SYNTHESIS AND CHARACTERIZATION OF ESTER-PROTECTED CAPSAICIN DERIVATIVES

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A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science Degree in Chemistry
May 2018

Thesis entitled "Synthesis and Characterization of Ester-Protected Capsaicin Derivatives"

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has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Arts Degree in English of Naresuan University

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ACKNOWLEDGEMENT

Firstly, I would like to thank and offer my sincere gratitude to my advisor Dr.Filip Kielar. I greatly appreciated the guidance, support, and encouragement throughout the completion of my research. I also would like to give a special thanks to all thesis committee members and deeply grateful for their helpful comments and suggestions.

I wish to acknowledge all staffs of Department of Chemistry, Faculty of Science, Naresuan University, especially Kielar Group for their assistance and friendship.

I would like to thank The Thailand Research Fund for stipend as well as research budgets and Center of Excellence in Biomaterial, Faculty of science, Naresuan University for presentation budgets.

I wish to acknowledge Dr.Uthai Wichai and Uthai's research group for the assistance and guidance in the research work.

The friendship and encouragement given to me by my classmate is also appreciated, and I want to give specially thank to my parents for their endless love, understanding and support.

Finally, I would like to express my gratefulness to all others for all supports to make me a complete this thesis but are not named in this acknowledgement.

Watcharapon Wongsupa

Title	SYNTHESIS AND CHARACTERIZATION OF ESTER-PROTECTED CAPSAICIN DERIVATIVES
Author	Watcharapon Wongsupa
Advisor	Filip Kielar, PhD.
Academic Paper	Thesis M.S. in Chemistry, Naresuan University, 2017
Keywords	Capsaicin, chili peppers, prodrug, ester-protected derivatives

ABSTRACT

Ester-protected derivatives of capsaicin (acetate, isobutyrate, carbamate, proline, and phosphate) were synthesized and characterized by ^{1}H-NMR and ESI-MS. The motive for performing these transformations was to prepare prodrug like derivatives of capsaicin with reduced pungency by blocking the phenolic group needed for capsaicin binding with the TRPV1 receptor. The derivatization were performed in crude chili extract and dried chili powder. It was also envisaged that these derivatives could be deprotected in-vivo by the action of suitable enzymes. The enzymatic hydrolysis of the three capsaicin derivatives (acetate, isobutyrate, and carbamate) was investigated using Porcine Liver Esterase. The cytotoxicity of these capsaicin derivatives was studied using the MTT assay in the LNCaP cell line (0.1-200 μM) and also studied using the Resazurin microplate assay in the human dermal fibroblast cell (3.13 - 100 μg/ml). Overall the results indicate that the synthesized compounds could indeed act as capsaicin prodrugs.

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CHAPTER I

INTRODUCTION

Rationale for the study

Capsaicin, capsiate, and their derivatives are compounds isolated from chili peppers and their nonpungent mutant CH-1 9 . The structure of capsaicin (CAP) and dihydrocapsaicin (DHC), the most dominant members of this class, are shown in Figure 1. The figure shows that capsaicin and dihydrocapsacin contain a phenolic functional group in their structure. Capsaicin, the compound responsible for the pungency of chili peppers, has numerous biological effects. Notably, it can act as an analgesic through the inactivation of transient receptor potential vanilloid 1 (TRPV1) after prolonged stimulation. It should be noted that the pungency of capsaicin is also related to its binding and activation of the TRPV1 channel [1].

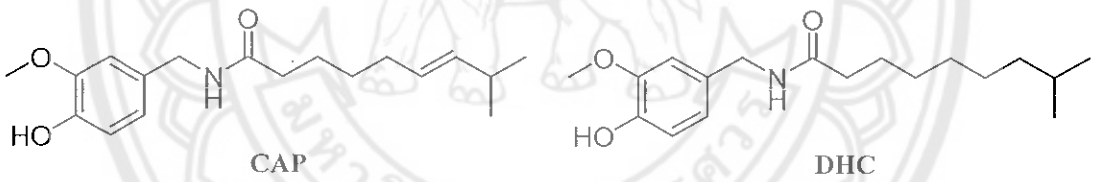


Figure 1 Structures of capsaicin (CAP) and dihydrocapsaicin (DHC)

However, prolonged stimulation of the TRPV1 receptor results in its inactivation. Capsaicin therefore possesses analgesic properties, for which it has been used for a long time. This use is however limited to topical application; probably due to the severe burning sensation it gives. Herein, we propose to prepare derivatives of capsaicin that should be inactive and be turned into capsaicin (i.e. be activated) inside the body. Furthermore, it should be possible to perform these modifications on crude capsaicin extracts. An additional expected benefit of developing this methodology would be applying it to capsiate and its derivatives. Capsiate is known to be suffering from instability in aqueous media and derivatizing it in the proposed directions could alleviate this problem. To solve the limitations in application of capsaicin and capsiate,

prodrug strategy is one the options. It is hypothesized that derivatives protected on the phenolic oxygen will have reduced adverse effects in the case of capsaicin, and will also have increased stability in the case of capsiate. Prodrugs are bioreversible derivatives of drug molecules, which are inactive after the administration and undergo enzymatic and/or chemical transformation in vivo by metabolic processes before becoming an active parent drug that can then exert the desired pharmacological effect (Figure 2). We aim to use this approach. Therefore, the protecting groups to be used in this work are to be removable in vivo by the action of appropriate enzymes.

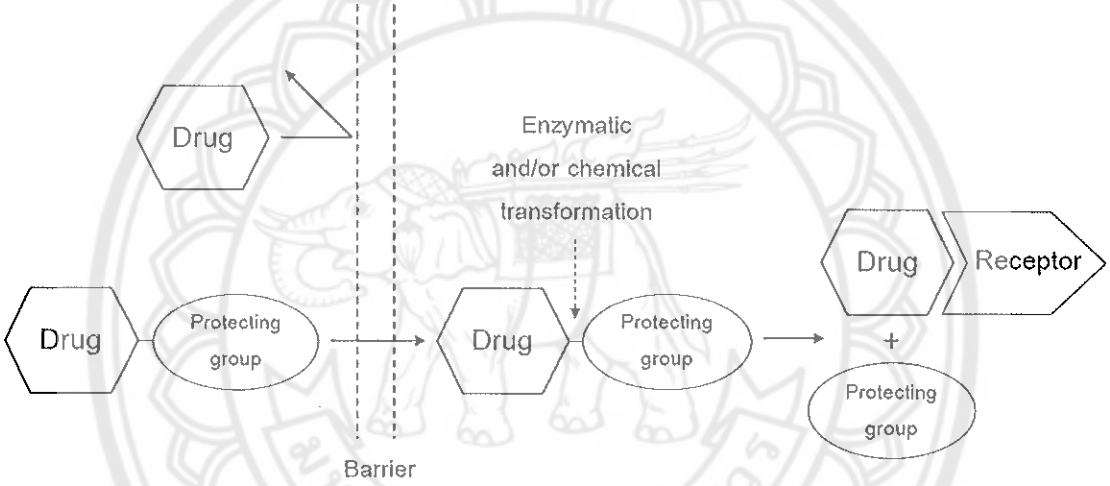


Figure 2 A simplified representative illustration of the prodrug concept

Research objectives

1. Synthesis of ester-protected capsaicin derivatives to reduce its pungency.
2. Evaluation of properties (e.g. stability, rate of deprotection) of capsaicin derivatives.
3. Evaluation of cytotoxicity of the procapsaicin derivatives.

Scope of the study

The purpose of this research is the development of capsaicin derivatives using the prodrug approach. The protecting groups to be used in this work include but are not limited to: acetyl, carbamate, isobutyl, L-proline, and phosphate. The possibility to

perform the protecting reactions in crude pepper extracts will be investigated. Solubility and stability in aqueous phase will be investigated as well as enzymatic deprotection reactions. Cytotoxicity will be investigated using human cells.

Research Hypothesis

Modified plant derived materials containing capsaicin that could have potential use as food additives, nutraceuticals etc. will be prepared and investigated. The derivatives can be divided into two groups; 1) Lipophilic (acetate, carbamate and isobutyrated): more suitable for transdermal administration. 2) Hydrophilic (phosphate and L-prolinate) more suitable for oral administration.

CHAPTER II

LITERATURE REVIEW

Prodrugs

Prodrugs have become an established tool for improving the undesirable properties of investigational or marketed drugs and are often the focus in the optimization of physicochemical, biopharmaceutical, or pharmacokinetic properties; such as absorption, distribution, metabolism, and excretion (ADME). About 5–7% of drugs approved worldwide can be classified as prodrugs, and the implementation of a prodrug approach in the early stages of drug discovery is a growing trend [2]. Many prodrugs are developed to improve drug delivery and oral bioavailability when a drug itself is poorly absorbed from the gastrointestinal tract. Furthermore, prodrugs may also alter tissue distribution, efficacy and reduce adverse effects of the drug, which can have severe unintended side effects (e.g. toxicity), as well as the selectivity for the promotion site-specific delivery of the drug [3].

The design of lipophilic ester prodrugs is a commonly used approach to enhance lipophilicity of the parent compound and the passive membrane permeability in oral drug delivery programs. For this study, a phenolic group (in general hydroxyl group) within a molecule can be esterified in order to improve the solubility and stability and to provide a slow release of the parent drug by the appropriate enzymes. The phenolic hydroxyl group is one of the most common functional groups found in therapeutic drug molecules (Figure 3), such as narcotic agonists, anticancer agents, antibacterial agents etc. However, bioavailability and effectivity usually present limitation for many phenolic drugs. Solubility limitations have also been found in the development of commercial phenolic drugs. On the other hand, the presence of phenolic groups in drug molecules leads to the possibility of prodrug approach, which can result in effective and efficient delivery of the parent drug to the blood circulation and to the targeted site. Esterification of the hydroxyl group has been one of the preferred prodrug strategies. Various phenolic protecting groups can be used for prodrugs preparation (Figure 4) and several alkyl and aryl ester prodrugs are in clinical

use. However, one significant challenge for ester prodrugs is the accurate prediction of pharmacokinetic disposition [4].

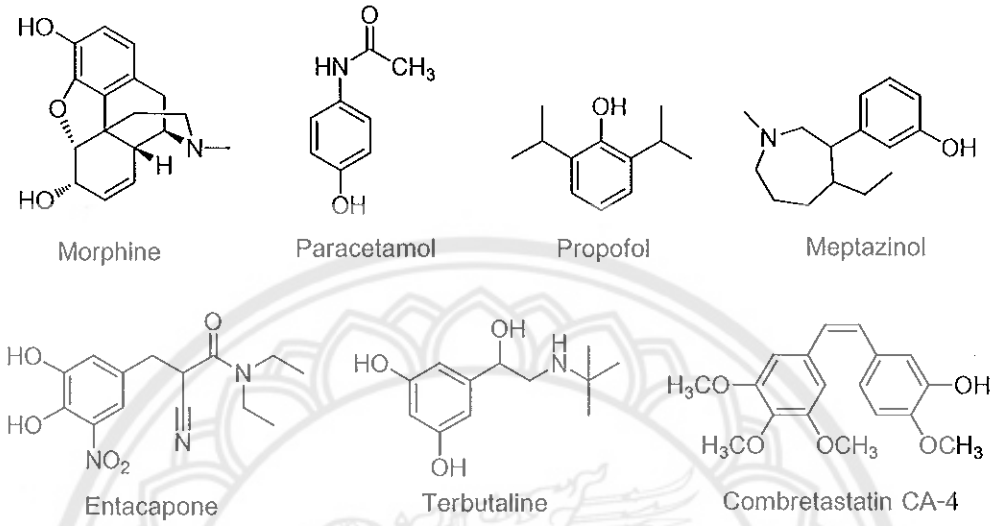


Figure 3 Some examples of phenolic drugs

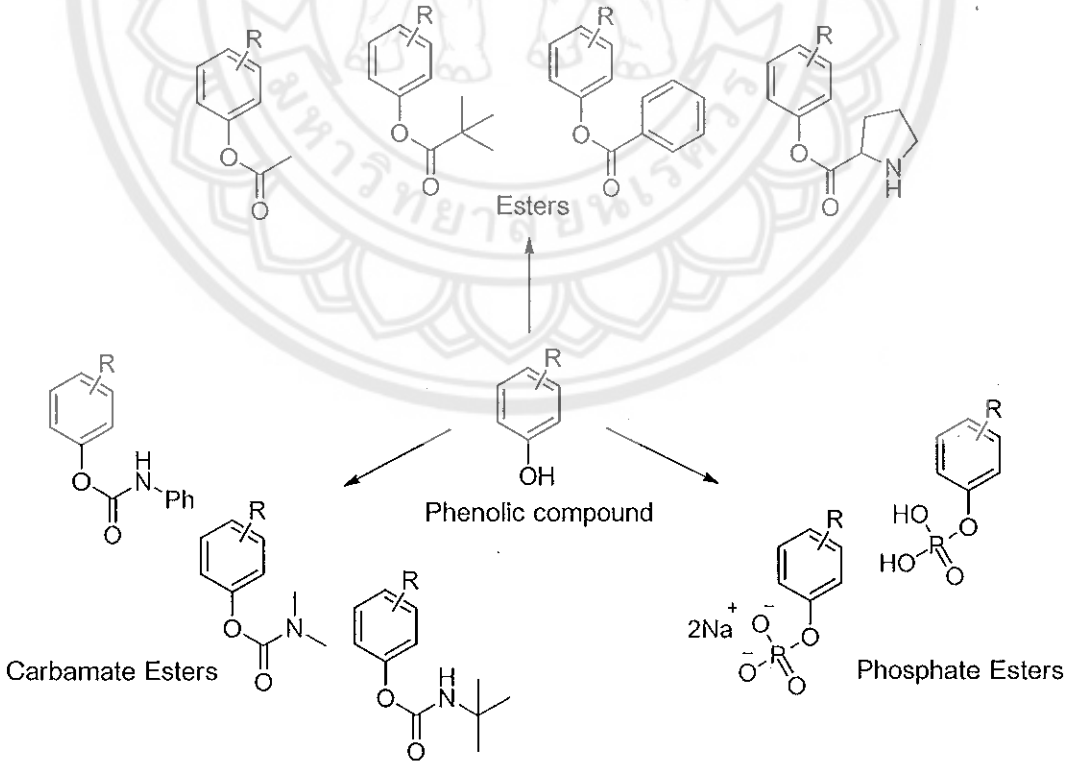


Figure 4 The ester protecting group on phenolic compound

Protecting groups

1. Acetates

The use of phenol acylation as a prodrug strategy is well documented in the literature. For example, esters of Nalbuphine have been investigated as prodrugs to be delivered across skin in 1999. The passive diffusion of its prodrugs increased with the drug lipophilicity [5]. In 2002, Naltrexone was also developed to increase the delivery rate of naltrexone across human skin by using lipophilic alkyl ester prodrugs [6]. *O*-acetyl prodrug of psilocin was prepared by David E. Nichols and co-workers. It increased the stability of parent compound and could be crystallized as the fumarate salt. This prodrug was proposed to study the psychopharmacology of psilocin and replace psilocybin [7]. In 2010, Tao Zhou and co-workers designed and evaluated L-dopa amide derivatives as potential prodrugs for the treatment of Parkinson's disease. The diacetyl derivative of L-dopa amide (1) (Figure 5) was found to be more active than L-dopa after its oral administration and generated plasma levels L-dopa in the therapeutic range for an antiparkinsonian effect in man [8]. Ester prodrugs of morphine were studied though enzymatic hydrolysis, the acetyl prodrug (2) (Figure 5) was found to be the most stable derivative in both plasma and saliva. The study demonstrates that the buccal delivery of morphine can be markedly improved by using ester prodrugs with higher lipophilicity than morphine itself [9].

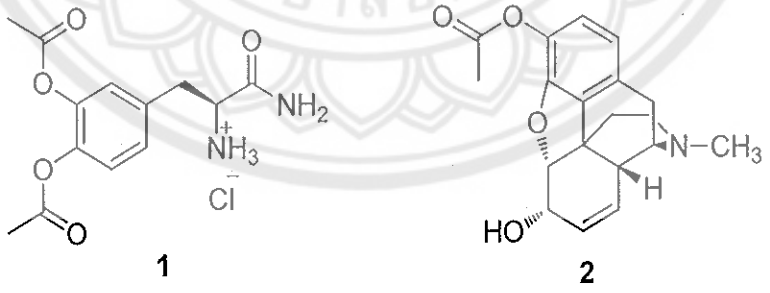


Figure 5 Some examples of acetate prodrug structures: L-dopa amide derivatives (1) and morphine-3-acetate (2)

2. Carbamates

Conversion of phenolic drugs into their carbamate derivatives is another well-documented prodrug strategy for phenolic compounds. In 1991, various phenyl carbamate esters were prepared by Joan Hansen and co-workers. These derivatives were expected to be protected against first-pass metabolism following oral administration [10]. Carbamate esters are also currently used in commercially available prodrugs. For example, *N*-Monoalkyl and *N,N*-dialkyl carbamate prodrugs of naltrexone and series of carbamates of capillarisin sulfur-analogue (3) (Figure 6) were prepared and evaluated in vivo as its prodrugs [11, 12]. In 2006, Antitumor prodrugs were synthesized and evaluated in vitro and in mouse tumor models by Yuqiang Wang and co-workers [13]. In 2014, Martin Kratky and co-workers designed and synthesized salicylanilide *N,N*-disubstituted carbamates and thiocarbamates (4) (Figure 6). The compounds were evaluated in vitro as potential antimicrobial agents and investigated for their inhibitory effect on mycobacterial isocitrate lyase and cellular toxicity [14]. In 2015, hydrophilic prodrug carbamate ester analogues of resveratrol were synthesized and evaluated. One of them was developed into a highly water-soluble molecule as low water solubility is a serious practical problem for resveratrol application. The findings suggest a possible use of glycosyl-resveratrol derivatives for the treatment of colitis and prevention of colon cancer [15].

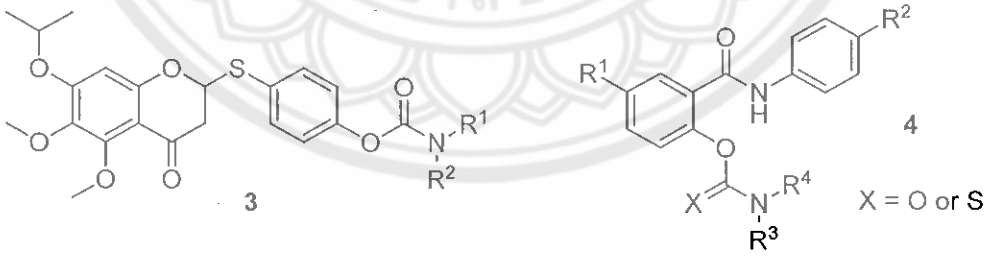


Figure 6 Examples of carbamate prodrug structures: Carbamate prodrug of capillarisin sulfur-analogue (3), Salicylanilide *N,N*-disubstituted carbamates and thiocarbamates (4)

3. Proline esters

The next method of prodrug modification is conversion to esters of *L*-proline. One example of this method is its use in the synthesis of prodrugs derived from the anesthetic agent propofol (5) (Figure 7). The interesting result of this work is the improved aqueous solubility of the proline modified propofol [16]. In 2010, Zhiqian Wu and co-workers synthesized and developed proline ester prodrug of acetaminophen (6) (Figure 7). The prodrug was evaluated for its stability in PBS buffer at various pH values as well as using Caco-2 cell homogenate in Table 1. Physicochemical characteristics such as melting point of the newly synthesized prodrug were determined by MDSC technique. The results have shown that the stability of Pro-APAP in comparison with APAP has increased [17].

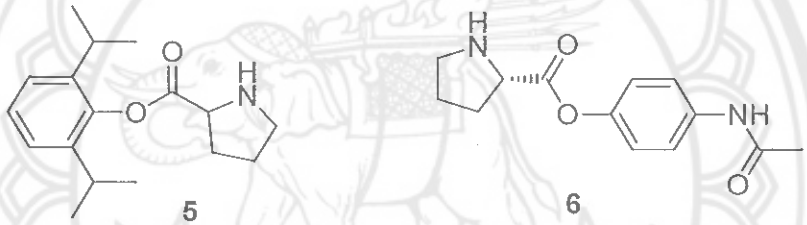


Figure 7 Example of proline esters as prodrugs: proline modified propofol (5) and proline ester prodrug of acetaminophen (6)

4. Phosphate esters

The fourth modification is conversion of phenols to their phosphate analogues. It is a method for the preparation of highly water-soluble prodrugs as discussed in the literature. An example is provided by the research Jukka Leppanen et al. published in 2000 discussing a phosphate ester of Entacapone, which was synthesized in order to increase its aqueous solubility and dissolution rate. The hydrolysis of the phosphate ester and the release of the parent drug was investigated in liver homogenate [18]. In 2002, a Propofol Phosphate was developed by Mariusz G. Banaszczyk and co-workers, to improve its water-solubility and increased a duration time. The prodrugs were used for sedative and anesthetic applications [19]. In 2010, George R. Pettit and co-workers developed a synthetic strategy for Combretastatin A-4

Phosphate (CA4P) (7) (Figure 8). They have succeeded in the development of a new CA4P synthesis suitable for 4-methoxy isotope labeling. This prodrug has been used in human cancer clinical trials [20]. In 2012, Vincent Gasparik and co-workers synthesized highly soluble analogues of chalcone 4 by using phosphate, L-seryl, and sulfate as protecting groups to overcome its poor solubility in aqueous buffers. As expected, the solubility of the phosphate prodrug (8) (Figure 8) is thus at least 3000 times higher than the solubility of chalcone 4. Investigation of the activity of chalcone 4 in comparison with phosphate 8 showed that the prodrug provides more efficient distribution of parent drug in the target tissue [21]. Further chalcone analogues purposed as anticancer agents

were synthesized and evaluated *in vitro* and *in vivo* by Cuige Zhua and co-workers. The compound has been modified using phosphate prodrug strategy to improve its water solubility and bioavailability [22]. In 2017, Erica N. Parker and co-workers have been synthesized new KGP94 analogues to obtain water-soluble phosphate prodrug salt. Its biological evaluation and enzymatic hydrolysis were studied and confirmed a conversion to the parent compound [23].

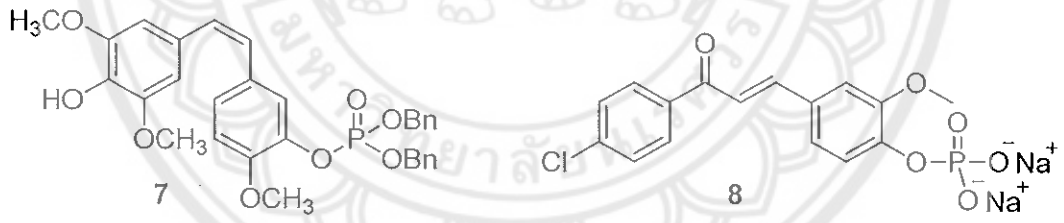


Figure 8 Example of phosphate esters as prodrugs: Combretastatin A-4 Phosphate (7) and phosphate prodrug of chalcone 4 (8)

5. Other esters protecting groups

In 1996, alkyl ester prodrugs of Buprenorphine were synthesized by Audra L. Stinchcomb and co-workers. The prodrugs were protected on the phenolic moiety using acetyl, propyl, butyl, and isobutyl group to improve its delivery through human skin [24]. In 2015, Nrupa Borkar and co-workers synthesized and purified lipophilic diesters of apomorphine, and investigated their in vitro degradation in biorelevant media before and after incorporating them into self-emulsifying drug delivery systems

(SEDDS) for oral delivery. Two apomorphine diester prodrugs were synthesized: dilauroyl apomorphine (9) and dipalmitoyl apomorphine (10) [25].

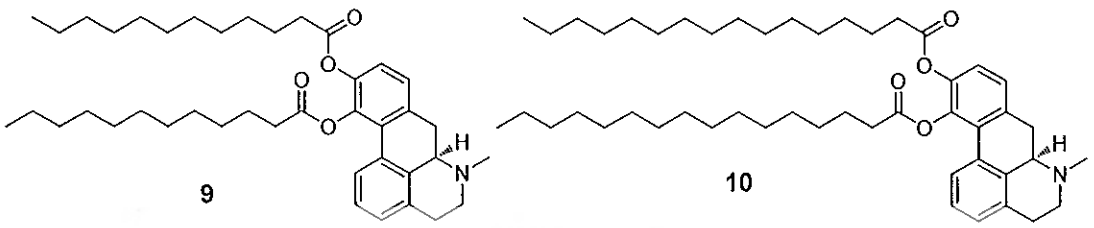


Figure 9 Structure of dilauroyl apomorphine and dipalmitoyl apomorphine

Capsaicin

Chilli peppers are an important plant product in therapeutic history. It was first cultivated on Tamaulipas mountains in Mexico around 5000 BC and was also grown in Peru since around 2000 BC. There is evidence that chilli peppers have been used as traditional medicine since then [26]. The active pungent compounds in red chilli pepers are capsaicinoids. Capsaicin is the major compound of capsaicinoids and shows the most potency in terms of pungency. In 1816, an impure form of capsaicin was discovered by Christian Friedrich Bucholz [27]. This active compound was isolated from plants in the genus Capsicum. It was initially isolated by John Clough Thresh in 1846 and named "capsaicin" [28]. The chemical structure of capsaicin was first determined by E. K. Nelson in 1919 [29]. The complete synthesis of 8-methyl-N-vanillyl-6-nonenamide (capsaicin's IUPAC name) was reported in 1930 by Ernst Spath and Stephen F. Darling [30]. In 1960s, other substances from capsicum extracts with similar chemical and pharmacological properties were isolated and named "capsaicinoids" by S. Kosuge and Y. Inagaki [31]. Capsiate, the non-pungent compound found in a special type of non-pungent chilli peppers (CH-19) was isolated in 1998 by Kenji Kobata and co-workers [32]. It has a chemical structure similar to that of capsaicin with the exception that capsiate is an ester while capsaicin is an amide. The biosynthetic pathway of capsiate was investigated by the same Japanese research group in 2006 [33]. There are three principal parts of the structure of

capsaicin and capsiate; i) aromatic region, ii) bond region (Amide bond for capsaicin/Ester bond for capsiate), and iii) aliphatic chain region (Figure 10).

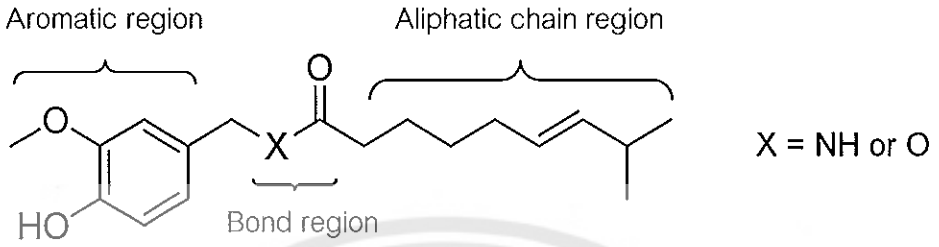


Figure 10 A component of capsaicin and capsiate structure

Capsaicin has been used for neuropathic pain treatments for a long time, but it has been determined useful for other specific applications as well, including in the gastrointestinal tract, for weight-loss, and as an analgesic. It is an important molecule in areas such as medicine but its adverse effects (pungency, burning, and skin irritation) limit its use in crinical trials [34]. The effects of capsaicin, especially in terms of its pungency and analgesic properties, are linked to its agonist properties for the transient receptor potential cation channel, subfamily V, member 1 (TRPV 1). This receptor is a non-selective ionic channel that responds to chemical ligands (e.g. capsaicin), heat above 43°C, and acidic conditions (below pH 5.5). Pharmatherapy of capsaicin was studied in many terms of symptoms such as pain relief, weight reduction, anti-cancer properties, cardiovascular effects, gastrointestinal effects, neurogenic bladder, and dermatological conditions. For example, an external analgesic capsaicin treatment was reported in many US patents [35, 36, 37]. However, capsaicin is limited to pain remedy in clinical applications, which can be attributed to the lack of action specificity and associated toxicity. Pharmacological challenges for use of capsaicin in several conditions point to the need to establish the efficacy of capsaicin through mechanical experiments to study the interaction of capsaicin with TRPV1 receptors and to investigate in human subjects are required [38, 39].

Walpole and co-workers have published a series of articles on the relationship between the structure of the capsaicin analogues and their ability to produce capsaicin like effects in 1993 [40, 41, 42]. Individual papers dedicated to the three regions of capsaicin structure indicated in Figure 10 were published. From the perspective of this work the most relevant is the paper dedicated to structure activity relationships in the aromatic part of the molecule. This work, which utilized monitoring of $^{45}Ca^{2+}$ uptake in neonatal rat cultured spinal sensory neurons, demonstrated the importance of the phenolic group and the resulting loss of activity in case of its protection. Specifically, the EC_{50} of capsaicin is 0.2 μM while that of an analogue where the hydroxyl group is replaced by a methoxy increases to 6.4 μM.

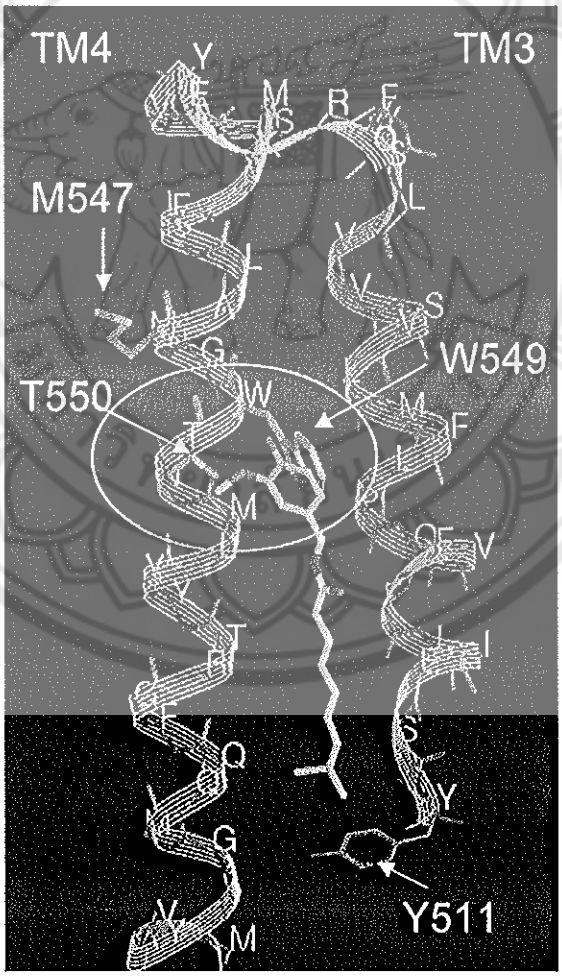


Figure 11 Structural model of capsaicin (light green) interacting with transmembrane helices of TRPV1

The idea of this work is to modify the phenolic group on the aromatic region was further supported by several modeling and structure determination studies, which identified this group as an important feature for TRPV1 binding. In 2004, Narender R. Gavva and co-workers performed a modelling study, which suggested that the phenolic position of capsaicin could interacted with TRPV1 receptor by hydrogen bonding interaction with Threonine (Thr 550) (Figure 11) [43], clearly indicating its importance for this interaction. This study was followed in 2013 by a cryo-EM structure determination of the TRPV1 receptor at 3.4 Å resolution by Liao and co-workers (Liao, M., Cao, E., Julius, D., & Cheng, Y. (2013). Structure of the TRPV1 ion channel determined by electron cryo-microscopy [44]. While providing valuable information this study could not aid in determining details about the interaction of TRPV1 and various ligands such as capsaicin.

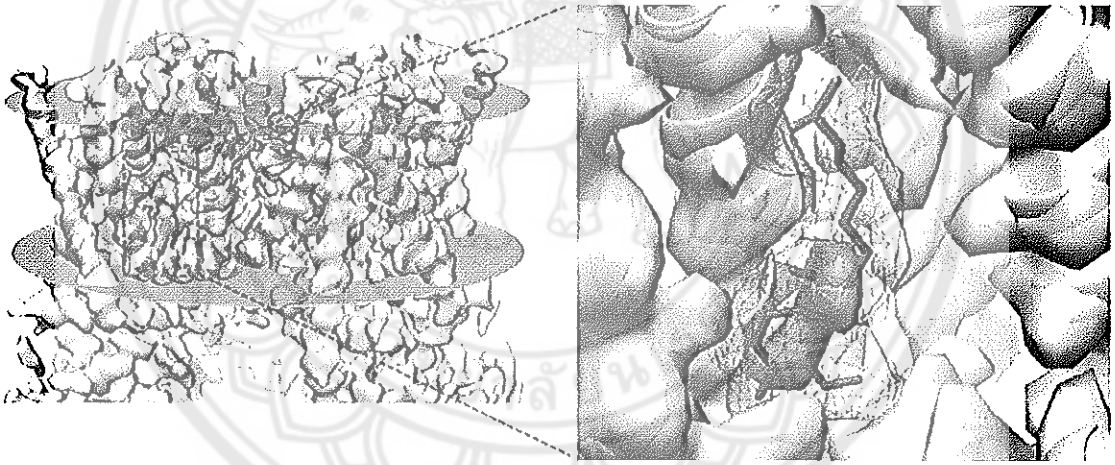


Figure 12 The formation and zoom in view of capsaicin-binding pocket; vanillyl, amide and aliphatic chain groups colored in red, blue and purple, respectively

Further refinement of the cryo-EM data and additional modelling was reported by Yang and co-workers in 2015 [45]. The work provided further details for structural mechanism of capsaicin docking on TRPV1 ion channel. The structure of capsaicin and its analogue were divided into three parts included head (vanillyl group), neck (amide group), and tail (aliphatic chain). The results have demonstrated that TRPV1

was activated using hydrogen bond interactions between the vanillyl group, likely involving the phenolic moiety, and the glutamic acid E571 in the S4-S5 linker. Furthermore, the oxygen atom of amide group interacted with T551 and non-specific Van der Waals interactions was suggested for the aliphatic chain. The authors have concluded that capsaicin bonding with TRPV1 is in the form of "tail-up, head-down" configurations (Figure 12) interactions. It is interesting to note that this arrangement is opposite to the "tail-down, head-up" suggested in the work of Gavva and coworkers. However, despite their differences both studies suggest important role for the phenolic group of capsaicin in its interaction with TRPV1.

CHAPTER III

RESEARCH METHODOLOGY

Material and chemicals

Capsaicin powder, chili extract of capsaicin, and chili powders containing capsaicin were received from Uthai research group (URG), Department of Chemistry, Naresuan University, Phitsanulok, Thailand. The chemicals used for synthesis and cell culture is shown in Table 1 and Table 2, respectively.

Table 1 Chemicals for synthesis

Chemical name	Abbreviation	Supplier	Grade/Purity
4-Dimethylaminopyridine	DMAP	Acros	99%
Acetic anhydride	Ac_2O	-	-
Acetonitrile	CH_3CN	Carlo Erba	Analysis
Acetone	C_3H_6O	-	Commercial
BOC-L-proline	-	Acros	99+%
Dichloromethane	CH_2Cl_2	Carlo Erba	Analysis
Dimethylcarbamyl chloride	C_3H_6ClNO	Sigma Aldrich	98%
Ethyl acetate	$CH_3COOC_2H_5$	Carlo Erba	Analysis
Isobutyric acid	$C_4H_8O_2$	Acros	99+%
Lipase acrylic resin	-	Sigma Aldrich	-
Methanol	CH_3OH	Carlo Erba	Analysis
n-Hexane	C_6H_{14}	Carlo Erba	Analysis
N-Hydroxysuccinimide	HOSu	Acros	98+%
N,N'-Dicyclohexylcarbodiimide	DCC	Acros	99%
Phosphoryl chloride	$POCl_3$	-	-
Potassium carbonate	K_2CO_3	Ajax Finechem	99%

Table 1 (cont.)

Chemical name	Abbreviation	Supplier	Grade/Purity
Pyridine	C_5H_5N	Carlo Erba	99%
Triethylamine	$(C_2H_5)_3N$	Carlo Erba	99.5%
Vinyl acetate	$C_4H_6O_2$	Acros	99+%

Table 2 Chemicals for cell culture

Chemical name	Abbreviation	Supplier	Grade/Purity
Dimethyl sulfoxide	DMSO	Vivantis	99.9%
Dulbecco's modified eagle medium – high glucose	DMEM	Sigma Aldrich	For cell culture
Ethanol	C_2H_5OH	RCI Labscan	AR.
Fetal bovine serum gold	FBS	GE Healthcare	-
Minimum essential medium	MEM	Gibco	-
Penicillin streptomycin	Pen Strep	Gibco	-
Phosphate buffered saline	PBS	Vivantis	Molecular biology
Trypsin-EDTA	-	Gibco	0.25%
In vitro toxicology assay kit, XTT based	-	Sigma Aldrich	-

Research instruments

^{1}H and ^{13}C NMR spectra were acquired on a Bruker model Avance 400 MHz nuclear magnetic resonance spectrometer. Mass spectra and LC-MS analyses were acquired using the Agilent 6540 ultra-high definition (UHD) accurate-mass Q-TOF LC/MS systems, and MassHunter workstation data mining tools (Agilent Technologies, Inc., Santa Clara, CA, USA).

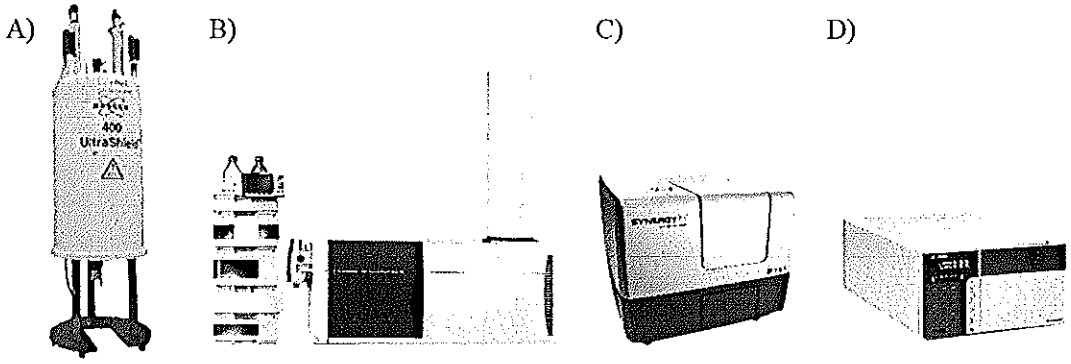


Figure 13 The model of research instruments; A) NMR spectrometer, B) LC-MS, C) microplate reader, and D) HPLC

BioTek model Synergy H1™ hybrid multi-mode microplate reader and Gen5 software used for controlling the reader and analysis of the measurement values were used to perform the cell viability assays. HPLC analyses were performed on a Shimadzu CORP., Japan. The HPLC method parameters are shown in Table 3.

Conditions for LC-MS and HPLC separation

Column: VertiSep UPS C18 HPLC column, 4.6x250 mm, 5 mm

Mobile phase A: Water/Acetonitrile 1:1 (0.2% Acetic acid)

Mobile phase B: Acetonitrile (0.1 % Formic acid)

Flow rate during separation: 0.5 ml/min

Table 3 Gradient conditions for LC-MS separation

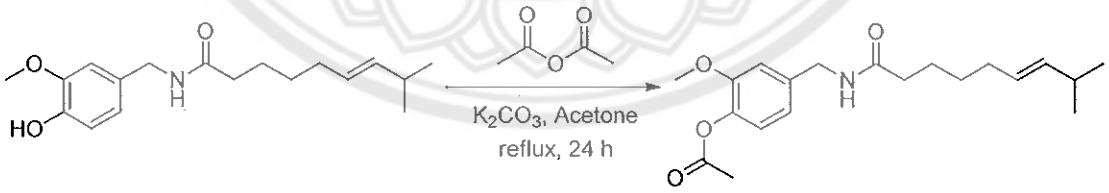
Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	100	0
10	0	100
20	0	100

Table 4 HPLC Conditions

Parameters	Conditions		
Column	Vertisep^{TM} UPS C18 HPLC column, 4.6x250mm, 5µm		
Mobile phase	Solvent A: Water/Acetonitrile 1:1 (0.2% Acetic acid) Solvent B: Acetonitrile (0.1% Formic acid)		
LC program	Time (minute)	Mobile phase A (%)	Mobile phase B (%)
	0	100	0
	10	0	100
	25	0	100
Flow rate	0.8 ml/minute		
Column Temp.	25 °C		
Injection volume	20 µl		

Experimental

1. Synthesis of acetylated capsaicin in acetone



Scheme 1 Acetylation of capsaicin in acetone

Capsaicinoids (200 mg, 0.65 mmol) were dissolved in 5 ml of acetone. Potassium carbonate (135.8 mg, 0.98 mmol) and acetic anhydride (618 µl, 6.55 mmol) was added to the solution and the reaction mixture was heated to reflux under nitrogen atmosphere for 24h. The reaction mixture was cooled to room temperature and

evaporated. The residue was purified by column chromatography on silica (20% ethyl acetate in dichloromethane). The product of acetylated capsaicinoids was obtained as a white solid (206 mg, 0.59 mmol, 91.4 %). m.p. 57 - 59 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 6.97-6.99 (d, J = 8.0 Hz, 1H), 6.90 (s, 1H), 6.83-6.85 (d, J = 8.0 Hz, 1H), 5.69 (s, 1H), 5.28-5.41 (qq, J = 12.3, 6.1 Hz, 1H), 4.41-4.42 (d, J = 5.7 Hz, 2H), 3.82 (d, J = 1.1 Hz, 3H), 2.31 (s, 3H), 2.19-2.23 (m, 3H), 1.97-2.02 (q, J = 7.3, 6.9 Hz, 1H), 1.62-1.68 (m, 4H),1.35-1.43 (p, J = 7.6 Hz, 1H), 1.26-1.31 (m, 2H), 1.13-1.17 (t, J = 6.7 Hz, 1H), 0.94-0.95 (d, J = 6.7 Hz, 3H), 0.84-0.86(d, J = 6.6 Hz,3H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 173.12, 173.00, 169.24, 151.36, 139.23, 138.24, 137.59, 137.57, 126.60, 122.98, 120.15, 112.31, 56.02, 43.55, 39.08, 36.94, 36.78, 32.34, 31.08, 29.75, 29.50, 29.43, 28.06, 27.35, 25.90, 25.37, 22.77, 22.74, 20.75. +ESI-MS: m/z 348 $[M_{CAP}+H]^+$, 350 $[M_{DHC}+H]^+$, 370 $[M_{CAP}+Na]^+$, 372 $[M_{DHC}+Na]^+$, 386 $[M_{CAP}+K]^+$, 388 $[M_{DHC}+K]^+$.

2. Acetylation of capsaicin with chili pepper extract in acetone

Crude extract from chilli peppers (200 mg), acetic anhydride (456 µl, 4.8 mmol), and potassium carbonate (100 mg, 0.72 mmol) were dissolved in acetone. The reaction mixture was stirred under reflux for 16 hours. Acetic anhydride (456 µl, 4.8 mmol) was added to the reaction mixture and reflux continued for further 24h. The reaction mixture was allowed to cool down. The reaction mixture was filtered and evaporated to dryness under vacuum. The residue was kept in fridge.

3. Two step acetylation of capsiate containing extracts

Dried chili pepper (CH-19) powder (1g) was suspended in acetone (10 ml). The reaction mixture was stirred at room temperature (or reflux) for 1h. The suspension was filtered. Acetic anhydride (0.25 ml) and potassium carbonate (100 mg) were added to the filtrate. The mixture was stirred at room temperature (or reflux) for 24h. The composition of the reaction mixture was analyzed by mass spectrometry.

4. Single step acetylation of capsiate containing extracts

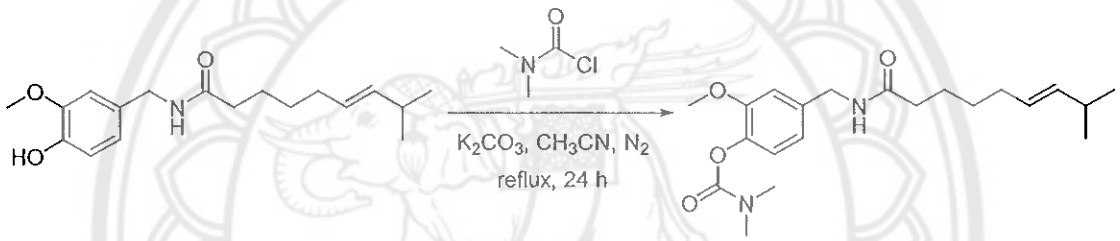
Dried chili pepper (CH-19) powder (1g) was suspended in acetone (10 ml). Acetic anhydride (0.25 ml) and potassium carbonate (100 mg) were added to the suspension. The mixture was stirred at room temperature (or reflux) for 24h. The composition of the reaction mixture was analyzed by mass spectrometry. The mixture

was filtered. Solvent was removed in vaccuo to yield an oil residue (175 mg), which was kept in the fridge.

5. Single step acetylation of capsaicin containing extracts

Extracts from pungent chili pepper (1g) were suspended in acetone (10 ml). Acetic anhydride (0.25 ml) and potassium carbonate (100 mg) were added to the suspension. The mixture was stirred at room temperature (or reflux) for 24h. The composition of the reaction mixture was analyzed by mass spectrometry. The mixture was filtered. After evaporation an oily residue was obtained (155 mg) and stored in the fridge.

6. Synthesis of carbamate protected capsaicin in acetonitrile

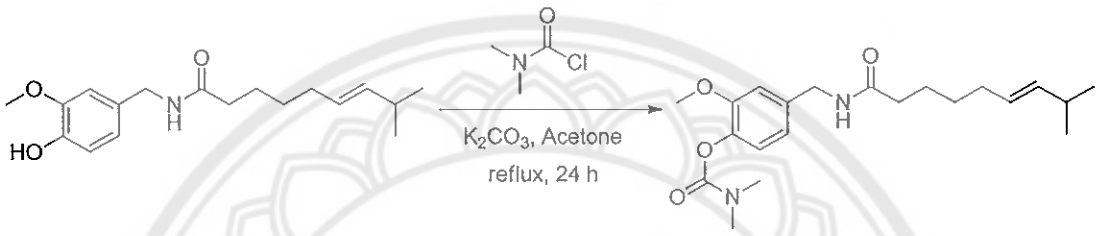


Scheme 2 Synthesis of carbamate protected capsaicin in acetonitrile

A mixture of capsaicin (150 mg, 0.49 mmol), dimethylcarbamoyl chloride (68 µl, 0.75 mmol), and potassium carbonate (102 mg, 0.74 mmol) in acetonitrile was stirred under reflux under nitrogen atmopsphere for 24 hours. Dimethylcarbamoyl choride (68 µl, 0.75 mmol) was added to the reaction mixture and the reaction mixture was stirred overnight. Further amount of dimethylcarbamoyl chloride (226 µl, 2.5 mmol) was added to the reaction mixture and reflux was continued for further 12h. The reaction mixture was cooled to room temperature and evaporated to dryness under vacuum. The residue was dissolved in water (50 ml) and extracted into diethyl ether (2x20 ml). The combined organic fractions were washed with 1 M of potassium hydroxide (25 ml) and water (25 ml). The organic phase was dried over magnesium sulfate, filtered, and evaporated to dryness in vacuum. The residue was purified by column chromatography on silica (20% ethyl acetate in dichloromethane). The product was obtained as an oil (27 mg, 0.07 mmol, 14.9%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ

(ppm): 6.99 - 7.01 (d, J = 7.6 Hz, 1H), 6.87 (s, 1H), 6.80-6.82 (d, J = 7.9 Hz, 1H), 5.91 (s, 1H), 5.42 – 5.25 (m, 1H), 4.39 (s, 2H), 3.81 (s, 3H), 2.99-3.11 (d, J = 44.4 Hz, 6H), 2.18 -2.23 (m, J = 9.4 Hz, 3H), 1.96 – 2.00 (q, J = 6.8 Hz, 1H), 1.79 (s, 5H), 1.65 (s, 2H), 1.36 – 1.42 (m, Hz, 1H), 1.10 – 1.14 (m, 1H), 0.95 (d, J = 6.8 Hz, 3H), 0.85 (d, J = 6.6 Hz, 3H). +ESI-MS: m/z 377 $[M_{CAP}+H]^+$, 399 $[M_{CAP}+Na]^+$, 415 $[M_{CAP}+K]^+$.

7. Synthesis of carbamate protected capsaicin in acetone



Scheme 3 Synthesis of carbamate protected capsaicin in acetone

Capsaicin (150 mg, 0.49 mmol) was dissolved in 5 ml of acetone. Potassium carbonate (102 mg, 0.74 mmol) and dimethylcarbamoyl chloride (452 µl, 4.91 mmol) were added to the solution and the reaction mixture was refluxed under nitrogen atmosphere for 24h. The reaction mixture was cooled to room temperature and evaporated to dryness. The residue was purified by column chromatography on silica (20% ethyl acetate in dichloromethane). The product was obtained as an oil (101 mg, 0.27 mmol, 54.7%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 6.98-7.01 (d, J = 8.0 Hz, 1H), 6.86 (s, 1H), 6.84 – 6.79 (d, 1H), 5.77 (s, 1H), 5.42 – 5.27 (m, 1H), 4.39 (d, J = 5.5 Hz, 2H), 3.81 (d, J = 1.1 Hz, 3H), 3.12 (d, J = 6.6 Hz, 4H), 3.00 (s, 3H), 2.81 (s, 2H), 2.20 (td, J = 7.6 Hz, 2H), 2.02 – 1.95 (m, 1H), 1.69 – 1.61 (m, 2H), 1.55 – 1.45 (m, 1H), 1.42 – 1.35 (m, 1H), 1.19 – 1.10 (m, 1H), 0.93 - 0.96 (dd, J = 6.7 Hz, 3H), 0.84 - 0.86 (dd, J = 6.6 Hz, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 173.13, 173.02, 154.88, 151.93, 140.06, 138.19, 136.88, 126.64, 123.45, 120.12, 112.35, 56.12, 43.59, 39.08, 36.93, 36.76, 36.68, 32.35, 31.08, 29.75, 29.50, 29.44, 28.06, 27.35, 25.92, 25.39, 22.77, 22.74. +ESI-MS: m/z 377 $[M_{CAP}+H]^+$, 399 $[M_{CAP}+Na]^+$, 415 $[M_{CAP}+K]^+$

8. Synthesis of carbamate protected capsaicin in chili pepper extract

Crude extract from chili peppers (200 mg) was dissolved in 5 ml of acetone. Potassium carbonate (102 mg, 0.74 mmol) and dimethylcarbamoyl chloride (450 µl, 4.83 mmol) were added to the solution and the reaction mixture was refluxed under nitrogen atmosphere for 24 h. The reaction mixture was cooled to room temperature and evaporated to dryness. The residue was kept in a fridge

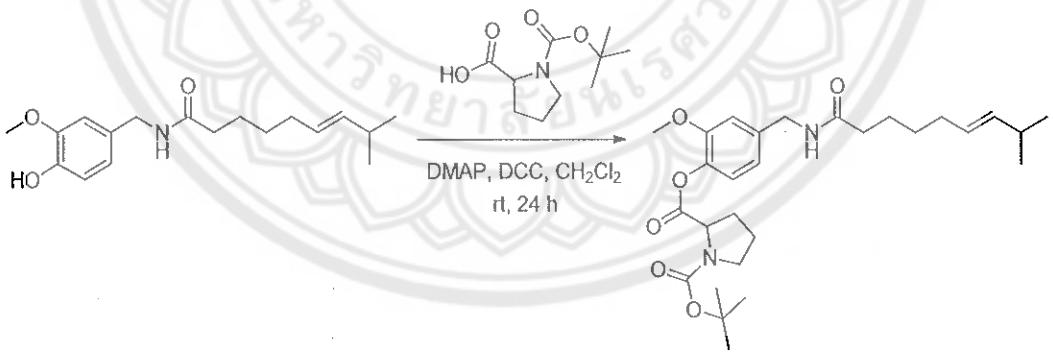
9. Single step carbamate derivantization of capsiate containing extracts

Dried chili pepper (CH-19) powder (1g) was suspended in acetone (10 ml). Dimethylcarbamoyl chloride (225 µl) and potassium carbonate (100 mg) were added to the suspension. The mixture was stirred at room temperature (or reflux) for 24h. The composition of the reaction mixture was analyzed by mass spectrometry.

10. Single step carbamate derivatization of capsaicin containing extracts

Dried chili pepper powder (1g) was suspended in acetone (10 ml). Dimethylcarbamoyl chloride (300 µl) and potassium carbonate (226 mg) were added to the suspension. The mixture was stirred at room temperature (or reflux) for 24h. The composition of the reaction mixture was analyzed by mass spectrometry.

11. Synthesis of BOC-L-proline protected capsaicin

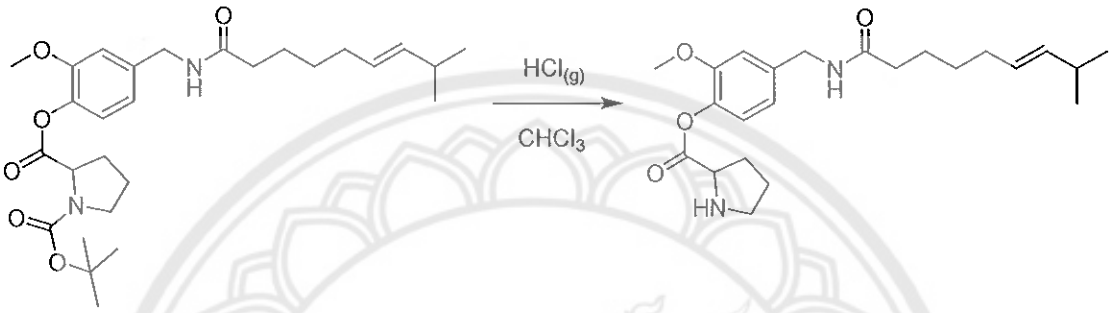


Scheme 4 Synthesis of BOC-L-proline protected capsaicin

Capsaicin (150 mg, 0.49 mmol), BOC-L-proline (116 mg, 0.54 mmol) and DMAP (24 mg, 0.2 mmol) were dissolved in 10 ml of dichloromethane. Solution of DCC (304 mg, 1.47 mmol) in dichloromethane (5 ml) was added to the reaction mixture. The reaction mixture was stirred at room temperature under nitrogen

atmosphere for 24h. The reaction mixture was filtered and evaporated to dryness. The residue was purified by column chromatography on silica (20%ethyl acetate in dichloromethane). +ESI-MS: m/z 525 $[M_{CAP}+H]^+$, 541 $[M_{CAP}+Na]^+$.

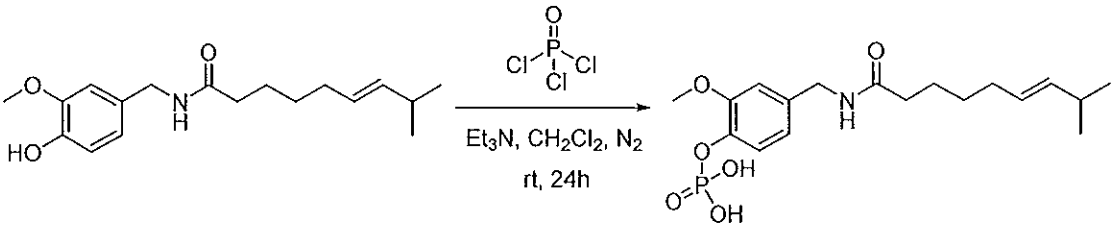
12. Synthesis of proline protected capsaicin



Scheme 5 Deprotection of BOC group to proline protected capsaicin

Concentrated H_2SO_4 was slowly dropped into a solution of NaCl/HCl to make HCl gas. The gas was then bubbled through the solution of BOC-L-proline protected capsaicin (0.1 g) dissolved in CHCl3 (50 ml) for 30 minutes. The solution was evaporated to dryness. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.37 (s, 2H) 6.75-6.87 (m, 3H), 5.94 (s, 1H), 5.32-5.36 (m, 1H), 4.36 (s, 2H), 3.88 (s, 3H), 3.80 (s, 1H), 3.48 (s, 1H), 2.24 (s, 3H), 2.17 (s, 1H), 1.77 (s, 1H), 1.66 (s, 3H), 1.47-1.51 (m, 1H),1.25 (s, 4H), 1.14 (s, 1H), 1.03-1.04 (d, 1H), 0.94-0.95 (d, 3H) 0.84-0.86(d, 3H). +ESI-MS: m/z 403 $[M_{CAP}+H]^+$, 425 $[M_{CAP}+Na]^+$.

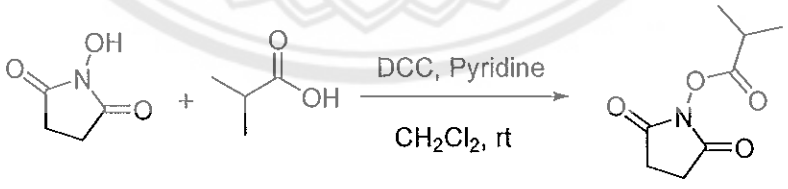
13. Phosphorylation of capsaicin



Scheme 6 Synthesis of phosphate capsaicin

Capsaicin (150 mg, 0.49 mmol) was dissolved in 15 ml of dichloromethane. Triethylamine (686 µl, 4.91 mmol) and phosphoryl chloride (125 µl, 1.47 mmol) were added to reaction mixture, which was then stirred at room temperature under nitrogen atmosphere for 24h. The reaction was quenched by the addition of water. The pH was adjusted to 12 with 1 M sodium hydroxide. The organic solvent was removed under vacuum and pH was adjusted to neutral using HCl. The solution was evaporated to dryness using a freeze drier. Reverse phase silica (C18) sorbent (SiliaBond® C18, Silicycle) was soaked in an acetonitrile/water mixture (50:50) for 24 h. The sorbent was loaded into a chromatography column and conditioned with water/acetonitrile mixture (98:2). The phosphorylated mixture of capsaicin and dihydrocapsaicin (100 mg) was dissolved in a mixture of water and acetonitrile (98/2) and loaded into a chromatography column. The column was eluted with a gradient of increasing acetronitrile concentration up to 50%. The fractions were analyzed by TLC. Fractions containing the separated products were combined and the solvent was removed in vaccuo. The residues were analyzed by ^{1}H-NMR and mass spectrometry. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.49-7.51 (d, 1H), 6.87 (s, 1H), 6.76-6.78 (d, 1H), 5.31-5.42 (m, 2H) 4.28 (s, 2H), 3.81 (s, 4H), 2.20-2.23 (t, 3H), 2.02-1.97 (dd, 2H), 1.59-1.66 (m, 2H),1.35-1.40 (m, 2H), 0.96-0.97 (d, 6H). -ESI-MS: m/z 384 $[M_{CAP}\text{-}H]^-$.

14. Synthesis of Isobutyric acid N-hydroxy succinimide ester

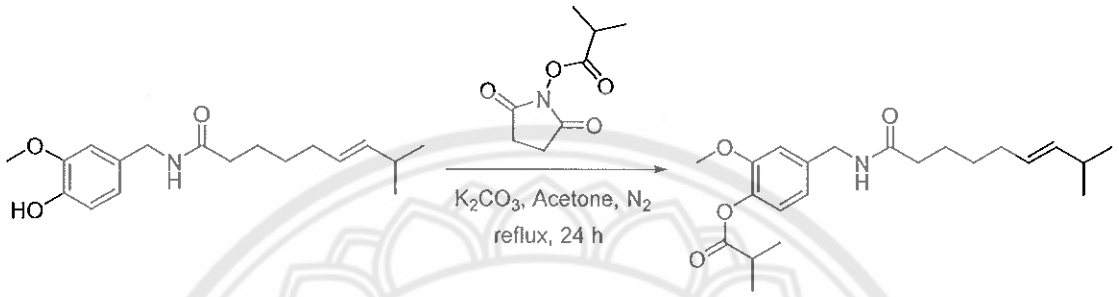


Scheme 7 Synthesis of Isobutyric acid N-hydroxy succinimide ester

N-hydroxy succinimide (313 mg, 2.72 mmol) was suspended in 3 ml of dichloromethane. Pyridine (220 µl, 2.72 mmol) and Isobutyric acid (210 µl, 2.27 mmol) were added to the mixture. *N,N'*-Dicyclohexylcarbodiimide (562 mg, 2.72

mmol) dissolved in 2 ml of dichloromethane was dropwise to give a white precipitate. The reaction mixture was evaporated to dryness and kept in a fridge.

15. Synthesis of isobutyrated capsaicin



Scheme 8 Protection of capsaicin with isobutyric acid performed in acetone

N-Hydroxysuccinimide (313 mg, 2.72 mmol) was suspended in 3 ml of dichloromethane. Pyridine (220 µl, 2.72 mmol) and isobutyric acid (210 µl, 2.27 mmol) were added to the reaction mixture. *N,N'*-dicyclohexylcarbodiimide (562 mg, 2.72 mmol) dissolved in 2 ml of dichloromethane was added drop wise to give a white precipitate. The reaction mixture was evaporated to dryness and kept in a fridge. The crude product was used in the subsequent reaction with capsaicinoids (150 mg, 0.49 mmol). The mixture was dissolved in 5 ml of acetone. Potassium carbonate (102 mg, 0.74 mmol) was added to the solution and the reaction mixture was refluxed under nitrogen atmosphere for 24h. The reaction mixture was cooled to room temperature and evaporated to dryness. The residue was purified by column chromatography on silica (20% ethyl acetate in dichloromethane). The product of isobutyrated capsaicinoids was obtained as a white solid (169 mg, 0.45 mmol, 91.8%), m.p. 63 - 64.5 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 6.95-6.97 (d, J = 8.0 Hz, 1H), 6.88 (s, 1H), 6.82-6.84 (d, J = 8.0 Hz, 1H), 5.69 (s, 1H), 5.28-5.40 (m, 1H) 4.41-4.42 (d, J = 5.6 Hz, 2H), 3.80 (s, 3H), 2.79-2.86 (m, 1H), 2.17-2.23 (m, 3H), 1.96- 2.02 (q, J = 7.0 Hz, 1H), 1.62-1.66 (m, 4H), 1.35-1.43 (q, J = 7.8 Hz 1H), 1.31-1.33 (d, J = 7.0 Hz, 7H), 1.26-1.29 (m, 2H), 1.15-1.16 (d, J = 6.7 Hz, 1H), 0.94-0.96 (d, J = 6.7 Hz, 3H), 0.85-0.86 (d, J = 6.6 Hz, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm):175.44, 173.13,

173.01, 151.46, 139.50, 138.23, 137.32, 126.61, 122.93, 120.15, 112.34, 56.05, 43.58, 39.09, 36.96, 36.79, 34.10, 32.35, 31.09, 29.76, 29.51, 29.43, 28.07, 27.37, 25.92, 25.39, 22.78, 22.75, 19.14. +ESI-MS: m/z 376 $[M_{CAP}+H]^+$, 378 $[M_{DHC}+H]^+$, 398 $[M_{CAP}+Na]^+$, 400 $[M_{DHC}+Na]^+$, 414 $[M_{CAP}+K]^+$. 416 $[M_{DHC}+K]^+$.

16. Enzymatic Deprotection of Acetylated Capsaicin

The stock solutions of the tested compounds (1mM) were prepared in DMSO. A stock solution of porcine liver esterase (10 mg/ml) in 50 mM sodium phosphate buffer pH 7.4 was prepared. The reaction mixture was prepared by mixing the stock solution (1.67 ml), sodium phosphate buffer (3.33 ml), and the enzyme stock solution (0.1 ml to give 20 µg/ml concentration). The reaction mixture was kept at 37°C and aliquots were withdrawn at predetermined time points and analyzed by LC-MS or HPLC with a UV-Vis detector.

17. Cell Viability Tests

Cell viability testing for the LNCaP cells was performed using the MTT assay [46]. LNCaP cells were maintained in a 75 cm^2 culture flask at 37 °C under 5% CO_2 humidified atmosphere. The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% of 10,000 U/ml Penicilin G and 10,000 µg/ml Streptomycin. To examine the effect of capsaicin, acetyl capsaicin, and carbamate protected capsaicin, LNCaP cells were seeded into 96-well plated at 104 cells/well and incubated for 48h. The medium was then changed to serum free RPMI-1640 medium (without phenol red) containing the test compounds. The treated cells were incubated for another 22 h. Then 10 µl of 5 mg/ml of MTT reagent was added and incubated for 2 h. The medium was then removed and the formazan produced in the viable cells was solubilized by adding DMSO:EtOH (1:1 v/v). The absorbance at 595 nm was measured using microplate reader. The % cell viability was determined by comparing the absorbance with the control (non-treated).

Cell viability testing of the human dermal fibroblast, neonatal (HDFn) cell line was performed using the Resazurin Microplate assay (REMA) at the bioactive screening laboratory of BIOTEC using the standard protocol.

CHAPTER IV

SYNTHESIS

The aim of this research was to synthesize derivatives of capsaicin (Figure 1), which would exhibit reduced pungency and act as capsaicin prodrugs. Various ester protecting groups are to be used for the modification of the free phenolic group of capsaicin as that is the only real alternative where a modifying group can be installed. Furthermore, the hydroxyl group plays an important role in the binding of capsaicin to the TRPV1 receptor and thus its modification was expected to have influence on the pungency. Pure capsaicin, chili pepper extract, and chili powders containing capsaicin have been used as starting materials. The proposed modifications were acetylation, formation of dimethyamino carbamate, formation of isobutyrate derivative, formation of proline ester, and phosphorylation (Figure 14).

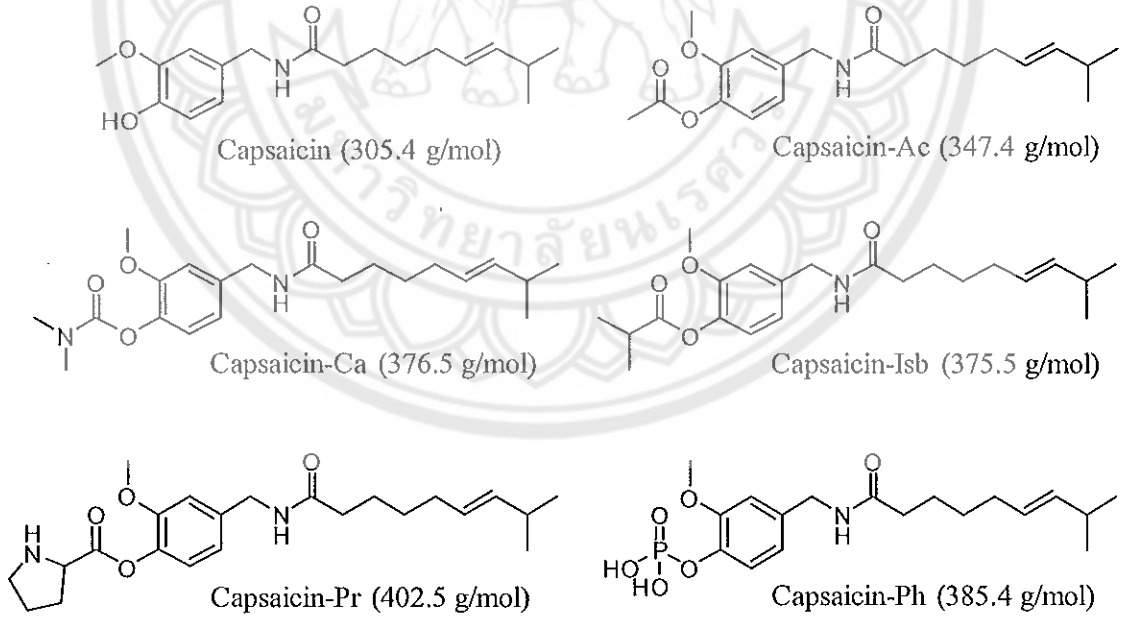
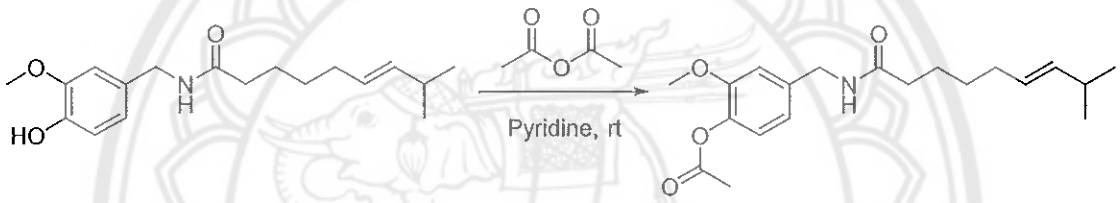


Figure 14 The proposed structures of capsaicin derivatives

Synthesis of pure derivatives

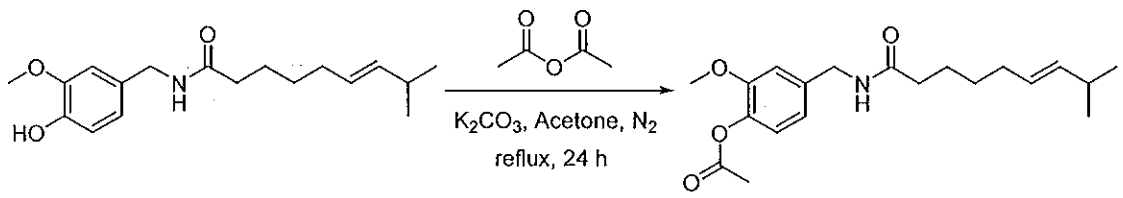
1. Acetate

Acetylated capsaicin was the first compound that has been synthesized during the course of this project as this functionalization has been viewed as the simplest and was also the only modification previously reported [47]. The reaction was initially conducted based on standard literature procedure involving reaction of capsaicin with acetic anhydride in refluxing pyridine (Scheme 9). However, pyridine was considered as a non-ideal solvent for a practical performance of this reaction. Improved reaction conditions were obtained based on literature precedent for the synthesis of the carbamate derivative.



Scheme 9 Acetylation of capsaicin obtained from reaction in pyridine

The reaction also utilizes acetic anhydride as reactant. However, potassium carbonate is used as base and the reaction is carried out in in refluxing acetone (Scheme 10). The appealing features of this reaction lie in that the base and the subsequent products are inorganic salts that can be removed by filtration and also the fact that acetone is one of the suitable solvents for the extraction of capsaicin. The product was obtained as clear oil (91.4%), and characterized by ^{1}H-NMR and mass spectrometry (Figure 15-16).



Scheme 10 Acetylation of capsaicin obtained from reaction in acetone

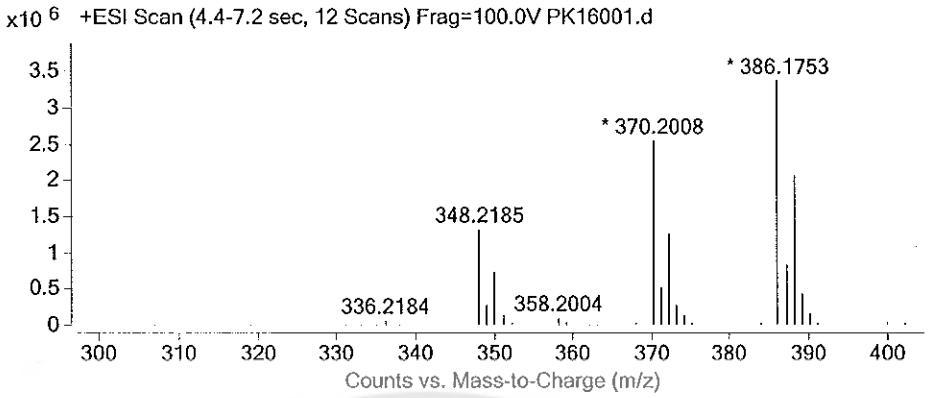


Figure 15 Mass spectrum of acetylated capsaicin showing the $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ peaks

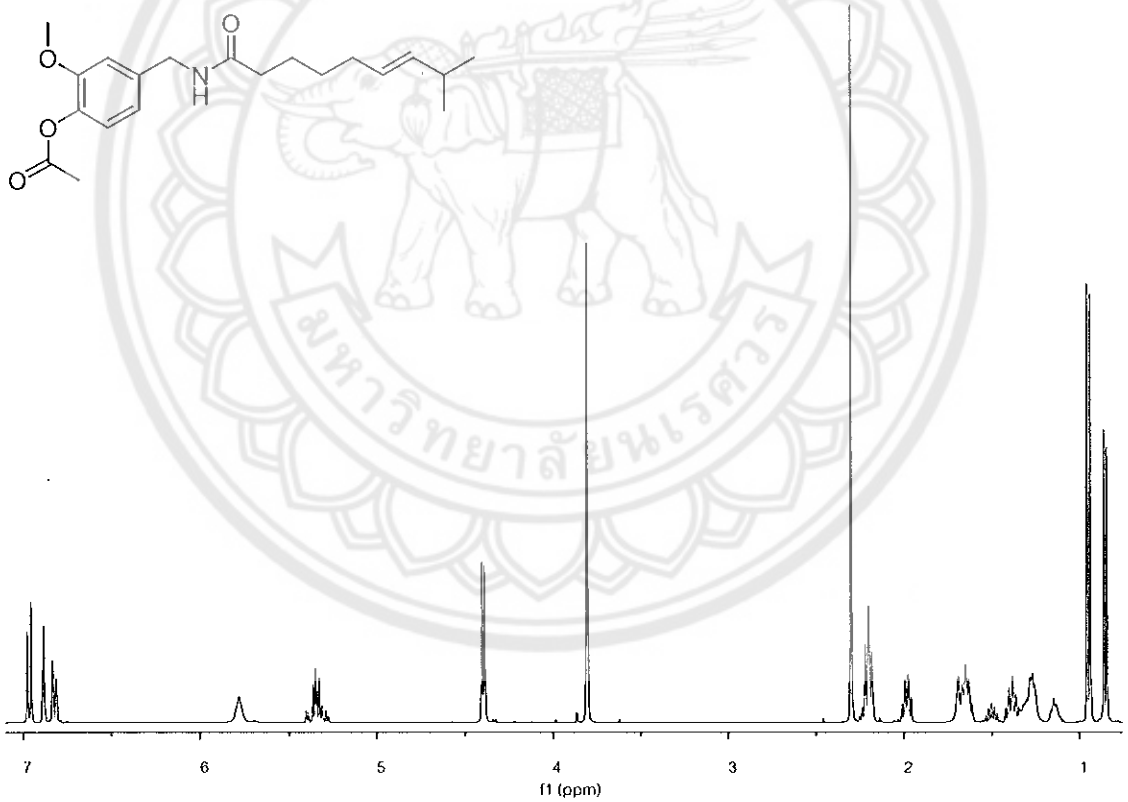
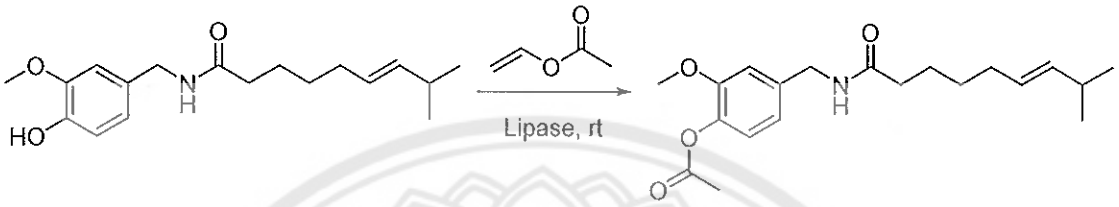


Figure 16 ^{1}H-NMR spectrum of acetylated capsaicin

Furthermore, an attempt was made to find biocatalytic conditons for this reaction (Scheme 11). The enzyme chosen for this work was lipase B immobilized on

polystyrene beads (Novozym 435) from *Candida antarctica*. The acyl donor in this case is vinyl acetate. Initial results have shown a promise for this reaction as the formation of the product (Figure 17). However, the reaction remained sluggish and insufficient conversion was observed despite these optimization efforts.



Scheme 11 Acetylation of capsaicin using lipase enzyme

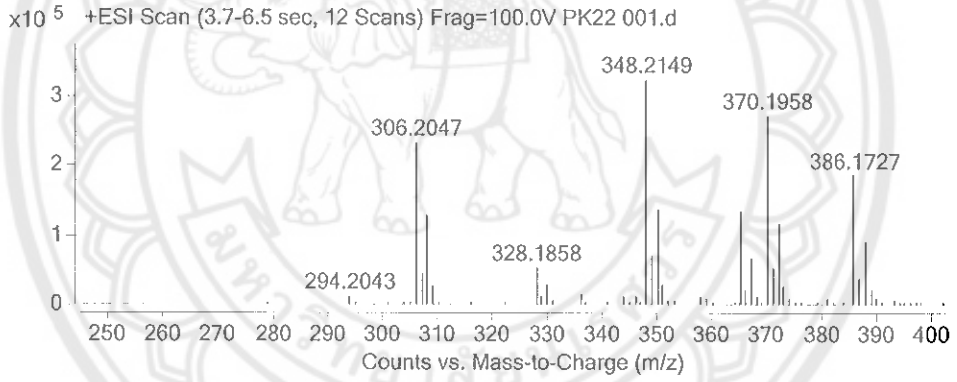
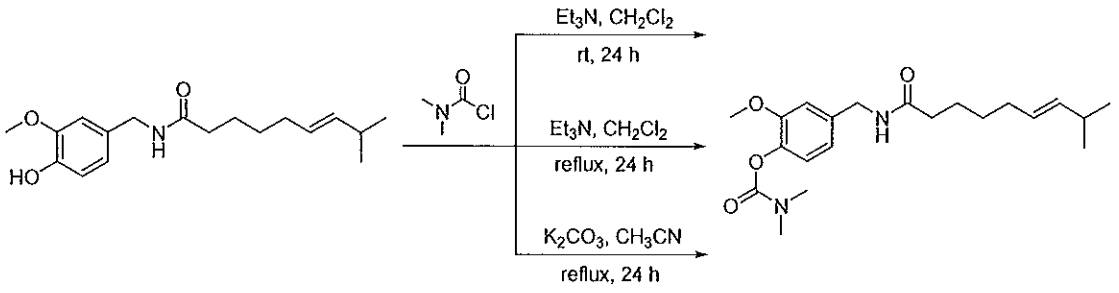


Figure 17 Mass spectrum of a reaction mixture for the acetylation of capsaicin catalyzed by a lipase enzyme



Scheme 12 Optimization of carbamate modification of capsaicin

2. Carbamate

Carbamate modification of capsaicin is an interesting reaction that was selected to give a lipophilic derivative, which should be deptrotected by acetylcholine esterase. The reaction was initially attempted using three different reaction conditions as is shown in Scheme 12.

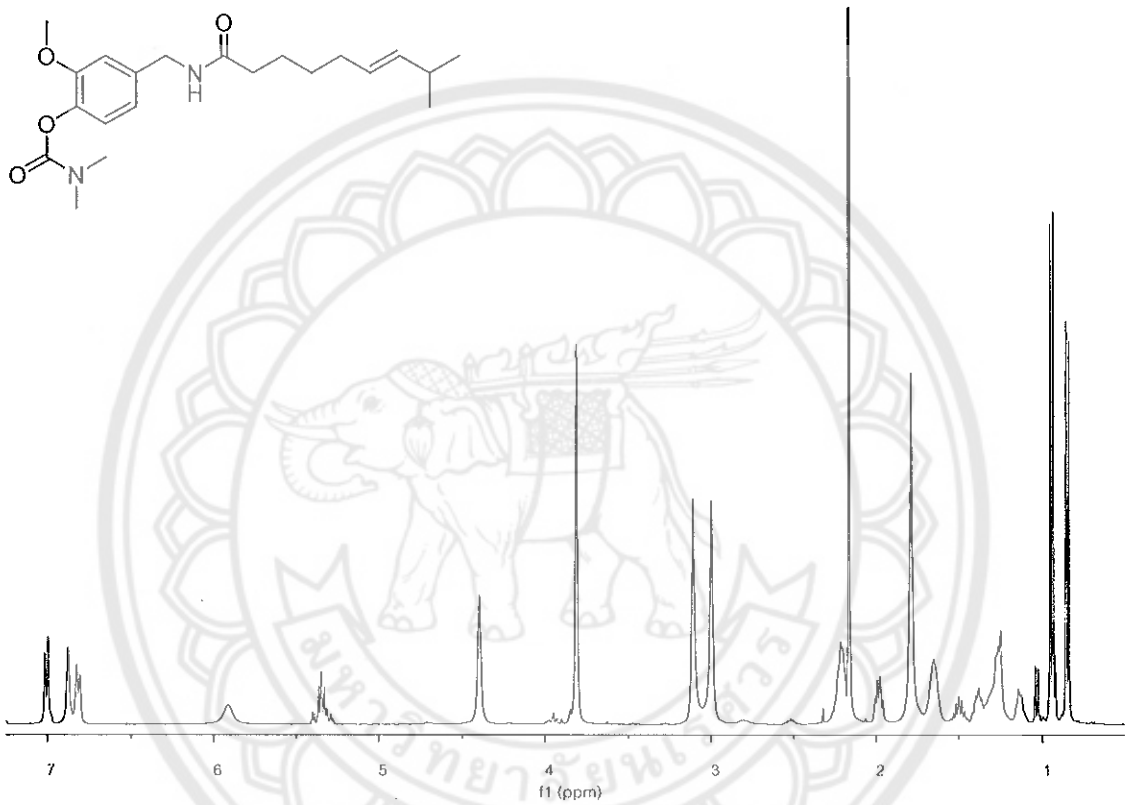
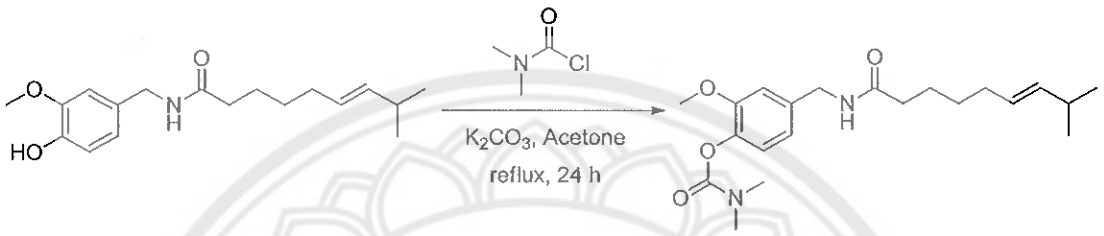


Figure 18 ^{1}H-NMR spectrum of carbamate capsaicin obtained from reaction in acetonitrile

The first two iterations were carried out with dichloromethane as the solvent and triethylamine as the base. The first reaction was carried out at ambient conditions while the second one was performed at reflux. The final iteration was carried out in refluxing acetonitrile with potassium carbonate as the base. The reaction was tried at both room temperature and reflux conditions (Scheme 12). The ^{1}H-NMR of crude carbamate capsaicin obtained from reaction in acetonitrile (14.9 % yield) confirmed that the capsaicin was protected by the carbamate group (Figure 18). However, the

reaction needed to be further investigated in order to be more useful. The optimization of the reaction included changing the temperature to reflux, using potassium carbonate as base, and using acetone as solvent (Scheme 13). The product was obtained as light yellow oil (54.7% yield). The structure was identified by ^{1}H-NMR spectrum and mass spectrometry (Figure 19-20).



Scheme 13 Carbamate modification of capsaicin in acetone

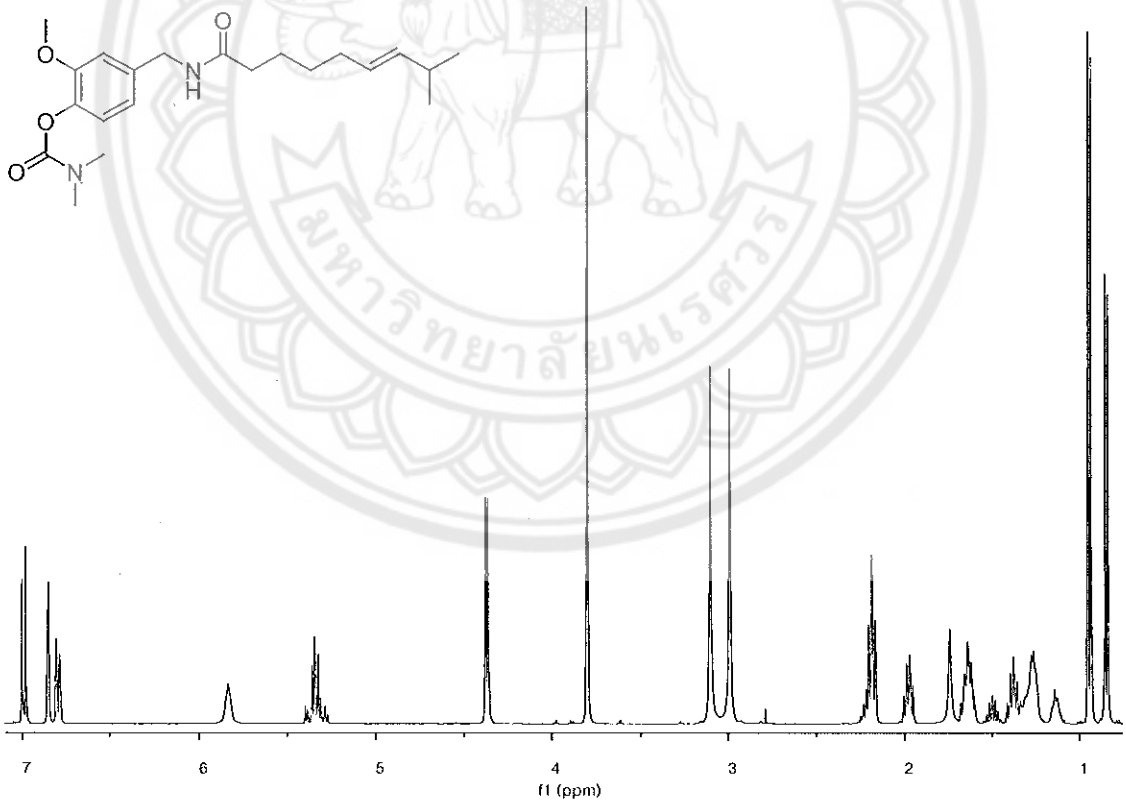


Figure 19 ^{1}H-NMR spectrum of carbamate capsaicin from reaction in acetone

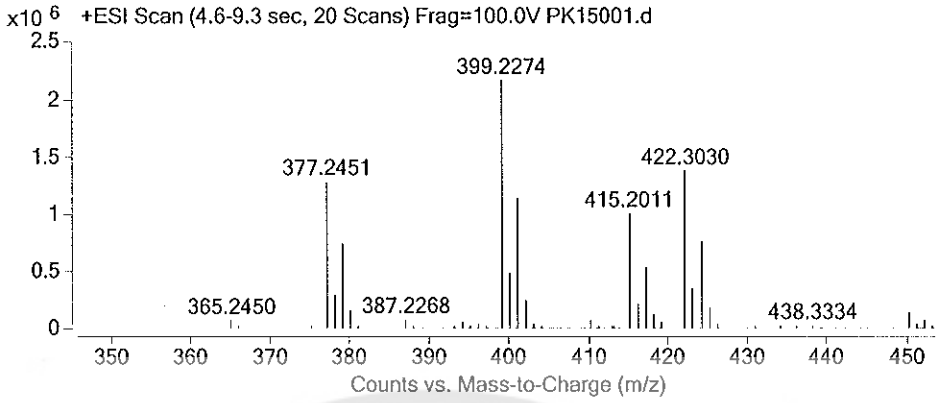
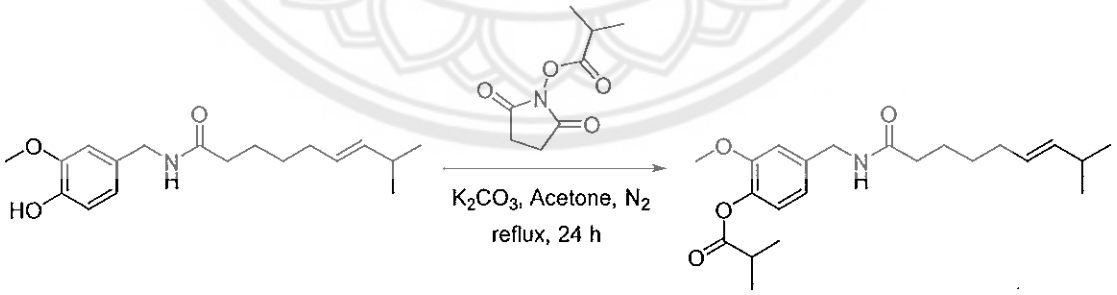


Figure 20 Mass spectrum of carbamate capsaicin showing the $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ peaks

3. Isobutyrate

Isobutyrated capsaicin, a derivative which was envisaged at a later stage of the project, was synthesized so that its properties could be compared to those of the acetylated capsaicin and carbamate capsaicin. It was prepared in a two steps procedure from isoburtyric acid via its *N*-hydroxy succinimide ester. The reaction of this activated ester was performed using the same reaction conditions as for capsaicin acetylation (Scheme 14). The product was obtained as white solid in 91.8% yield and confirmed by ^{1}H-NMR spectrum (Figure 21) and mass spectrometry (Figure 22).



Scheme 14 Isobutyrate modification of capsaicin in acetone

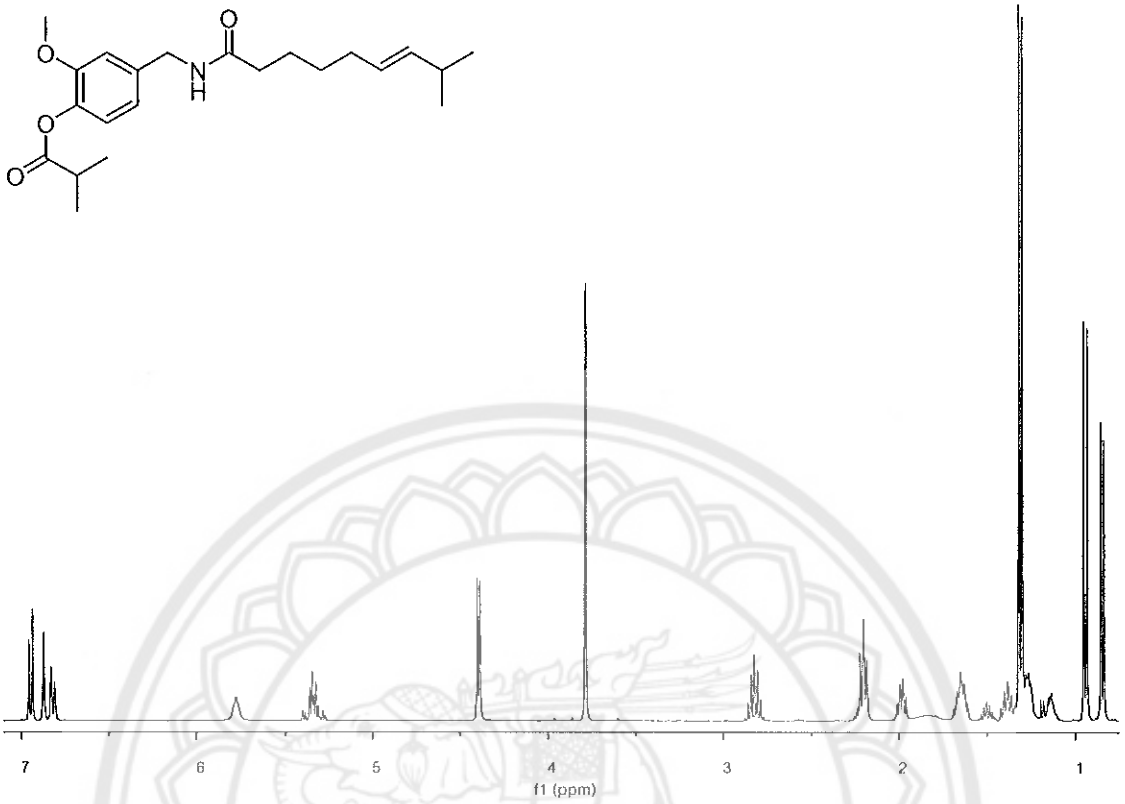


Figure 21 ^{1}H-NMR spectrum of isobutyrated capsaicin

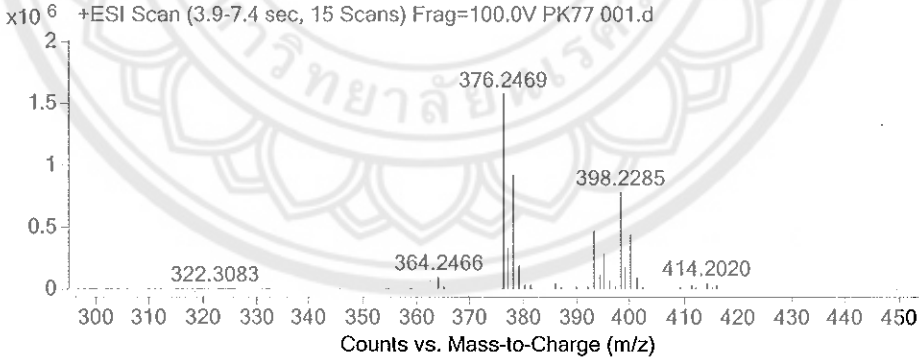
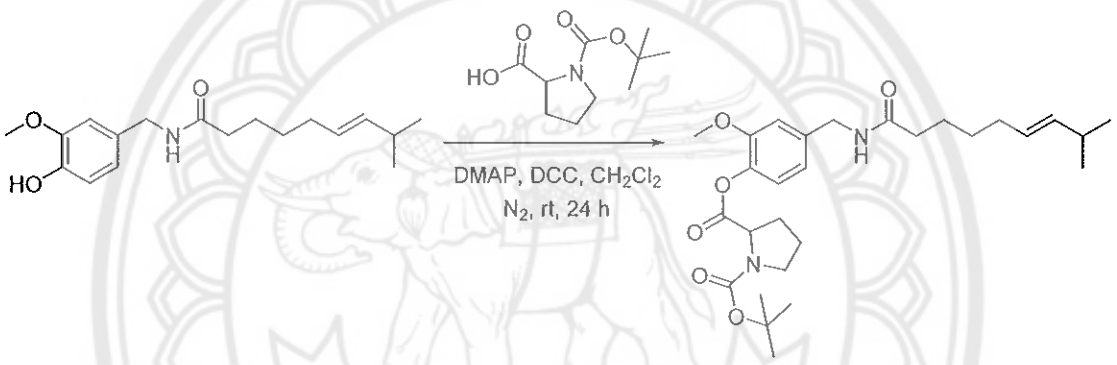


Figure 22 Mass spectrum of isobutyrated capsaicin showing the $[M+H]^{+}$ and $[M+Na]^{+}$ peaks

4. Proline derivative

The capsaicin derivatives described above are actually more lipophilic than capsaicin itself. Therefore an attempt was also made to develop less lipophilic

and more water soluble derivatives. Protecting capsaicin with L-proline was chosen as the first approach to achieve this goal. L-proline was selected as the amino acid protecting group instead of D-proline because, L-proline is one of natural amino acids used in living organisms as the building blocks of proteins. The protection was performed with BOC-L-proline as the starting material and DCC as the coupling agent in dichloromethane (Scheme 15). The protected intermediate was isolated in 39% yield. The result was confirmed by mass spectrometry and ^{1}H-NMR spectrum (Figure 23-24). However, when an attempt was made to reproduce this result using the crude extract it was not successful.



Scheme 15 Modification of BOC-L-proline protected capsaicin

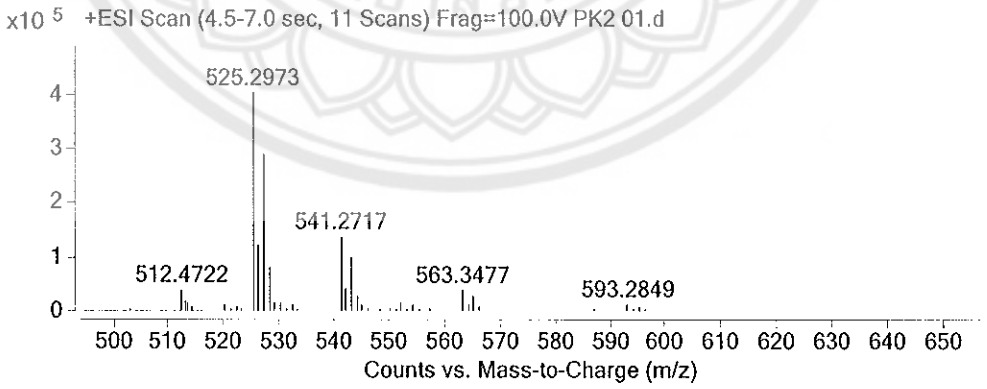


Figure 23 Mass spectrum of BOC-L-proline protected capsaicin

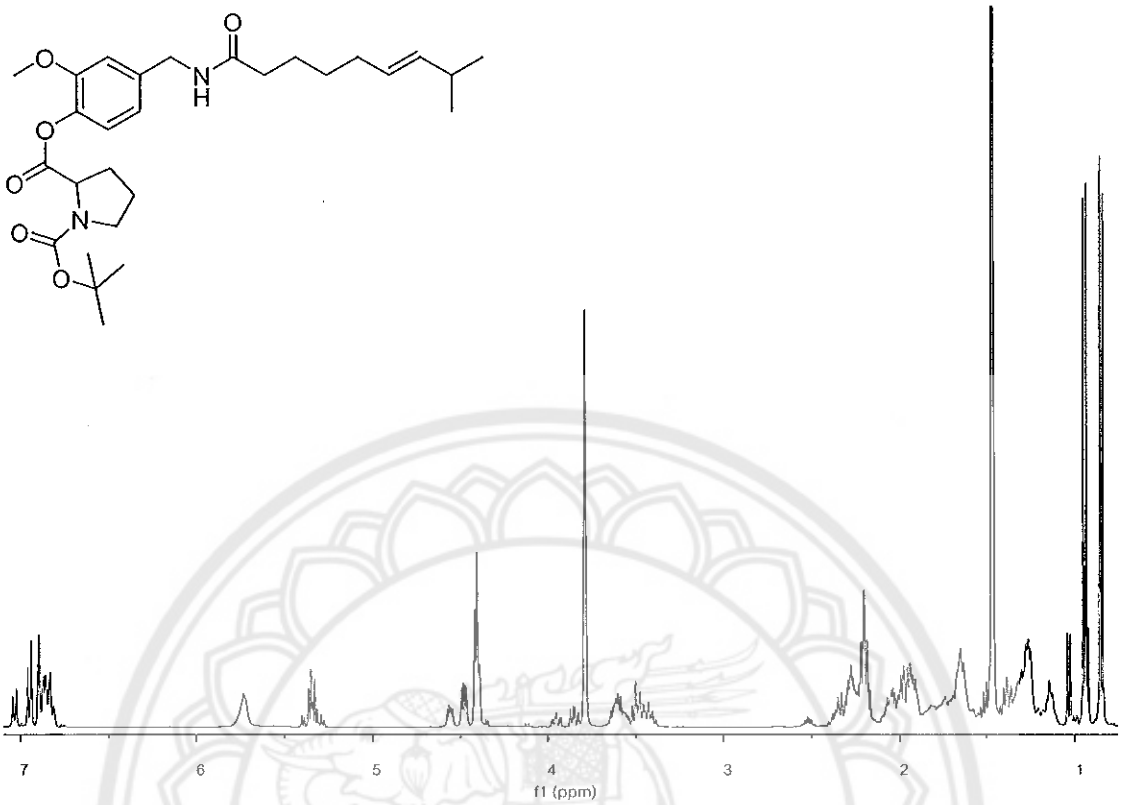
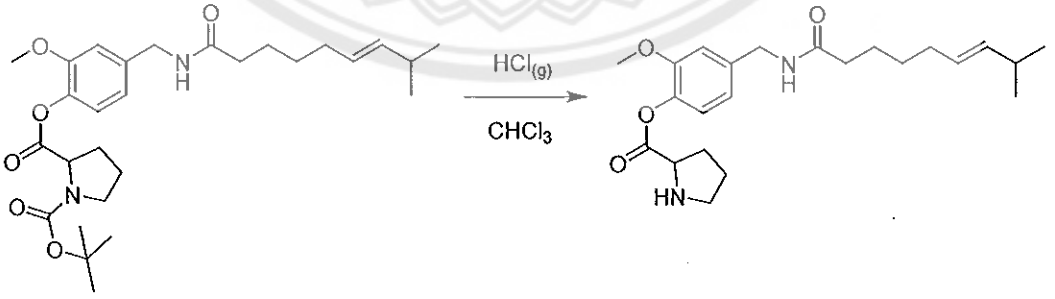


Figure 24 ^{1}H-NMR spectrum of BOC-L-proline protected capsaicin

The removal of the protecting BOC group was performed using HCl gas (Scheme 16). The structure of proline protected capsaicin was confirmed by mass spectrometry and ^{1}H-NMR spectrum (Figure 25-26).



Scheme 16 The removal of BOC group to proline protected capsaicin

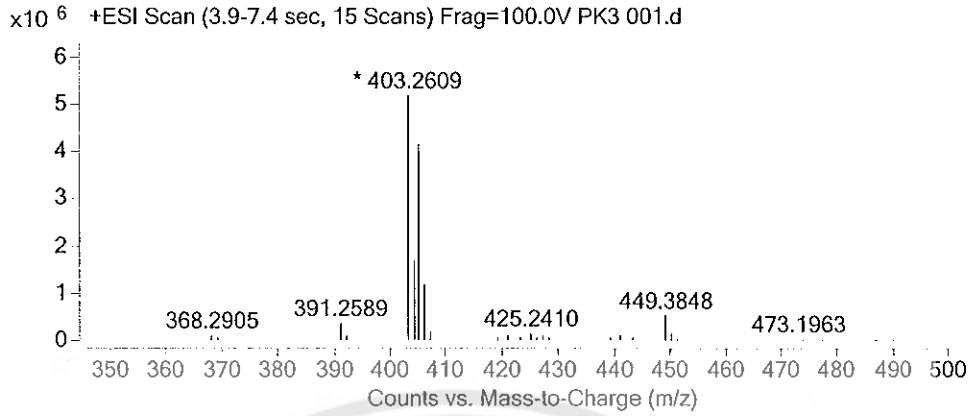


Figure 25 Mass spectrum of L-proline protected capsaicin

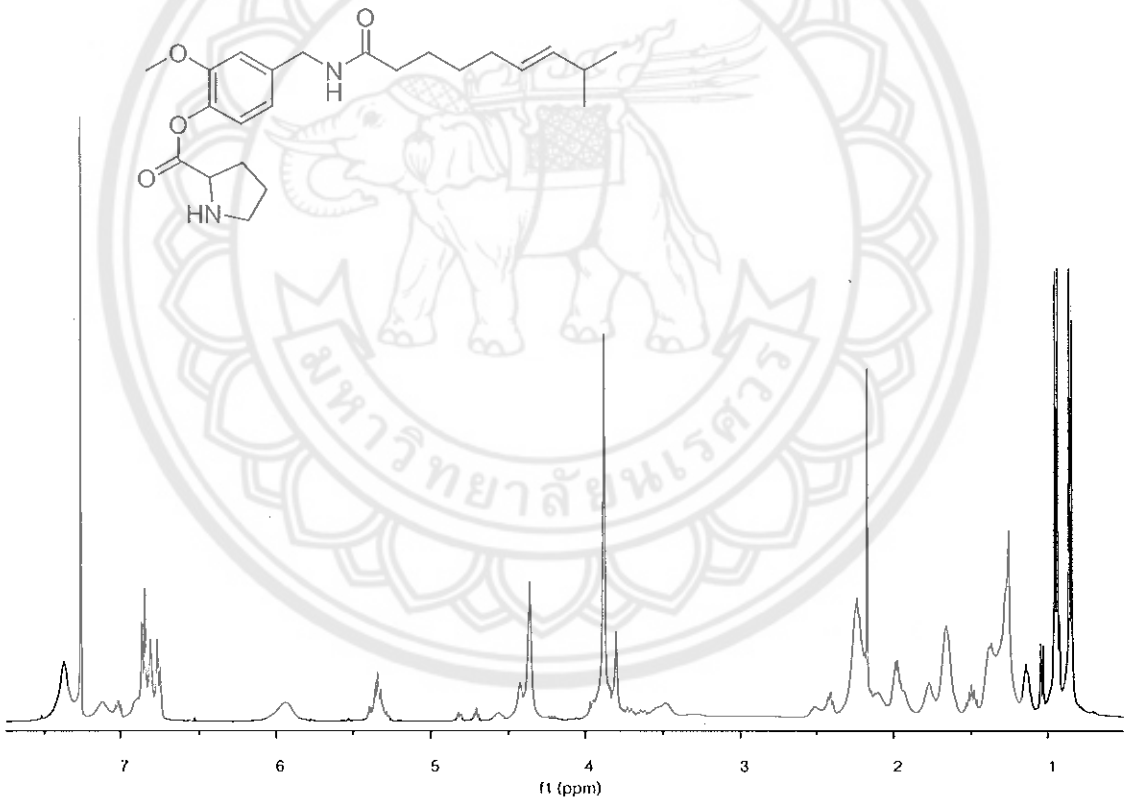
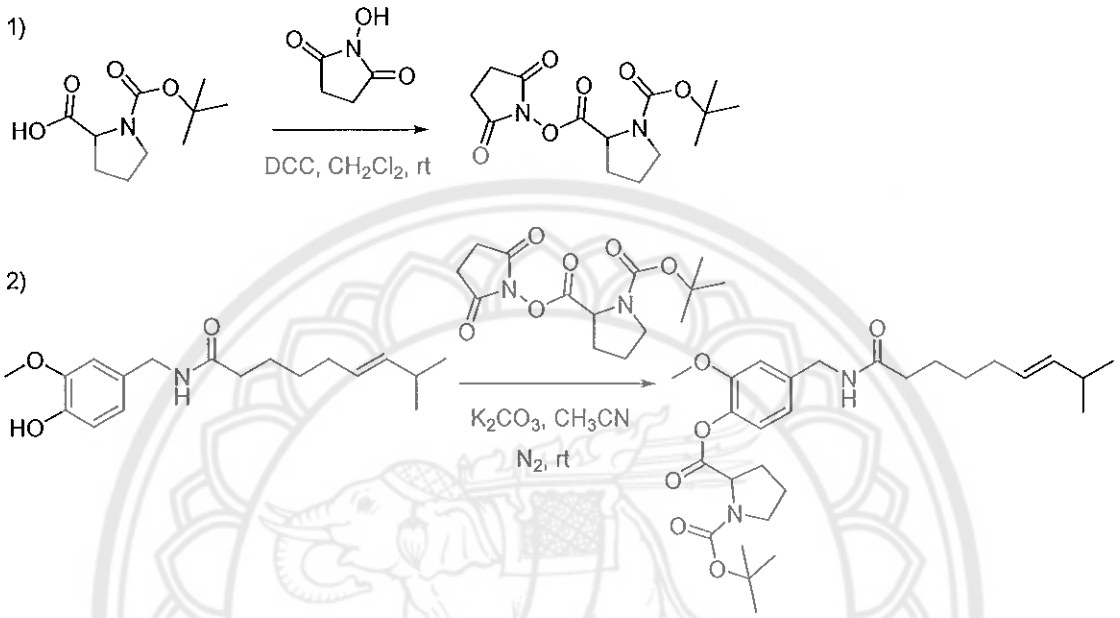


Figure 26 ^{1}H-NMR spectrum of L-proline protected capsaicin

In an attempt to address the fact that it was not possible to modify capsaicin in crude chilli pepper extracts using the DCC coupling with BOC-L-proline, an alternative pathway for this reaction was tested. The BOC-L-proline was first converted into its

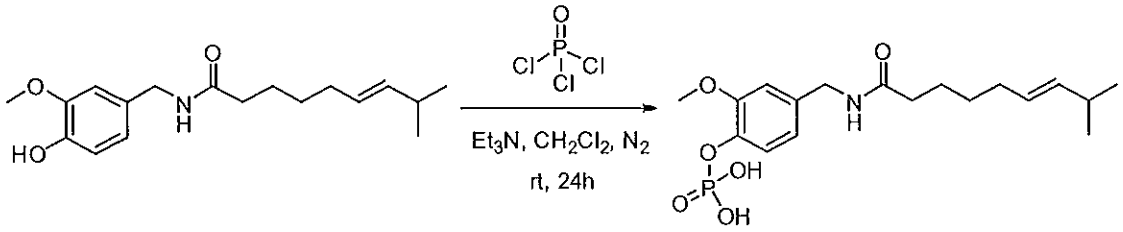
N-hydroxysuccimidyl ester, which was then used in a reaction with capsaicin (Scheme 17). The reaction was successful when performed with pure capsaicin but failed once again when applied to the extract.



Scheme 17 Synthesis of BOC-L-proline protected capsaicin via an NHS ester

5. Phosphate

Phosphorylation was selected as the second approach to making a more hydrophilic and water soluble capsaicin derivatives. This reaction was carried out using phosphoryl chloride ($POCl_3$) in dichloromethane and using triethylamine as base (Scheme 18). Conversion was observed with both pure capsaicin and crude extract from capsaicin containing chili peppers. Given the high solubility of this derivative in water, its purification was carried out using reverse phase silica, and using water/acetonitrile as mobile phase. The product was obtained as yellow solid. The structure was confirmed by ^{1}H-NMR spectrum and mass spectrometry: -ESI-MS: m/z 384 $[M-H]^-$ (Figure 27-28). Actually in this case the protected dihydrocapsaicin was isolated separately, unlike in the cases of the other derivatives where both the capsaicin and dihydrocapsaicin were obtained together.



Scheme 18 Phosphorylation of capsaicin

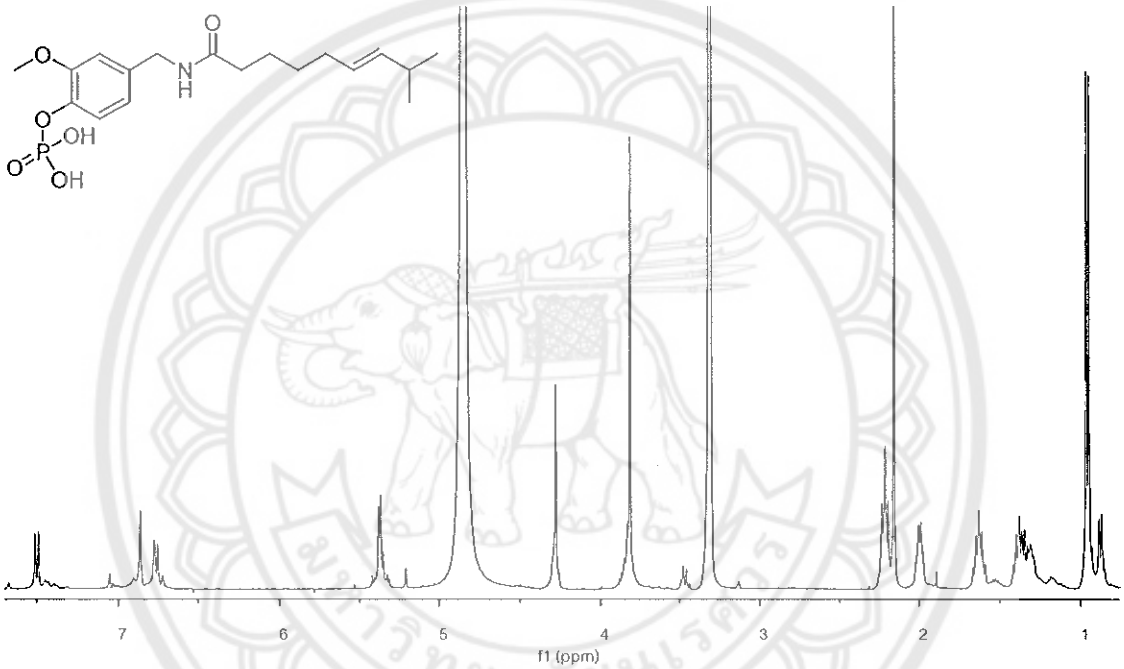


Figure 27 ^{1}H-NMR spectrum of phosphate protected capsaicin

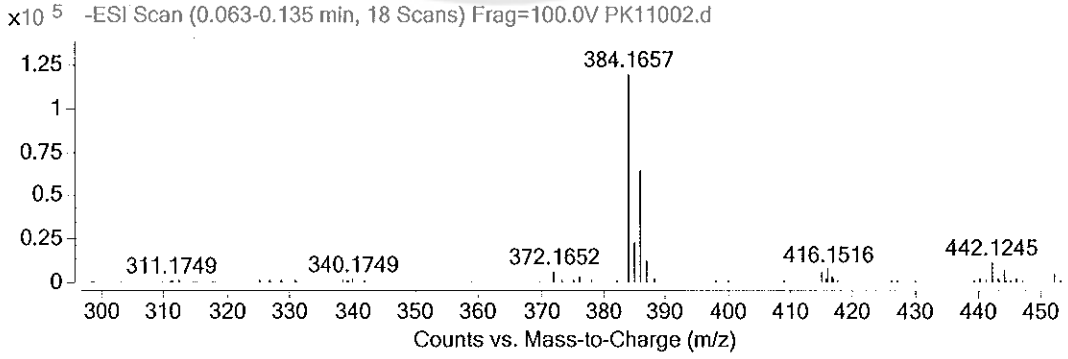
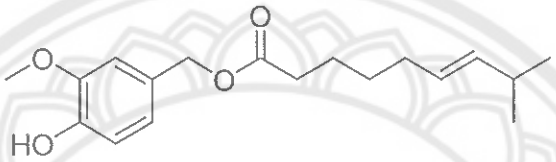


Figure 28 Mass spectrum of phosphorylated capsaicin showing the $[M-H]^-$ peak

6. Attempted capsiate synthesis

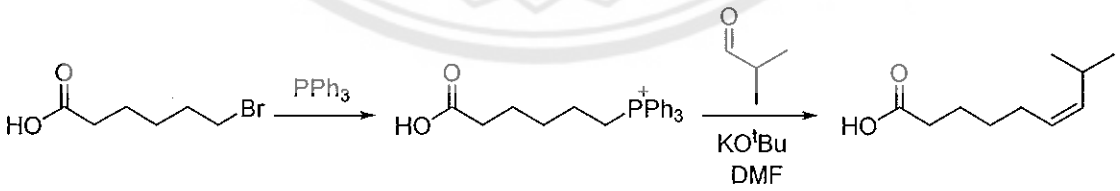
Capsiate is an analogue of capsaicin that has been isolated from a special type of peppers (Figure 29). The difference from capsaicin lies in the fact that the central amide of capsaicin is changed to an ester. One of the issues of capsiate is its instability. It was hypothesized that this instability could also be addressed by protecting the phenolic functional group in its structure. However, due to its unstable nature the capsiate needed to be synthesized for the purposes of this project.



Capsiate (306.4 g/mol)

Figure 29 Molecular structure of capsiate

This synthesis of capsiate was planned in two stages: 1) Synthesis of the carboxylic acid part and 2) Coupling of the carboxylic acid with vanillyl alcohol. Several attempts of performing this synthesis have been made, however, formation of the double bond using Wittig reaction was unsuccessful in all of these (Scheme 19). Given the other discouraging results with materials containing capsiate, these attempts have been halted



Scheme 19 Attempted synthesis of capsiate

Conversion in chili extract and LC-MS analysis

One of the key objectives of this project was to demonstrate the possibility to perform the selected capsaicin modifications not only using pure capsaicin but also using crude chilli pepper extracts. Therefore, the modifications of pure capsaicin described above served two goals. Firstly, they were proof of concept experiments demonstrating the possibility to perform these transformations. Secondly, the products of these transformations were used as standards for the development of LC-MS methods for the analysis of the reaction mixtures obtained when the modifications were performed with crude chilli extract. The modifications performed with the crude extracts are described below. Reference LC-MS chromatograms for pure capsaicin, derivatives obtained from pure capsaicin, and chilli pepper extract are shown in the appendix.

1. Acetates

Acetylation of chili extract was carried out using similar conditions as for the derivatization of pure capsaicin. The acetylated chilli pepper extract was purified by column chromatography on silica and ^{1}H-NMR of fractions containing acetylated capsaicin was recorded (Figure 30). This spectrum, despite exhibiting a number of impurities, lends further support to the formation of the desired product as it contains the corresponding peaks. Figure 31 shows the TIC and EIC traces for this modified chilli extracts. The results show that the reaction has taken place as peaks for acetylated capsaicin (Peak 1) and acetylated dihydrocapsaicin (Peak 2) can be observed. Their identity was confirmed from the EIC traces for m/z 348 (acetylated capsaicin) and 350 (acetylated dihydrocapsaicin). The chromatograms also indicate only partial conversion as peaks for capsaicin (Peak 3) and dihydrocapsaicin (Peak 4).

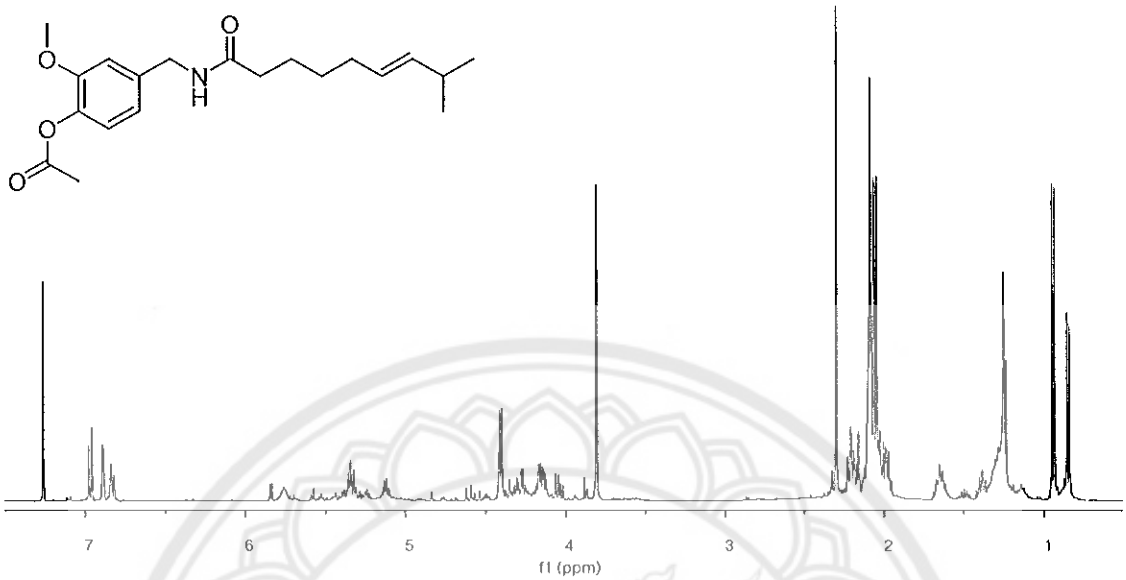


Figure 30 ^{1}H-NMR spectrum of acetylated crude extract

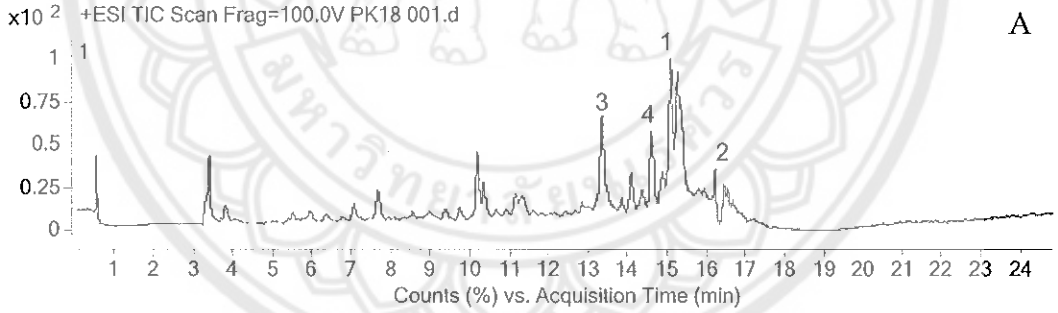


Figure 31 (A) LC-MS chromatogram for acetylated chili pepper extract showing the presence of acetylated capsaicin (Peak1) and acetylated dihydrocapsaicin (Peak 2) as well as residual capsaicin (Peak 3) and dihydrocapsaicin (Peak 4). (B) Extracted ion chromatogram for m/z 348 (Capsacin-Ac). (C) Extracted ion chromatogram for m/z 350 (Dihydrocapsaicin-Ac). This data is for the reaction carried out in acetone

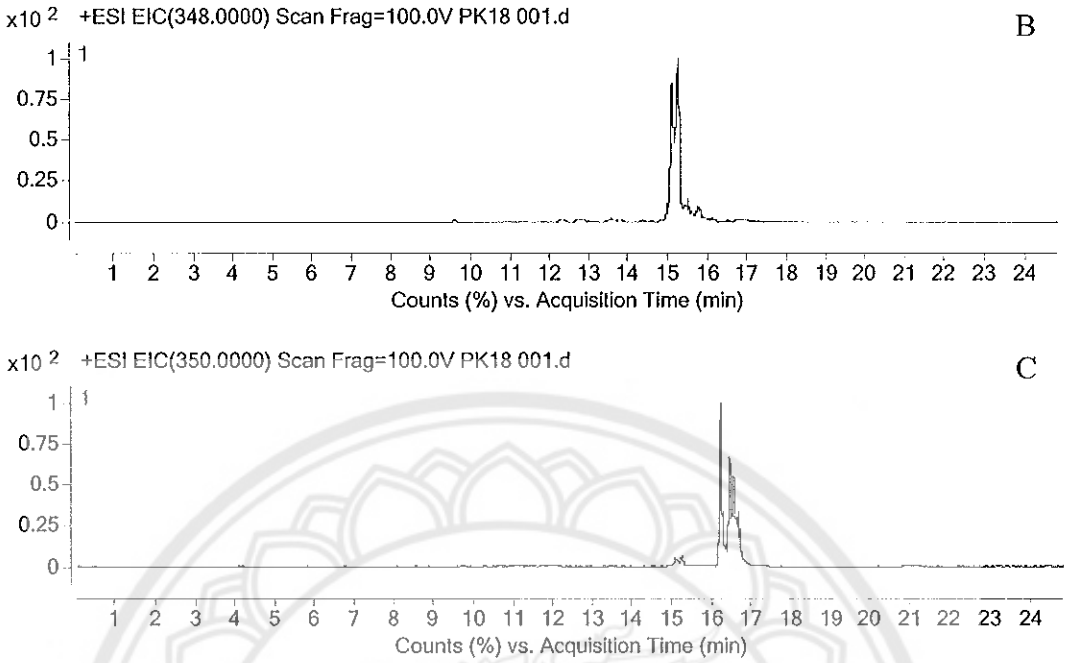
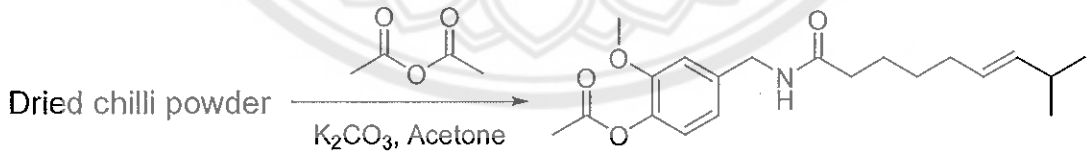


Figure 31 (cont.)

The modification of capsaicin described above was performed with material obtained after extraction and solvent evaporation. However, given the fact that acetone is a solvent that can extract capsaicin from chilli peppers, it was envisioned that a direct, single pot extraction/acetylation process could be performed as well (Scheme 20). The formation of the desired product was confirmed using mass spectroscopy (Figure 32).



Scheme 20 Single step extraction and acetylation of capsaicin

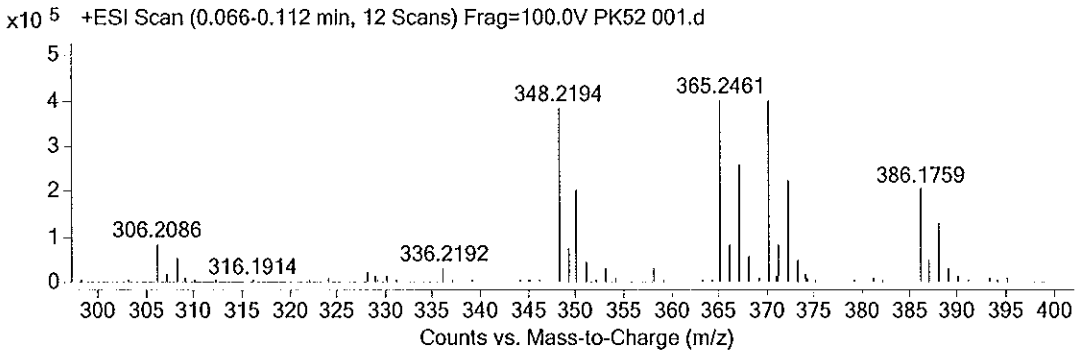


Figure 32 MS spectrum of acetylated capsaicin obtained after one pot extraction and acetylation procedure. The spectrum shows peaks for acetylated capsaicin at m/z 348 $[M+H]^+$, 370 $[M+Na]^+$, and 386 $[M+K]^+$. A peak for residual capsaicin is present at m/z 306 $[M+H]^+$

2. Carbamate

Carbamate modification of chili extract was initially performed using the conditions established for pure capsaicin derivatization. However, the protection was not successful and significant amounts of capsaicin remained in the reaction mixture as determined by mass spectroscopy (Figure 33). The problem was solved by adding 10-fold excess of dimethylcarbamoyl chloride to the reaction mixture. The successful transformation was confirmed by LC-MS (Figure 34). Figure 34 shows the TIC traces and the EIC traces of the carbamate modified chilli extract. The presence of carbmate protected derivatives is confirmed by the EIC traces for m/z 377 (carbamate capsaicin) and 379 (carbamate dihydrocapsaicin), which correspond to peaks 1 and 2 in the TIC chromatogram respectively. The ^{1}H-NMR spectrum for carbamate protected chilli pepper extract purified by column chromatography is shown in Figure 35. Key peaks of the product can be seen despite the presence of various impurities.

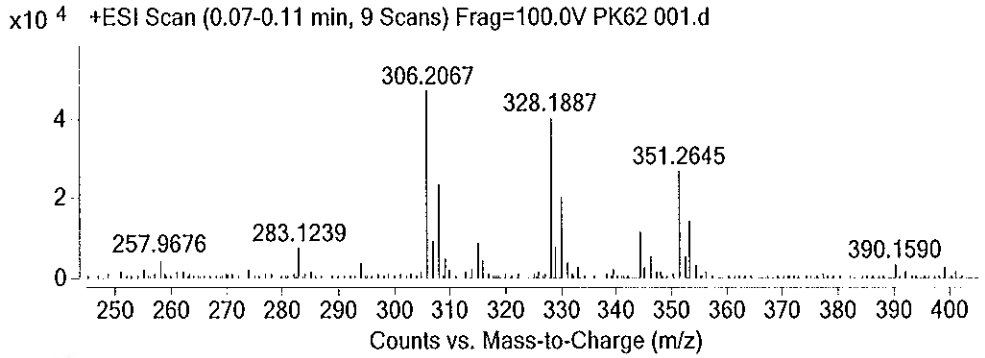


Figure 33 Mass spectrum of carbamate crude extract

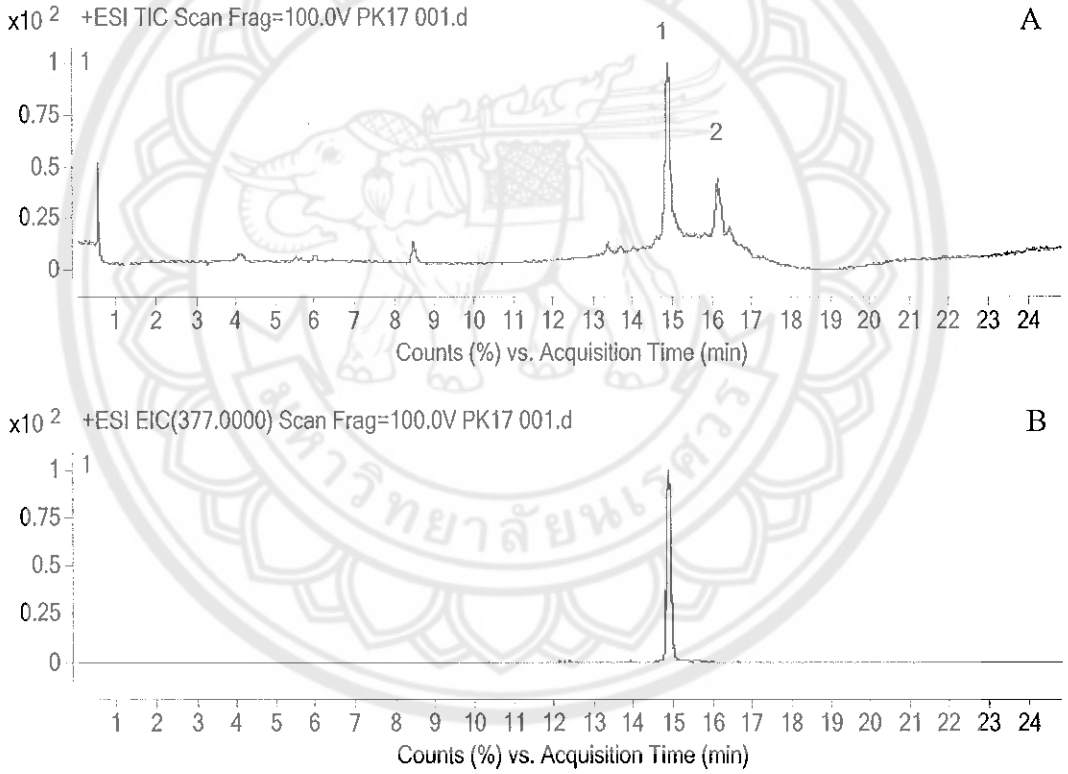


Figure 34 (A) LC-MS chromatogram for carbamate derivatized chili extract (Peak 1) showing the presence of carbamate derivatzed dihydro-capsaicin (Peak 2). (B) Extracted ion chromatogram for m/z 377 (Capsacin-Ca). (C) Extracted ion chromatogram for m/z 379 (Dihydrocapsaicin-Ca). This data is for the reaction carried out in acetone

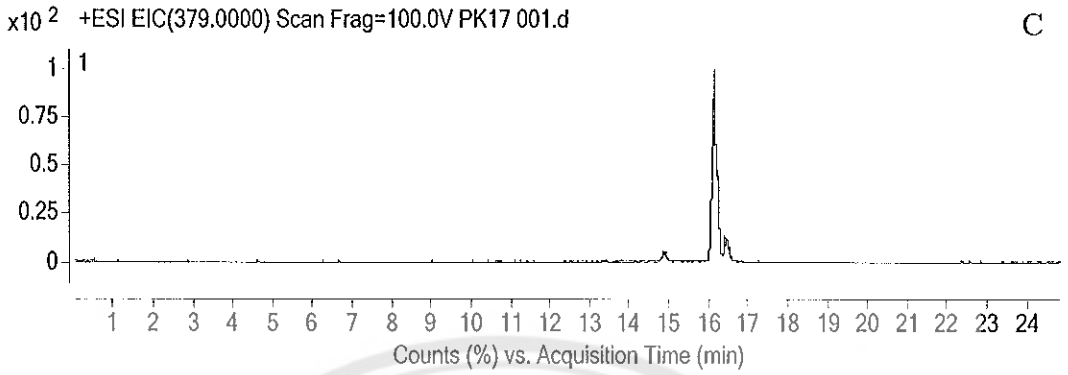


Figure 34 (cont.)

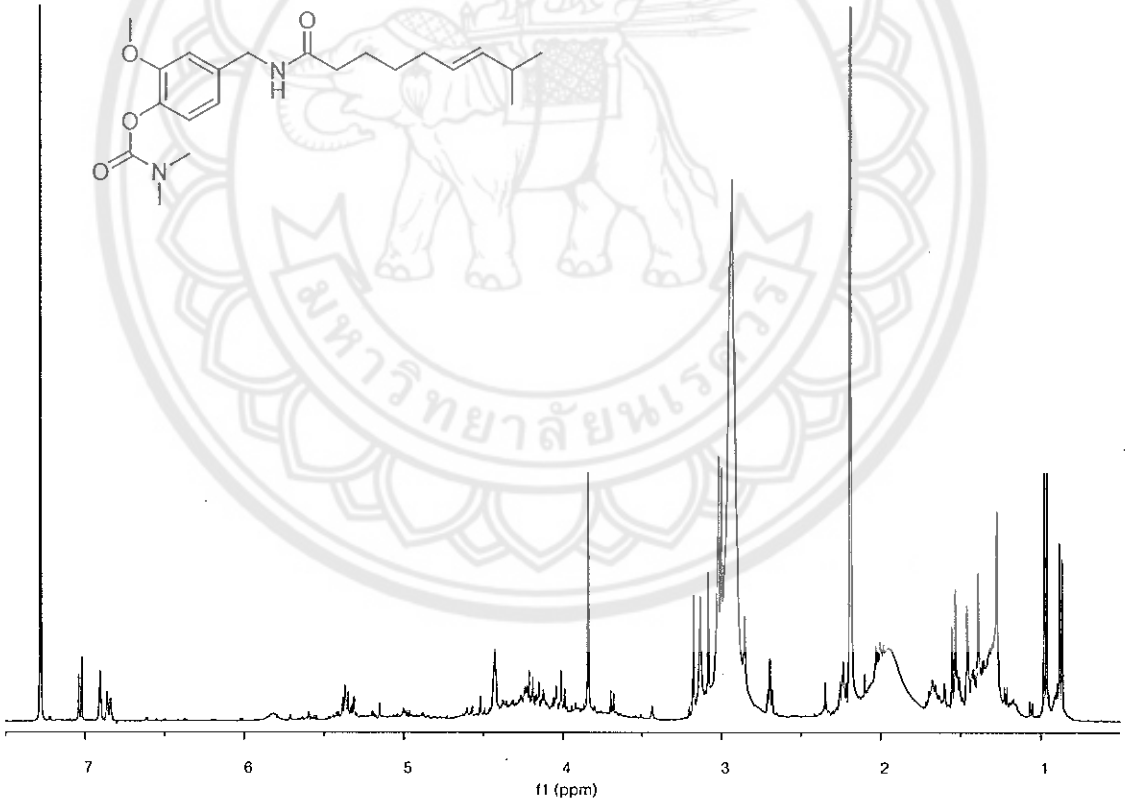


Figure 35 ^{1}H-NMR spectrum of carbamate crude extract

3. Isobutyrate

Isobutyratation of capsaicin in chili extract was also carried out using similar conditions as for the derivatization of pure capsaicin. The isobutyrate capsaicin in the extract was confirmed from +ESI-MS spectra where the peaks at m/z 376 $[M+H]^+$ and 414 $[M+K]^+$ (Figure 36) were observed.

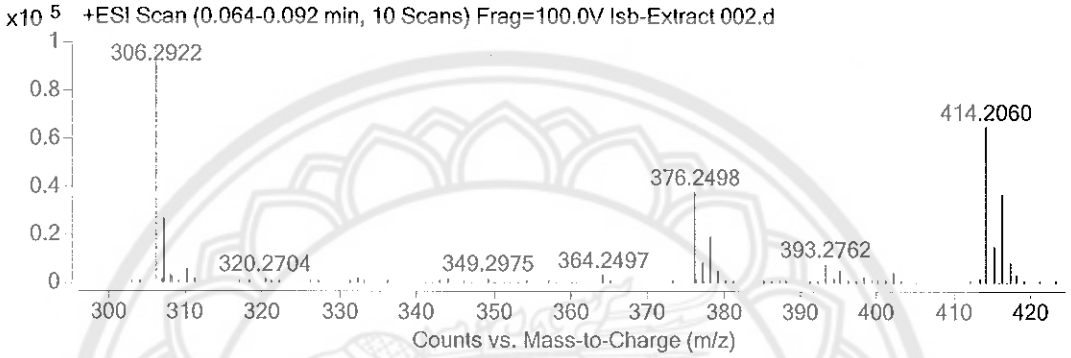


Figure 36 Mass spectrum of isobutyrated capsaicin from chili extract showing the [M+H]+ and [M+K]+ peaks

4. Phosphate

Phosphorylation of capsaicin in the crude chilli pepper extract was attempted utilizing the conditions identified for phosphorylation of pure capsaicin. The phosphorylation of capsaicin in the extract was confirmed from -ESI-MS spectra where a peak at m/z 384 $[M-H]^-$ (Figure 37) was observed.

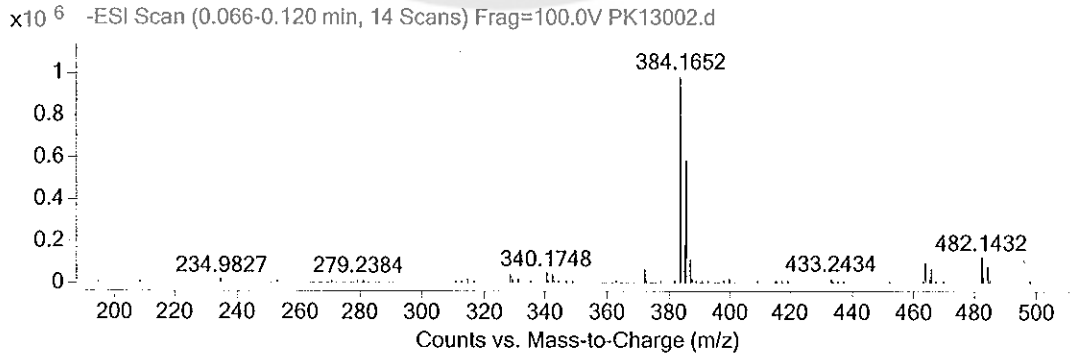
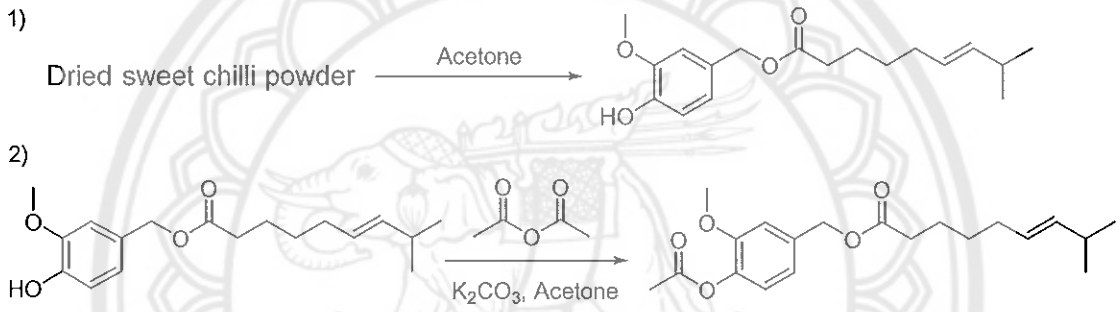


Figure 37 Mass spectrum of phosphorylated capsaicin from chili extract

Modification of capsiate in chili extract

Despite the fact that synthesis and modification of capsiate were unsuccessful during the course of this project, it was hypothesized, based on the results obtained with chilli pepper extracts, that modification of capsiate in extracts from its biological source (CH-19 peppers) could be possible. Initially, this modification was attempted in a two-step process involving obtaining the extract in the first step and its modification in the second step (Scheme 21). However, the desired product could not be found in the reaction mixture using mass spectrometry (Figure 38). Furthermore, there was no evidence of capsiate presence in the modified extract either (Figure 39).



Scheme 21 Two steps extraction and acetylation procedure for capsiate modification

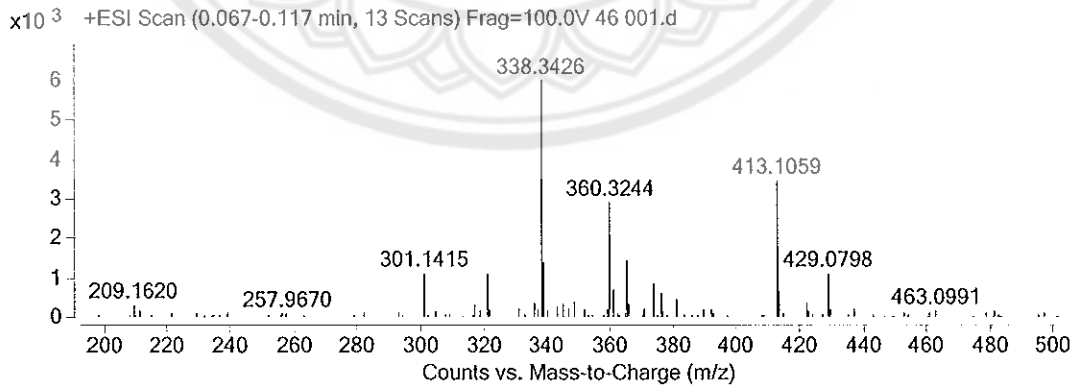
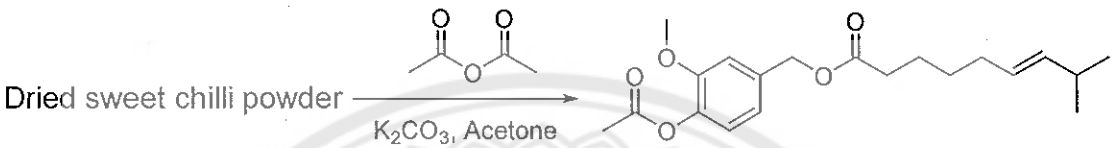


Figure 38 MS spectrum of reaction mixture after two step extraction acetylation of capsiate

Given the results of the previous experiment, and the fact that we have demonstrated the possibility to modify capsaicin in a direct extraction and acetylation experiment, it was decided to perform an analogous single step process using the dried sweet chilli (CH-19) powder (Scheme 22). In this case the evidence of the presence of acetylated capsiate was found in the MS spectra (Figure 39).



Scheme 22 Single step extraction and acetylation of capsiate

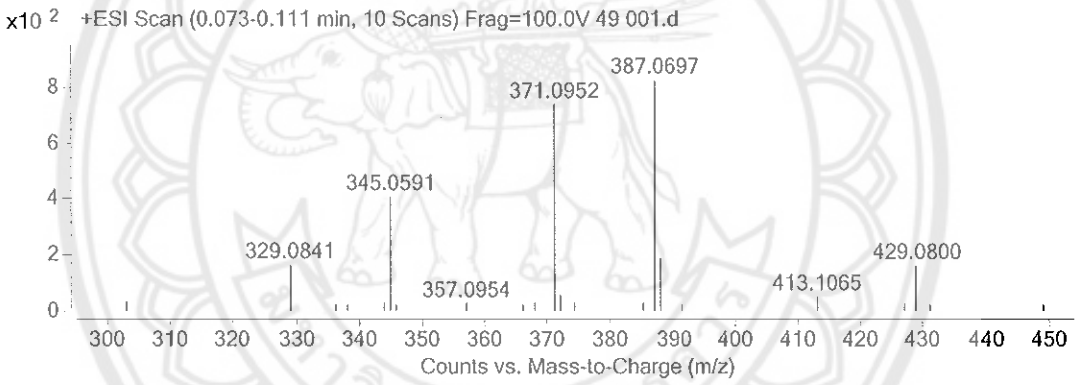


Figure 39 MS spectrum of acetylated capsiate obtained after one pot extraction and acetylation procedure. The spectrum shows peaks for acetylated capsiate at m/z 371 $[M+Na]^+$, and 387 $[M+K]^+$. A peak for residual capsaiate is present at m/z 345 $[M+K]^+$.

In summary, it was demonstrated that capsaicin can be modified by various groups on its phenolic moiety and that some of these modifications (e.g. acetylation, carbamate formation, phosphorylation) can even be performed in crude extracts from chilli peppers. It was also demonstrated that capsaicin and even capsiate can be acetylated in a direct, one step extraction-modification procedure.

CHAPTER V

INVESTIGATION OF PROPERTIES

As mentioned above the modified capsaicin derivatives were synthesized with the aim of them acting as prodrugs or precursors for capsaicin in biological systems. In order to evaluate the suitability of these derivatives for this purpose their properties were investigated. This investigation focused on two key areas: 1) The possibility to deprotect the capsaicin derivatives by enzymes and 2) The cytotoxicity of the synthesized capsaicin derivatives. The results of these investigations are discussed below.

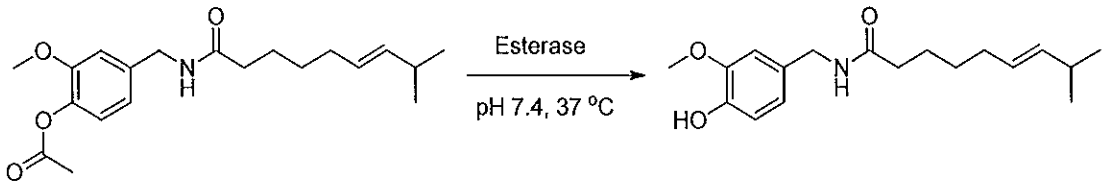
Enzymatic hydrolysis

1. Initial detection by LC-MS

Four derivatives of capsaicin (acetylated capsaicin, carbamate capsaicin, isobutyrated capsaicin, and phosphorylated capsaicin) were studied in terms of stability and potential of their deprotection by enzymatic hydrolysis. Initial investigation of these hydrolysis reactions were carried out using LC-MS. These experiments were focused and detecting formation of capsaicin utilizing the high sensitivity of MS detection. Further experiments focused on the investigation of the kinetics of the deprotecion reactions were carried out with detection using HPLC equipped with an UV-Vis detector. The deprotection reactions with the lipophllic capsaicin derivatives were carried out in reaction media containing 30 % v/v of DMSO to ensure their solubility. The concentrations of capsaicin derivatives in these experiments were 1 mM.

The deprotection of acetylated capsaicin was tested using porcine liver esterase. The experiment was carried out at 37 °C and using phosphate buffer pH 7.4 (Scheme 23) to simulate physiological conditions. The result show that acetylated capsaicin is stable in the solution without enzyme (Figure 40) but it was hydrolyzed fast in the presence of the enzyme (Figure 41). Actually, the reaction was so fast that it was complete within 10 minutes when the enzyme was used at a concentration of 20

µg/ml. The results shown in Figure 40 are for an experiment where enzyme concentration of 2 µg/ml was used.



Scheme 23 Enzymatic deprotection of acetylated capsaicin

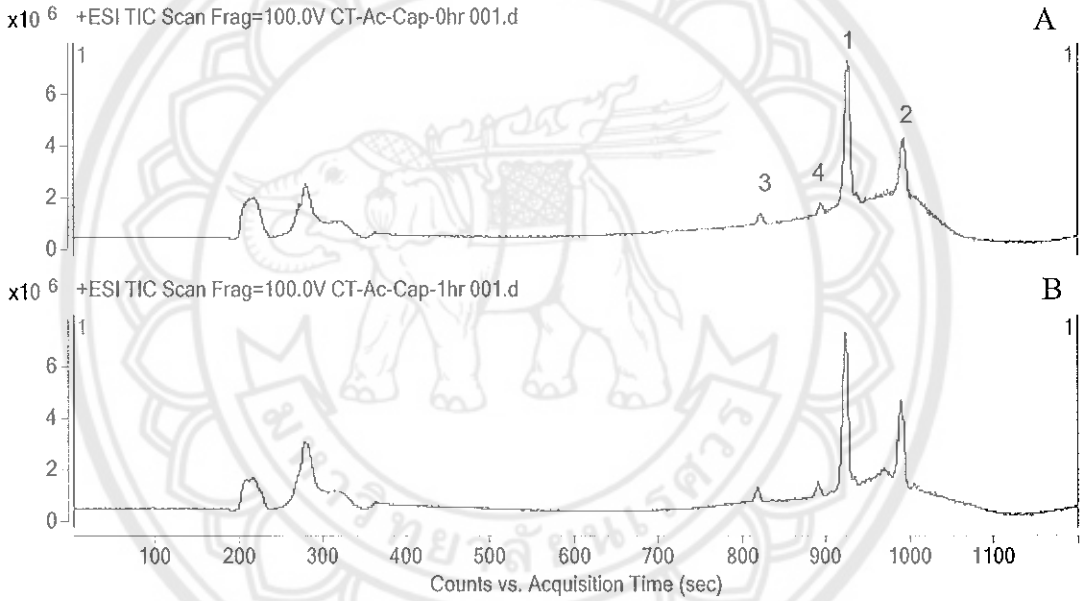


Figure 40 LC-MS chromatogram for acetylated capsaicin (Peak 1) in aqueous buffer at the beginning of the hydrolysis and stability testing without enzyme, showing the presence of acetylated dihydrocapsaicin (Peak 2), residual capsaicin (Peak3), and residual dihydrocapsaicin (Peak 4). The samples of the reaction mixture were taken immediately after reaction start (A) and at 1h after the start of the reaction (B)

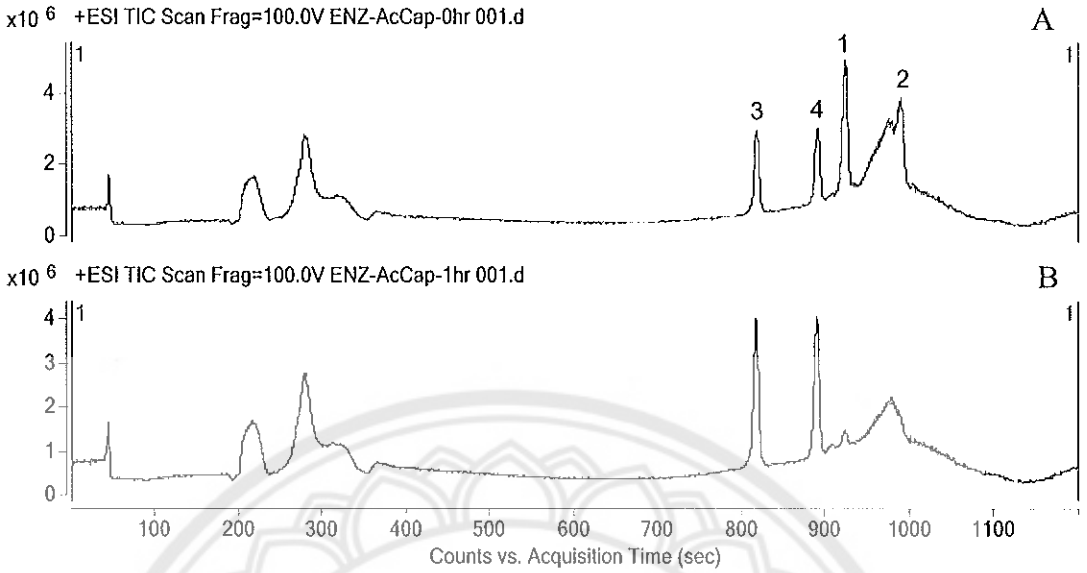


Figure 41 LC-MS chromatogram for acetylated capsaicin (Peak 1) in aqueous buffer at the beginning of the hydrolysis and stability testing with enzyme, showing the presence of acetylated dihydrocapsaicin (Peak 2), residual capsaicin (Peak3), and residual dihydrocapsaicin (Peak 4). The samples of the reaction mixture were taken immediately after the addition of enzyme (A) and at 1h after the start of the reaction (B)

The stability and hydrolysis of carbamate protected capsaicin were tested under similar conditions (pH 7.4, 37 °C) using Porcine Liver Esterase (Scheme 24).

Scheme 24 Enzymatic deprotection of carbamate protected capsaicin

The initial results have shown that the carbamate capsaicin was stable in the absence of porcine liver esterase (Figure 42) and was not hydrolyzed by the enzyme within the 1 h time frame as observed for acetylated capsaicin (Figure 43).

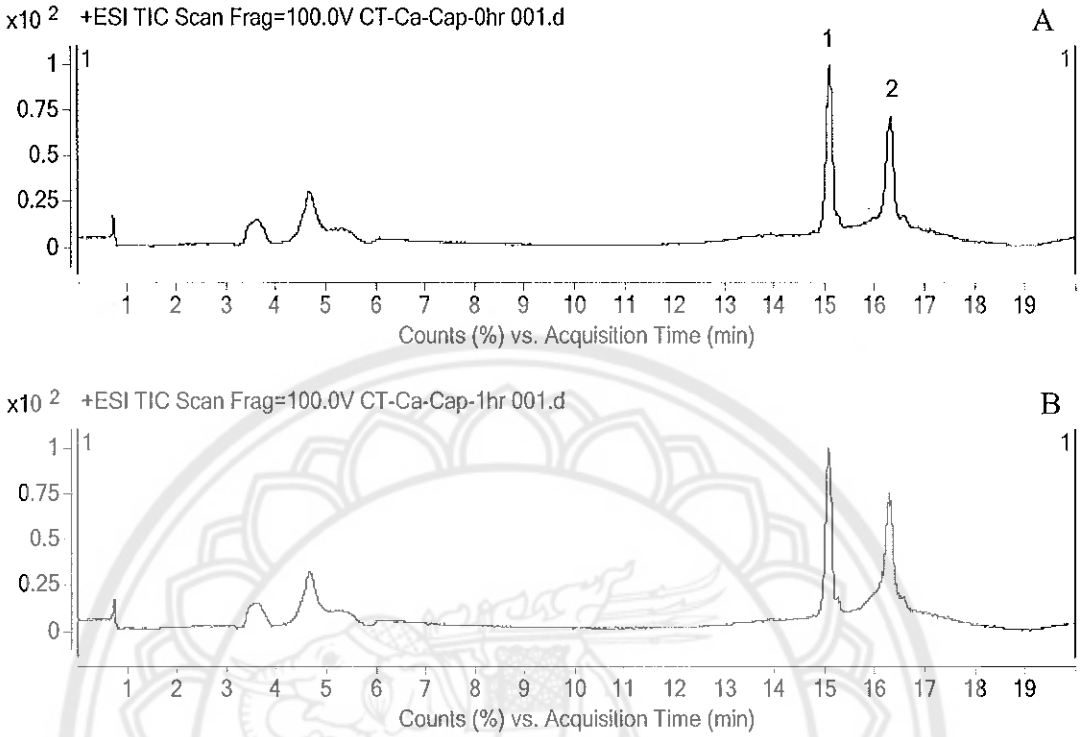


Figure 42 LC-MS chromatogram for carbamate capsaicin (Peak 1) in aqueous buffer at the beginning of the hydrolysis and stability testing without enzyme, showing the presence of carbamate dihydrocapsaicin (Peak 2). The samples of the reaction mixture were taken immediately after reaction start (A), at 1h after the start of the reaction (B).

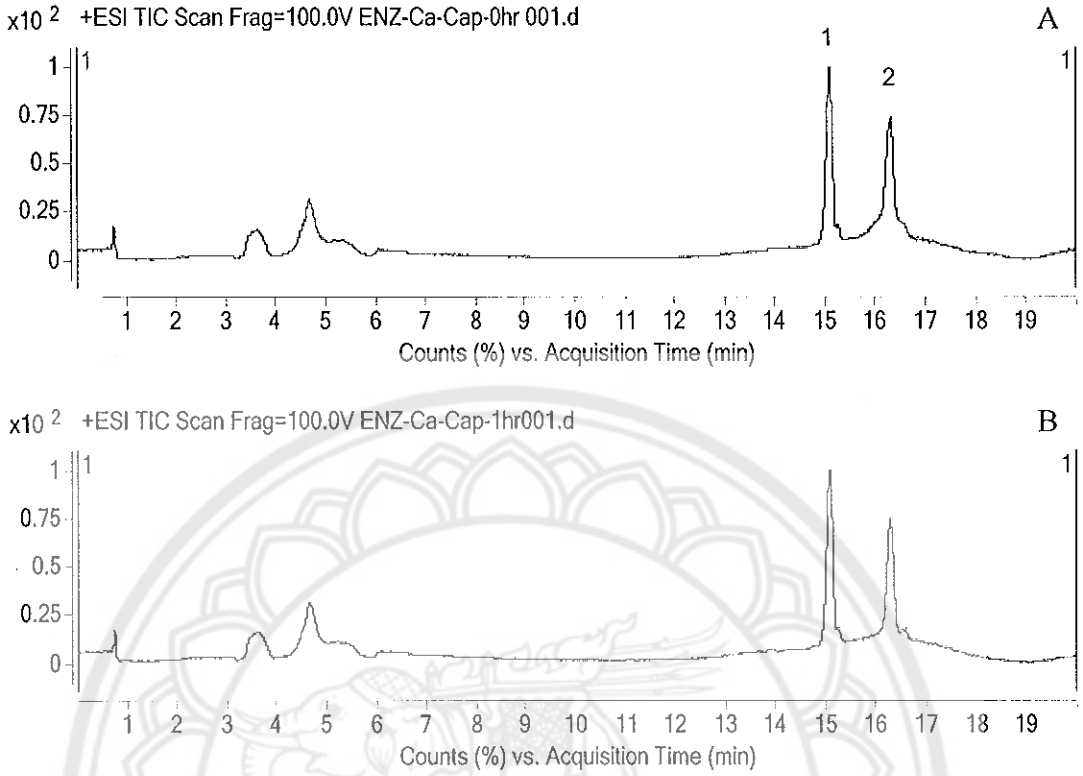


Figure 43 LC-MS chromatogram for carbamate capsaicin (Peak 1) in aqueous buffer at the beginning of the hydrolysis and stability testing with enzyme, showing the presence of carbamate dihydrocapsaicin (Peak 2). The samples of the reaction mixture were taken immediately after the addition of enzyme (A), at 1h after the start of the reaction (B).

However, when the reaction was carried out for 28 h it was possible to observe the capsiacin peak in the LC-MS chromatogram using EIC despite the fact that it was not possible to obsrve it in the TIC chromatogram (Figure 44). The formation of capsaicin in this reaction became even more apparent, with a minor peak appering even on the TIC, when the enzyme was used at a concentration of 200 μg/ml (Figure 45).

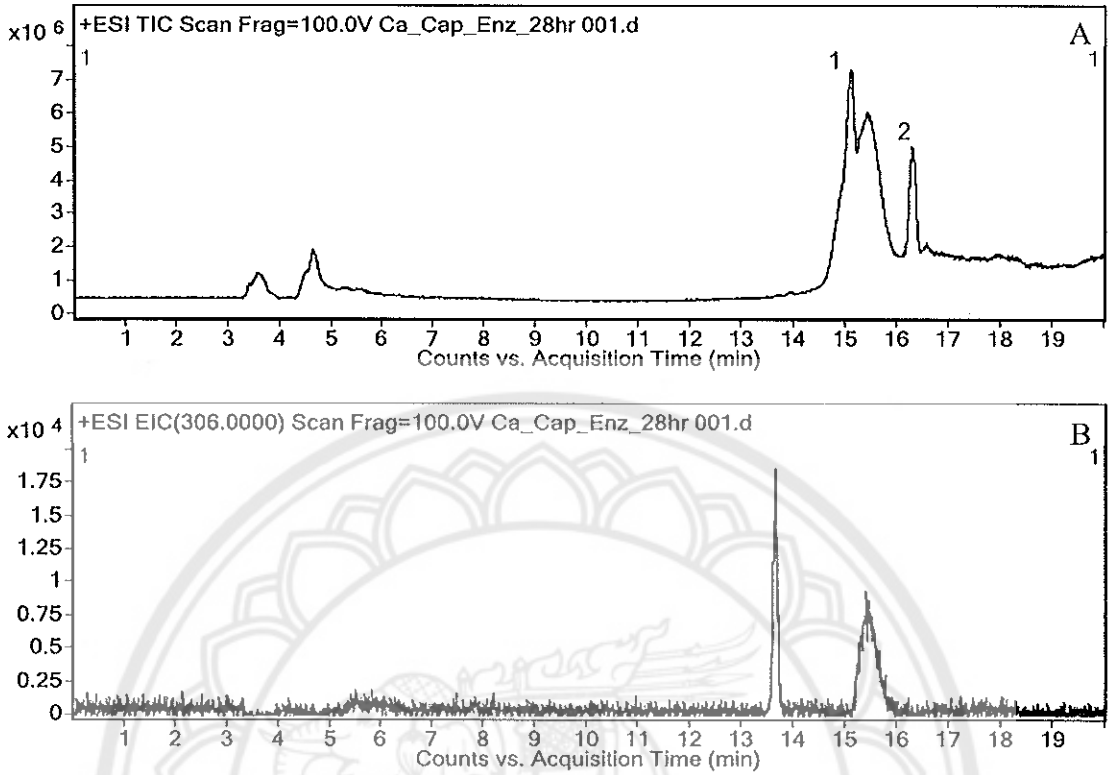


Figure 44 LC-MS chromatogram for carbamate capsaicin (Peak 1) in aqueous buffer after 28 h in the presence of porcine liver esterease, showing the presence of carbamate dihydrocapsaicin (Peak 2). Both the TIC trace (A) and EIC trace for capsaicn (m/z 306) are shown (B).

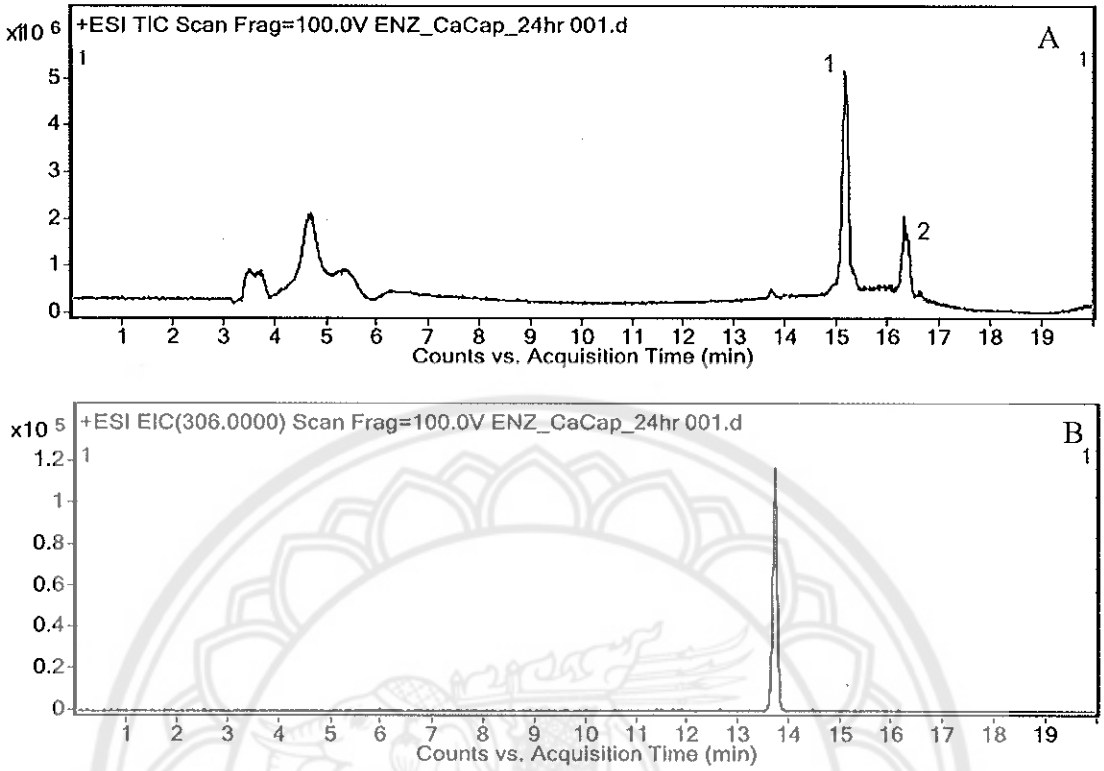


Figure 45 LC-MS chromatogram for carbamate capsaicin (Peak 1) in aqueous buffer after 24 h in the presence of porcine liver esterease (200 μg/ml), showing the presence of carbamate dihydrocapsaicin (Peak 2). Both the TIC trace (A) and EIC trace for capsaicn (m/z 306) are shown (B).

The reaction was then carried out in the presence of acetyl choline esterease. As in the reactions performed with porcine liver esterase, very limited extent of hydrolysis was observed as capsaicin was only detected using EIC (Figure 46). It should be noted that carbamates are actually known to covalently modify acetyl choline esterase and thus the reaction in this case is not truly catalytic [48].

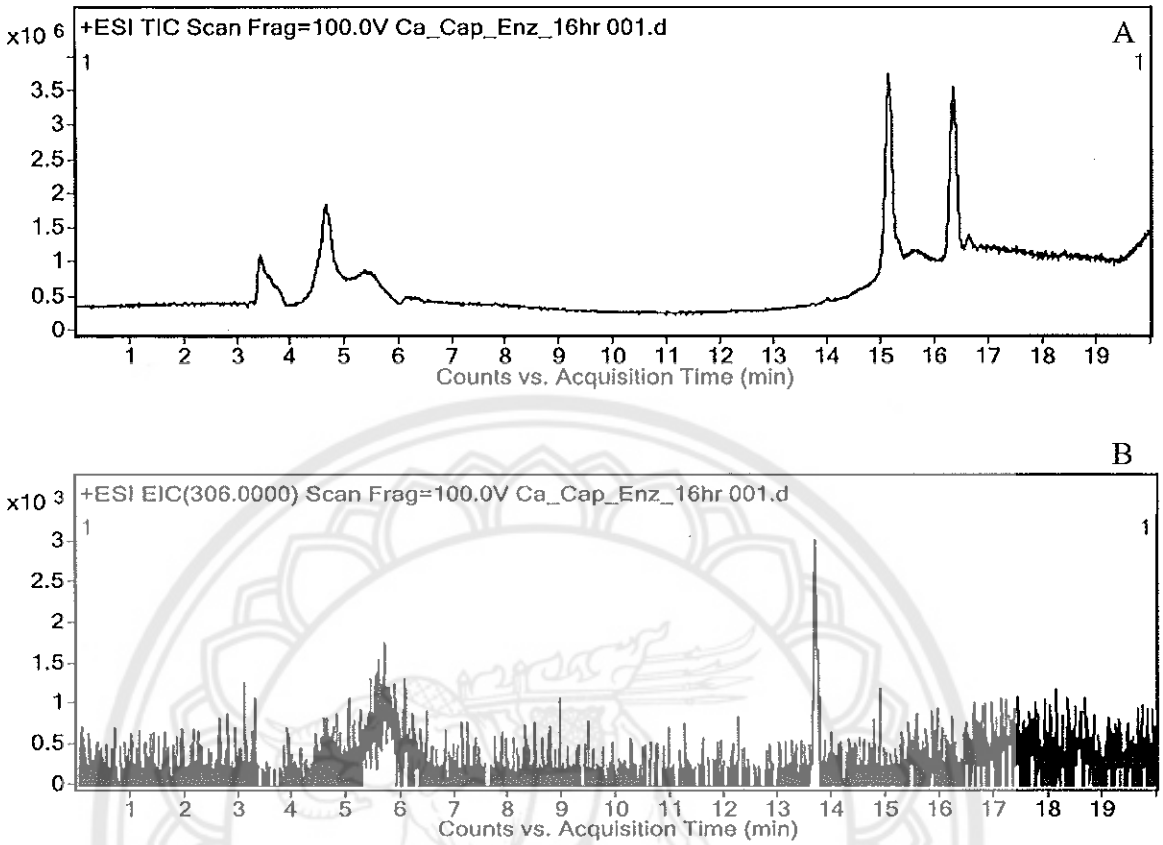
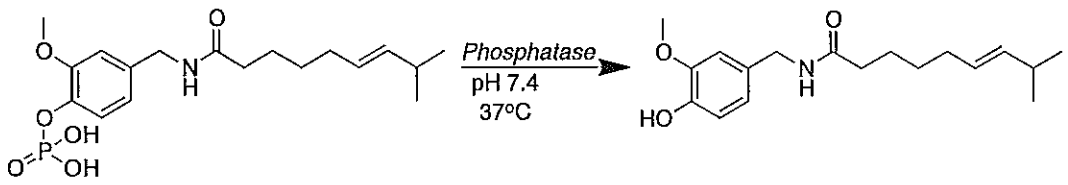


Figure 46 LC-MS chromatogram for carbamate capsaicin (Peak 1) in aqueous buffer after 24 h in the presence of acetyl choline esterase showing the presence of carbamate dihydrocapsaicin (Peak 2). Both the TIC trace (A) and EIC trace for capsaicn (m/z 306) are shown (B)

Finally, the phosphate modified capsaicin was tested for its susceptibility to hydrolysis by alkaline phosphatase. The reaction was monitored by MS in negative mode only.



Scheme 25 Enzymatic deprotection of phosphate protected capsaicin

Using EIC for both phosphorylated capsaicin (m/z 384) and capsaicin (m/z 304) it could be seen that in the presence of enzyme the signal for phosphorylated capsaicin could not be detected in the reaction mixture after 16 h of reaction time (Figure 47)

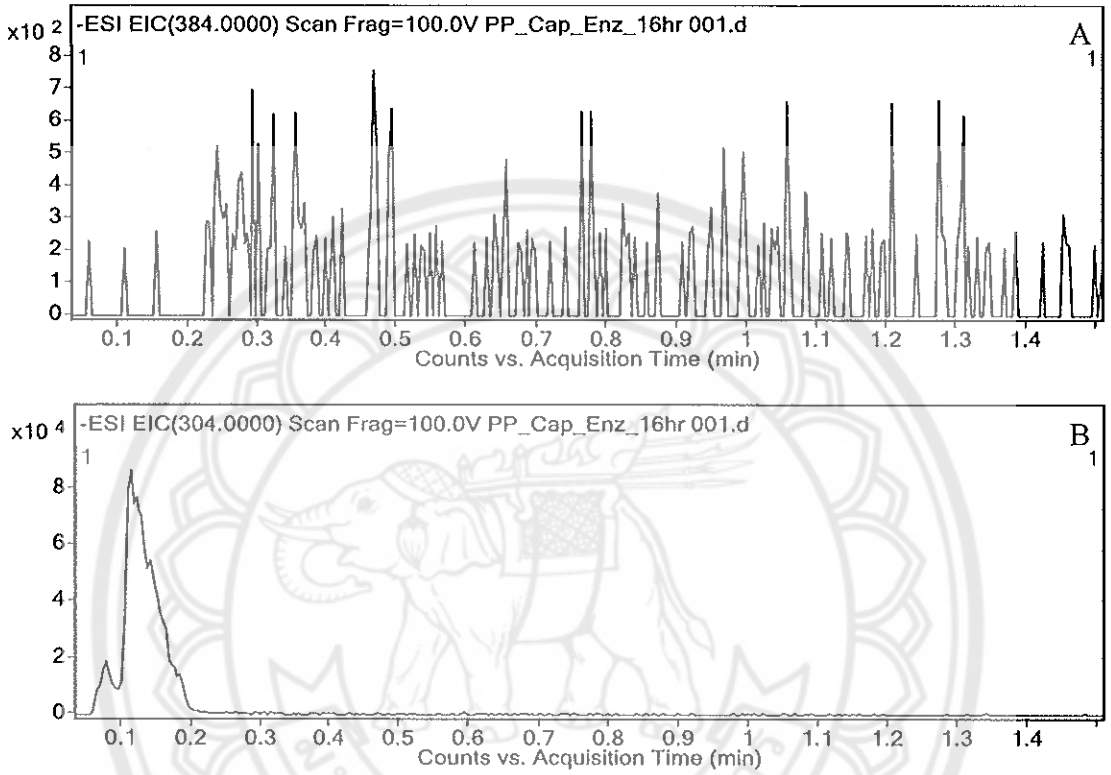


Figure 47 EIC trace for direct injection of phosphorylated capsaicin incubated in the presence of alkaline phosphatase showing trace for m/z 384 (phosphorylated capsaicin, A) and m/z 304 (capsaicin, B)

2. Study of enzymatic deprotection kintetics using HPLC

Given the results for enzymatic hydrolysis of protected capsaicin derivatives obtained using LC-MS it was decided to perform the reaction using HPLC with UV-Vis as the method of detection in order to obtain kinetic data about the reactions. This investigation was carried out with capsaicin modified with acetate, isobutyrate, and dimethyl carbamate protecting groups. The HPLC chromatograms for enzymatic deprotection reaction of acetylated capsaicin using porcine liver esterase are shown in Figure 48. Chromatograms for samples taken at 0, 30, 60, and 120 min can be seen and the peaks of capsaicin, dihydrocapsaicin, acetylated capsaicin, and

acetylated dihydrocapsaicin are indicated. It should be noted that this reaction was carried out with enzyme concentration of 0.5 μg/ml given the fast reaction kinetics observed during the LC-MS study.

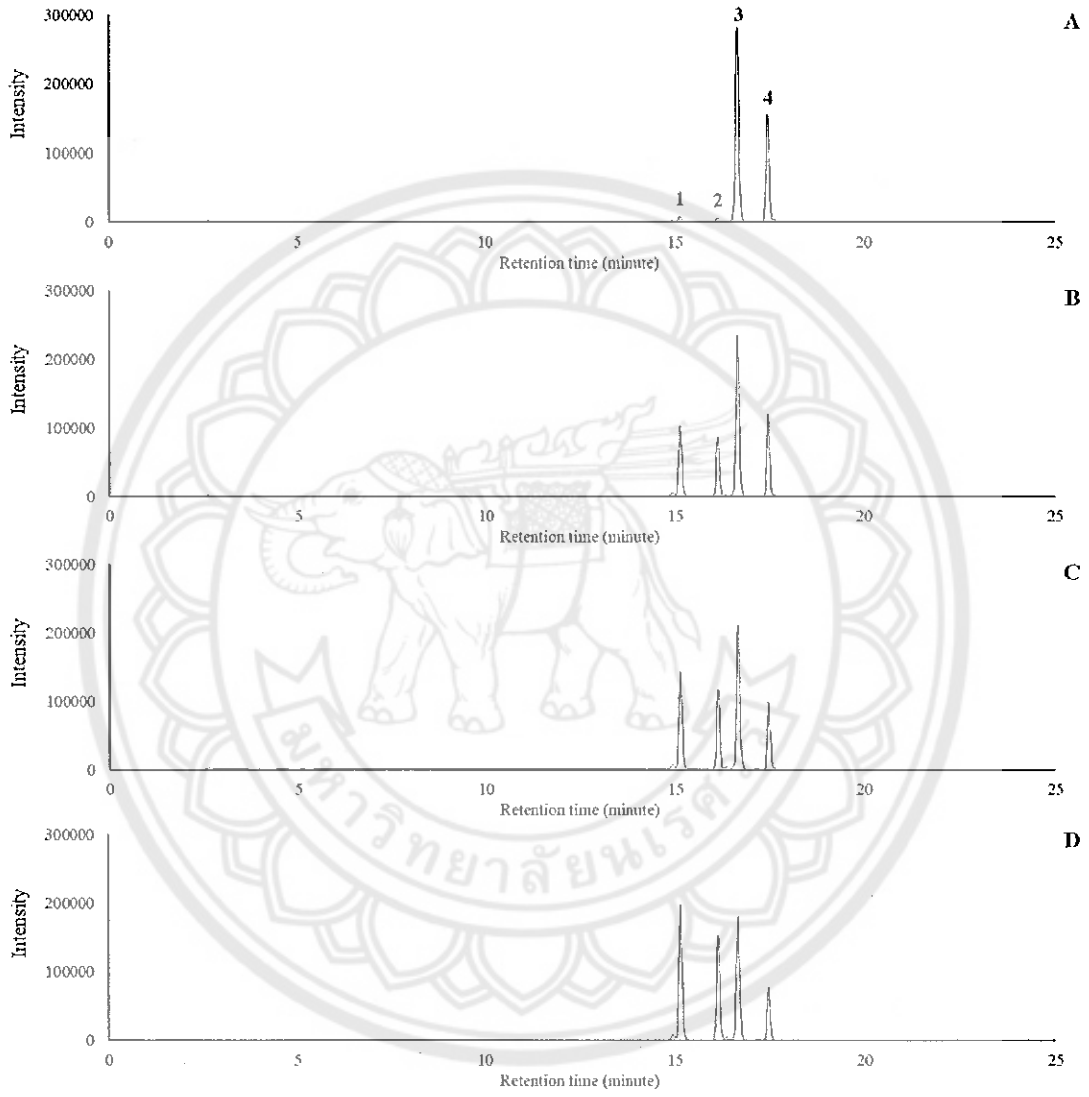


Figure 48 HPLC chromatograms for the enzymatic deprotection reaction showing capsaicin (1), dihydrocapsaicin (2), acetylated capsaicin (3), and acetylated dihydrocapsaicin (4) after 0 min (A), 30 min (B), 60 min (C), and 120 min (D)

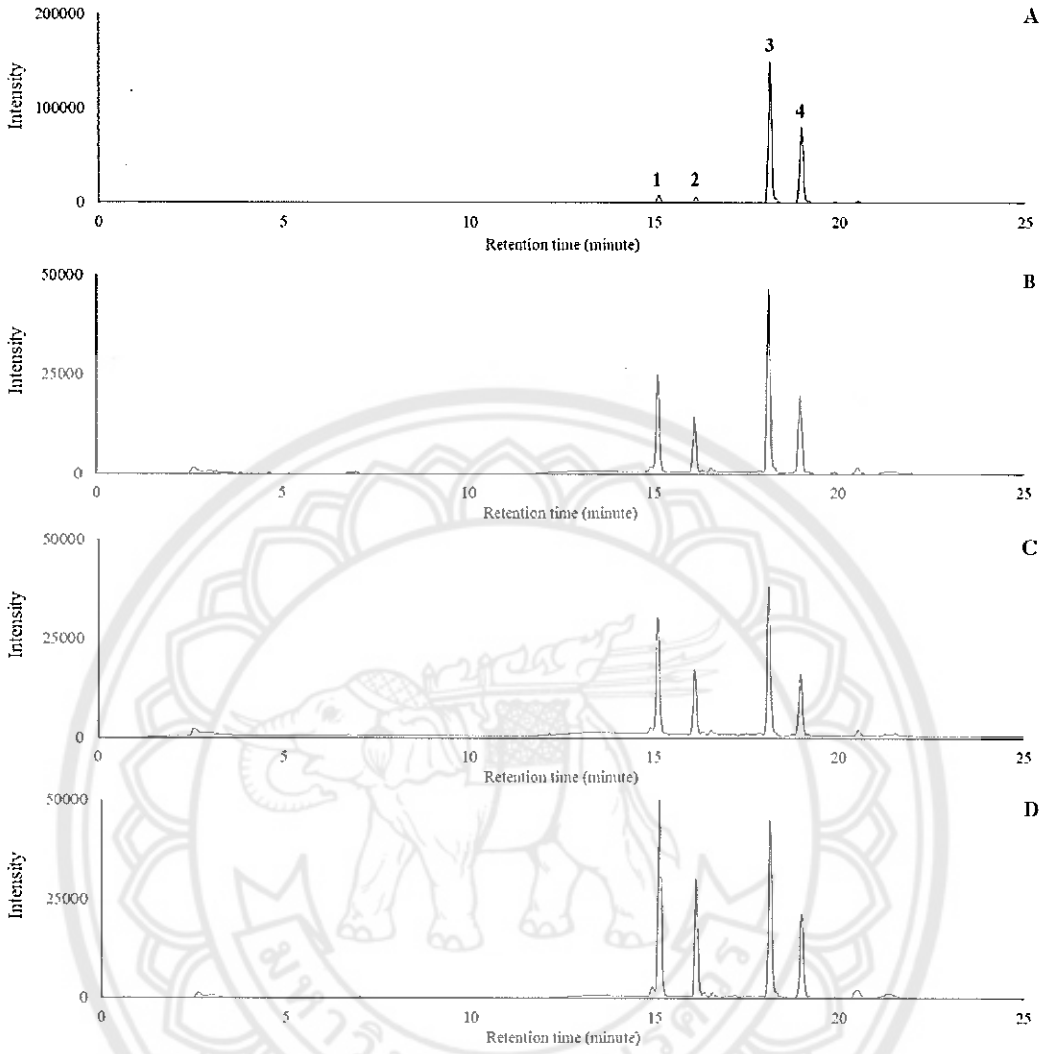


Figure 49 HPLC chromatograms for the enzymatic deprotection reaction showing capsaicin (1), dihydrocapsaicin (2), isobutylated capsaicin (3), and isobutylated dihydrocapsaicin (4) after 0 min (A), 30 min (B), 60 min (C), and 120 min (D)

The results for the enzymatic deprotection reaction of isobutyated capsaicin are shown in Figure 49, which shows the HPLC chromatograms of the reaction mixtures after 0, 30, 60, and 120 min of the enzymatic deprotection reaction indicating the retention times of capsaicin, dihydrocapsaicin, isobutyrated capsaicin and isobutyrated dihydrocapsaicin. The reaction was carried out with enzyme concentration of 1 μg/ml as precipitation was observed at the concentration used for acetylated capsaicin.

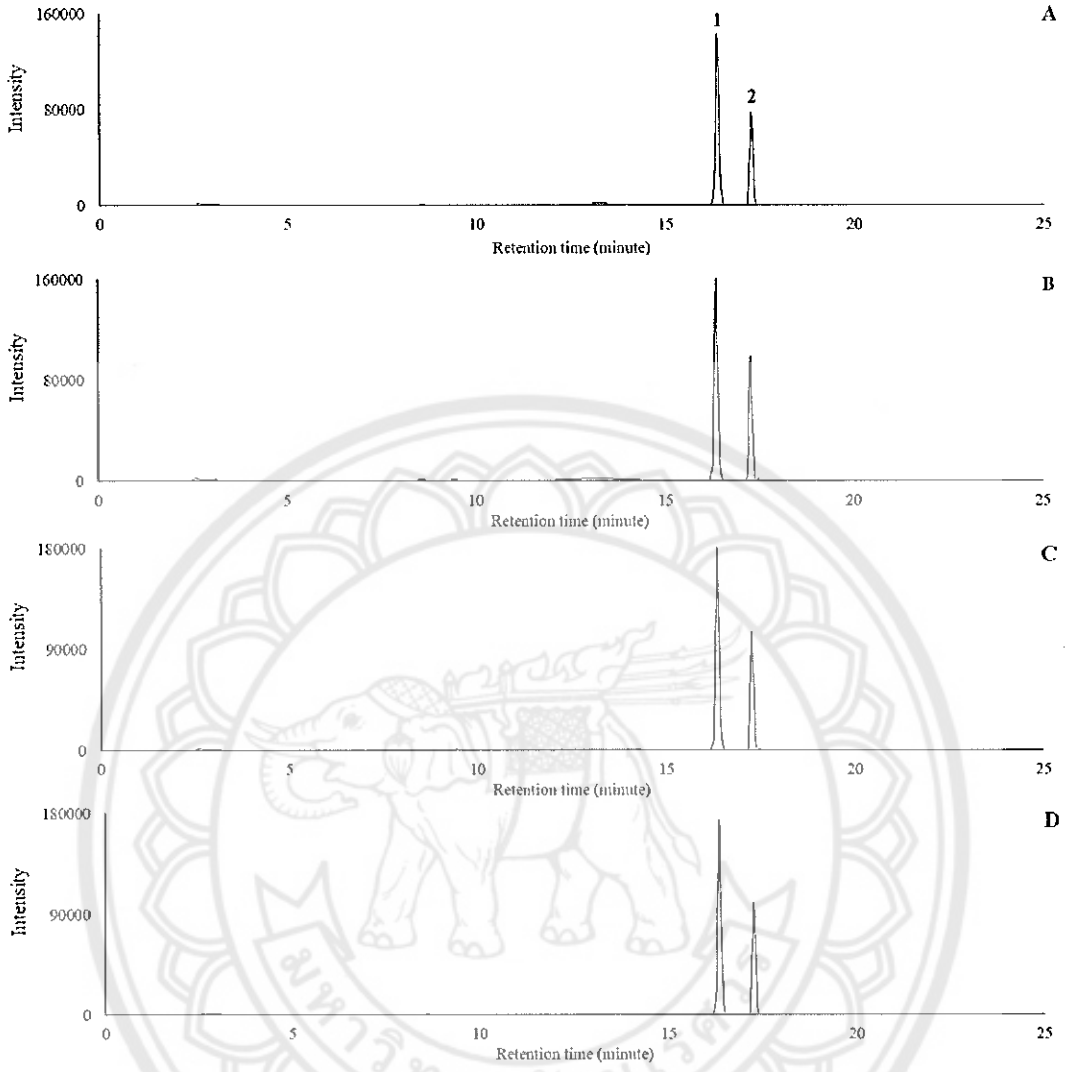


Figure 50 HPLC chromatograms for the enzymatic deprotection reaction showing carbamate capsaicin (1) and carbamate dihydrocapsaicin (2) after 0 min (A), 30 min (B), 60 min (C), and 120 min (D)

The results for the enzymatic deprotection reaction of carbamate capsaicin are shown in Figure 50, which shows the HPLC chromatograms of the reaction mixtures after 0, 30, 60, and 120 min of the enzymatic deprotection reaction indicating the retention times of capsaicin, dihydrocapsaicin, carbamate capsaicin, and carbamate dihydrocapsaicin. The reaction was carried out with enzyme concentration of 0.5 μg/ml as in the case of acetylated capsaicin.

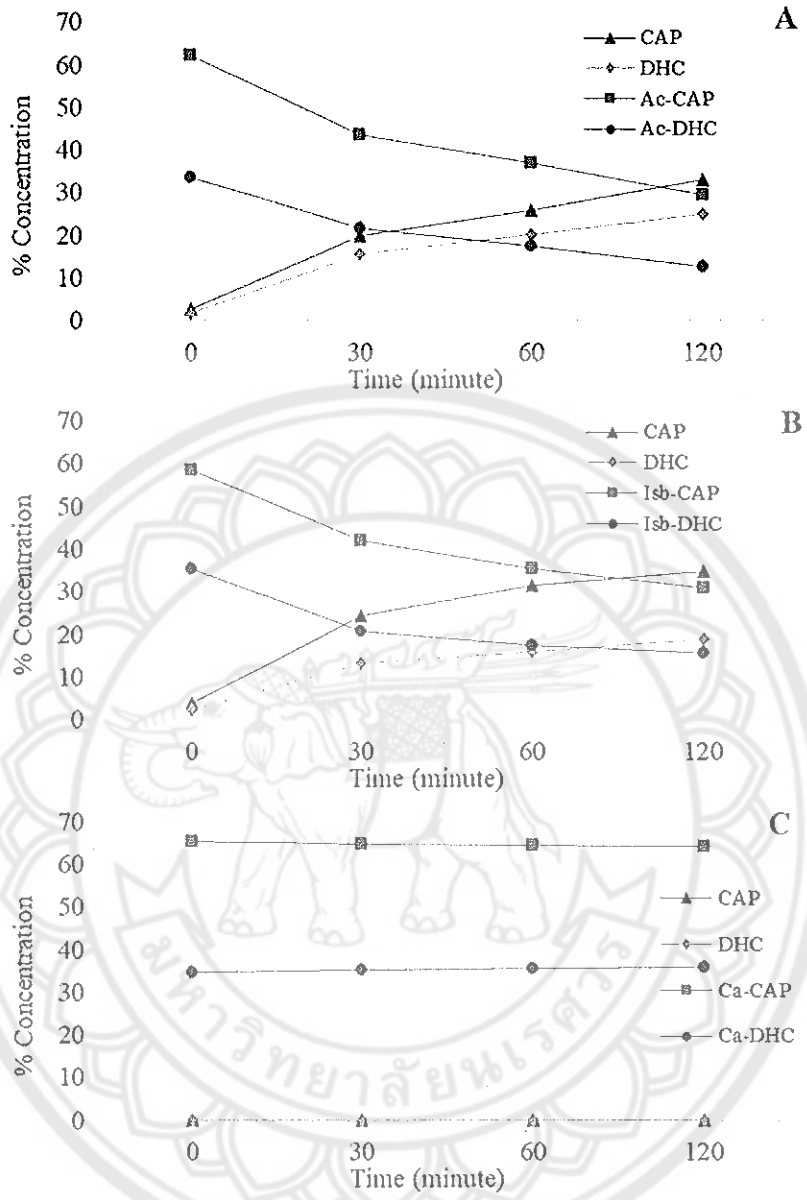


Figure 51 A) Percentage of capsaicin (CAP), dihydrocapsaicin (DHC), acetylated capsaicin (Ac-CAP), and acetylated dihydrocapsaicin (Ac-DHC) in enzymatic deprotection reaction of acetylated capsaicin, B) Percentage of capsaicin, dihydrocapsaicin, isobutyrated capsaicin (Isb-CAP), and isobutyrated dihydrocapsaicin (Isb-DHC) in enzymatic deprotection reaction of isobutyrated capsaicin, and C) Percentage of capsaicin (CAP), dihydrocapsaicin (DHC), carbamate capsaicin (Ca-CAP), and carbamate dihydrocapsaicin (Ca-DHC) in enzymatic deprotection reaction of carbamate capsaicin

Plots of the relative contents of the protected and free capsaicin in the reaction mixtures as a function of time are shown in Figure 51. The results show that the enzymatic reaction is fast for the acetylated capsaicin with the aggregate content of capsaicin and dihydrocapsaicin in the reaction mixture reaching 58.0% after 120 minutes (Figure 51A). The aggregate content of capsaicin and dihydrocapsaicin in the reaction mixture of isobutyrated capsaicin after 120 minutes was 53.7%. This result indicates, given the higher concentration of enzyme used, that the hydrolysis of the isobutyrated capsaicin is indeed slower as anticipated based on its higher steric demand. No hydrolysis is observed in the case of carbamate capsaicin as was anticipated based on the results from the LC-MS experiments.

Taken together, these results indicate the possibility to deprotect the capsaicin derivatives under physiological conditions by the action of various enzymes

3. Cytotoxicity testing

Given the fact that the intended use of the synthesized derivatives of capsaicin is as capsaicin pro-drug/precursor to be used by human subjects, initial investigation of the cytotoxicity of the derivatives was performed as part of this project. This testing was performed with two cell lines; the human dermal fibroblast, neonatal (HDFn) cell line and the LNCaP cell line.

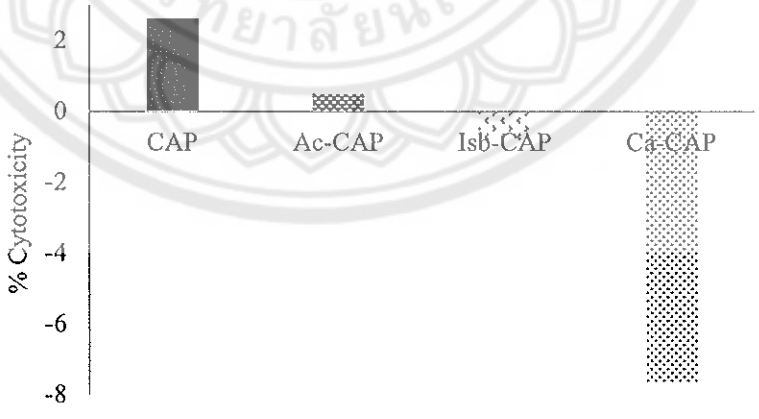


Figure 52 Cytotoxicity against human dermal fibroblast at 100 µg/ml of capsaicin, acetylated capsaicin, isobutylated capsaicin, and carbarmate capsaicin

The evaluation of the cytotoxicity of capsaicin, acetylated capsaicin, isobutyrated capsaicin, and carbamate capsaicin in the human dermal fibroblast, neonatal (HDFn) cell line was performed using the Resazurin Microplate assay (REMA) and were reported as % cytotoxicity. The tested concentrations were in the range of 3.13 - 100 µg/ml. Figure 52 show the results for the highest concentration tested indicating that the compounds are not cytotoxic in the whole concentration range tested (Figure 51).

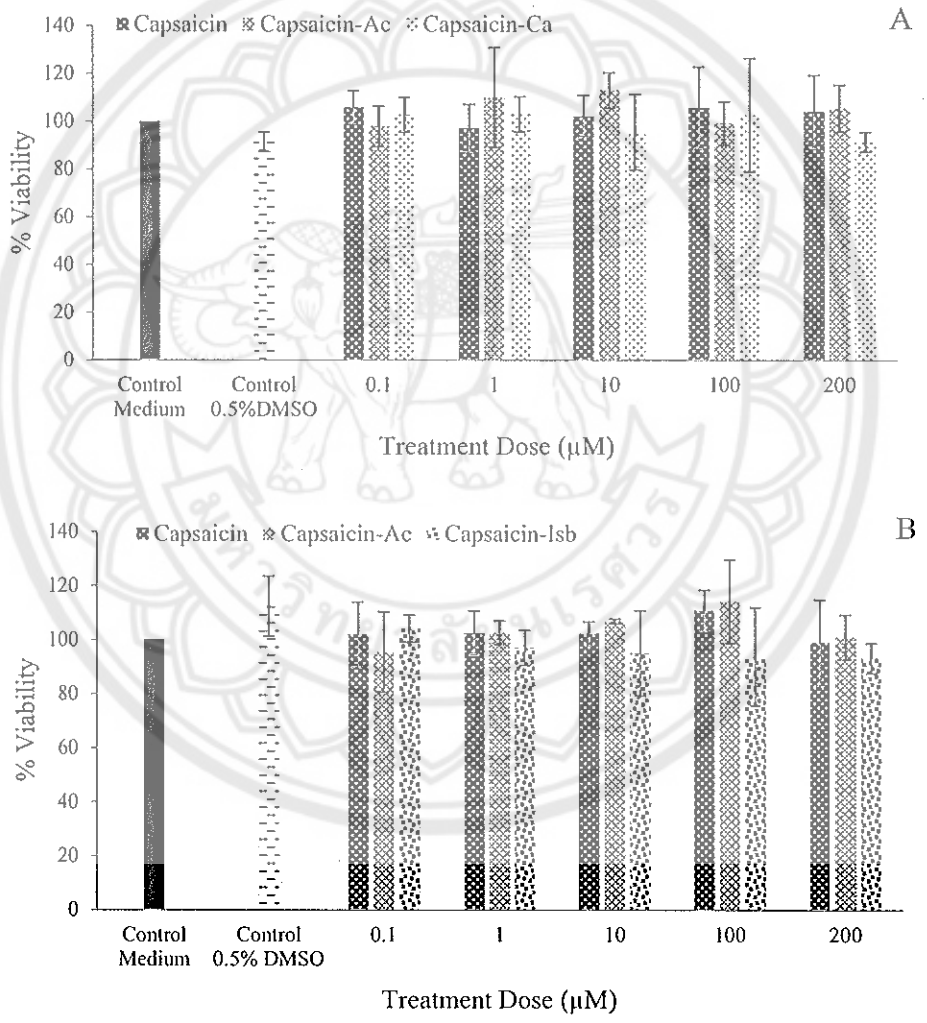


Figure 53 Results of toxicity testing obtained using the MTT assay; A) cytotoxicity compared of capsaicin, acetylated capsaicin, and carbamate capsaicin and B) cytotoxicity compared of capsaicin, acetylated capsaicin, and isobutylated capsaicin

In addition, the cytotoxicity of capsaicin, acetylated capsaicin, carbamate protected capsaicin and isobutyrated capsaicin was evaluated using the LNCaP cell line. The testing was performed using the MTT assay. The tested concentrations were in the range of 0.1 - 200 μM. The results for this test can be seen in Figure 53 and they again indicate absence of cytotoxicity in the whole concentration range.

Thus the limited testing of cytotoxicity of the capsaicin derivatives prepared in this work does not raise any concerns in terms of the cytotoxicity of these compounds. Obviously further tests are necessary to demonstrate the safety of these compounds.

CHAPTER VI

CONCLUSION

Several esterified derivatives of capsaicin have been synthesized and characterized during the course of this project. The main focus has so far been given to the acetate, isobutyrate, and carbamate derivatives. Acetylated capsaicin, carbamate capsaicin and isobutyrated capsaicin were synthesized with yield, 91.4 %, 54.7% and 91.8%, respectively. All structures were confirmed by ^{1}H-NMR, ^{13}C-NMR, and mass spectroscopy. Furthermore, derivatization of capsaicin in crude chilli pepper extract and dried chili powder have been achieved for acetylation and carbamate formation. The conversion of water soluble derivatives, proline protected capsaicin and phosphate capsaicin were observed and the products have been characterized by ^{1}H-NMR and mass spectroscopy. Phosphorylation has also been achieved in crude chilli pepper extracts. Deprotection of the capsaicin derivatives was investigated and a kinetic study of the enzymatic deprotection reaction has been performed in reaction with porcine liver esterase with the acetate, isobutyrate and carbamate derivatives. The acetated and isobutyrate capsaicin derivatives are clearly deprotected, at different rates, by porcine liver esterase to afford capsaicin. Deprotection products have also been observed for the carbamate and phosphate derivatives, even though they were only observed using MS detection. Thus, it can be concluded that acetylated capsaicin, isobutyrated, carbamate protected, and phosphorylated capsaicin do have a prodrug like character. Cytotoxicity testing of capsaicin, acetylated capsaicin, carbamate capsaicin, and isobutyrated capsaicin with the LNCaP cell line shows that three derivatives are not toxic in the concentration range of 0.1-200 μM. The same compounds were tested cytotoxicity against human dermal fibroblast, neonatal (HDFn) in the concentration range of 3.13 - 100 μg/ml. The results are thus positive, especially for the acetate and isobutyrate derivative, and warrant their further study in terms of their pungency.

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APPENDIX

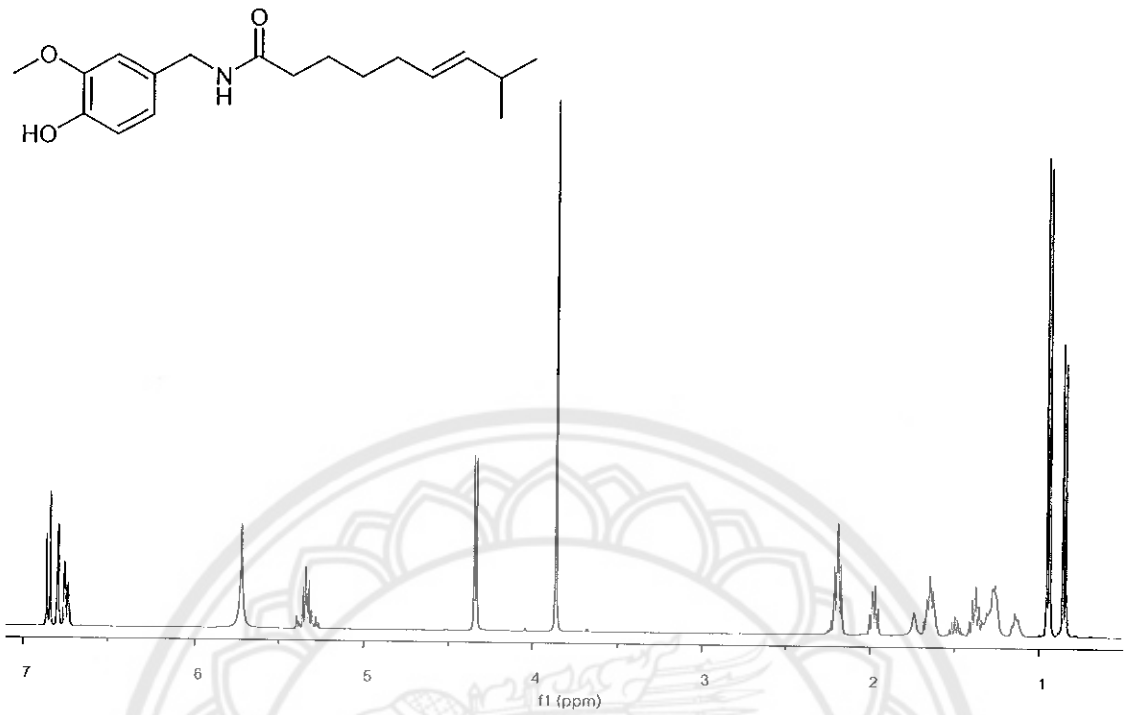

Figure 54 ^{1}H NMR spectrum of capsaicin

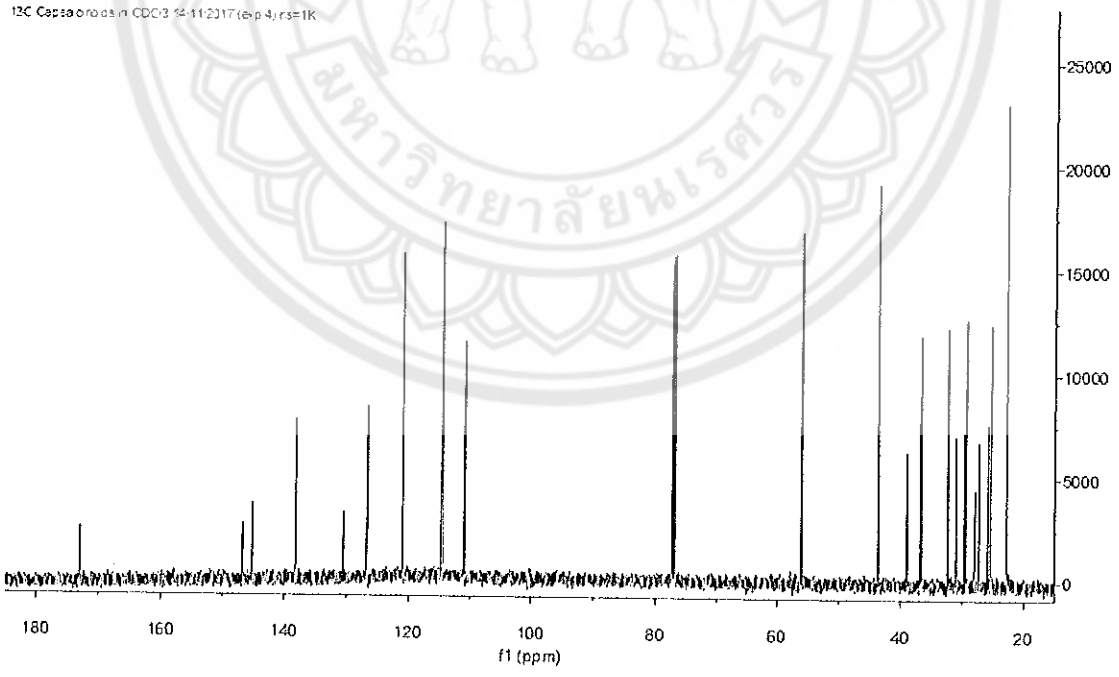

Figure 55 ^{13}C NMR spectrum of capsaicin

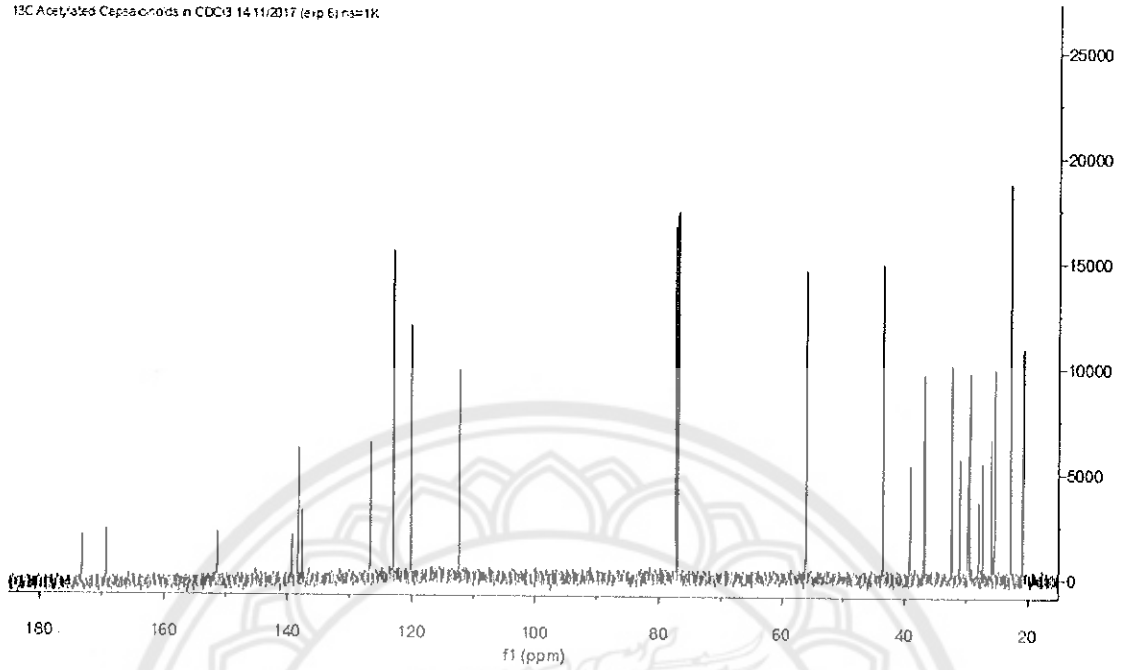

Figure 56 ^{13}C NMR spectrum of acetylated capsaicin

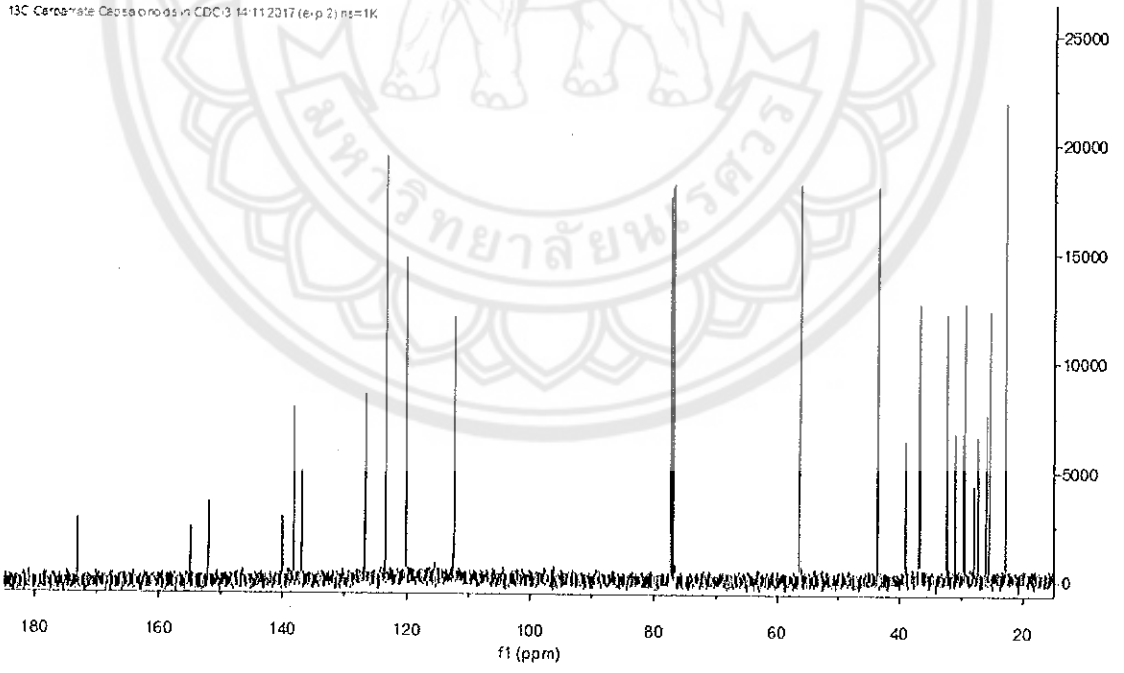

Figure 57 ^{13}C NMR spectrum of carbamate capsaicin

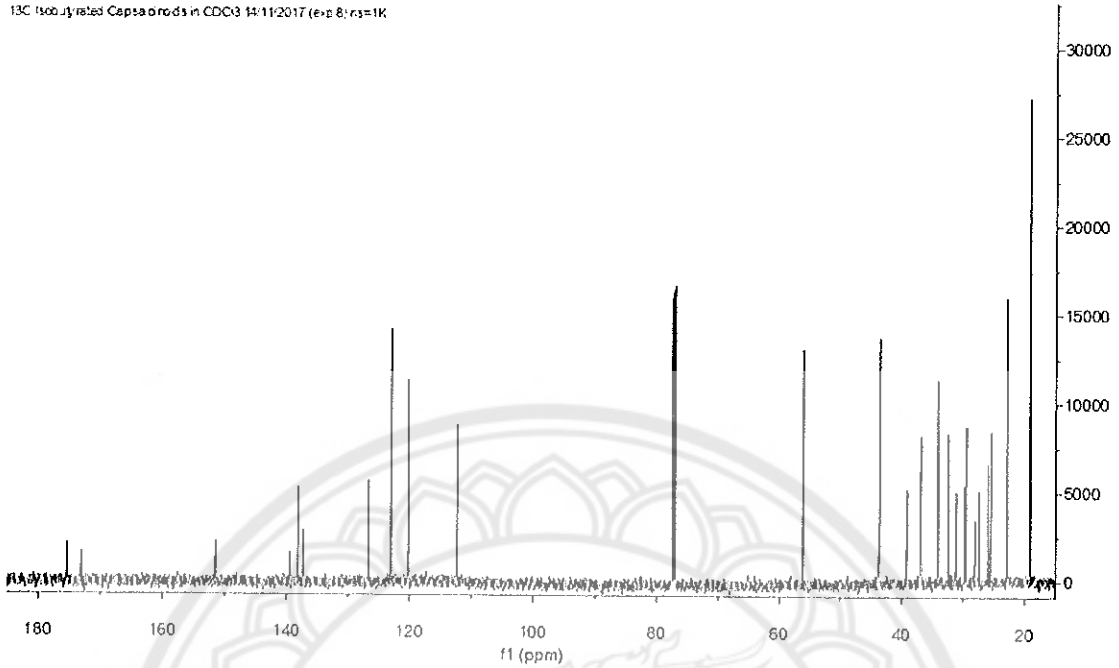

Figure 58 ^{13}C NMR spectrum of isobutyrated capsaicin

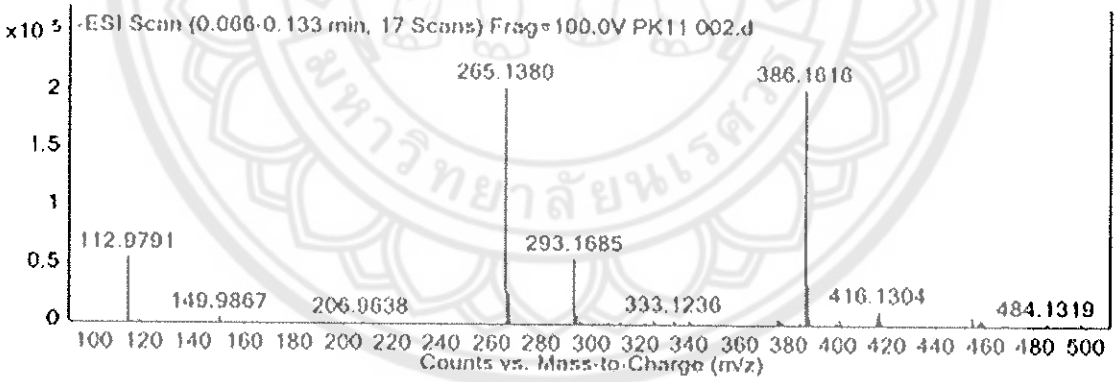

Figure 59 MS spectrum of phosphorylated dihydrocapsaicin (m/z 386, $[M-H]^-$)

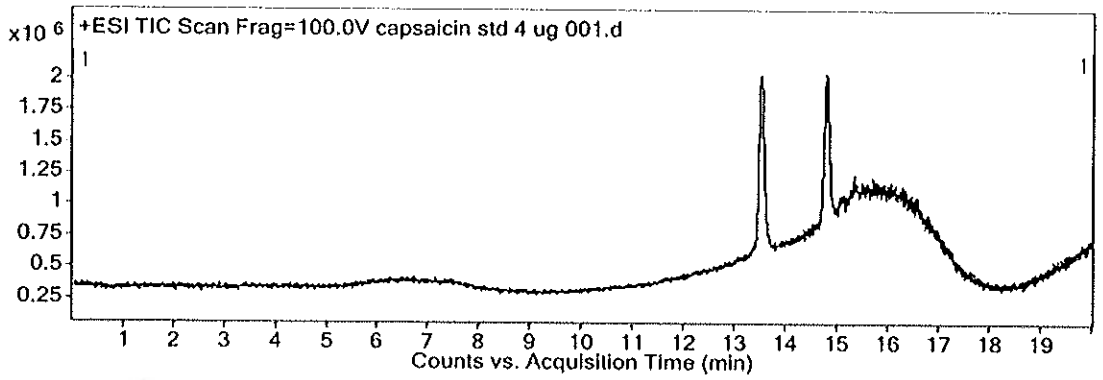

Figure 60 LC-MS chromatogram for capsaicin used in this study. Peak 1 at 13.5 minutes is capsaicin (CAP) and peak 2 at 14.7 minutes is dihydrocapsaicin (DHC)

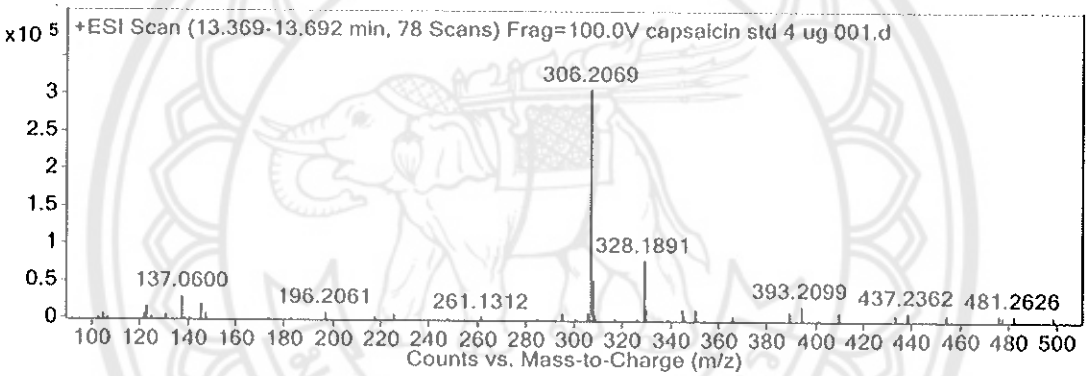

Figure 61 MS for peak at 13.5 min (Peak 1) in chromatogram (CAP)

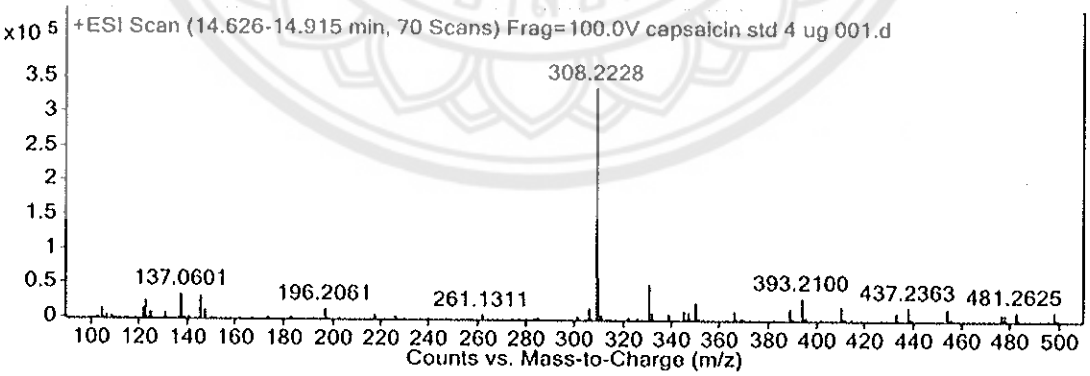

Figure 62 MS for peak at 14.7 min (Peak 2) in chromatogram (DHC)

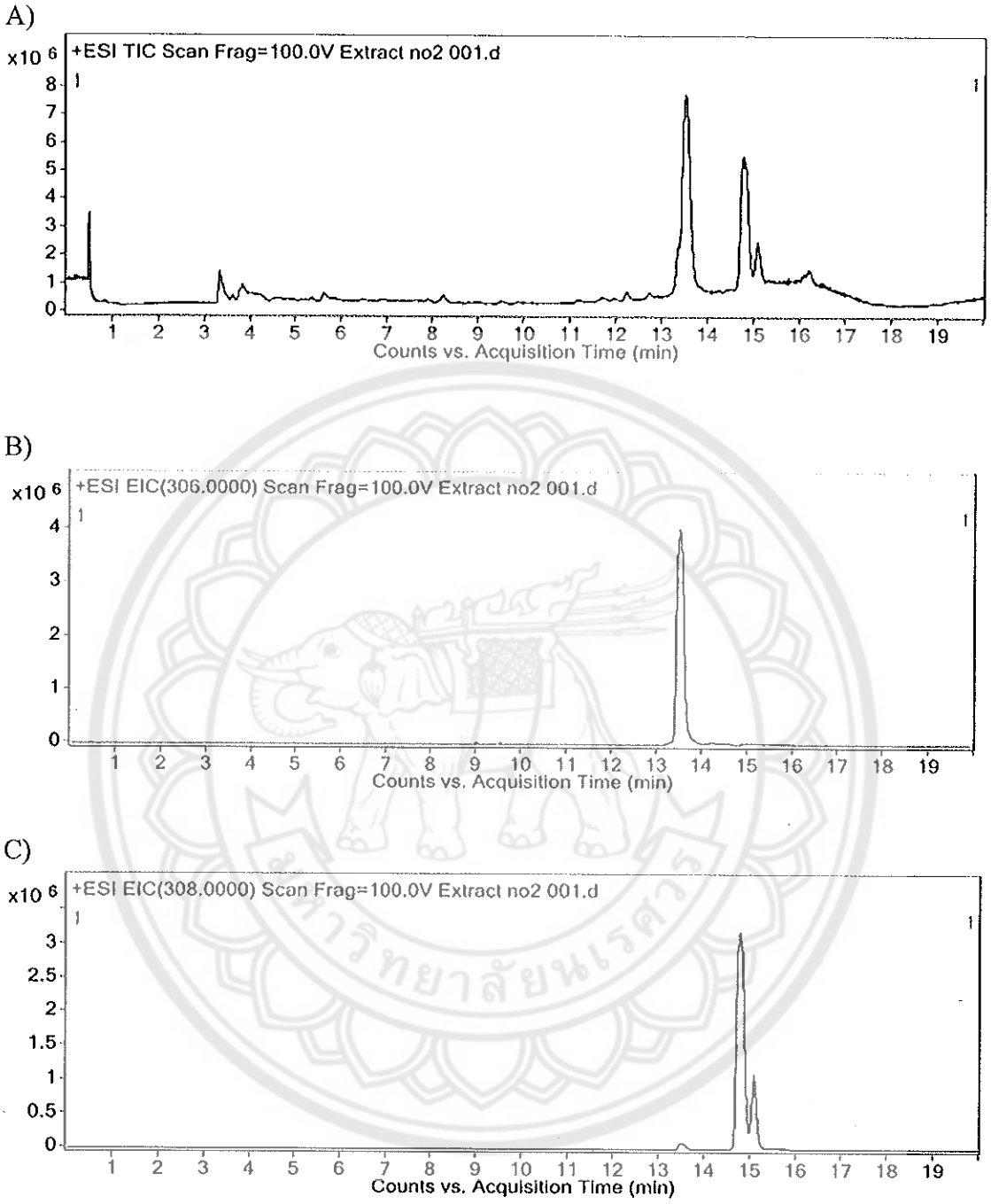

Figure 63 (A) LC-MS chromatogram for crude chili pepper extract showing the presence of both capsaicin (Peak 1) and dihydrocapsaicin (Peak 2). (B) Extacted ion chromatogram for m/z 306 (capsaicin). (C) Extracted ion chromatogram for m/z 308 (dihydrocapsaicin)

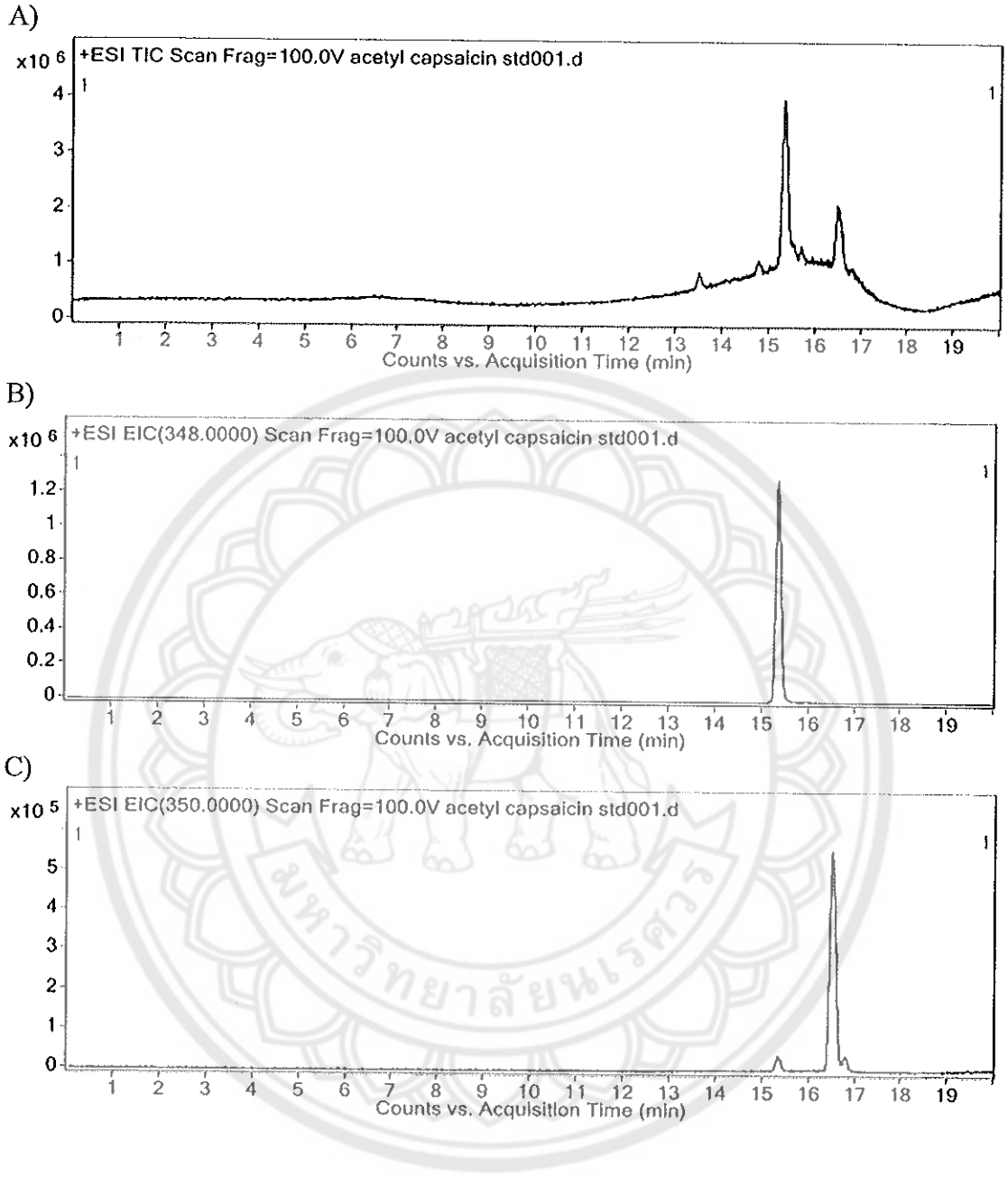

Figure 64. (A) LC-MS chromatogram for acetylated capsaicin (Peak 1-Capsaicin-Ac) showing the presence of acetylated dihydrocapsaicin (Peak 2-DihydroCapsaicin-Ac), residual capsaicin (Peak 3), and residual dihydrocapsaicin (Peak 4). (B) Extracted ion chromatogram for m/z 348 (Capsacin-Ac). (C) Extracted ion chromatogram for m/z 350 (Dihydrocapsaicin-Ac)

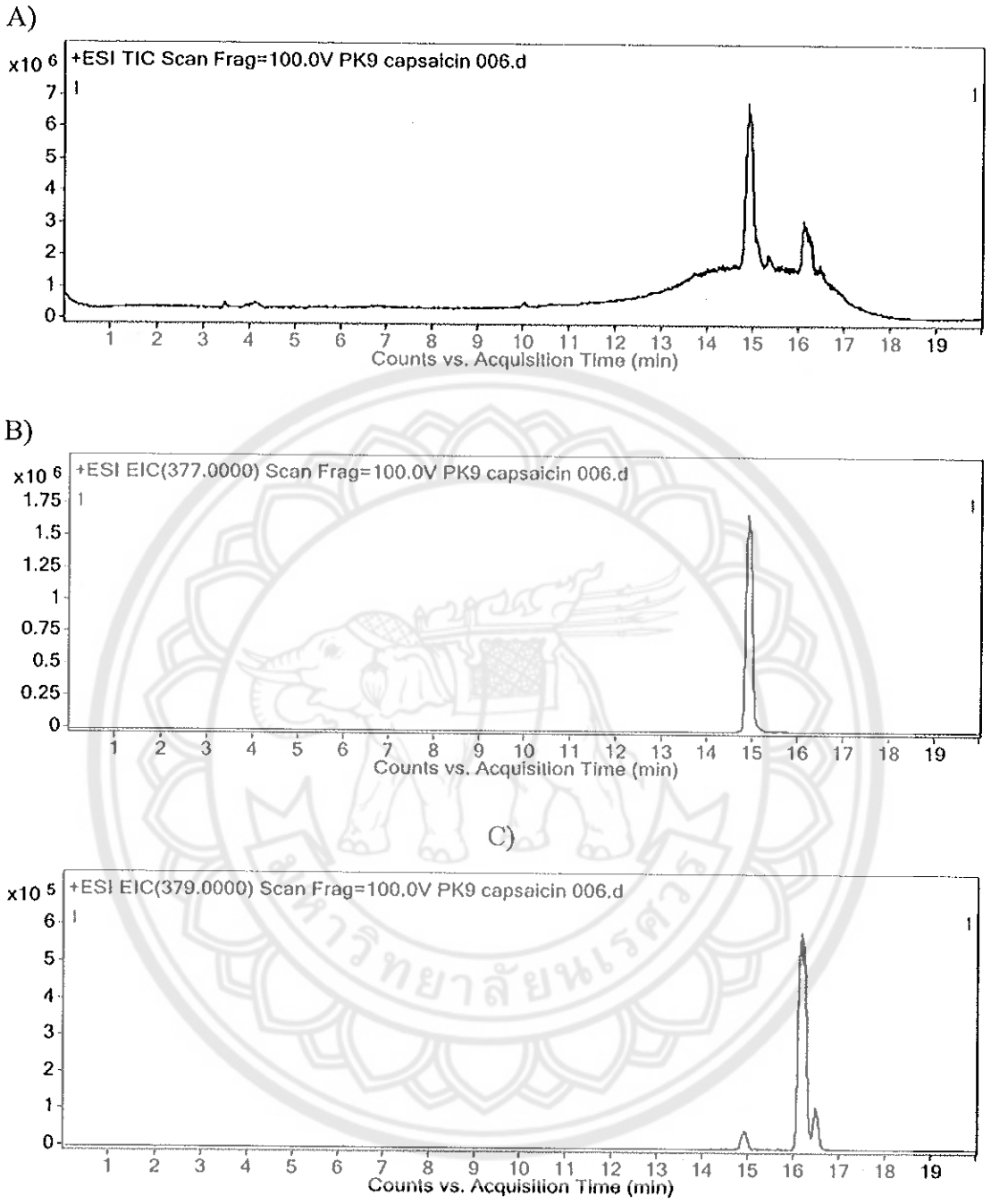

Figure 65 (A) LC-MS chromatogram for carbamate protected capsaicin showing the presence of carbamate protected capsaicin (Peak1-Capsaicin-Ac) and carbamate protectd dihydrocapsaicin (Peak 2-DihydroCapsaicin-Ac). (B) Extracted ion chromatogram for m/z 377 (Capsaicin-Ca). (C) Extracted ion chromatogram for m/z 379 (Dihydrocapsaicin-Ca)

ABBREVIATIONS

ABBREVIATIONS

Ac_2O	=	Acetic anhydride
CH_3CN	=	Acetonitrile
C_3H_6O	=	Acetone
ADME	=	Absorption, distribution, metabolism, and excretion
AR	=	Analytical grade
CAP	=	Capsaicin
DHC	=	Dihydrocapsaicin
$CDCl_3$	=	Deuterated chloroform
DMAP	=	4-Dimethylaminopyridine
CH_2Cl_2	=	Dichloromethane
C_3H_6ClNO	=	Dimethylcarbamyl chloride
DMSO	=	Dimethyl sulfoxide
DMEM	=	Dulbecco's modified eagle medium – high glucose
$CH_3COOC_2H_5$	=	Ethyl acetate
FBS	=	Fetal bovine serum gold
$C_4H_8O_2$	=	Isobutyric acid
CH_3OH	=	Methanol
MEM	=	Minimum essential medium
C_6H_{14}	=	n-Hexane
DCC	=	N,N'-Dicyclohexylcarbodiimide
HOSu	=	N-Hydroxysuccinimide
$POCl_3$	=	Phosphoryl chloride
K_2CO_3	=	Potassium carbonate
C_5H_5N	=	Pyridine
Pen Strep	=	Penicillin streptomycin
PBS	=	Phosphate buffered saline
$(C_2H_5)_3N$	=	Triethylamine
$C_4H_6O_2$	=	Vinyl acetate

ABBREVIATIONS (CONT.)

ADME	=	Absorption, distribution, metabolism, and excretion
HDFn	=	Human dermal fibroblast, neonatal
REMA	=	Resazurin Microplate assay
TRPV1	=	Transient receptor potential cation channel subfamily V member 1
EIC	=	Extracted ion chromatogram
TIC	=	Total ion chromatogram
HPLC	=	High-performance liquid chromatography
LC-MS	=	Liquid chromatography-mass spectrometry
NMR	=	Nuclear magnetic resonance
Q-TOF	=	Quadrupole-time of flight
UHD	=	Ultra-high definition
UV-Vis	=	Ultraviolet–visible
m/z	=	Mass-to-charge ratio
J	=	Coupling constant
δ	=	Chemical shift
s	=	Singlet
d	=	Doublet
t	=	Triplet
q	=	Quartet
p	=	Pentet
m	=	Multiplet
g	=	Gram
µg	=	Microgram
mg	=	Milligram
MHz	=	Megahertz
min	=	Minute

ABBREVIATIONS (CONT.)

µl	=	Microliter
ml	=	Milliliter
mmol	=	Millimole
µM	=	Micromolar
mM	=	Millimolar
°C	=	Degree of Celsius
pH	=	Potential of Hydrogen ion