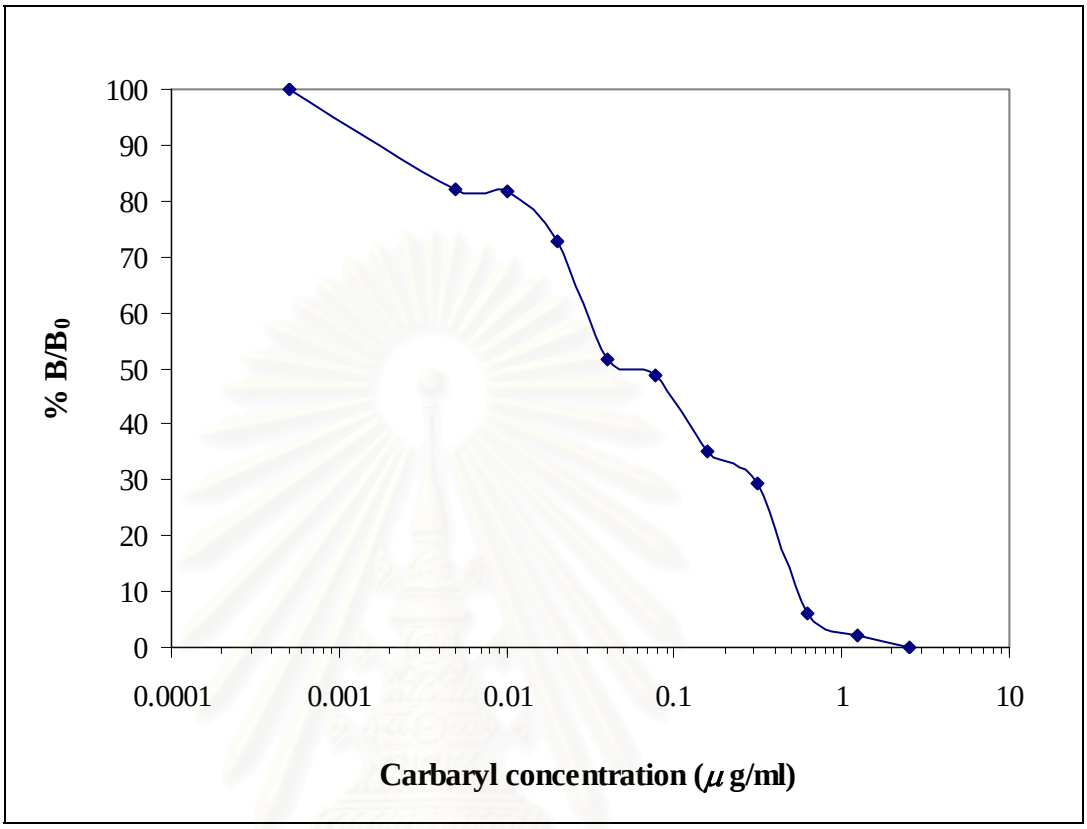


Figure 4. Normalized standard curve of carbaryl in the presence of matrix by competitive encapsulation with carbaryl tracer into immobilized CD on prepared amino plate.

BIOGRAPHY

Miss Wiramsri Sriphochanart was born on January 26, 1978. She graduated with the Bachelor Degree of Science in Chemistry from Silapakorn University in 2000 and studying for Master in Biochemistry Program, Faculty of Science, Chulalongkorn University.