การกักเก็บสารสกัดพริกขี้หนู *Capsicum frutescens* ในอนุภาคแอลจิเนต-ไคโตซาน

นางสาวภัทริน สุทธินุ่น

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ENCAPSULATION OF EXTRACT FROM *Capsicum frutescens* IN ALGINATE-CHITOSAN PARTICLES

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ภัทริน สุทธินุ่น: การกักเก็บสารสกัดพริกขี้หนู *Capsicum frutescens* ในอนุภาคแอลจิเนต-ไคโตซาน (ENCAPSULATION OF EXTRACT FROM *Capsicum frutescens* IN ALGINATE-CHITOSAN PARTICLES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ดร. พัฒทรา สวัสดี, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร.กฤษณา ศิรเลิศมุกุล, 71 หน้า.

ได้เตรียมอนุภาคขนาดไมโครของแอลจิเนต-ไคโตซานโดยใช้เทคนิคคอมเพล็กซ์โคเอเซอเวชัน อนุภาคไมโครขึ้นรูปด้วยพอลิเมอร์ในอัตราส่วนต่างๆ จากภาพ SEM อนุภาคไมโครที่อัตราส่วนแอลจิเนตต่อไคโตซานที่ 1:1 มีลักษณะทางกายภาพที่ดีที่สุด มีลักษณะเป็นทรงกลม ผิวเรียบ และมีขนาดเฉลี่ย 560±38.9 ไมโครเมตร นอกจากนี้อนุภาคที่เชื่อมขวางด้วยโซเดียมไทรพอลิฟอสเฟต (ทีพีพี) มีขนาดเล็กกว่า อนุภาคที่ไม่ได้เชื่อมขวางด้วยทีพีพี ระดับการบวมของอนุภาคแอลจิเนต-ไคโตซาน ในสภาวะเลียนแบบกระเพาะอาหาร (พีเอช 1.2) (ประมาณ 50%) ต่ำกว่าอนุภาคไมโครแอลจิเนต-ไคโตซานในสภาวะเลียนแบบลำไส้เล็ก (พีเอช 7.4) (ประมาณ 400%) อนุไมโครแอลจิเนต-ไคโตซานที่กักเก็บสารสกัดพริก ถูกเตรียมที่อัตราส่วนโดยมวลของแอลจิเนต-ไคโตซานที่ 1:1 และใช้ทีพีพีเป็นสารเชื่อมขวาง อัตราส่วนโดยมวลของอนุภาคไมโครแอลจิเนต/ไคโตซาน/สารสกัดพริกถูกเตรียมที่ 1/1/0.5 มีประสิทธิภาพการกักเก็บสูงสุด 73.90 เปอร์เซ็นต์ และมีความจุในการกักเก็บ 10.44 เปอร์เซ็นต์ รูปแบบการปลดปล่อยยาของอนุภาคไมโครแอลจิเนต-ไคโตซานที่กักเก็บสารสกัดพริก ในสภาวะเลียนแบบกระเพาะอาหารและสภาวะเลียนแบบลำไส้เล็ก หาได้จากปริมาณการปลดปล่อยของแคปไซซิน พบว่า ในสภาวะเลียนแบบกระเพาะอาหาร 3 ชั่วโมงแรก สารแคปไซซินค่อยๆปลดปล่อยออกมาจากอนุภาคไมโคร ระดับการปลดปล่อย 32% เมื่ออนุภาคไมโครถูกเคลื่อนย้ายจากสภาวะเลียนแบบกระเพาะอาหารไปยังสภาวะเลียนแบบลำไส้เล็ก สารแคปไซซินถูกปลดปล่อยเพิ่มขึ้นอย่างรวดเร็วจนถึง 78 เปอร์เซ็นต์ ภายใน 4 ชั่วโมง และปลดปล่อยอย่างสมบูรณ์ใน 8 ชั่วโมง ดังนั้นการเก็บสามารถป้องกันปริมาณแคโรที่นอยด์ในสารสกัดพริกจากแสง ช่วยลดความเผ็ด และลดการระคายเคืองต่อระบบทางเดินอาหารและกระเพาะอาหาร โดยอนุภาคแอลจิเนต-ไคโตซานที่กักเก็บสารสกัดพริกที่เตรียมได้สามารถใช้เป็นอาหารเสริมได้

สาขาวิชา ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก….....

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5272473623 : MAJOR PETROCHEMISTRY AND POLYMER SCIENCE
KEYWORDS : CHILI OLEORESIN/ CAPSAICIN/ALGINATE/ CHITOSAN/ MICROPARTICLES/ COMPLEX COACERVATION

PATTARIN SUTTHINOON: ENCAPSULATION OF EXTRACT FROM *Capsicum frutescens* IN ALGINATE-CHITOSAN PARTICLES. ADVISOR: PATTARA SAWASDEE, Ph. D., CO-ADVISOR: KRISANA SIRALERTMUKUL, Ph. D., 71 pp.

Alginate-chitosan microparticles were prepared using complex coacervation technique. These microparticles were formed with different ratio of alginate and chitosan. From the SEM images, microparticles using a chitosan:alginate (CS:ALG) mass ratio of 1:1 showed the best morphology which was spherical and smooth-surface with the average size of 560 ± 38.9 μm. Moreover, particles cross-linked with sodium tripolyphosphate (TPP) had the smaller sizes than those without TPP. The swelling degree of alginate-chitosan microparticles in simulated gastric fluid (SGF, pH 1.2) (about 50%) was lower than that (about 400%) in simulated intestinal fluid (SIF, pH 7.4). Chili oleoresin-loaded alginate-chitosan microparticles were prepared with the CS:ALG mass ratio of 1:1 and using TPP as a cross-linked agent. The highest encapsulation efficiency (73.90%) and loading capacity (10.44%) were obtained from the chitosan/alginate/chili oleoresin mass ratio of 1/1/0.5. The release profile of entrapped chili oleoresin in SGF and SIF was constructed by determining the amount of release capsaicin. At the initial three hours in SGF, capsaicin was slowly release from the microparticles with the release level of 32%. When microparticles were transferred from SGF into SIF medium, capsaicin releasing level increased to be 78% within 4 hours and complete release was not attained in 8 hours. Therefore, the encapsulation can be protected the carotenoids content in chili oleoresin from light, masking pungent taste and reduce irritation to the stomach. Chili extract-loaded alginate-chitosan microparticles can be used as supplementary food.

Field of Study: Petrochemistry and Polymer Science Student's Signature.....
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LIST OF ABBREVIATIONS

%	percentage
^{o}C	degree Celsius (centrigrade)
ALG	alginate
CS	chitosan
CS:ALG	the ratios of chitosan and alginate
DD	degree of deacetylation
EE	encapsulation efficiency
g	gram
hr	hour
HPLC	High performance liquid chromatography
kDa	kilodalton
LC	loading capacity
mg	milligram
mm	millimeter
min	minute
mL	milliliter
µm	micrometer
OAC	chili loaded alginate-chitosan
pH	the negative logarithm of the hydrogen ion concentration
rpm	round per minute
SEM	Scanning Electron Microscope
S.D.	Standard deviation
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
TPP	sodium tripolyphosphate
UV	ultraviolet
v/v	volume by volume
w/w	weight by weight
w/v	weight by volume

CHAPTER I

INTRODUCTION

1.1 Introduction

Bird's eye chili (*Capsicum frutescens* L.) is a flavor spice for Thai food. Chili has been widely used as a food flavoring, a coloring agent in food and pharmaceutical industries. Chili oleoresin is a complex mixture, which is extracted from dried, ripe fruit of chili. It is a thick, dark reddish-brown liquid concentrate. The active compounds in chili oleoresin are capsaicinoids, carotenoids, including several vitamins and minerals [1]. The capsaicinoids are the compounds responsible for the pungent or hot spicy flavor in chili oleoresin. Among of the capsaicinoids, capsaicin is the major component which demonstrates a variety of the pharmacological activities such as anti-tumor, anti-mutagenic activities [2], anti-inflammatory activities [3-4], decrease in plasma glucose levels [5], antioxidant [6], anti-bacterial [7] including acetylcholinesterase and butyrylcholinesterase inhibitory activities [8].

However, the main disadvantages of using capsaicin are the side effects. Natural capsaicin is a strong irritant for the skin, eyes, and upper respiratory tract.

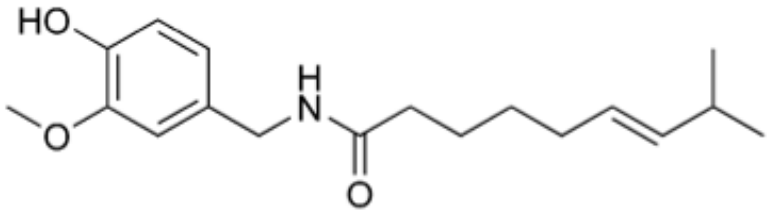


Figure 1.1 The chemical structure of capsaicin

(*trans*-8-methyl-*N*-vanillyl-6-nonenamide)

Encapsulation of drug into particles can reduce its accidental. The encapsulation is the technique which one material or a mixture of materials is coated with or entrapped within another material or system. The coated material was called active or core material, and the coating material was called shell, wall material, carrier

or encapsulant. The previous studies, the encapsulation of capsaicin into polymeric materials shell; gelatin and acacia which can reduce its side effects [9].

At the present time, polymers have become attractive materials in the pharmaceutical advancement. A number of natural polymers have been used for the microparticles arrangement such as cellulose, starch, chitosan, pectin and alginate. Alginate is anionic polysaccharides which was gained a lot of attention because its simplicity to form the microparticles by adding a sodium alginate solution into a calcium chloride solution. However, this method was found the drug-loss during the microparticles arrangement. This problem can prevent by blending with other polymers such as chitosan [10], gelatin [11]. Alginate and chitosan are biopolymers that were obtained lots of attention and have been widely study for such use.

Alginate-chitosan (ALG-CS) polyionic complexes are formed through the ionic gelation via interactions between the carboxylic groups of alginate and the amine groups of chitosan. The complex protects the encapsulant, has biocompatible and biodegradable and biodegradable characteristics, and limits the release of encapsulated materials which more effectively than either alginate or chitosan alone [12].

Alginate is a naturally occurring anionic polymer typically obtained from brown seaweeds, It consisted of linear block copolymer of (1,4)-linked β-D-mannuronate and α-L-gurulonate residue [13]. Alginate has been widely investigated and used for many biomedical applications, due to its biocompatibility, low toxicity, relatively low cost and mild gelation by addition of divalent cations such as Ca^{2+}.

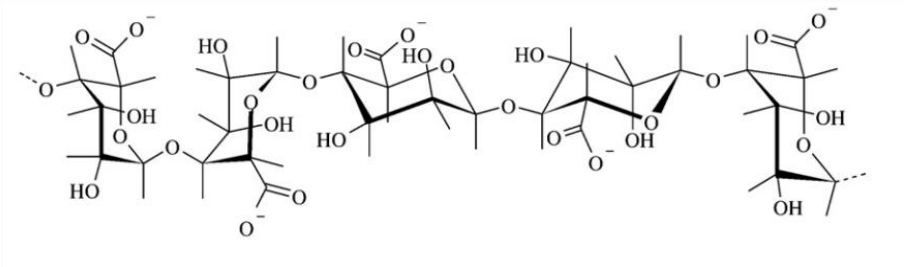


Figure 1.2 Structure of Alginate.

Chitosan is a cationic natural linear polysaccharide composed of copolymers of D-glucosamine and N-acetyl-D-glucosamine units linked by β-(1-4)-glycosidic linkages. Chitosan is receiving importance in the pharmaceutical field because of its unique polymeric cationic character, non-toxicity, good biocompatibility and biodegradability. Its cationic nature allows formation of complexes with negatively charged polymers, and with ionic gelation alginate and chitosan spontaneously form a complex that is biocompatible and non-toxic [14]. Chitosan is frequently reacted with tripolyphosphate in the preparation of micro/nanoparticles.

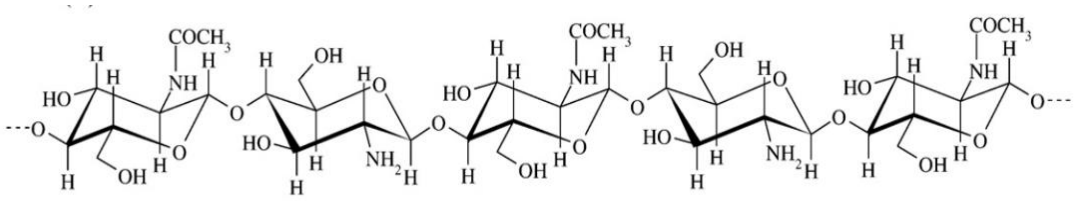


Figure 1.3 Structure of Chitosan.

1.2 The objectives of this research

The aim of the present work is to prepare microparticles of chili oleoresin, using the matrix polymer consisting of alginate, chitosan as a retarding material, by complex coacervation technique. The details of these objectives were described as follows:

1) To evaluate the effect of alginate concentration and the effect of sodium tripolyphosphate (TPP) on alginate-chitosan microparticles.

2) To prepare alginate-chitosan microparticles loaded chili oleoresin by complex coacervation technique.

3) To study the release profile of alginate-chitosan microparticles in simulated gastric fluid (pH1.2) and simulated intestinal fluid (pH 7.4).

4) To study the stability of caroteniods in alginate-chitosan microparticles loaded with chili oleoresin and chili oleoresin.

1.3 The scope of research

The scope of this research was carried out by stepwise investigation as follows:

1) Literature review of related works

2) Preparation of chili oleoresin

3) Preparation of alginate-chitosan microparticles with parameters including alginate concentration and the effect of sodium tripolyphosphate on alginate-chitosan microparticles.

4) Characterization of obtained microparticles in terms of morphology and size.

5) Study the swelling behavior of the microparticles in simulated gastric fluid (pH1.2) and simulated intestinal fluid (pH7.4).

6) Preparation of alginate-chitosan microparticles loaded with chili oleoresin

7) Evaluation of chili oleoresin encapsulation efficiency.

8) In vitro chili oleoresin release study from alginate-chitosan microparticles in simulated gastric fluid (pH1.2) and simulated intestinal fluid (pH7.4).

9) Study the stability of caroteniods in alginate-chitosan microparticles loaded with chili oleoresin and chili oleoresin.

10) Report preparation of the summarized results.

CHAPTER II

BACKGROUND AND LITERATURE REVIEWS

Bird's eye chilli (*Capsicum frutescens* L.) is the pungent fruit of the genus Capsicum, belongs to the Solanaceae family. Chili extract or capsicum oleoresin is manufactured by solvent extraction from chili which is used as a food flavoring, a coloring agent and pharmaceutical industries. Chili oleoresin is composed of capsaicinoids, caroteniods, vitamins and minerals. The major compounds in chili are capsaicinoids and among of these, capsaicin is the major component which demonstrates a variety of the pharmacological activities such as anti-tumor, anti-mutagenic, anti-inflammatory and anti-cholinesterase activities. However, capsaicin causes irritation to the contact areas and gastro-intestinal tract. These problems could be solved by the encapsulation technology.

2.1 Microencapsulation

Microencapsulation is a process by which small distinct solid particles or small liquid droplets are completely enclosed by an intact shell. The method selected to prepared microparticles depends to a great scope on the core and wall materials used and the ultimate application of the product. In pharmaceutical, the most commonly used wall materials include gelatin, ethyl cellulose, methyl cellulose, alginate and chitosan. Generally, core materials are liquids or solids including polymers, waxes and resin.

The pharmaceutical application of microencapsulation showed as below. It also concludes some of the problems that drugs display to the pharmaceutical scientist which must be solved during formation into the finished dosage form:

Pharmaceutical applications of microencapsulation [15].

1. Sustained or prolonged-release of drugs
2. Masking taste and odor of drugs
3. Stabilizing drugs sensitive to atmospheric conditions
4. Prevent vaporization of volatile substance
5. Elimination of incompatibilities via physical separation
6. Turn liquids into solids
7. Prevent degradation due to exposure to light or oxygen

A main advantage of microencapsulated drug products is with drugs which irritate linings in the stomach and intestines. The drug concentration is controlled by slow release characteristics of the microparticles. Therefore, the safety of potential inflammation - producing drugs is increased.

2.2 Controlled release system [16]

The concept of controlled or sustained release of biologically active agents has existed for over three decades. Early commercial applications of the technology occurred in both the pharmaceutical and agricultural industries.

Controlled-release technology emerged during the 1980s as a commercial sound methodology. The achievement of predictable and reproducible release of an agent into a specific environment over an extended period of time has much significant merit. It creates a desired environment with optimal response, minimum side-effects and prolonged efficacy.

Controlled drug delivery occurs when a polymer is combined with the drug or other active agents in such a way that the active agent is released from the material in a predesigned manner.

2.2.1 Advantages of controlled release

Controlled release system provides numerous benefits over conventional dosage form. Controlled release dosage forms are able to control the rate of drug delivery, the target area of drug administration and maintain therapeutic levels of drug with narrow fluctuations (Figure 2.1). That can reduce toxic and/or undesirable side effects of the drug. The serum concentration of drug released from controlled release dosage form fluctuates within the therapeutic range over a long period of time. That makes it possible to reduce the frequency of drug administration to encourage patients to comply with dosing instructions and improvement in treatment efficiency.

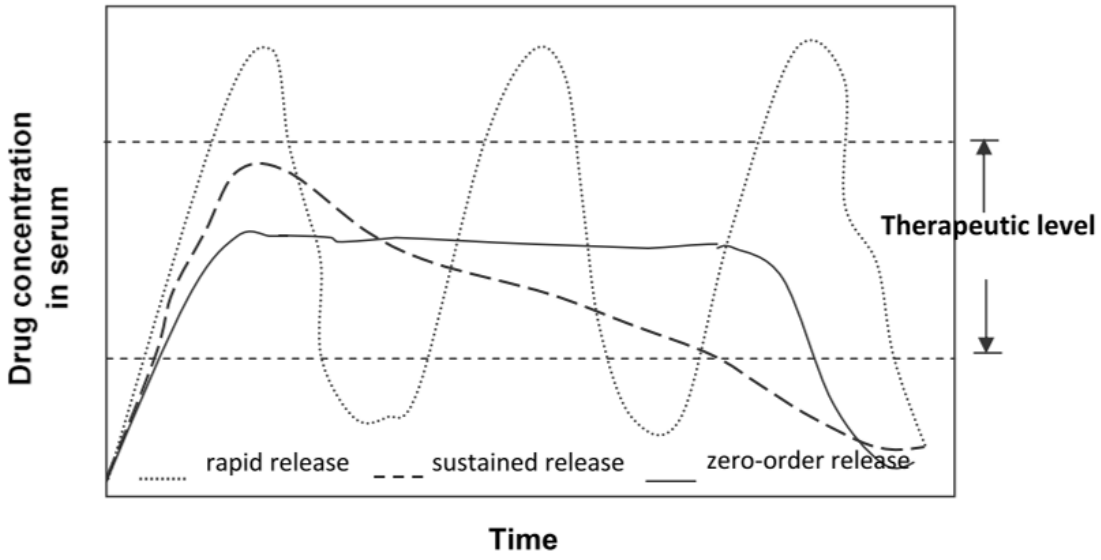


Figure 2.1 Hypothetical serum drug concentrations of various oral dosage forms [16]

2.2.2 Controlled release mechanism

The drug can be released from the system by 3 mechanisms.

1. Diffusion Controlled Release

Diffusion occurs when drug molecules pass from the polymer matrix to the external environment. As the release continues, its rate normally decreases with this type of system, since drug has progressively longer distance to travel and therefore requires a longer diffusion time to release.

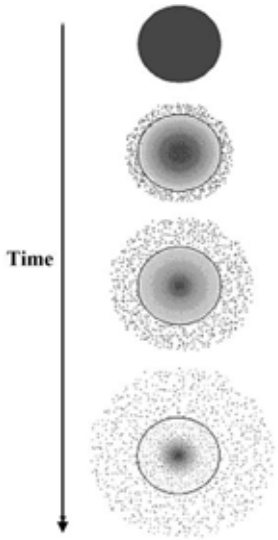


Figure 2.2 Presentation of diffusion controlled release [17]

2. Swelling Controlled Release

The swelling of the carrier increases the aqueous solvent content within the polymer matrix, enabling the drug to diffuse through the swollen network into the external environment. Most of materials used are based on hydrogel. The swelling can be triggered by a change in the environment surrounding such as pH, temperature, ionic strength, etc.

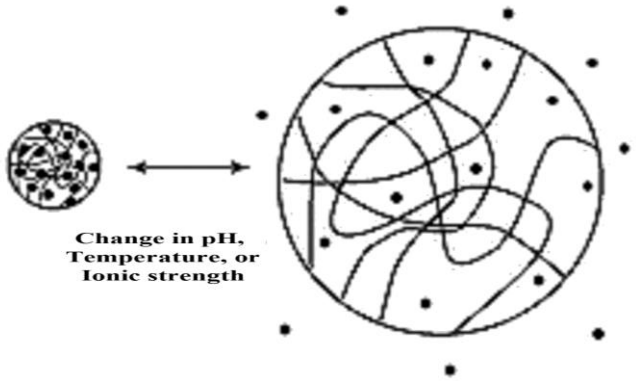


Figure 2.3 Presentation of swelling controlled release [17]

3. Erosion Controlled Release

The drug can be released from the matrix due to erosion of polymers, which can be classified into 2 types.

Bulk erosion: The polymer degrades in a fairly uniform manner throughout the polymer matrix.

Surface erosion: The degradation occurs only at the surface of the polymer device.

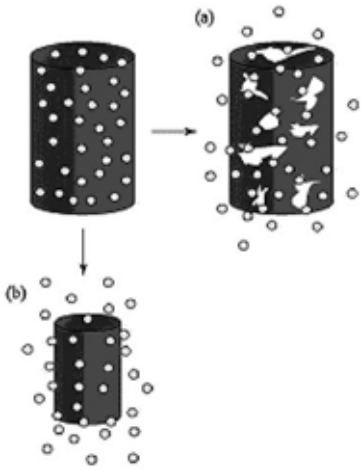


Figure 2.4 Presentation of erosion controlled release-(a) bulk erosion and (b) surface erosion [17]

2.3 Polyelectrolyte complex (PECs)

Polyelectrolytes are polymers containing ionizable groups. As charged macromolecules, they can form polyelectrolyte complexes (PECs) with oppositely charged molecules or polymers through intermolecular interactions, such as hydrogen bonding, Coulomb forces, van der Waals forces, and transfer forces. These properties have been applied to the encapsulation of therapeutic proteins, cells, enzymes [18] and drug [19].

A number of natural polyelectrolyte based colloidal systems are being described as promising carriers for bioactive molecules applying simple and mild

encapsulation processes free of heating and organic solvents. Polymers like chitosan (Chit), alginate (Alg) and dextran sulfate (DS) have been described as biocompatible, biodegradable and mucoadhesive, enabling numerous pharmaceutical and biomedical applications [20].

2.3.1 Chitosan-alginate polyelectrolyte complex

Alginate is a polysaccharide consisted of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid monomers in varying proportions. The negatively charged carboxylic acid groups of manuronic and guluronic acid units in alginate interact electrostatically with the positively charged amino groups of chitosan to form a polyelectrolyte complex. This complex can reduce the porosity of alginate beads and decreases the leakage of the encapsulated drugs. Chitosan complex with alginate was studied as a coating on alginate beads, alginate – chitosan coacervates, etc. The easy solubility of chitosan in low pH is prevented by the alginate network since alginate is insoluble in low pH conditions. The possible dissolution of alginate at higher pH is prevented by the chitosan which is stable at higher pH ranges [21].

2.3.2 Alginate

Alginate is a natural biopolymer extracted from marine brown algae. It consists of linear chains of α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues joined by 1,4-glycosidic linkages. Alginate is used as a biopolymer to prepare microparticle due to its good biocompatibility, biodegradability, non-toxicity, mucoadhesion, gelation, and film formation properties.

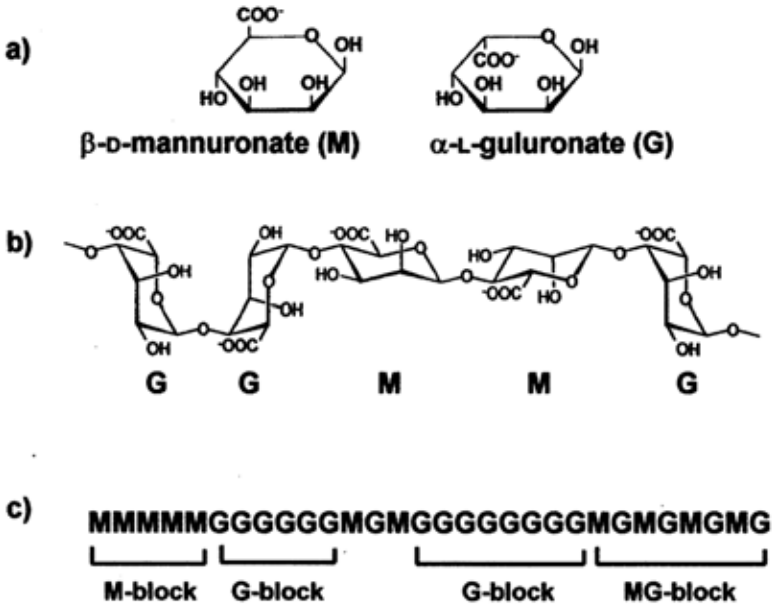


Figure 2.5 Alginate monomer (a) and chain (b) conformations and a schematic alginate chain sequence (c) [22].

2.3.2.1 Gel formation [21]

The gelation of alginate can be carried out under an extremely mild environment and uses non-toxic reactants. The most important property of alginates is their ability to form gels by reaction with divalent cations such as Ca^{2+}. Alginate beads can be prepared by extruding a solution of sodium alginate containing the desired protein, as droplets, in to a divalent cross-linking solution such as Ca^{2+}, Sr^{2+}, or Ba^{2+}. The gelation and cross-linking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations, and the stacking of these guluronic groups to form the characteristic egg-box structure shown in Figure 2.6.

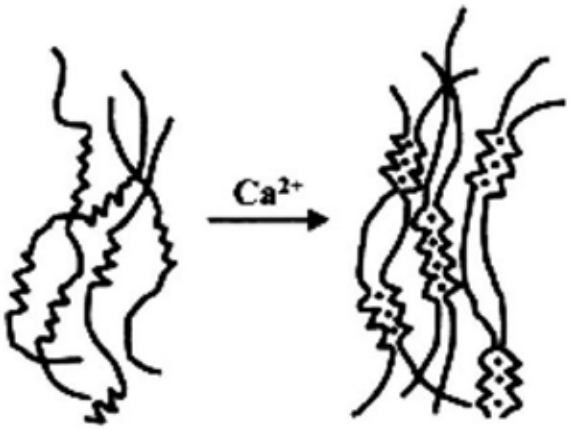


Figure 2.6 Egg-box structure of an alginate gel formed by chelation of Ca^{2+}ions.

2.3.2.2 Properties of alginate [21]

1. Biocompatibility

Alginate is used extensively in food industry as a thickener, emulsifier and as a stabilizer. Alginates are included in a group of compounds that are generally regarded as safe (GRAS) by the FDA. The oral administration of alginate has not been shown to provoke much immune responses unlike the intravenously administered forms and it is reported that alginate is non-toxic and biodegradable when given orally.

2. Bioadhesiveness

Alginate, being an anionic polymer with carboxyl end groups, is a good mucoadhesive agent. Studies have shown that alginate has the highest mucoadhesive strength as compared to polymers such as polystyrene, chitosan, carboxymethylcellulose and poly(lactic acid). Due to the adherence of alginate particles to the mucosal tissues, protein transit time is delayed and the drug is localized to the absorptive surfaces. It improves drug bioavailability and effectiveness.

3. pH sensitivity

In theoretical, alginate shrinks at low pH (gastric environment) and the encapsulated drugs are not released. In gastric fluid, the hydrated sodium alginate is converted into a porous, insoluble called alginic acid. Once passed into the higher pH

of the intestinal tract, the alginic acid skin is converted to a soluble viscous layer. This pH dependent behavior of alginate can be exploited to customize release profiles.

2.3.2.3 Alginate uses [23]

The uses of alginates are based on three main properties. The first property, when dissolved in water, the resulting solution was thickened. The second property, they can form gels; gels form when a calcium salt is added to a solution of sodium alginate in water. The gel forms by chemical reaction, the calcium displaces the sodium from the alginate, holds the long alginate molecules together and a gel is the result. Last property of alginates can form films of sodium or calcium alginate and fibres of calcium alginates.

1. Textile printing

In textile printing, alginates are used as thickeners for the paste containing the dye. These pastes may be applied to the fabric by either screen or roller printing equipment. Alginates became important thickeners with the advent of reactive dyes. These combine chemically with cellulose in the fabric. Many of the usual thickeners, such as starch, react with the reactive dyes, and this leads to lower color yields and sometimes by-products that are not easily washed out. Alginates do not react with the dyes; they easily wash out of the finished textile and are the best thickeners for reactive dyes. Textile printing accounts for about 50 percent of the global alginate market.

2. Food

The thickening property of alginate is useful in both sauces and syrups. By thickening pie fillings with alginate, softening of the pastry by liquid from the filling is reduced. Alginate can make icings non-sticky and allow the baked goods to be covered with plastic wrap. Water-in-oil emulsions such as mayonnaise and salad dressings are less likely to separate into their original oil and water phases if thickened with alginate. Moreover, Alginates have some applications that are not related to either their viscosity or gel properties. They act as stabilizers in ice cream; addition of alginate reduces the rate at which the ice cream will melt.

Additionally, Calcium alginate films and coatings have been used to

help preservation of frozen fish. The oils in oily fish such as herring and mackerel can become rancid through oxidation even when quick frozen and stored at low temperatures. If the fish is frozen in a calcium alginate jelly, the fish is protected from the air and rancidity from oxidation is very limited. The jelly thaws with the fish so they are easily separated. If beef cuts are coated with calcium alginate films before freezing, the meat juices released during thaw are re-absorbed into the meat and the coating also helps to protect the meat from bacterial contamination. If desired, the calcium alginate coating can be removed by re-dissolving it with sodium polyphosphate.

3. Pharmaceutical and medical uses

The fibers of calcium alginate are used in wound dressings. They have very good wound healing and haemostatic properties and can be absorbed by body fluids because the calcium in the fiber is exchanged for sodium from the body fluid to give a soluble sodium alginate. This also makes it easy to remove these dressings from the large open wounds or burns since they do not adhere to the wound. Furthermore, removal also can be assisted by rinsing the dressing with saline solution to ensure its conversion to soluble sodium alginate.

Alginate is used in the controlled release of medicinal drugs and other chemicals. In some applications, the active ingredient is placed in a calcium alginate bead and slowly released as the bead is exposed in the appropriate environment. More recently, oral controlled-release systems involving alginate microspheres, sometimes coated with chitosan in order to improve the mechanical strength.

2.3.3 Chitosan

Chitosan is a cationic natural linear polysaccharide consisting of copolymers of D-glucosamine and N-acetyl-D-glucosamine units linked by β-(1-4)-glycosidic linkages (Figure 2.7). It is produced commercially by deacetylation of chitin, which present in outer structure in marine crustaceans such as crabs and shrimp. Chitosan is soluble in dilute acidic solution. The degree of deacetylation (%DD) has a significant effect on the solubility and rheological properties of polymer. The degree of deacetylation is one of the more important chemical characteristics of chitosan. This

determines the content of free amino groups in the polysaccharide. Methods for checking the removal of acetyl groups in chitosan include UV spectrophotometry, IR spectrometry, gas chromatography and dye adsorption [24].

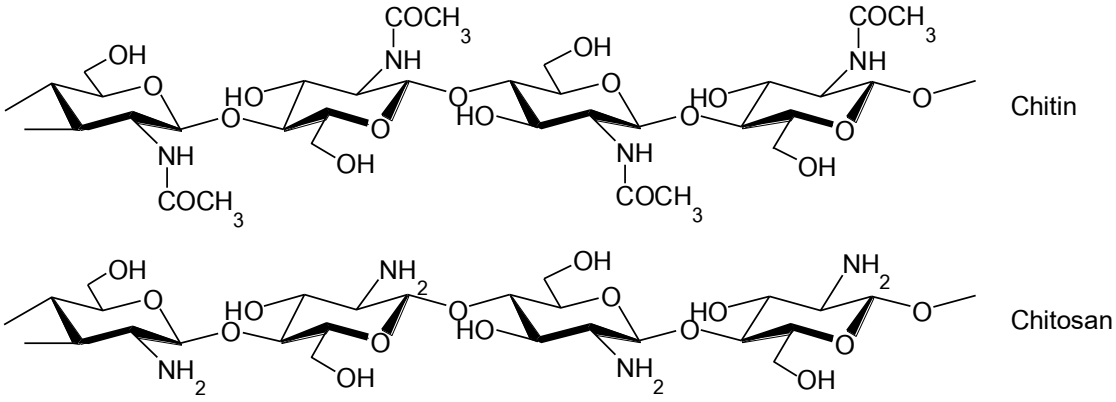


Figure 2.7 Structure of chitin and chitosan

2.3.3.1 Properties of chitosan[5]

1. Biocompatibility and biodegradability

Chitosan has been studied in biomedical field and has been found to be highly biocompatible. Also, chitosan is metabolized by certain human enzymes, especially lysozyme, and is considered biodegradable.

2. Mucoadhesiveness

The mucoadhesive properties of chitosan have been illustrated by its ability to adhere to porcine gastric mucosa in vitro, and hence it could be useful for in site-specific drug delivery. Moreover, chitosan has been suggested that residence time of formulations at sites of drug action or absorption could be prolonged through the use of chitosan. It has also been suggested that chitosan might be valuable for delivery of drugs to specific regions of the gastrointestinal tract like the stomach, small intestine, and buccal mucosa.

3. pH sensitiveness

Chitosan exhibits a pH-sensitive behavior as a weak polybase due to the large quantities of amino groups on its chain. Chitosan dissolves easily at low pH while it is insoluble at higher pH ranges. The mechanism of pH sensitive swelling involves the protonation of amine groups of chitosan under low pH conditions. This

protonation leads to chain repulsion, diffusion of proton and counter ions together with water inside the gel and dissociation of secondary interactions.

4. Mild gelation condition

The hydrogel preparation and drug entrapment can be done under relatively mild gelation conditions. Chitosan solution can be formed into a gel upon contact with a cross-linking agent. One of the commonly used cross-linking agents for the ionic gelation of chitosan is tripolyphosphate (TPP) (see figure 2.8). It is a non-toxic polyanion which can interact with chitosan via electrostatic forces to form ionic cross-linked networks. It can be used for the preparation of chitosan beads and microspheres because of its quick gelling ability. The cross-linking procedure helps to reinforce the chemical and mechanical properties of chitosan, making it a more stable network. Thus it can perform controlled protein release at higher pH of intestine instead of rapidly releasing the protein drugs by rapid dissolution in the stomach.

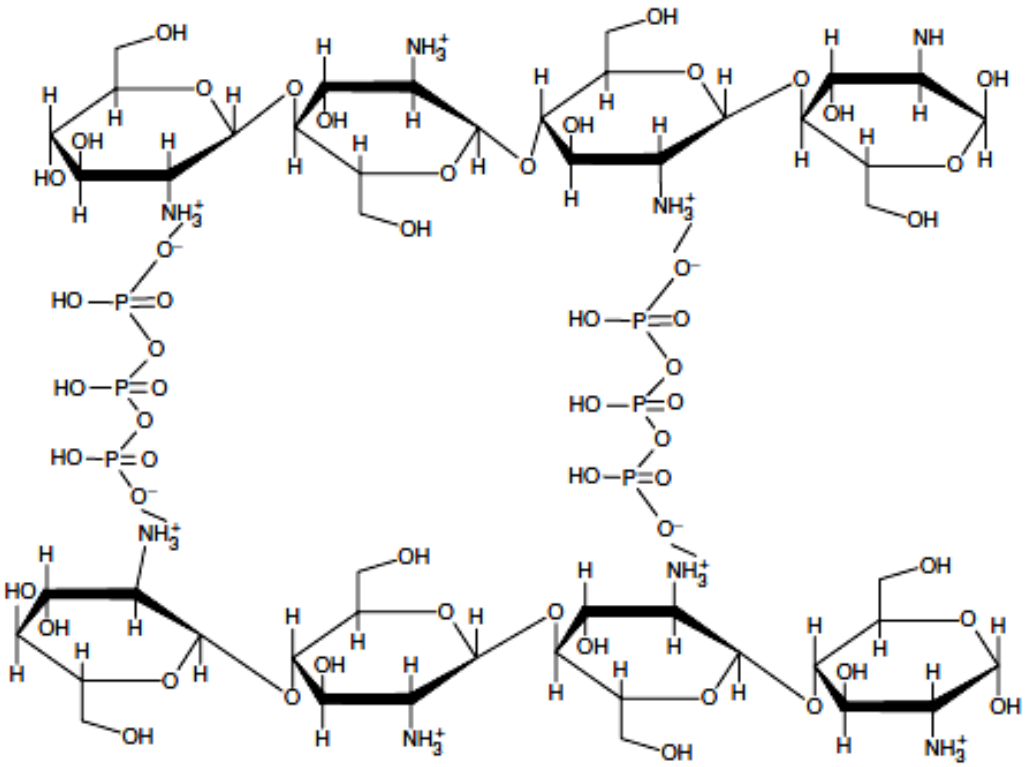


Figure 2.8 Ionic reaction of chitosan with aqueous TPP solution [25].

2.3.3.2 Chitosan uses [26]

1. Cosmetics

Chitosan forms a protective tensor film on the skin's surface that can fix other active principles for the skin. Thus other hydrating agents, solar filters, organic acids or other active principles can be combined with the derivatives of chitin. Chitin facilitates their effects. Chitin and its derivatives allow active principles to be placed in close contact with the skin by means of a medium that is not only a film-forming tensor but is especially hydrating. This is a new double advantage that makes chitosan of great interest in cosmetics. Therefore, chitosan are now widely used in skin creams, shampoos, lacquers, varnishes, etc.

2. Agriculture

Chitosan has many potential applications in agriculture because the Polymer is essentially naturally occurring and biodegradable; therefore it should not cause pollution problems. Chitosan can be used as seed coating; it has many beneficial effects, such as inhibition of fungal pathogens in the vicinity of the seeds and enhancement of plant-resistant response against diseases.

3. Water treatment

Chitosan has been gaining interest for industry and nature conservation. They are remarkable as chelating agents and heavy metal traps such as copper, lead, mercury and uranium from wastewater.

4. Pharmaceutical and medical uses

Chitosan have demonstrated their effectiveness for all forms of dressings-artificial skin, bandages and sponges for surgical treatment because its biocompatibility with human body tissue, Chitosan is an excellent medium for reducing side effect, delivering lower concentration and slow releasing of drugs in the body.

2.4 Preparation of polymeric particles

The main types of preparation method of micro/nanoparticle, as follow:

1. Emulsion cross-linking
2. Coacervation

3. Spray-drying
4. Ionic gelation
5. Electrospray ionization method
6. Inclusion complex

Various methods for preparation of alginate-chitosan micro/nanoparticles have been reported such as complex coacervation [9], emulsification/internal gelation [15], o/w emulsification, gelification and solvent removal [14].

This work focus on a technique for preparation of chili oleoresin loaded alginate-chitosan microparticles by using complex coacervation.

2.5 Complex Coacervation

Complex coacervation is the one of the oldest and simplest method of encapsulating drugs for sustained drug delivery. The Coacervation process may be divided into two types: simple and complex ones, depending on the number of polymeric ingredients. Because of simple preparation conditions such as use of non-toxic solvent and low agitation, they are also employed in the encapsulation of oil [27], drug [9] and protein [17].

Complex coacervation involves reaction between two oppositely charged polymers to yield a polymer rich and polymer poor region. The polymer rich region used to coat the core (drug) particles. Various negatively charged polysaccharides such as acacia, pectin, alginate and carboxy methyl cellulose were used with positively charged protein molecules such as gelatin and chitosan [27]. For example, Wittaya-areegul [28] prepared alginate/chitosan microparticles containing prenisolone by complex cocervation method.

2.6 Capsicum oleoresin

Capsicum oleoresin or chili extract is a complex mixture of a variety of straight-chained and branched-chained alkyl vanillyamides. Capsicum oleoresin is a thick, dark reddish-brown liquid concentrate produced by the extraction of fruits with volatile solvents. It is extensively used in food industry as natural flavouring and used

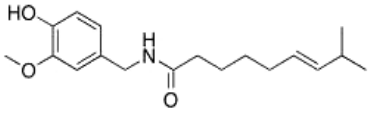
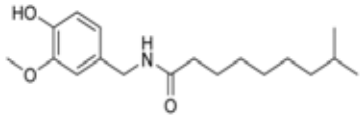
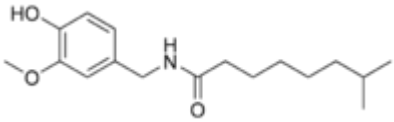
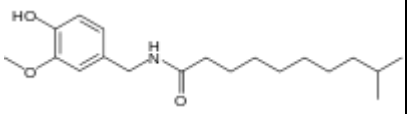
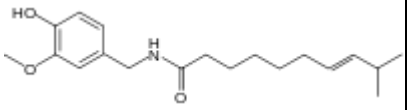
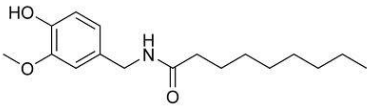
in various pharmacological preparations. The active compounds in this mixture, such as capsaicinoids, carotenoids, vitamins, minerals, flavonoids etc.

Carotenoids are naturally occurring colors compounds that are abundant as pigments in chili fruits and vegetables. The major carotenoids in chili fruits are capsanthin, capsorubin and β-carotene. β-carotene has an important nutritional role as the principal precursor of vitamin A, which is involved in vision, cell differentiation. Carotenoids have been linked with the enhancement of the immune system and decreased risk of degenerative disease such as cancer, cardiovascular disease. Moreover, carotenoids have also been identified as a potential inhibitor of Alzheimer's disease [29].

Capsaicinoids are the pungent compounds of the capsicum fruit. Among of the capsaicinoids, capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) (see figure 2.9) is the major component which demonstrates a variety of the pharmacological activities such as anti-tumor, anti-mutagenic, anti-inflammatory and anti-cholinesterase activities.

The name and chemical structure of the six natural capsaicinoids are shown in Table 2.1.

Table 2.1 The six natural capsaicinoids [30]

Capsaicinoid name	Abbrev.	Typical relative amount	Scoville heat units	Chemical structure
Capsaicin	C	69%	16,000,000	
Dihydrocapsaicin	DHC	22%	15,000,000	
Nordihydrocapsaicin	NDHC	7%	9,100,000	
Homodihydro-capsaicin	HDHC	1%	8,600,000	
Homocapsaicin	HC	1%	8,600,000	
Nonivamide	PAVA	-	-	

2.6.1 Physicochemical properties

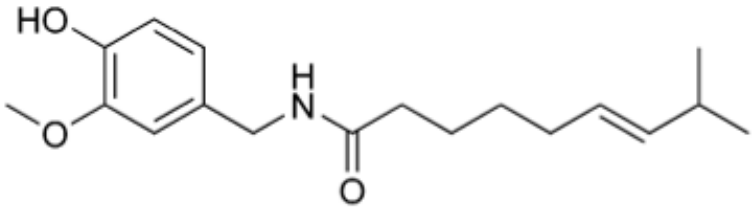


Figure 2.9 Chemical structure of Capsaicin

IUPAC name : 8-Methyl-N-vanillyl-trans-6-nonenamide

Molecular formula : $C_{18}H_{27}NO_3$

Molecular weight : 305.4 g/mol

Melting point : 62–65 °C

Boiling Point : > 187°C

Vapor pressure : Very low

Solubility (water) : 10.3 mg/L at 25 °C

Odor: Spicy odor with an odor threshold limit of approximately 10 ppm

Solvent solubility: Practically insoluble in water; freely soluble in benzene, alcohol, ketone, ether, chloroform and parafin oils.

The aim of this work is to prepare chili oleoresin-loaded alginate-chitosan microparticles using TPP as a cross-linked agent by complex coacervation technique. The model of alginate-chitosan microparticles was showed in figure 2.10.

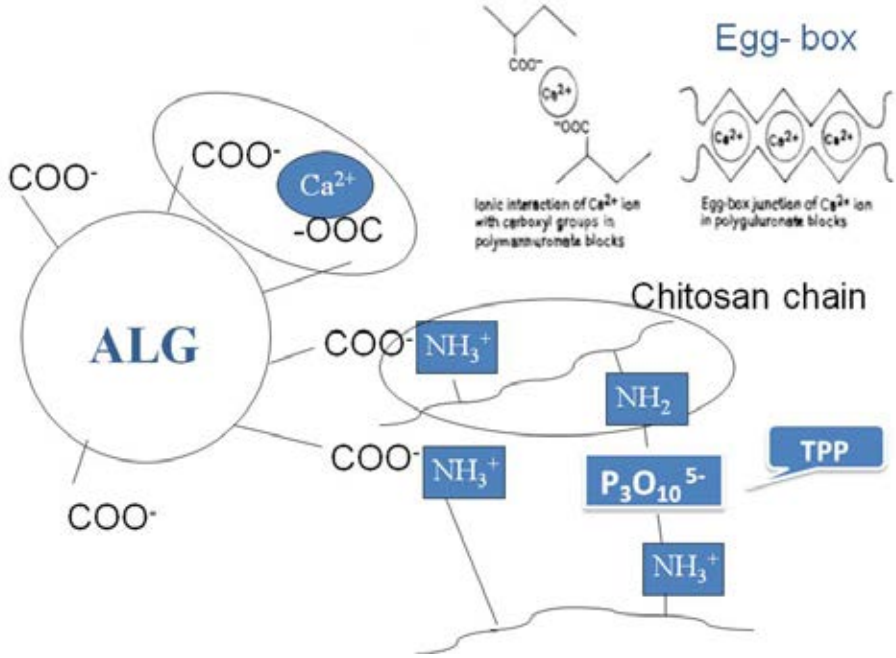


Figure 2.10 The model of alginate-chitosan microparticles

2.7 Literature reviews

Wang and coworkers [9] used gelatin and acacia as wall and capsaicin as core substance by using simple coacervation methods which has the concept of encapsulated capsaicin in polymer to hiding pungent odor, biocampatibility and biodegradation. The results showed that the nanocapsules was prepared has a mean particles size about 100 nm. Moreover, the melting point and thermal stability of nanocapsule were improved.

Because of solvent extracted spice oleoresin are sensitive to light, heat and oxygent. This problem can be solved by encapsulation. Shaikh and coworkers [30] prepared microcapsules loaded with black pepper oleoresin by spray-drying technique, using gum arabic and modified starch as wall materials. The microcapsules were evaluated for the content and stability of volatiles, non-volatiles, total piperidine and entrapped piperine for six weeks. The results revealed that microcapsules prepared from modified starch had granule sizes ranging from 5-15 μm, while that using gum arabic had slightly bigger size of 7-20 μm. Moreover, gum arabic was greater protection to the pepper oleoresin than modified starch.

Pasparakis and coworkers [31] studied the swelling behavior and the in vitro release of the antihypertensive drug verapamil hydrochloride from calcium alginate and calcium alginate-chitosan beads. The results indicated that the swelling of calcium alginate or calcium alginate-chitosan beads is dependent on the presence of the polyelectrolyte complex between alginate and chitosan, the pH of the aqueous media and the initial physical state of the beads. The encapsulation efficiency of verapamil in calcium alginate and calcium alginate-chitosan mixed beads exceeds 80%. Moreover, chitosan was found to retard the release from both wet and dry beads.

Ribeiro and coworkers [32] prepared chitosan-coated alginate microspheres loaded with lipophilic drugs by emulsification/internal gelation. This work used chitosan as a membrane coat to increase the mechanical strength and stabilize the microspheres in simulated intestinal media. The results showed that the microspheres were prepared with diameters ranging from 500-800 μm and encapsulation yield ranging from 60 to 80%. The release profiles of lipophilic drug were observed under simulated gastric condition, and simulated intestinal condition. Minimal release was

observed initially under gastric condition but rapid release was triggered by transfer into simulated intestinal fluid.

Kim and coworkers [33] prepared calcium alginate (CA), chitosan-coated calcium alginate (CCA-I) and chitosan-calcium alginate complex (CCA-II) gel beads entrapping allyl isothiocyanate (AITC) by oil-in-water emulsion. The entrapment efficiency of AITC was 81 and 30% for CA gel beads and chitosan treated gel beads, respectively. The swelling studies showed that all the beads immediately shrunken in simulated gastric fluid (pH1.2). In simulated intestinal fluid (pH7.4), CA and CCA-I gel beads rapidly disintegrated. On the other hand, CCA-II gel beads highly swelled without degradation owing to the strong chitosan-alginate complexation. The release profiles showed that most entrapped AITC was released during the shrinkage, degradation or swelling of the gel beads. Moreover, CCA-II gel beads showed the highest bead stability and AITC retention in pH1.2.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Active components

Dried chili (Chin-Da variety) of 200g was mercerated in ethanol at room temperature. The extract solution was filtered and evaporated in vacuo to afford a chili extract or chili oleoresin (24.0 g) (12.0% yield).

3.1.2 Polymers

- Chitosan with $\overline{M}_w$ of 200-250 kDa and a degree of deacetylation (DD) of 85% (Bio-line,Thailand)
- Sodium alginate (Carlo Erba Reactifs SA) (The viscosity of 1.5% solution is 192.4 cP at 25 °C)

3.1.3 Chemicals

- Calcium chloride (Carlo Erba Reactifs SA)
- Acetonitrile, HPLC grade (RCI Labscan Limited, TH)
- Methanol, HPLC grade (Merck, Germany)
- Hydrochloric acid (Carlo Erba Reactifs SA)
- Acetic acid glacial (Carlo Erba Reactifs SA)
- Sodium tripolyphosphate (Sigma-Aldrich, USA)
- Sodium hydrogen phosphate (Carlo Erba Reactifs SA)
- Potassium dihydrogen phosphate (Carlo Erba Reactifs SA)
- Sodium chloride (Carlo Erba Reactifs SA)
- Ethyl acetate (Carlo Erba Reactifs SA)
- Ethanol (Carlo Erba Reactifs SA)
- Standard capsaicin

3.2 Instruments

The instruments used in this study are listed in Table 3.1

Table 3.1 Instruments

Instrument	Manufacture	Model
Syringe pump	New Era Pump Systems	NE-300
Analytical balance	Mettler Toledo	AG204
Scanning electron microscope	Philips	XL30CP
Micropipette (100-10000 μl)	Mettler Toledo	Volumate
pH-meter	Thermo Fisher Scientific	Orion* 2 star
Magnetic stirrer	LMS	HTS-1003
High performance liquid chromatography	Waters Alliance	600
UV-VIS Spectrophotometer	Agilent	8453(G1103A)
Horizontal shaking water bath	Memmert	WNB 7-45
Evaporator	Buchi	R114
UV/VIS lamp	Synvania ultraviolet	G 10 W

3.3 Preparation of microparticles

3.3.1 Preparation of alginate-chitosan microparticles

Alginate-chitosan microparticles were prepared by complex coacervation method using alginate as a gel core. The alginate solution was prepared by dissolving different ratio of sodium alginate in 100 mL of distilled water. A coagulation fluid was prepared by mixing 100 mL of different ratio of chitosan solution dissolved in 1% (v/v) acetic acid and 100 mL of 1.5% (w/v) calcium chloride solution. The

alginate solution was extruded dropwise into 200 mL of a coagulation fluid using a 26 gauge syringe needle with a dropping rate 1.0 mL/min. The microparticles were gently stirred for 2 hrs, the particles were further cross-linked by adding 2 mL of 1% (w/v) sodium tripolyphosphate (TPP). The particles were allowed to harden for at least 30 min before washing twice with distilled water and dried at room temperature for 24 hours.

The ratios of chitosan and alginate (CS:ALG) in this study were 1:0.1, 1:0.3, 1:0.5, and 1:1 (total solid 3 g), as shown in Table 3.2. In addition, the effect of TPP on microparticle size was examined by comparing alginate-chitosan microparticles of ratio CS:ALG = 1:1 between with and without TPP. Moreover, the alginate microparticles with the exception of chitosan in the coagulation fluid were prepared as previously described. These particles were studied the swelling behavior comparing with alginate-chitosan particles to prove that chitosan was coated the surface of particle.

Table 3.2 The ratios of chitosan and alginate for preparing alginate-chitosan microparticles

Ratios of chitosan and alginate (w/w)	**Total solid (chitosan and alginate) 3 g**	
	Chitosan (g)[a]	**Alginate (g)[b]**
1:0.1	2.73	0.27
1:0.3	2.31	0.69
1:0.5	2.00	1.00
1:1	1.50	1.50

[a]: dissolved in 100mL of 1% acetic acid
[b]: dissolved in 100mL of distilled water

3.3.2 Preparation of chili oleoresin-loaded alginate-chitosan (OAC) microparticles

The microparticles were prepared by using a CS:ALG:chili oleoresin mass ratio of 1:1:0.5 (OAC-4). Chili oleoresin 0.5 g was dissolved with 1 mL of ethanol and then added in 100 mL of 1.5% (w/v) alginate solution under magnetic stirring at 300 rpm for 30 min. This solution was extruded into 200 mL of a coagulation fluid (100 mL of 1.5% (w/v) chitosan and 100 mL of 1.5% (w/v) calcium chloride solution) using a 26 gauge syringe needle with a dropping rate 1.0 ml/min. The microparticles were gently stirred for 2 hrs and then the particles were cross-linked by adding 2 mL of 1% (w/v) sodium tripolyphosphate (TPP). The particles were allowed to harden for at least 30 min before washing twice with distilled water and dried at room temperature for 24 hours.

3.4 Characterization of alginate-chitosan microparticles

3.4.1 Scanning electron microscopy (SEM)

The shape and surface morphology of the microparticles were examined using scanning electron microscopy (Philips, XL30CP). The samples were mounted directly onto the SEM sample holder using double-side sticking tape and were gold spray-coated.

3.4.2 Particle size determination

Measurements of mean diameters of the microparticles were carried out with SemAfore Programme. Fifty randomly microparticles were taken to measure their individual sizes.

3.5 Swelling studies

3.5.1 Preparation mediums

3.5.1.1 Simulated gastric fluid

The simulated gastric fluid (SGF; pH 1.2, 0.1 M HCl) was prepared by dissolving hydrochloric acid (HCl) 4.16 ml in a 500 mL of distilled water and adjusted to pH 1.2 by 1 M HCl.

3.5.1.2 Simulated intestinal fluid (SIF) of phosphate buffer saline

The phosphate buffer saline (pH 7.4) was prepared by dissolving sodium chloride 2.4 g, potassium chloride 2 g, disodium hydrogen phosphate 14.4 g and potassium dihydrogen phosphate 2.4 g in a 1000 ml of distilled water and adjusted to pH 7.4 by 1 M HCl.

3.5.2 Swelling studies

The swelling measurement was performed according to the method reported in the previous report [34]. The alginate and alginate-chitosan microparticles were examined their swelling behaviors in SGF and SIF fluids.

The accurately weighed amounts of microparticles 0.1 g were bathed in 10 mL of buffer fluid. At time intervals, 0, 0.5, 1, 2, 3, 4, 5 and 24 hours, the microparticles were separated from the medium, gently wiped with paper and weighed. The dynamic weight change of the microparticles with respect to time was calculated according to formula below:

$$\% \text{ Weight change} = \frac{W_t - W_i}{W_i} \times 100$$

Where W_t is the weight of the microparticles in swelled state and W_i is the initial weight of the microparticles. The data was plotted the relationship between percent weight change and time.

3.6 Determination of chili oleoresin content and encapsulation efficiency (EE)

3.6.1 Calibration curve of capsaicin

A stock solution of 1000 mg/L was prepared by dissolving 100 mg of standard capsaicin in ethanol and adjust the volume to be 100 mL in a volumetric flask. This stock solution was individually pipetted 5, 4, 3, 2, 1, 0.2 and 0.1 mL, respectively, into 10 mL volumetric flasks and adjusted the volume with ethanol. The final concentrations of each solution were 500, 400, 300, 200, 100, 20 and 10 mg/L, respectively.

The peak area of each stock solution was determined by HPLC using a semipreparative reversed phase C18 column (250 × 4.6 mm.). The mobile phase was acetonitrile and 1% of acetic acid with a ratio of 42:58 v/v. The flow rate was 1 mL/min and the effluent was monitored at wavelength of 280 nm.

3.6.2 Determination of encapsulation efficiency (EE) and loading

3.6.2.1 Capsaicin content in chili oleoresin

Chili oleoresin (100 mg) was dissolved in ethanol and adjust the volume to be 100 mL in a volumetric flask. Then, 1 mL of this solution was filtered through nylon filters (0.45 μm) and determined capsaicin content by HPLC technique with the aid of a calibration curve.

3.6.2.2 Encapsulation efficiency

The encapsulation efficiency of chili oleoresin in this study was done. The accurately weight amount of microparticles (0.5 g) was mercerated in 50 mL of 0.3 M of sodium chloride solution for 24 hours. The microparticles immediately swelled. It is due to the Na^+ ions appearing in the sodium chloride solution undergo ion-exchange process with Ca^{2+} ions which are binding with COO^- groups mainly in the polymanuronate sequences of sodium alginate. This phenomenon results in the electrostatic repulsion among negatively charged COO^- groups which causes the chain relaxation and enhances the swelling properties [35]. Then, microparticles were extracted with 50 ml of ethyl acetate and remove solvent by vacuum evaporate. The

extract was re-dissolved with ethanol and adjusted a volume to be 10 mL in a volumetric flask. Solution was filtered through nylon filters (0.45 μm, Whatman, England) and determined capsaicin content by HPLC technique. The actual amount of chili oleoresin content was determined by using the data from 3.6.2.1 and the capsaicin content from HPLC analysis. All experiments were performed in triplicates. The encapsulation efficiency (EE) and loading capacity (LC) were calculated as follows:

$$\%EE = \frac{\text{actual amount of chili oleoresin content (mg)}}{\text{total amount of chili oleoresin content (mg)}} \times 100$$

$$\%LC = \frac{\text{actual amount of chili oleoresin content (mg)}}{\text{total amount of microparticles (mg)}} \times 100$$

3.7 *In vitro* release studies

The capsaicin release were carried out consequently in two aqueous media; a simulated gastric fluid (SGF, pH 1.2 HCl) for 3 hours and a simulated intestinal fluid (SIF, pH 7.4) for 8 hours, respectively.

Alginate-chitosan microparticles of 2 g were placed in a conical flask containing 50 mL of SGF. The sample flask was shaken in a shaking water bath at 37± 1°C. Solution of 10 mL was withdrawn from the SGF at the time intervals of 10, 20, 30, 60, 120, and 180 min. After 180 min, transferred the microparticles into 50 mL of SIF and then placed the flask in the shaking water baths at 37± 1°C. Solution of 10 mL was withdrawn from the SIF at the time intervals of 30, 60, 120, 180, 240, 300, 360, 420 and 480 min. An equal volume of the fresh medium was replaced immediately after each sampling in order to keep a constant volume of the medium in the flask throughout the experiment.

Each sampling solution was extracted with 50 mL of ethyl acetate and remove solvent by vacuum evaporator. The extract was then re-dissolved with ethanol and

adjusted the volume to be 10 mL in a volumetric flask. Each solution was filtered through nylon filters (0.45 μm, Whatman, England) and determined the capsaicin released by HPLC analysis. Each experiment was performed in triplicate. The amount of capsaicin was calculated from the calibration curve. The percentage of cumulative capsaicin release at time intervals was calculated from the following equations:

$$\% \text{ Cumulative release} = \frac{\text{Amount of capsaicin from releasing}}{\text{Amount of capsaicin before releasing}} \times 100$$

3.8 Stability of caroteniods

The stability of caroteniods was determined by measuring the absorbance by using UV/VIS spectrophotometry at λ_{max} = 454 nm. The stability of carotenoids between chili oleoresin and OAC microparticles were compared**.**

3.8.1 Determining stability of carotenoids in chili oleoresin

Five gram of chili oleoresin were spread on a petri dish (diameter 60 mm) and placed under a ultraviolet radiation. Chili extract of 30 mg was sampled under period of time at 0, 0.5, 12, 3, 4, 5, 6, 7, 8, 24, 48, 72, 96 and 120 hours, respectively, to determined the carotenoid content. The sampling was dissolved in 5 mL of ethyl acetate and filtered through nylon filters (0.45 μm, Whatman, England). The solution was then determined the absorbance and evaluated the carotenoids content as follow:

$$\% \text{ Relative} = \frac{\text{the initial absorption - the absorption at various time}}{\text{the initial absorption}} \times 100$$

3.8.2 Determining stability of carotenoids in OAC microparticles

Dried OAC microparticles of 0.5 g were spread on a petri dish (diameter 60 mm) and placed under a ultraviolet radiation. The microparticles of 30 mg were

sampled under period of times at 0, 0.5, 12, 3, 4, 5, 6, 7, 8, 24, 48, 72, 96 and 120 hours, respectively. Then, the sampling particles were suspended in 5 mL of 0.3 M of NaCl solution for 24 hours and extracted with 10 mL of ethyl acetate. The extract was filtered through nylon filters (0.45 μm, Whatman, England).The solution was then determined the absorbance and evaluated the carotenoids content from the difference between the initial absorption and the absorption at various time as follow:

$$\% \text{ Relative} = \frac{\text{the initial absorption - the absorption at various time x100}}{\text{the initial absorption}}$$

The procedures for detemining carotenoid content are summarized in Figure 3.1.

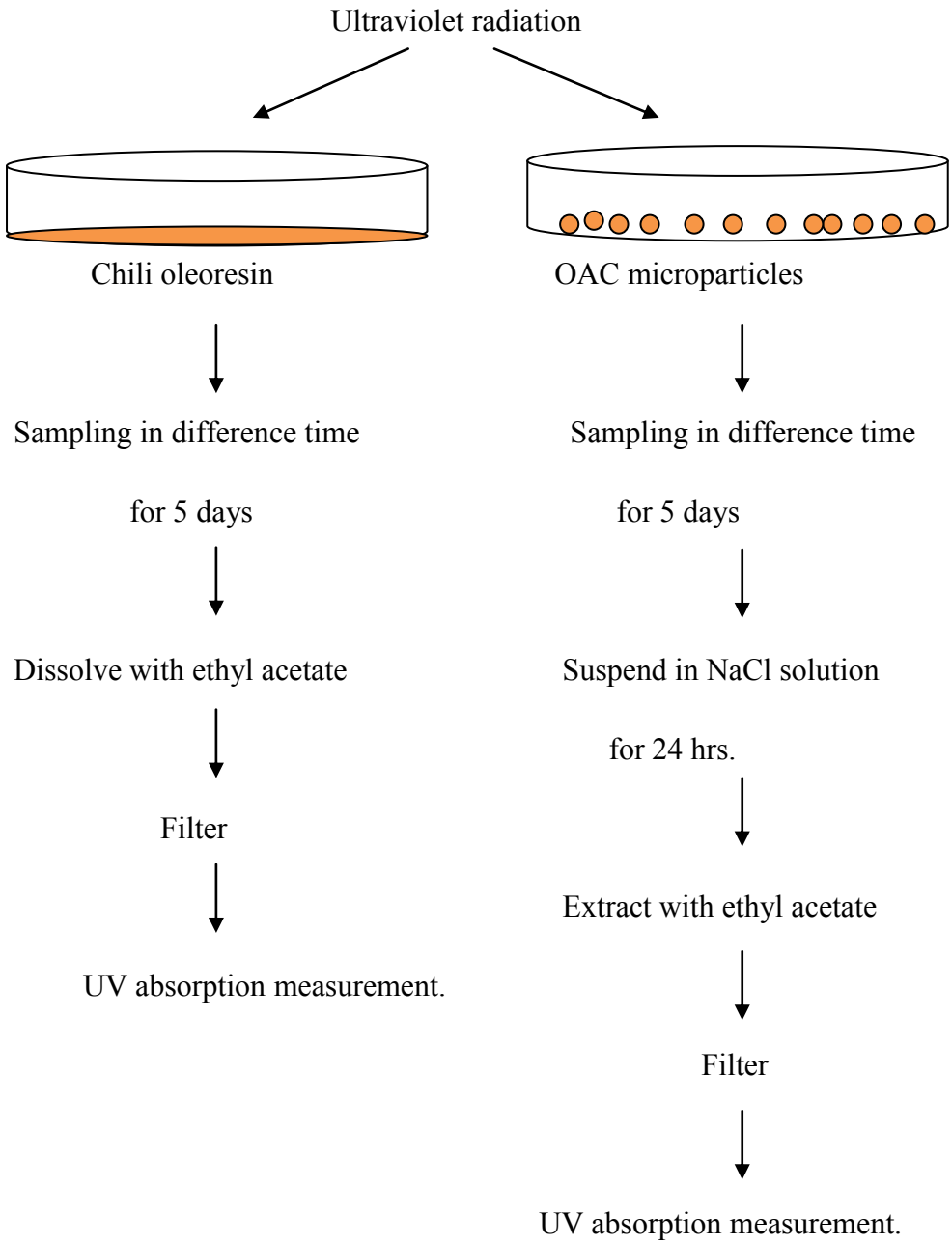


Figure 3.1 The procedure flowchart of carotenoid stability study.

3.9 Statistical analysis

All measurements were performed in triplicate for each experiment. Results are presented as means ± SD. Statistical analysis was performed by one-way ANOVA using Microsoft Excel (Microsoft Corporation) with $P < 0.05$ considered to indicate statistical significance.

CHAPTER IV

RESULTS AND DISCUSSION

In this work, alginate-chitosan microparticles were formed using complex coacervation technique. The effects of alginate and chitosan mass ratio and sodium tripolyphosphate (TPP) on morphology and size of a microparticle were studied and characterized by scanning electron micrographs (SEM). Moreover, the swelling behaviors, control releasing and carotenoid stability of microparticles were evaluated.

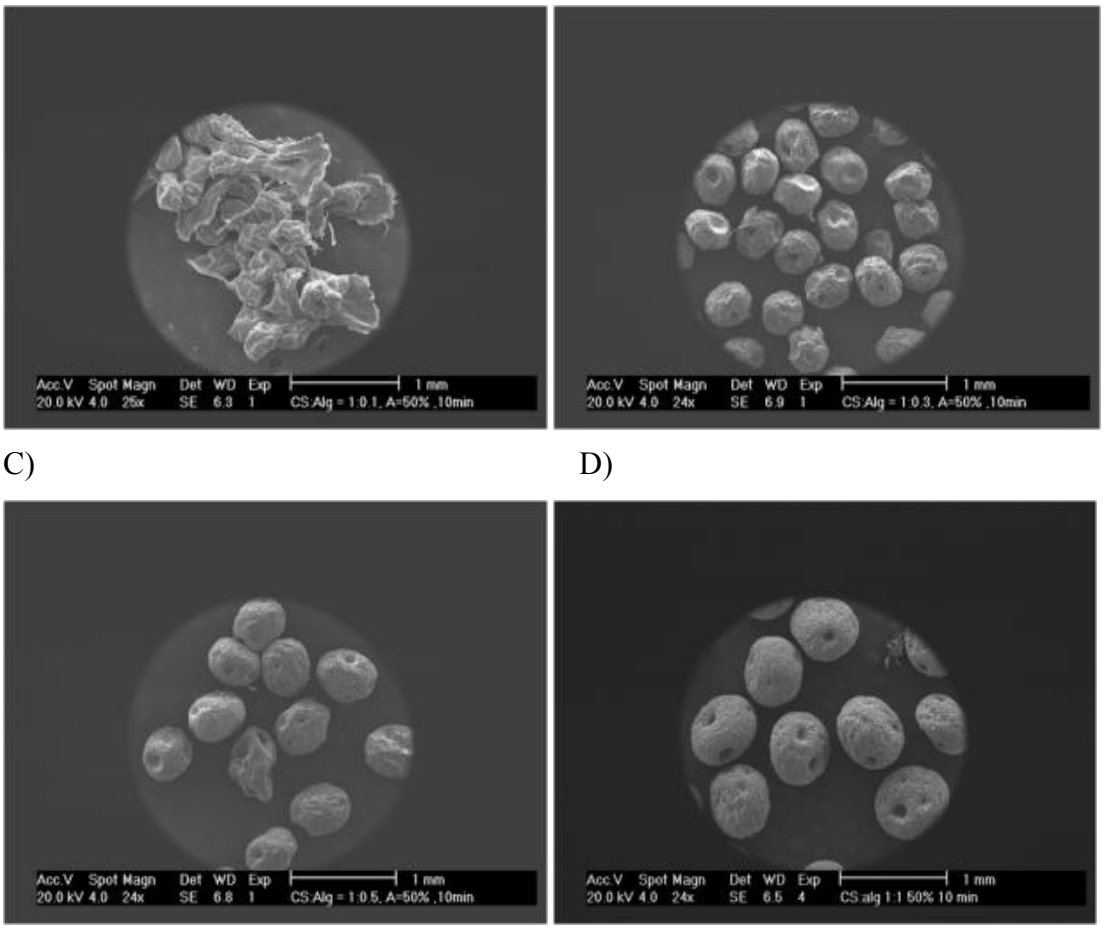
4.1 Effect of alginate concentration

The different chitosan and alginate mass ratios; 1:0.1, 1:0.3, 1:0.5 and 1:1, were used to prepare alginate-chitosan microparticles. The SEM micrographs and the average sizes of these microparticles were shown in Figure 4.1 and Table 4.1, respectively. At chitosan and alginate ratio (CS:ALG) of 1:0.1, it could not be formed the spherical bead whereas at other ratios the particles were formed and the size of microparticles increased by increasing the alginate concentration. The results can be explained that when alginate solution was added dropwise into a coagulation fluid, both the positively charged of chitosan and calcium ions are competing with the negative charges on the surface of the alginate core. At low concentration of alginate, the binding degree between alginate and chitosan/calcium chloride was quite low and not enough to form spherical particle. However, CS:ALG of 1:1 gave the best morphology, spherical shapes and smooth surfaces, of microparticles. Thus, this ratio was chosen for study the effect of TPP on the morphology of alginate-chitosan microparticles.

Table 4.1 The average sizes of alginate-chitosan microparticles with different ratios of chitosan and alginate.

Ratios of CS:ALG (w/w)	Average size (μm)
1:0.1	-
1:0.3	365.3 ± 25.2
1:0.5	421.2 ± 29.1
1:1	560.0 ± 38.9

A) B)



C) D)

Figure 4.1 Representative SEM images of the microparticles at CS:ALG ratios of A) 1:0.1, B) 1:0.3, C) 1:0.5, and D) 1:1

4.2 Effect of sodium tripolyphosphate (TPP)

Chitosan solution can be formed into a gel upon contact with a cross-linking agent. Sodium tripolyphosphate (TPP) is commonly used as cross-linking agents for the ionic gelation of chitosan. It is a non-toxic polyanion which can interact with chitosan *via* electrostatic forces to form ionic cross-linked networks. It can be used for the preparation of chitosan beads and microspheres because of its quick gelling ability [36].

The effect of TPP on the morphology and sizes of alginate-chitosan microparticles at CS:ALG ratio of 1:1 were examined. The SEM images of alginate-chitosan microparticles with and without TPP in a coagulation fluid were shown in Figure 4.2. The results indicated that microparticles cross-linked with TPP (Figure 4.2 B) had smooth surface and significantly smaller than those without TPP (Figure 4.2 A) (ANOVA, $P = 1.60 \times 10^{-3}$). The average-sizes of microparticles with and without TPP were 560±38.8 and 585±52.1 μm, respectively. The formulation ratio of CS:ALG at 1:1 and cross-linked with TPP was selected for further studies due to this formulation showed good morphology.

A) B)

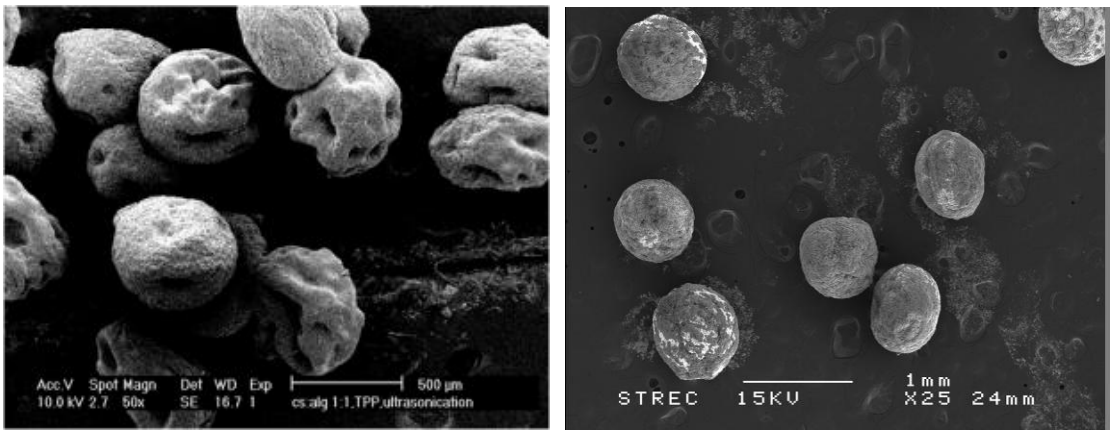


Figure 4.2 Representative SEM images of the microparticles of CS:ALG ratio of 1:1 A) without TPP, B) with TPP.

4.3 The swelling behaviors of microparticles

The alginate and alginate-chitosan particles were examined their swelling profiles in two medium, simulated gastric fluids (SGF, pH 1.2, HCl) and simulated intestinal fluid (SIF, pH 7.4).

The swelling behaviors of microparticles indicate the speed and easiness of a liquid to penetrate into the polymer matrix leading to the microparicles swell which can be used to deliver an active compound into a target organ.

4.3.1 The swelling behaviors in SGF

The swelling behaviors of particles; 1% alginate (ALG), 1.5% alginate (ALG), CS:ALG 1:0.5 and CS:ALG 1:1, in SGF were shown in Figures 4.3-4.4. Under acidic condition, alginate microparticles (without chitosan) exhibited the swelling degree significantly lower than alginate-chitosan microparticles. When alginate particles expose to low pH (less than 4.0), some calcium ions in the alginate network are replaced by the protonated carboxyl group of alginate which is alginic acid. Alginic acid, a water-insoluble residue, lead to the disruption of the network structure and particles are slightly swell. This phenomena occur in both alginate and alginate-chitosan microparticles. In case of highly swelling alginate-chitosan microparticles, the dissolution of chitosan on the surface of particles occur due to the weak interaction between the positively charged amino groups and alginic acid at low pH.

The swelling behaviors of 1% ALG and 1.5% ALG particles were not different (ANOVA, $P = 0.1$). However, the swelling effects of alginate-chitosan micropaticles prepared between CS:ALG mass ratios of 1:0.5 and 1:1 are different (ANOVA, $P = 0.007$). This might be caused by the different chitosan concentrations. The microparticles of CS:ALG 1:0.5 showed a higher swelling degree than the microparticles of CS:ALG 1:1. It is due to a high alginate concentration leading to a high carboxylic acid groups which interact electrostatically with amino groups of chitosan on the surface of microparticles resulting in higher swelling degree.

According to the contradiction the swelling behaviors of alginate microparticles and alginate-chitosan microparticles in SGF could be proved that there is chitosan coated the outer surface of alginate-chitosan particle

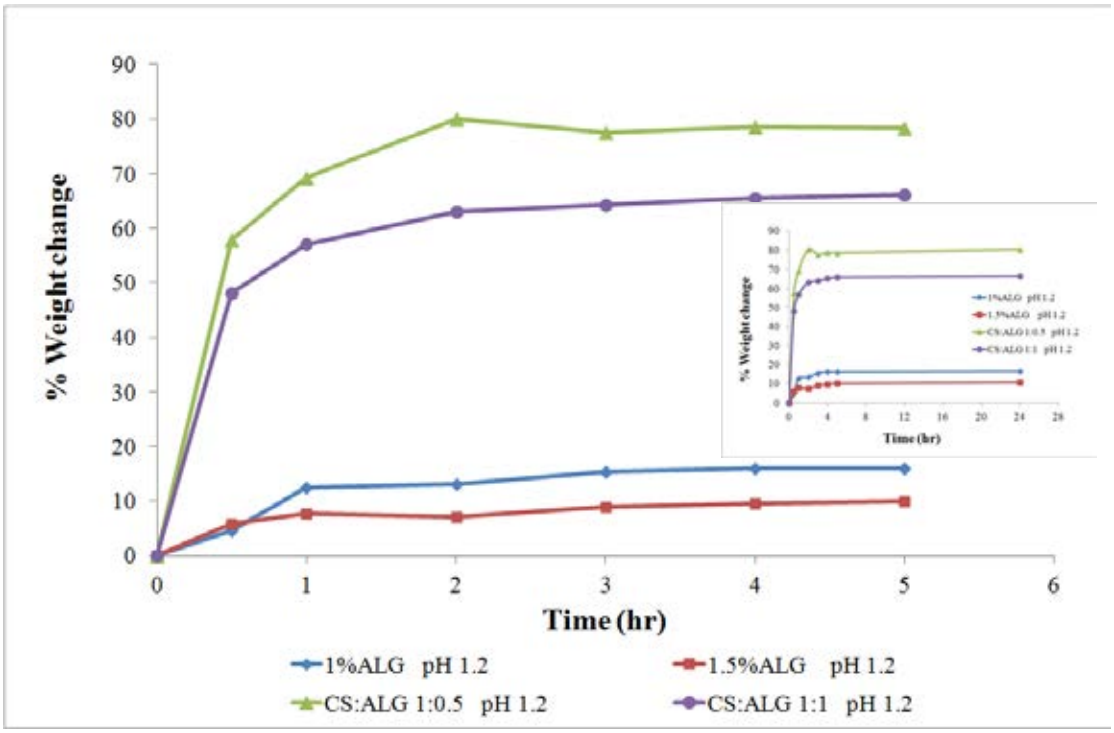


Figure 4.3. Swelling profiles of alginate-chitosan microparticles in simulated gastric fluid (pH 1.2, HCl)

A) B)

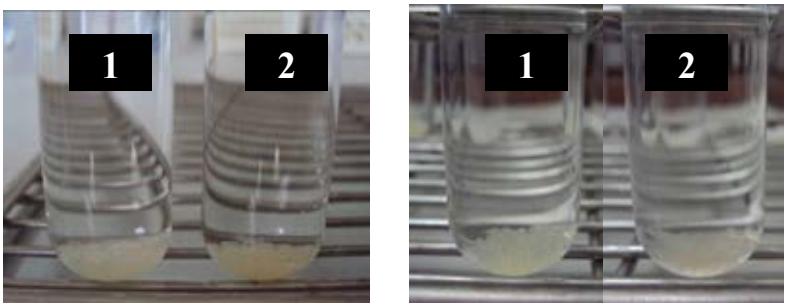


Figure 4.4. Representative images of the microparticles of 1A) 1% ALG, 2A) 1.5%ALG, 1B) CS:ALG 1:0.5 and 2B) CS:ALG 1:1.in simulated gastric fluid (pH 1.2, HCl) at 24 hours.

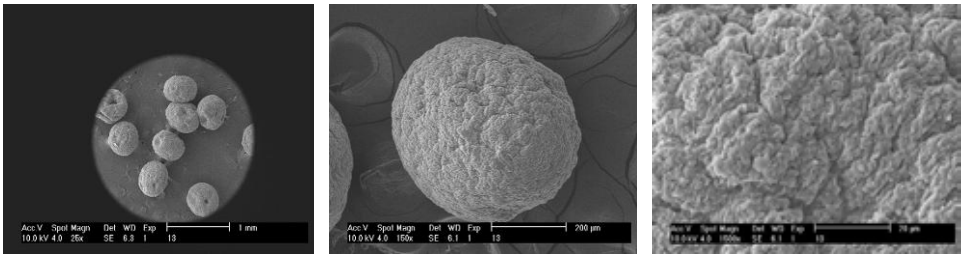
The morphology of microparticles in simulated gastric fluid (pH 1.2, HCl) at 24 hours was examined by SEM (Figures 4.5). The SEM was shown in Figure 4.5, suggesting that 1% ALG (Figure 4.5A) and 1.5%ALGmicroparticles (Figure 4.5B) did not disintegrated due to alginate converted into the insoluble alginic acid.

Whereas, CS:ALG 1:0.5 (Figure 4.5C) and CS:ALG 1:1 (Figure 4.5D) showed the surface of microparticles resembled with alginate microparticles due to the chitosan on the surface of microparticles was dissolved in acidic solution.

A)

B)

C)



D)

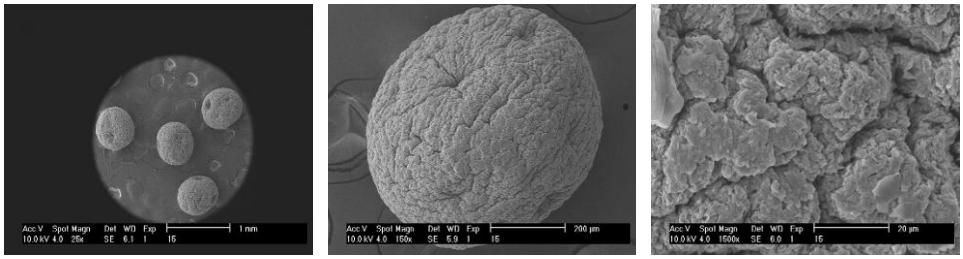


Figure 4.5. Scanning electron micrographs (SEM) of A) 1% ALG, B) 1.5%ALG, C) CS:ALG 1:0.5 and D) CS:ALG 1:1 in simulated gastric fluid (pH 1.2, HCl) at 24 hours.

4.3.2 The swelling behaviors in simulated intestinal fluid (phosphate buffer, pH 7.4)

The swelling behaviors of alginate and alginate-chitosan microparticles in SIF showed in Figures 4.6-4.7. When the alginate microparticles (without chitosan) were placed in simulated intestinal fluid (phosphate buffer, pH 7.4), the alginate microparticles were swollen immediately. In this basic medium, the Na^+ ions appearing in the external solution undergo ion-exchange process with Ca^{2+} ions which are binding with COO^- groups mainly in the polymanuronate sequences of sodium alginate. The phenomenon results in the electrostatic repulsion among negatively charged COO^- groups which causes the chain relaxation and increases the swelling properties [35]. Whereas, when inclusion the alginate microparticles with chitosan (CS:ALG 1:0.5, CS:ALG 1:1), the swelling degree of microparticles significantly lower than the alginate microparticle (without chitosan) (ANNOVA, $P = 1.94 \times 10^{-10}$). Because of chitosan was insoluble in basic medium, chitosan layer could protect the microparticle from outer fluids as well as envelop the whole microparticle with a fair firmness. Therefore, the swelling degree of the microparticles with chitosan lower than the swelling degree of microparticles without chitosan.

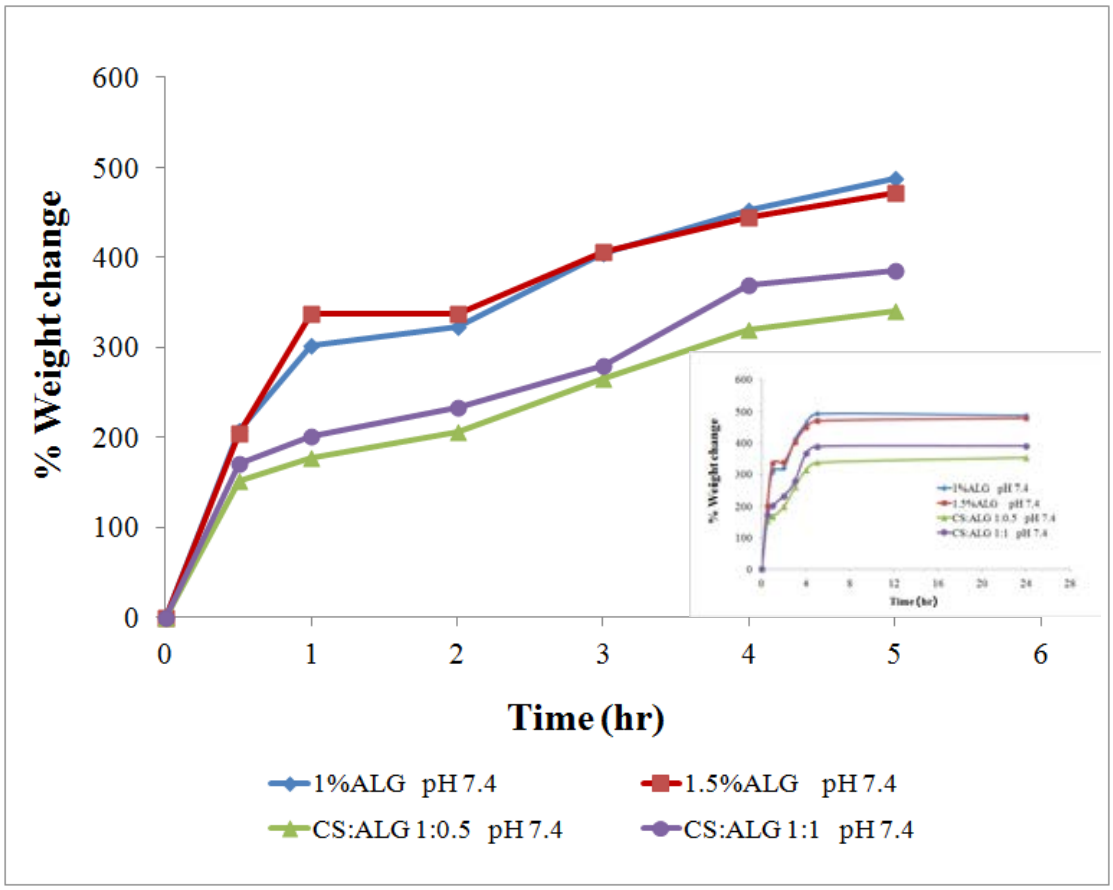


Figure 4.6. Swelling profiles of alginate-chitosan microparticles in simulated intestinal fluid (phosphate buffer, pH 7.4)

A) B)

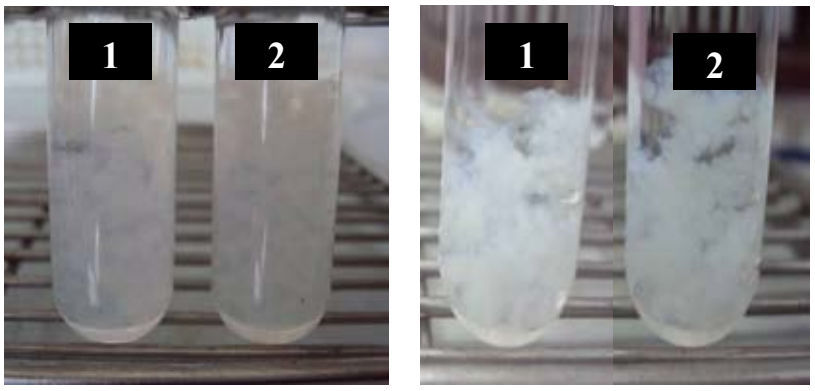


Figure 4.7. Representative images of the microparticles in simulated gastric fluid (pH 1.2, HCl) at 24 hours. , 1A) 1% ALG, 2A) 1.5%ALG, 1B) CS:ALG 1:0.5 and 2B) CS:ALG 1:1.

The SEM of microparticles in simulated intestinal fluid (phosphate buffer, pH 7.4) at 24 hours (Figures 4.8). The SEM showed that 1% ALG (Figure 4.8A) and 1.5%ALG microparticles (Figure 4.8B) were disintegrated due to calcium alginate loose and soluble under basic medium. The alginate microparicles (without chitosan) showed the smooth surface whereas, CS:ALG 1:0.5 (Figure 4.8C) and CS:ALG 1:1 (Figure 4.8D) were not. This may be due to the chitosan on the surface of microparticles.

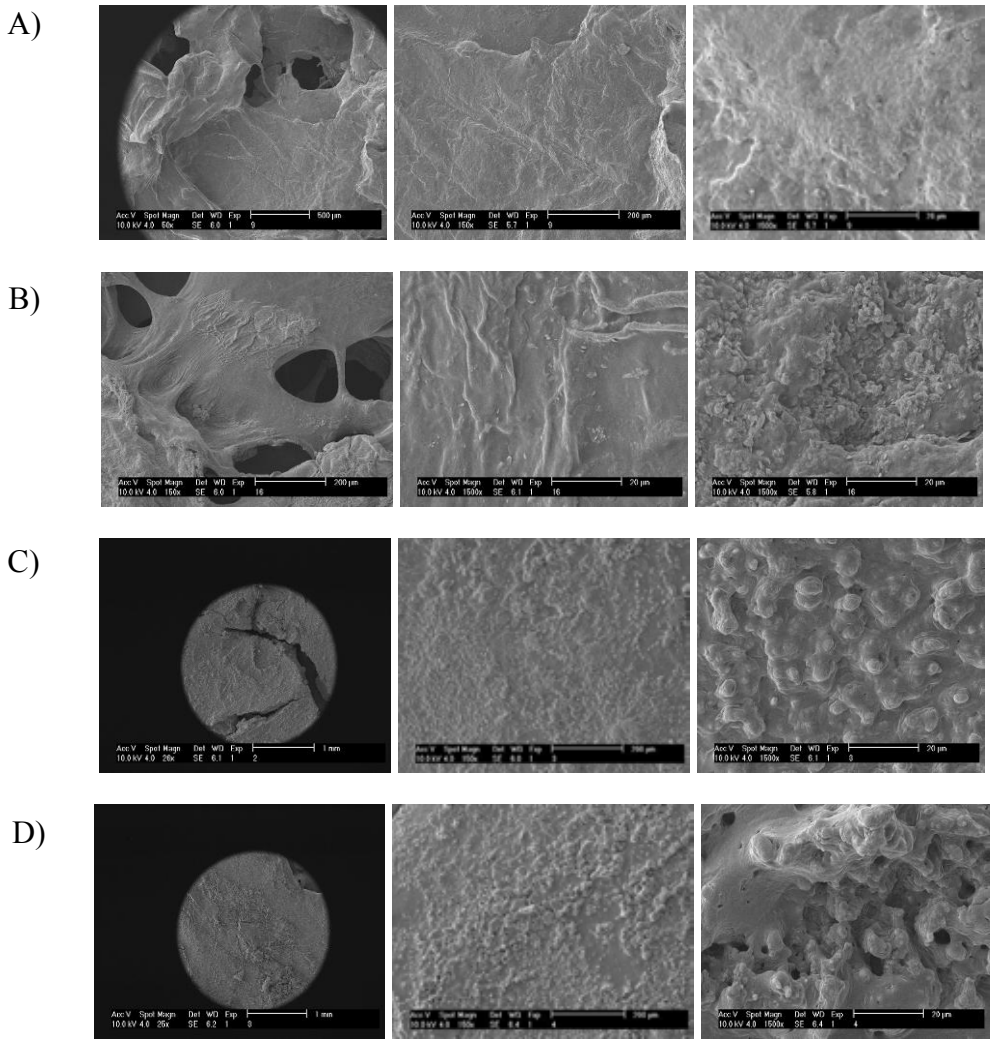


Figure 4.8. Scanning electron micrographs (SEM) of A) 1% ALG, B) 1.5%ALG, C) CS:ALG 1:0.5 and D) CS:ALG 1:1 in simulated intestinal fluid (phosphate buffer,pH 7.4) at 24 hours.

4.4 Encapsulation efficiency

The chili oleoresin loaded in the alginate-chitosan microparticle of the CS:ALG = 1:1 was prepared using amounts of oleoresin 0.2-0.5 g (OAC-1 to OAC-4). Encapsulation efficiency and loading capacity of each formula were shown in Table 4.2. When increasing the amount of chili oleoresin, The encapsulation efficiency was significantly increased from 59.43% to 73.05% (ANOVA, P = $2.99x10^{-6}$) and loading capacity significantly increased from 3.74% to 10.44% (ANOVA, P = $1.83x10^{-4}$). Nevertheless, the OAC-4showed highest encapsulation efficiency was chosen for in vitro chili oleoresin release studies.

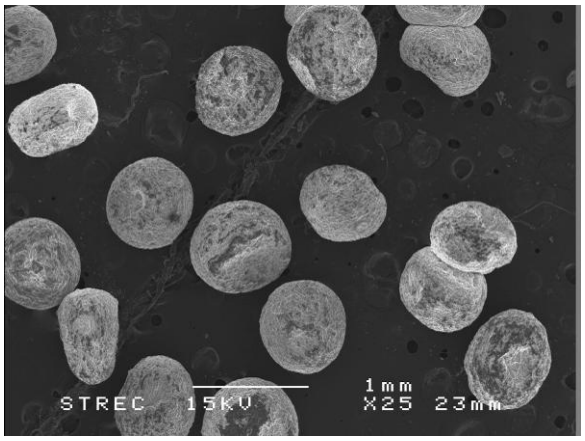
The morphology for OAC microparticles examined by SEM was shown in Figure 4.9. OAC-4 was given spherical microparticles with the average size of 758±58 μm.

Table 4.2 Encapsulation efficiency (%EE) and loading capacity (%LC) of chili oleoresin loaded alginate-chitosan microparticles (mean±SD, n=3)

Ratios of chitosan : alginate (w/w)[a]	Chili oleoresin (g)	Encapsulation efficiency (%EE)	Loading capacity(%LC)
1:1	0.2	59.43±1.78	3.74±1.26
1:1	0.3	60.41 ±0.67	5.55±1.02
1:1	0.4	63.14 ±0.93	7.57±0.80
1:1	0.5	73.05 ±1.24	10.44±0.76

[a] : total solid (chitosan and alginate) 3 g

A)



B)

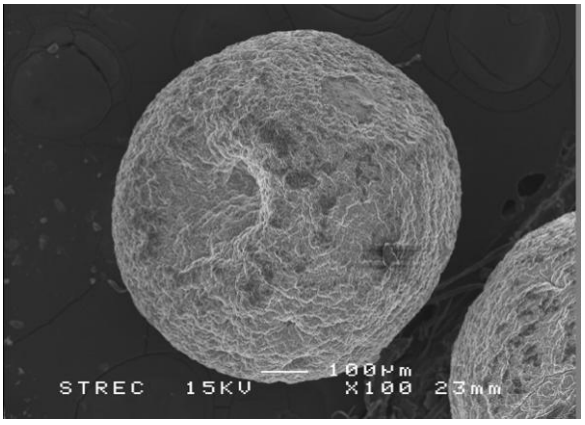


Figure 4.9. Representative SEM images of OAC-4

4.5 *In vitro* release

In vitro release of the OAC microparticles was carried out in SGF and SIF. The ratio of OAC-4 was chosen for in vitro chili oleoresin release studies owing to this ratio demonstrated highest encapsulation efficiency.

The release profile of alginate-chitosan microparticles was loaded with chili extract as shown in Figure 4.10. The release profile of the microparticles in SGF indicated that the chili oleoresin was slowly released about 32%. When the microparticles were transferred from SGF into SIF, the chili extract release rapidly increased from about 35 to 78% for 3 hours due to the fast microparticles degradation and then slowly increased up to 89%.

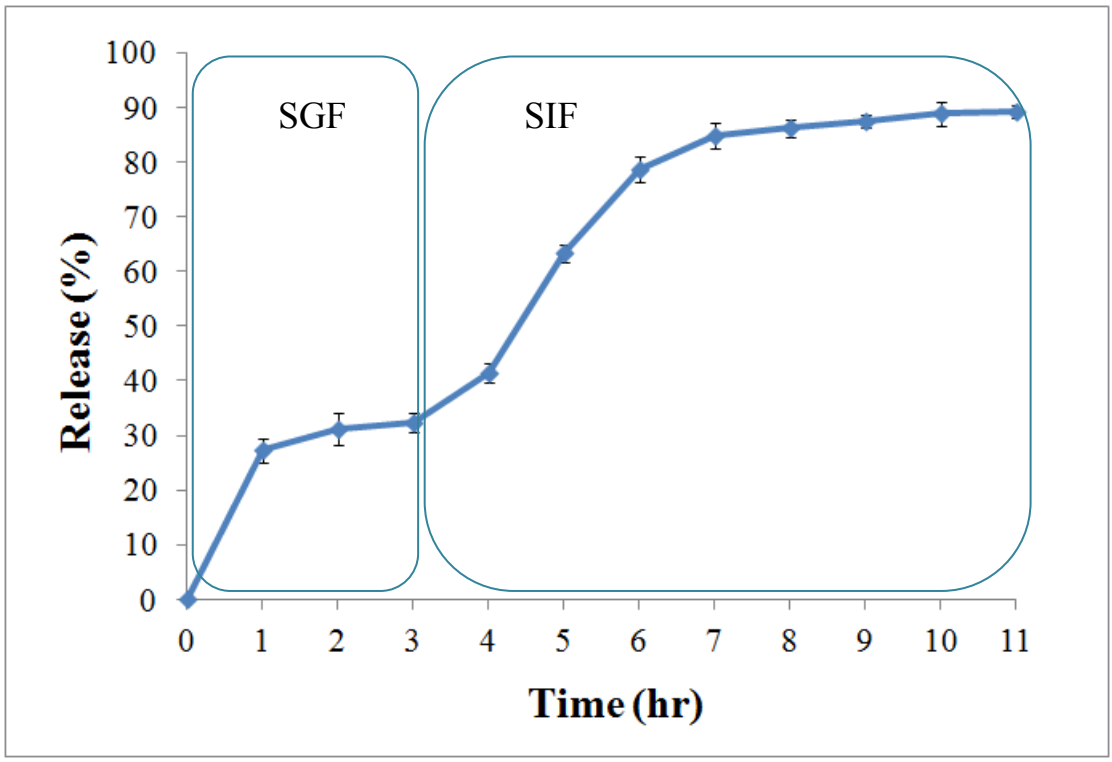


Figure 4.10. Release profile of OAC microparticles in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), 37 ± 0.1 °C (average ± SD, n = 3).

4.6 Stability of caroteniods

Cis-trans isomerization of the carotenoids easily occurs at room temperature and exclusion of light. Light promote isomerization of tran-carotenoids to the cis – form [29]. The possible scheme for carotenoid degradation is showed in Figure 4.11.

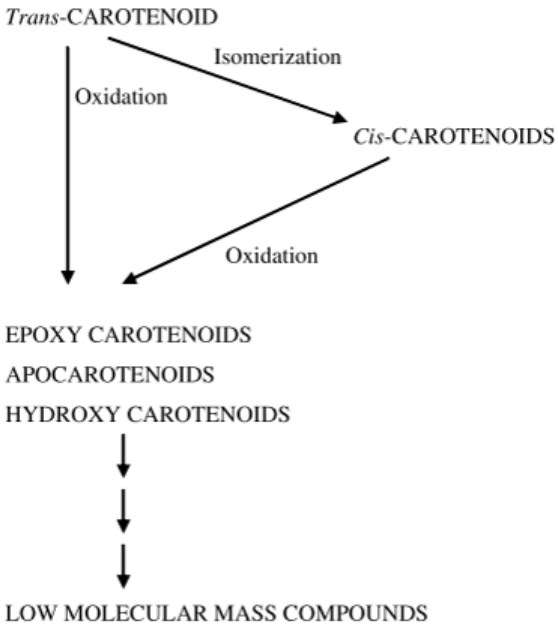


Figure 4.11. Possible scheme for carotenoid degradation [29].

The stability of caroteniods was determined by measuring the absorbance by using UV/VIS spectrophotometry at λ_{max} = 454 nm and evaluated the carotenoids content from the difference between the initial absorption and the absorption at various times. This experiment was compared between chili oleoresin and OAC microparticles.

In Figure 4.12, the result showed that the relative precent of chili oleoresin was significantly higher than the relative percent of OAC microparticles (ANOVA, $P = 1.28 \times 10^{-6}$). When increasing time, the absorption was decreased. It is due to chili oleoresin was encapsulated in polymer matrices which can be protected the carotenoids from light, heat and oxygen. On the other hand, chili oleoresin was not encapsulated in polymer matrices and also showed rapidly decreased. It is due to chili oleoresin was directly contacted with light, heat and oxygen.

In conclusion , the encapsulation can be protected carotenoids content in chili oleoresin from degradation and oxidation.

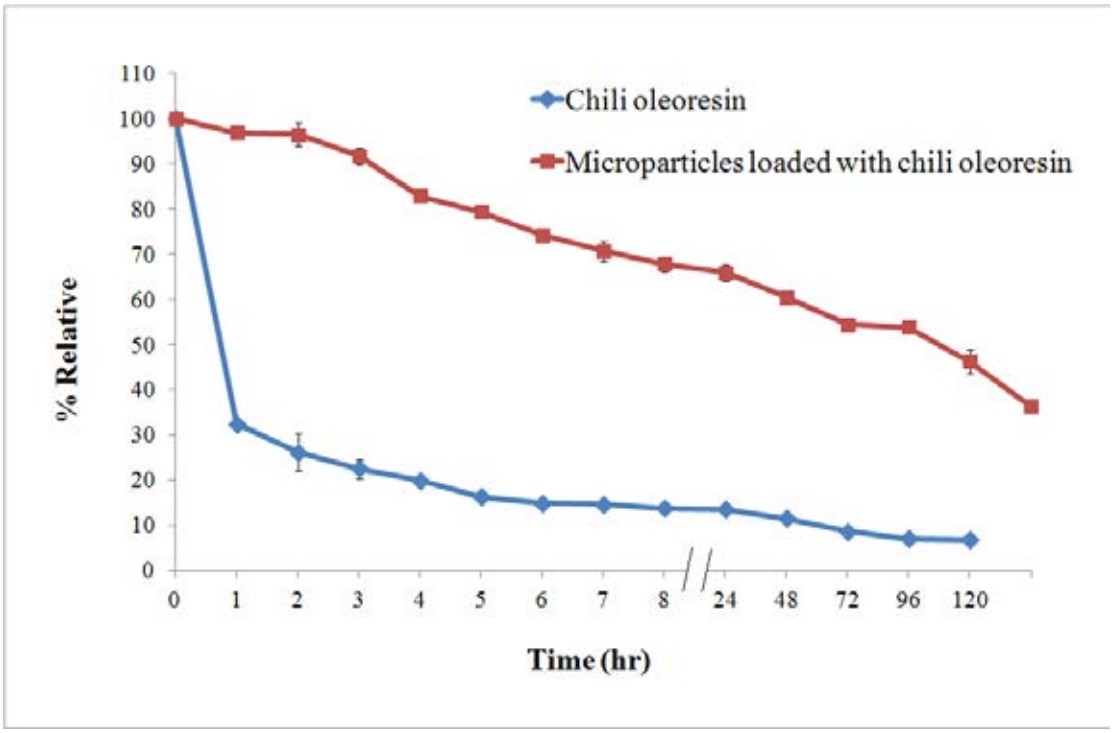


Figure 4.12. Stability of carotenoids in chili oleoresin and OAC microparticles.

CHAPTER V

CONCLUSION

5.1 Conclusion

Alginate-chitosan microparticles were prepared using complex coacervation technique. These microparticles were formed with different ratio of both polymers. From the SEM images, microparticles using the chitosan:alginate (CS:ALG) ratio of 1:1 showed the best morphology which was spherical and smooth-surface with the average size of 560 ± 38.9 μm. Moreover, the microparticles cross-linked with had smooth surface and significantly smaller than those without TPP. The average-size of microparticles with and without TPP were 560±38.8 and 585±52.1 μm, respectively. The swelling degree of alginate-chitosan microparticles in simulated gastric fluid (SGF, pH 1.2) (about 50%) was lower than that (about 400%) in simulated intestinal fluid (SIF, pH 7.4). This result might be indicated that the swelling-controlled releasing of this system was an ideal for the oral delivery in which the active compound is slightly released in the stomach but rather release in the intestinal. Chili oleoresin-loaded alginate-chitosan microparticles were prepared with the CS:ALG mass ratio of 1:1 and using TPP as a cross-linked agent. The highest encapsulation efficiency (73.90%) and loading capacity (10.44%) were obtained from the chitosan/alginate/chili oleoresin mass ratio of 1/1/0.5. The chili oleoresin-loaded alginate-chitosan microparticles was given spherical microparticles with the average size of 758±58 μm. At the initial three hours in SGF, capsaicin was slowly release from the microparticles with the release level of 32%. When microparticles were transferred from SGF into SIF medium, capsaicin releasing level increased to be 78% within 4 hours and complete release was not attained in 8 hours.

In additionally, chili oleoresin encapsulated in alginate-chitosan particles exhibited higher stability than free chili oleoresin. Therefore, the encapsulation can be protected the carotenoids content in chili oleoresin from light, masking pungent taste and reduce irritation to the stomach. Moreover, chili oleoresin usage instead of

pure capsaicin can reduce production costs. Lastly, chilli extract-loaded alginate-chitosan microparticles can be used as supplementary food.

5.2 Suggestion for future work

The suggestion for future work is to study thermal stability of carotenoids content in chili oleoresin.

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APPENDICES

Appendix A

Calibration Curve

The concentration versus peak area data of capsaicin in ethanol at 280 nm are presented in Table 1A. They show a linear relationship with the correlation coefficient = 0.9986.

Table A1 Peak area of standard capsaicin in ethanol determined by HPLC

Concentration (ppm)	Peak area
10	87984
20	310302
100	1481827
200	3025493
300	4667217
400	5998518
500	7921894

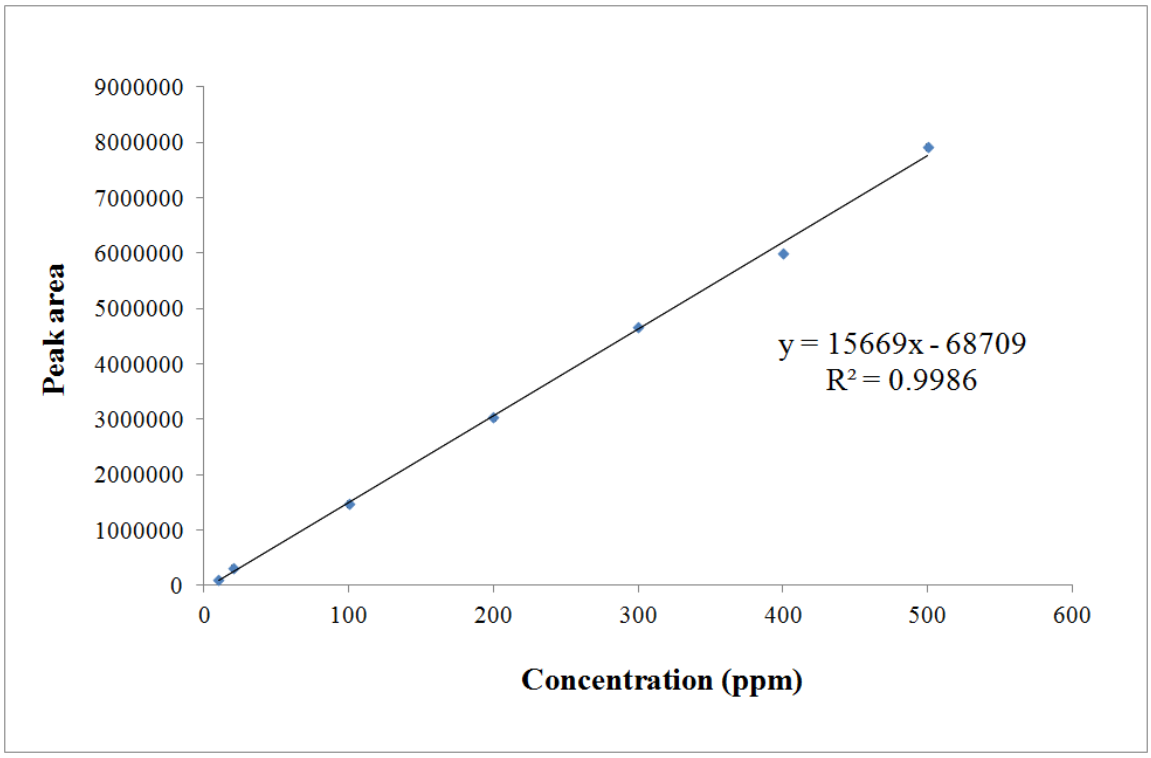

Figure A1 Standard calibration curve of peak area of capsaicin in ethanol.

Appendix B

Swelling degree

Table B1 The weight change percent of microparticles in SGF (pH1.2 HCl)

Weight change percent ± SD				
Time (hr)	**1%ALG**	**1.5%ALG**	**CS:ALG 1:0.5**	**CS:ALG 1:1**
0	0	0	0	0
0.5	4.67±2.17	5.92±1.02	57.85±0.62	48.03±0.41
1	12.55±2.23	7.74±1.73	69.22±1.83	57±0.26
2	13.12±2.35	7.16±2.54	80.01±1.64	63.12±0.32
3	15.32±1.98	8.89±1.94	77.45±1.81	64.20±0.11
4	15.96±2.03	9.51±1.85	78.54±2.64	65.45±0.22
5	15.93±2.19	9.95±1.94	78.30±3.21	66.04±0.53
24	16.21±2.48	10.47±1.45	80.01±2.58	66.67±0.78

Table B2 The weight change percent of microparticles in SIF (pH 7.4 phosphate buffer saline)

Weight change percent ± SD				
Time (hr)	**1%ALG**	**1.5%ALG**	**CS:ALG 1:0.5**	**CS:ALG 1:1**
0	0	0	0	0
0.5	207.87±2.14	205.07±6.89	151.18±2.77	171.1±1.10
1	302.4±10.06	336.92±1.76	177.6±9.11	201.2±2.4
2	323.22±3.83	336.55±3.51	205.73±6.28	233.09±2.66
3	403.73±6.15	405.28±3.79	264.55±2.54	279.35±6.68
4	452.77±9.2	445.04±4.24	319.87±5.65	369.97±5.07
5	488.31±4.78	471.1±7.58	339.72±1.68	385.93±4.47
24	485.38±4.76	477.40±3.51	356.98±3.08	387.93±4.39

Appendix C

Percentage of drug release

Table C1 Cumulative of capsaicin release in SGF (pH1.2 HCl) for 3 hours and in SIF (pH 7.4 phosphate buffer saline) for 8 hours

Medium	Time (hr)	% Cumulative capsaicin release				
		1	2	3	Mean	SD
SGF	0	0	0	0	0	0
	1	25.3800	29.7300	26.8900	27.3333	2.2086
	2	28.1300	31.7630	33.8670	31.2533	2.9022

Table C1 (continued) Cumulative of capsaicin release in SGF (pH1.2 HCl) for 3 hours and in SIF (pH 7.4 phosphate buffer saline) for 8 hours

Medium	Time (hr)	% Cumulative capsaicin release				
		1	2	3	Mean	SD
SGF	3	30.5788	32.3560	34.0145	32.3164	1.7181
SIF	4	41.1630	43.0796	39.9760	41.4062	1.5660
	5	62.8900	65.9075	61.0986	63.2987	2.4303
	6	79.9693	80.1239	76.0945	78.7292	2.2830
	7	84.9900	86.3903	83.0956	84.8253	1.6535
	8	86.6000	87.0045	84.8997	86.1681	1.1169

Table C1 (continued) Cumulative of capsaicin release in SGF (pH1.2 HCl) for 3 hours and in SIF (pH 7.4 phosphate buffer saline) for 8 hours

Medium	Time (hr)	% Cumulative capsaicin release				
		1	2	3	Mean	SD
SIF	9	87.3995	89.5468	85.3545	87.4336	2.0963
	10	89.0400	89.9043	87.5035	88.8159	1.2159
	11	89.1400	90.2342	88.0589	89.1444	1.0876

Appendix D

Stability of carotenoids

Table D1 Relative percent of stability of carotenoids in chili oleoresin

Time (hr)	Absorbance			Relative percent			Mean	SD
	1	2	3	1	2	3		
0	1.8775	1.8220	1.7875	100	100	100	100	0
1	0.5926	0.5911	0.5898	31.5648	32.4418	32.9958	32.3342	0.7215
2	0.5727	0.4620	0.3999	30.5017	25.3551	22.3737	26.0768	4.1118

Table D1 (continued) Relative percent of stability of carotenoids in chili oleoresin

Time (hr)	Absorbance			Relative percent			Mean	SD
	1	2	3	1	2	3		
3	0.4637	0.3746	0.3942	24.6961	20.5615	22.0537	22.4371	2.0938
4	0.3782	0.3426	0.3679	20.1454	18.8019	20.5790	19.8421	0.9266
5	0.3006	0.2893	0.3026	16.0128	15.8760	16.9309	16.2732	0.5737
6	0.2762	0.2702	0.2692	14.7089	14.8299	15.0590	14.8659	0.1778
7	0.2749	0.2629	0.2627	14.6397	14.4276	14.6965	14.5879	0.1418
8	0.2507	0.2512	0.2519	13.3502	13.7892	14.0901	13.7432	0.3721
24	0.2397	0.2489	0.2499	12.7691	13.6614	13.9804	13.4703	0.6279

Table D1 (continued) Relative percent of stability of carotenoids in chili oleoresin

Time (hr)	Absorbance			Relative percent			Mean	SD
	1	2	3	1	2	3		
48	0.2111	0.2099	0.2075	11.2415	11.5187	11.6056	11.4553	0.1901
72	0.1585	0.1564	0.1586	8.4399	8.5840	8.8727	8.6322	0.2204
96	0.1270	0.1267	0.1278	6.7654	6.9555	7.1497	6.9569	0.1921
120	0.1185	0.1259	0.1258	6.3137	6.9078	7.0389	6.7535	0.3864

Table D2 Relative percent of stability of carotenoids in chili oleoresin loaded alginate-chitosan microparticles

Time (hr)	Absorbance			Relative percent			Mean	SD
	1	2	3	1	2	3		
0	0.7386	0.7743	0.7753	100.0000	100.0000	100.0000	100.0000	0.0000
1	0.6901	0.7540	0.7645	93.4375	97.3757	98.6096	96.4742	2.7013
2	0.6705	0.7014	0.7256	90.7865	90.5888	93.5844	91.6532	1.6754
3	0.6170	0.6355	0.6434	83.5376	82.0765	82.9808	82.8649	0.7374
4	0.5875	0.6095	0.6188	79.5380	78.7134	79.8078	79.3531	0.5702
5	0.5399	0.5730	0.5853	73.0947	74.0084	75.4972	74.2001	1.2127

Table D2 (continued) Relative percent of stability of carotenoids in chili oleoresin loaded alginate-chitosan microparticles

Time (hr)	Absorbance			Relative percent			Mean	SD
	1	2	3	1	2	3		
6	0.5040	0.5486	0.5640	68.2354	70.8507	72.7422	70.6094	2.2630
7	0.4987	0.5130	0.5389	67.5165	66.2517	69.5086	67.7589	1.6419
8	0.4770	0.5042	0.5264	64.5852	65.1177	67.8898	65.8643	1.7743
24	0.4489	0.4588	0.4729	60.7820	59.2530	61.0009	60.3453	0.9523
48	0.3953	0.4162	0.4335	53.5155	53.7551	55.9190	54.3965	1.3239
72	0.3907	0.4118	0.4277	52.8967	53.1881	55.1593	53.7480	1.2308
96	0.3236	0.3551	0.3799	43.8078	45.8665	49.0043	46.2262	2.6168

Table D2 (continued) Relative percent of stability of carotenoids in chili oleoresin loaded alginate-chitosan microparticles

Time (hr)	Absorbance			Relative percent			Mean	SD
	1	2	3	1	2	3		
120	0.2773	0.2760	0.2756	37.5377	35.6494	35.5514	36.2462	1.1196

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