EFFECT OF CHITOSAN ON THE STABILITY OF FERULIC ACID ENTRAPPED LIPOSOME

TUANGTHIP PONGPRAYOON

MASTER OF SCIENCE
IN
COSMETIC SCIENCE

SCHOOL OF COSMETIC SCIENCE
MAE FAH LUANG UNIVERSITY
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Author	Tuangthip Pongprayoon
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ABSTRACT

This study intended to prolong the stability of liposome with chitosan. Containing positive charges from ammonium groups of its structure, chitosan can easily self-assemble with negatively charge liposome. Ferulic acid, a heat and light sensitive ingredient with several benefits for aesthetic purposes, e.g. sunscreening agent, whitening and antioxidant was chosen to be entrapped. Applied simple hand-shaking method was established with 65.52% entrapment efficiency and 94.38% yield recovery determined by spectrophotometry at maximum wavelength of 316 nm. Temperature controlling sonication system improved entrapment efficiency for 10.28%. The diameter of normal liposome significantly expanded when the temperature and storage time increased as well as the leakage of ferulic acid. Coating with chitosan prolonged the stability of ferulic acid in liposome and delayed the loss of ferulic acid in high temperature. Chitosan coated liposome had capability to maintain vesicle size and internal active ingredient content for a month.

Keywords: Chitosan/Ferulic acid/Liposome/Stability

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ABBREVIATIONS AND SYMBOLS

D	day
min	minute
mg	milligram
mL	milliliter
N.A.	not available
nm	nanometer
SD	standard deviation
W	week

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

The emerging trend of functional products from naturally occurring plants becomes stronger and stronger with eco-friendly perceptions and multifunctional benefits applied in food, drug and cosmetic industries. The worldwide growth of natural and organic cosmetic market has a rosy future (Matthews, 2013; Nichol, 2012). Phytochemicals stay in the limelight, tremendous scientific researches study about those plant derived substances and their advantages for health, wellness and beauty purposes. Ferulic acid is a ubiquitous phenolic phytochemical found in fruits, vegetables and cereal crops (Rybka, Sitarski & Raczynska-Bojanowska, 1993). It demonstrates a huge dimension of the valuable functional effects such as antioxidant, anti-inflammatory, antimicrobial and photoprotective properties. Most of researches have been studied about its therapeutic effects against diseases (Srinivasan, Sudheer & Menon; 2007). Experiments applied ferulic acid for cosmetic aspect gradually increased, particularly its benefits as a sunscreen and sunscreen enhancer (Lin et al, 2005). In vitro and in vivo transdermal studies support the topical application and safety of ferulic acid; however the further experiment for enhancement of its absorption is needed (Zhang, Al-Suwayeh, Hsieh & Fang, 2010). Ferulic acid decomposition induced by light, air, pH and temperature affected the appearance and efficacy of this substance in cosmetic formulations (Ouimet et al., 2013; Wang et al, 2011). Several experiments have been explored to stabilize the ingredient such as intercalation in anionic clay (Ricci et al., 2005), adding of photosensitizer like linoleic acid (Carlotti et al., 2008), insertion in vehicles like cyclodextrin (Anselmi et al., 2008), solid-lipid nanoparticle (Trombino et al., 2013) and liposome (De-Ling, Ke-

Long, Wei-Guo, Jing-Yi, & Fei-Peng, 2007), etc. Liposome is a remarkable delivery system with high attentions from scientists and clinicians due to its safety and compatibility with human body, but short stability (Sharma & Sharma, 1997). Chitosan is a naturally harmless compound with spontaneous ability to bind with liposome so several researchers tried to strengthen liposome and expanded its lifespan with chitosan (Gibis, Rahn & Weiss; 2013; Karn, Vanic, Pepic & Škalko-Basnet, 2011; Ngo, Umakoshi, Shimanouchi, Sugaya & Kuboi, 2012). The effect of chitosan, a nontoxic cationic polymer derived from crustaceans and fungi on the stability of liposome-entrapped ferulic acid is observed in this study. The loaded liposomes were coated with chitosan and monitored their stability in different conditions compared with uncoated liposome. The encapsulation efficiency, active ingredient content, morphology, and size of liposomes are also analyzed.

1.2 Research Objectives

1.2.1 To prepare the chitosan coated liposome-entrapped ferulic acid and compare with the uncoated liposome.

1.2.2 To prolong stability and increase efficiency of ferulic acid for further utilization.

1.3 Scope of Research

1.3.1 To identify the maximum wavelength of ferulic acid and determine its amount by using spectrophotometric method

1.3.2 To prepare ferulic acid entrapped liposomes and their chitosan coated form (chitosomes)

1.3.3 To study the morphology and physical appearance of liposomes and chitosomes.

1.3.4 To investigate liposome and chitosome stabilities in different conditions by monitoring their ferulic acid content and particle size in various temperatures and pHs.

1.4 Expected Outcome

Stability and efficiency increment of liposome loaded ferulic acid for further application in cosmetic product formulation.

CHAPTER 2

LITERATURE REVIEW

2.1 Ferulic Acid

Ferulic acid is a pervasive phenolic compound inherently present in plant tissue and plant cell walls, especially in the bran of cereal crops such as rice, wheat, oats, corn (Rybka et al., 1993). Extraction from waste water and residue of grain industrial with alkaline hydrolysis and microbial ferulic acid esterases have been achieved for commercial production (Kikuzaki, Hisamoto, Hirose, Akiyama & Taniguchi, 2002; Tomaro-Duchesneau, 2012). There is a bright prospect of ferulic acid in cosmetic world due to its healthful and helpful effects including antioxidant (Kikuzaki et al., 2002), antimicrobial (Varvaresou, 2009), UV protective (Carlotti, 2008; Lin et al., 2005, Oresajo et al., 2008) and whitening actions (Wang et al., 2011). Antioxidant is the main property of this bioactive ingredient that has been interested for cosmetic application while the perky arising of UV sunscreen and sunscreen amplifier advantages becomes more important.

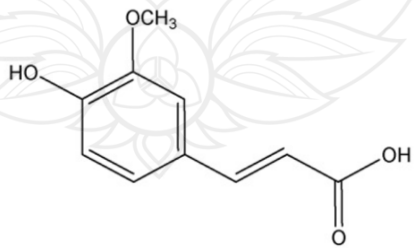


Figure 2.1 Structural formula of ferulic acid

Ferulic acid or 4-hydroxy-3-methoxycinnamic acid (Figure 2.1) is a monocarboxylic phenolic group with a contiguous conjugated double bonds and a carboxylic group which harmoniously facilitate free radical scavenging action, protection of lipid peroxidation and UV absorber imparting its potent antioxidant activity (Srinivasan et al., 2007). Several studies measured free radical scavenging activity of ferulic acid, its derivatives and many phenolic compounds on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) to determine the antioxidant ability (Kikuzaki et al., 2002; Stratil, Klejdus & Kubán, 2006). According to the UV spectrum pattern of ferulic acid (Figure 2.2) done by Jadhav, Kareparamban, Nikam & Kadam (2012), ferulic acid can absorb UV in a broad spectrum from 220 – 340 nm This supported the discoveries of protection against UV by this functional substance (Carlotti, 2008; Oresajo et al., 2008). Thus, spectrophotometry is one of methods applied to quantify ferulic acid content (Marković, Petranović & Baranac, 2000; Jadhav et al., 2012; Jankovska, Copikova & Sinitsya, 2001; Sohn & Oh, 2003; Woranucha & Yoksan, 2013).

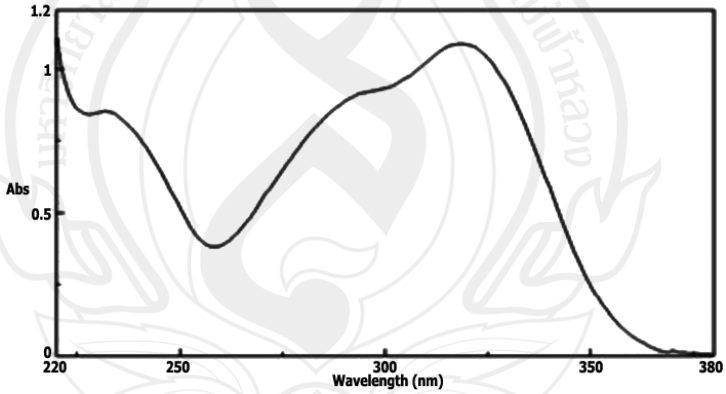


Figure 2.2 UV-Visible absorption spectrum of ferulic acid

A considerable amount of cosmetics incorporating ferulic acid have been launched to the market with good feedback, nevertheless, some comments about color change in skin care product containing this active ingredient have been posted in consumer review blogs. Instability and decomposition of this multifunctional compound were explored. Thermal decomposition of ferulic acid was observed and

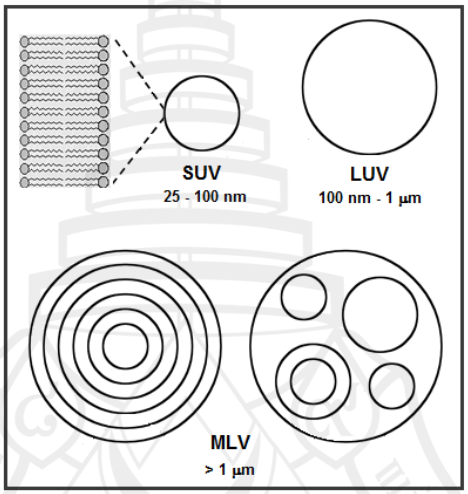
the mechanism of decarboxylation involving nitrogen and oxygen was proposed (Fiddler, Parker, Wasserman & Doerr, 1967). Absorbance shift of ferulic acid in alkaline condition was demonstrated (Friedman & Jürgens, 2000). Sohn & Oh (2003) suggested that ferulic acid should be stored below 76% relative humidity to prevent the adsorption of water vapor, which may lead to a potential instability. Chemical stability and degradation mechanism of ferulic acid blending cosmetic were also investigated confirming its unreliability related to pH and temperature (Wang et al., 2011). The study of ferulic acid efficacy and safety concluded that topical application was a safe and effective route for ferulic acid and its derivatives against photodamage, however its low permeation across the skin is not enough to provide effective results. (Zhang et al., 2010).. Various vehicles have been explored to convey and release ferulic acid at the final destination, i.e., cyclodextrin (Anselmi et al., 2008), chitosan graft (Woranucha & Yoksan, 2013), gel microspore (Zhang, Yuan, Du, Tnag & Xu, 2011), niosome (Chen, Liu & Fahr, 2010), ethosome (Chen et al., 2010), poly anhydride-ester (Ouimet et al., 2013), and liposome (De-Ling et al., 2007; Qin et al., 2008), etc.

2.2 Liposomes

Liposome is the hollow sphere container carrying active ingredient(s) with lipid bilayer shell imitating human skin barrier, can merge and pass through stratum corneum and bear the element safely to the right target (Torchilin, 2005). Carrying more than an active ingredient in a time, either and/ or both hydrophilic and lipophilic agent diversifying from micron to nano size is another impressive benefit of liposome (Egbaria & Weiner, 1990). It is safe and compatible to human, thus a tremendous amount of manipulation has been done using liposomes as nanodelivery system for oral, parenteral & topical administration (Egbaria & Weiner, 1990; Gangwar, 2012). Stability is limitation of liposome since it is sensitive to pH, temperature and shear force (Sulkowski et al., 2005; Sabin et al., 2006). Exertions to extend performance of liposomes have been done such as type and ratio varying of phospholipids, cholesterol

level adjusting, grafting, microencapsulation and polymer coating, etc. (Egbaria & Weiner, 1990; Riaz, 1995; Woranucha & Yoksan, 2013).

According to Manosroi & Manosroi (2007), Liposomes can be classified by their size and number of lamella. Their size is range from the smallest of approximately 25 nm in diameter to 1000 nm or greater in micrometer. They may contain single or multiple bilayer or lamella (Figure 2.3).



Source Lopes et al. (2013)

Figure 2.3 Types of liposomes depending on size and number of lamella

1. Multilamellar Vesicles (MLV): This is the simplest type of liposomes obtaining by hand shaken technique. They consist of more than one set of bilayer with diameter range around 0.05 – 10 micrometer.

2. Large Unilamellar Vesicles (LUV): The large size liposomes (0.1-4.0 micron.) compose of single lamella. They are suitable for entrapment of water soluble substance. LUV can be prepared by RVE (Reverse Phase Evaporation) method.

3. Small Unilamellar Vesicles (SUV): They are the smallest size liposomes with a sole bilayer. Their ranges of size are 0.025-0.1 micron or 25-50 nm

2.3 Chitosan

Chitosan (Figure 2.4) is a safely biodegradable copolymer, derived from chitin. Chitin is the second most plentiful natural polysaccharide on earth after cellulose (Dasha, Chiellinia, Ottenbriteb & Chiellinia, 2011). It is found in external integument of crustaceans (crabs, lobsters, shrimps, krills, etc.), insects as well as fungi (Aranaz et al., 2009). Transformation of chitin to be chitosan is naturally occurred only in Mucoraceae fungi by thermochemical deacetylation, whereas industrial production from crustacean shell obtained as food industrial waste needs protein and calcium elimination processes and several alkaline treatments (Dasha et al., 2011). Chitosan is considered as modern functional biomaterial that has been applied in many fields such as food, biomedicine, agriculture, textile, waste water treatment, food supplementary and cosmetic businesses (Kalyan, Sharma, Garg, Kumar & Varshney, 2010).

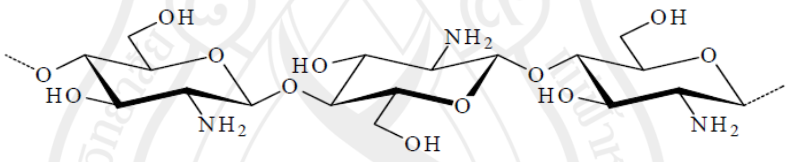


Figure 2.4 Chemical structure of chitosan

The properties of chitosan employed in cosmetics are antimicrobial agent, moisture retaining, film forming, and controlled release of active agent (Dutta, Dutta & Tripathia, 2004; Kim et al., 2009). Not only biodegradable, it is also bio-compatible and nontoxic therefore it is free of harm to human body. Polyelectrolyte is the outstanding characteristic applied for cosmetics since the positive charges from ammonium groups (NH_3^+) aligning along the structure of chitosan are swiftly bind to negative charges in mucopolysaccharides, proteins and fats of skin and hair forming coated thin film to keep moisture and provide flexibility (Dutta et al., 2004). Cosmetic products containing chitosan in present market are skin care cream,

shampoo, conditioner, hair styling, face powder, nail enamel, toothpaste, moisturizer, etc (Kalyan et al., 2010).

Binding of cationic chitosan to the negatively charge of simple liposome is self-assembly interaction (Sonvico et al., 2006; Bang et al., 2011). A handful of researches using of chitosan to coat the entrapped liposome in order to control the release of functional ingredient, strengthen those liposomes, and prolong their stability (Gibis et al., 2013; Karn et al., 2011; Ngo et al., 2012).

CHAPTER 3

EXPERIMENTAL METHOD

3.1 Instruments and Equipments

3.1.1 Analytical Balance (Precisa/ XT220A, Switzerland)

3.1.2 Rotary Evaporator (EYELA/ CCA 1110, Japan)

3.1.3 Sonicator (CREST 690 DAE, USA)

3.1.4 Refrigerator (SHARP/ SJ-D30M, Japan)

3.1.5 Hot Air Oven (MMM/ Ecocell 222C, Germany)

3.1.6 pH Meter (EUTECH pH/Ion 510, Netherlands)

3.1.7 UV-Visible Spectrophotometer (Genesys10UV, USA)

3.1.8 Zetasizer (Nano ZS90, England)

3.1.9 Hot Plat and Magnetic Stirrer (UMIMAG/ AREX2, USA)

3.1.10 Water bath (MEMMERT/ WB29, Germany)

3.1.11 Microscope (MOTIC/ BA300, USA)

3.2 Chemical Reagents and Standards

3.2.1 Hydrogenated Soya Lecithin (Nikko Chemicals Co., Ltd., Japan)

3.2.2 Cholesterol (Sigma-Aldrich Co., Ltd., Thailand)

3.2.3 Chloroform (Merk Ltd., Thailand)

3.2.4 Ferulic Acid (Tsuno Rice Fine Chemicals Co., Ltd., Japan)

3.2.5 Chitosan is a gift from Marine Bio Resource Co., Ltd., Thailand

3.2.6 Acetic acid (Merk Ltd., Thailand)

3.2.7 Triton X-100 (Panreac, Dow Chemical Co., Ltd., Thailand)

3.2.8 Folin-Ciocalteu reagent (Merk Ltd., Thailand)

3.2.9 Sodium Carbonate (Merk Ltd., Thailand)

3.3 Determination of Ferulic Acid by Spectrophotometric Method

3.3.1 Maximum Wavelength Identification

Standard ferulic acid solution was precisely prepared by weighing the pure chemical with 4 digit analytical balance, dissolving in deionized water and adjusting the final volume in a volumetric flask. The solution was diluted at proper concentration, and then scanned for maximum wavelength (λ_{max}) between 200-400 nm (1 nm interval) against deionized water as blank.

3.3.2 Standard Curve of Ferulic Acid by Spectrophotometry

The standard ferulic acid solution in deionized water was prepared in ten concentrations at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25 and 2.50 mg/100mL. The absorbance was measured at the acquired maximum wavelength (λ_{max}). The standard graph was plotted using the retrieved absorbance and concentrations.

3.4 Liposome and Chitosome Preparation

3.4.1 Preparation of Liposome

The preparation of primary liposome is an adapted method from hand shaking technique or chloroform film method (Manosroi, 2013) by dissolving 0.15 g hydrogenated soya lecithin and 0.08 g cholesterol in 20 ml chloroform. The solution was dried and formed a thin film in a round bottom flask using vacuum rotary evaporator at 47^{o}C under reduced pressure. Adding 20 ml ferulic acid in the flask shook in the 47^{o}C water bath for 20 minutes, and then transferred to the sonicator. Ferulic acid loading concentration and sonication time were varied in this experiment. The divergences of ferulic acid concentration were 0.05, 0.10, 0.20, 0.30, 0.40 and 0.50%. The sonication alternate periods were ten minute interval from 10 to 60 minutes. The best condition for entrapment will be adapted and applied along the

study. Empty liposome was assembled with the identical method at the chosen sonication pace, yet using deionized water in place of ferulic acid solution.

3.4.2 Preparation of Chitosan Coating Liposome (Chitosome)

0.1% (w/w) chitosan in 1% (v/v) acetic acid was processed and adjusted pH to 5.5 with 1N NaOH and kept at 4^{o}C in the refrigerator. Primary liposome from 3.4.1 (5 ml) was continuously dropped to chitosan solution (5 ml) while gently agitated with magnetic stirrer (Leelapornpisid, Leesawat, Natakarnkitkul & Rattanapanadda, 2010; Karn et al., 2011).

3.5 Entrapment Efficiency and Recovery Calculation

The absorbances of liposomes were measured at the selected wavelength (λ_{max}) against empty liposomes to determine the external (untrapped) ferulic acid reckoning from standard graph. Triton-X 100 (1%) was added ahead to each sample at the same amount then sonicated for 2 minutes to disintegrate vesicles for total bulk of internal and external ferulic acid mixture before identification of total (trapped + untrapped) ferulic acid by spectrophotometry. Internal (trapped) ferulic acid content was calculated by subtraction of external amount from total bulk (Qin et al., 2008). Repeated the same method was applied for chitosomes and empty chitosome was used as blank.

$$\% \textit{ Entrapment Efficiency} = \frac{(A_{actual} - A_{exterior})}{A_{initial}} \textit{ x } 100$$

$$\% \textit{ Recovery} = \frac{A_{actual}}{A_{initial}} \textit{ x } 100$$

$A_{exterior}$: the content of untrapped ferulic acid remained in the external phase

A_{actual} : the sum of untrapped ferulic acid and the trapped ferulic acid obtained by breaking of liposomal system

$A_{initial}$: the initial content of ferulic acid loaded in the system

3.6 Physical Characterization of Liposome and Chitosome

3.6.1 Morphology

A minute drop of blue water soluble dye was added to the miniature amount of each sample of liposomes and chitosomes on a slide with cover slip on top as wet mounting before observing their feature under microscope. The objective lens (100X) with a drop of oil was used to get the maximum magnification.

3.6.2 Determination of Particle Size

All particles were measured their size with Zetasizer (Nano ZS90) and the average diameters were compared. The analysis was performed at a 25^{o}C using samples diluted with de-ionized water.

3.7 Stability of Liposome and Chitosome in Different Conditions

3.7.1 Stability in Different Temperatures

The stability of primary liposome and chitosan coated liposome (chitosome) keeping in various temperatures (4±2^{o}C, ambient temperature and 45±2^{o}C) were monitored at different periods. Their particle sizes were measured by Zetasizer and the change of ferulic acid content was determined by spectrophotometric method.

3.7.2 Stability in Different pHs

Both liposomes and chitosome were tested for stability in discriminative pH values at 5.5, 6.5, 7.5, and 8.5. Since the maximum wavelength of ferulic acid is changed in different pH (Friedman & Jürgens, 2000), the determination of ferulic acid by Folin-Ciocalteu reagent was applied instead of direct spectrophotometry for stability investigation of liposome and chitosome at each pH.

3.7.3 Determination of Ferulic Acid by Folin-Ciocalteu Method

The eight concentration series of standard ferulic acid solution were prepared from 0.1 mg % - 0.8 mg % and completed the adapted Folin-Ciocalteu method

(Jadhav et al., 2012) for standard curve. The same method was done with liposome and chitosome samples.

Folin-Ciocalteu reagent 0.25 ml (1:2 diluted with DI) was mixed with 0.5 ml of each sample of liposomes and chitosomes. Sodium carbonate (1 ml of 15 %) was then added and the final volume was adjusted to 5 ml with deionized water. The absorbance was measured at 718 nm against blank (empty liposome or empty chitosome).

CHAPTER 4

RESULTS

4.1 Determination of Ferulic Acid by Spectrophotometric Method

Wavelength scanning of ferulic acid from 200-400 nm (1nm interval) showed the highest peak at 316 nm (Figure 4.1). The maximum wavelength was used to set the standard calibration graph (Appendix A). The spectrum pattern was similar to other previous studies, but slightly different in λ_{max} (Friedman & Jürgens, 2000; Jadhav et al., 2012). The ferulic acid scanning pattern was a broad spectrum curve covering especially from 280-340 nm which conforms to sun screening ability of the ingredient against UVA and UVB (Carlotti, 2008; Oresajo et al., 2008).

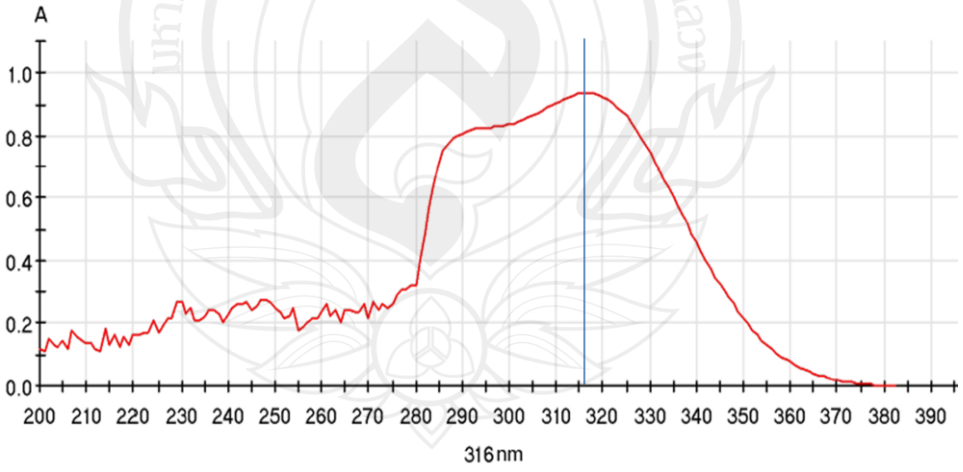


Figure 4.1 Absorption chromatogram of ferulic acid

4.2 Liposome and Chitosome Preparation

4.2.1 Preparation of Primary Liposome

The method for liposome preparation in this study was adapted from Manosroi (2013) with new loading amount and sonication time. The selected 100 mg/100mL ferulic acid loading amount was the best entrapment efficiency at 57.90% and 98.82% recovery (Table 4.1).

Table 4.1 Entrapment efficiency and recovery of liposomes with different ferulic acid loading amount

Ferulic acid content (mg/100mL)				%Entrapment Efficiency	%Recovery
Initial Load	Untrapped	Untrapped + Trapped	Trapped		
50.00	23.05	34.28	11.23	22.47	68.57
100.00	40.92	98.82	57.90	57.90	98.82
200.00	48.14	154.90	106.75	53.38	77.45
300.00	14.28	260.34	117.54	39.18	86.78
400.00	21.26	356.82	144.19	36.05	89.20
500.00	14.75	396.00	248.51	49.70	79.20

Temperature of the sonication tank after 10 min sonication period was 25^{o}C, but it climbed up to be 32 ^{o}C, 40 ^{o}C , 49 ^{o}C, 59 ^{o}C and 65 ^{o}C when the period was extended to 20, 30, 40, 50 and 60 min, respectively. Deionized ice was added to sonicator tank to avoid overheating during sonication and help controlling temperature around 20-22^{o}C. To prove the benefit of cooling sonication system, liposome preparations (20 min sonication) with ice and without ice during sonication were compared. Cooling sonication liposome had higher entrapment efficiency and recovery than the without ice system (Table 4.2). The experiment of sonication time variation was repeated with temperature controlling system and found that 30 min sonication time provided maximum entrapment efficiency of 65.52% with 94.38%

recovery (Table 4.3). The liposome preparation of 100 mg/100mL ferulic acid loading and 30 min sonication with cooling system was finalized as the selected condition.

Table 4.2 Comparison of liposomes (20 min sonication) with and without ice during sonication

Ice condition	Ferulic acid content (mg/100mL)			% Entrapment Efficiency	% Recovery
	Initial Load	Untrapped	Untrapped +Trapped		
No ice	100.00	46.10	68.57	22.47	68.57
With Ice	100.00	39.75	97.08	57.33	97.08

Table 4.3 Comparison of liposomes (cooling system) with various sonication times

Sonication time (min)	Ferulic acid content (mg/100mL)			% Entrapment Efficiency	% Recovery
	Initial Load	Untrapped	Untrapped +Trapped		
10	100.00	44.45	99.69	55.24	99.69
20	100.00	39.75	97.08	57.33	97.08
30	100.00	28.86	94.38	65.52	94.38
40	100.00	40.27	93.25	52.98	93.25
50	100.00	57.12	93.25	36.13	93.34
60	100.00	52.24	93.60	41.36	93.60

4.2.2 Preparation of Chitosan Coated Liposome (Chitosome)

After coating chitosan on the liposome, the entrapment efficiency barely changed from 65.52% to 64.12% (Table 4.4).

Table 4.4 Comparison of entrapment efficiency and recovery of liposome and chitosome

Particle Type	Ferulic acid content (mg/100mL)			% Entrapment Efficiency	% Recovery
	Initial Load	Untrapped	Untrapped +Trapped		
Liposome	100.00	28.86	94.38	65.52	94.38
Chitosome	100.00	29.30	93.42	64.12	93.42

4.3 Physical Characterization of Liposome and Chitosome

4.3.1 Appearance

Mixing of chitosan and liposome together changed the physical appearance of the solution. Similar to the study of Gibis, et al (2013), the empty liposome had least turbidity while the solution became denser in loaded liposome and chitosome, respectively (Figure 4.2).

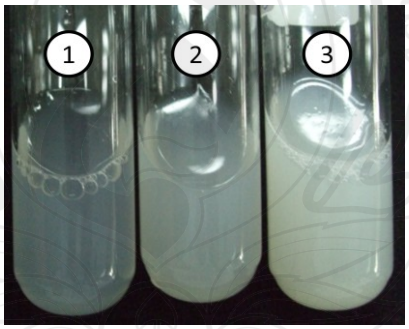


Figure 4.2 Appearance of empty liposome, loaded liposome and loaded chitosome.
① = Empty Liposome ② = Ferulic Acid Loaded Liposome
③ = Ferulic Acid Loaded Chitosome

4.3.2 Morphology

The liposomes and chitosomes were studied under binocular microscope. The dense chitosome solution was laborious to observe comparing to liposome one.

Figure 4.3 demonstrated the spherical or ellipsoidal shape of multi-vesicular liposome loaded ferulic acid and its chitosan coated nanoparticles (chitosomes) using 100X objective lens with oil. The differentiation of each particle in various temperatures and times by microscopic inspection was difficult to examine empirically. The results of particle size shift and change in ferulic acid content in the next part were done in parallel showed clearer evidences. However, the picture of microscopic liposomes showed some signs of irregular and expanded shapes in higher temperature on the third week (Figure 4.3).

4.3.3 Particle Size

The average particle size of 10 min sonication liposomes was the biggest, consecutively followed by 30 min sonication liposome (selected model) and chitosome. Their diameters were 1253.7±311.1 nm, 840.9±151.7 nm, and 469.6± 48.4 nm, respectively.

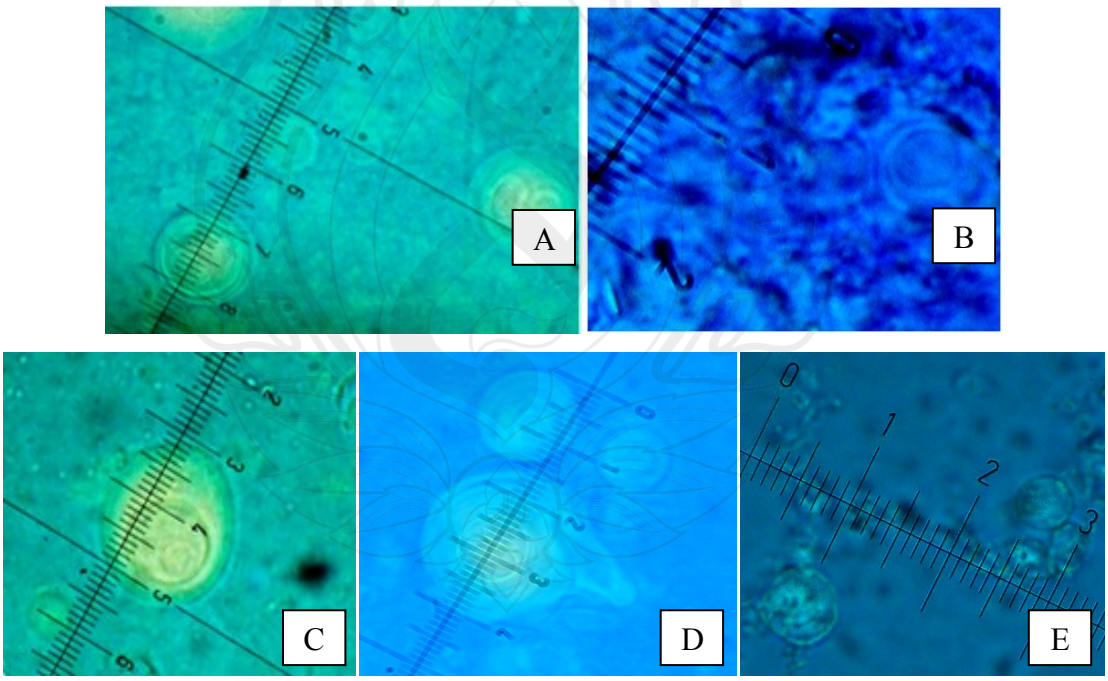


Figure 4.3 Microscopic images of liposomes and chitosomes (x1000)
A: Fresh liposomes B: Fresh chitosomes C: Wk3 liposome, ambient
D: Wk3 liposome; 45°C E: Wk3 chitosome; ambient

4.4 Stability of Liposome and Chitosome in Different Conditions

4.4.1 Stability in Different Temperatures

Ferulic acid content and particle size were two factors to monitor the stability of all particles in various temperatures (Table 4.5).

Table 4.5 Ferulic acid content and average size of vesicles in various temperatures

Type	Period	Temperature	Ferulic acid content (mg/100 mL)	% Remaining of ferulic acid within vesicles	Average Diameter (nm)
Liposome	D0	25 °C	65.52	100.00	N.A.
	D5	4 °C	61.16	93.35	840.9±151.7
	D5	Ambient	60.77	92.75	1,103.8±125.8
	D5	45 °C	55.46	84.65	1,160.0±198.7
	W4	4 °C	40.84	62.33	764.47±36.5
	W4	Ambient	22.09	33.72	1225.67±151.3
	W4	45 °C	14.76	22.53	1709.3±374.9
	W5	4 °C	18.26	27.87	N.A.
	W5	Ambient	1.18	1.80	Size too big to measure
	W5	45 °C	0.07	0.11	
Chitosome	D0	25 °C	64.12	100.00	N.A
	D5	4 °C	62.79	97.93	469.6±48.4
	D5	Ambient	61.29	95.59	467.7±40.8
	D5	45 °C	61.30	95.60	486.9±54.9
	W4	4 °C	61.93	96.58	661.5±94.0
	W4	Ambient	58.27	90.88	793.8±26.4
	W4	45 °C	56.07	87.45	350.1±31.7
	W5	4 °C	59.32	92.51	N.A.
	W5	Ambient	55.99	87.32	793.1±252.2
	W5	45 °C	52.33	81.61	N.A.
	W7	4 °C	52.79	82.33	N.A.
	W7	Ambient	21.82	34.03	N.A.
	W7	45 °C	13.13	20.47	N.A.

Note. 3 replications of each conditions were done and reported

4.1.1.1 Ferulic acid content of particles in different temperatures

After five day retention, ferulic acid within liposomes kept at 4°C and ambient temperature slightly descended comparing to newly prepared liposomes (4°C = 6.66%; ambient = 7.25%), while 45°C liposome showed a remarkable subsidence of 15.35%. The reduction of ferulic acid in liposomes continued by the storage time and clearly reflected on week 4 and week 5. The top loss of ingredient from vesicles on the fifth week was 45°C liposome (99.90%), followed by ambient temperature (98.21%) then 4°C (72.13%). The % remaining of ferulic acid within liposomes in different temperatures by storage time was compared in Figure 4.4.

Chitosomes of all conditions in day 5 had outstanding ability to hold ferulic acid closely to the fresh one. Ferulic acid content of 4 week aged chitosomes at 4°C and ambient temperature hardly fell from the first day they were produced, but gradually declined in the next week. The content of chitosomes kept at 45°C decreased at the fourth week and apparently decreased at week 5. The additional observation of chitosomes for another 2 weeks found that the 4°C chitosome contained 54.71 mg ferulic acid per 100 mL when ferulic acid of ambient and 45°C ones were 21.82 and 13.13 mg/100 mL, respectively. Figure 4.4 demonstrated the % remaining of ferulic acid within chitosomes in different temperatures by storage time.

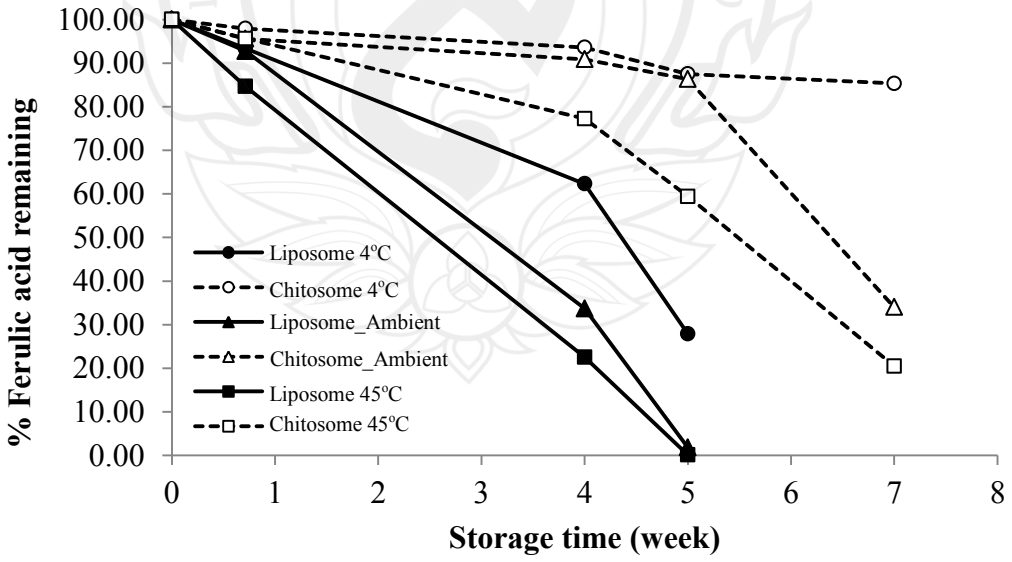


Figure 4.4 The % remaining of liposomal and chitosomal ferulic acid kept in various temperatures

4.1.1.2 Size of particles in different temperatures

There was a minor change in particle sizes among primary liposomes on day 5 with a tendency to be larger when the temperature rose. 4°C Liposome had not change within 4weeks, while the size of the other two conditions expanded. The particle proportion of all temperature chitosomes were the same at day5 and slightly transformed at week 4, but not as much as liposomes. Their particle sizes of different temperatures on day 5 and week 4 were compared in Figure 4.5.

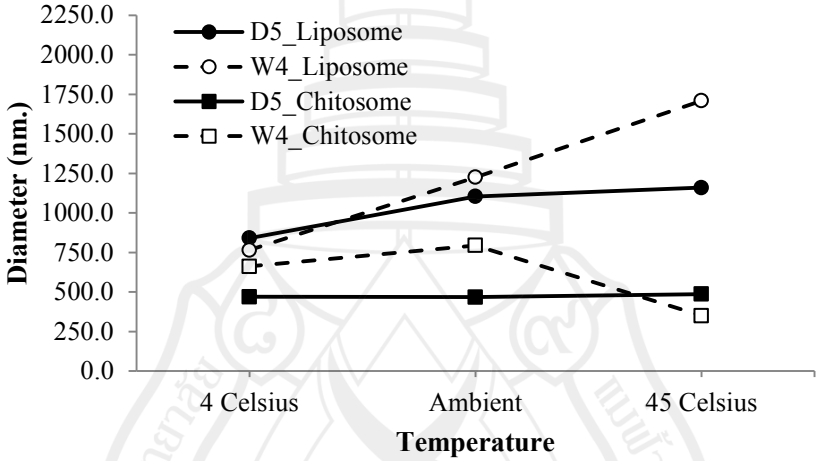


Figure 4.5 Particle size of liposomes and chitosomes kept at different temperature on day 5 and week 4

4.4.2 Stability in Different pHs

Both liposomes and chitosomes solutions had average pH around 4.5, their ferulic acid effluence under various pH conditions (5.5, 6.5, 7.5, and 8.5) were compared (Table 4.6). Since ferulic acid spectrum was changed by pH (Friedman & Jürgens, 2000), Folin-Ciocalteu method (Jadhav et al., 2012) was used to determine the external ferulic acid content in variety of pH. Liposome was unable to maintain ferulic acid within the particle when pH rose. The higher the pH, the more leakage it had. Chitosomes had better ability to maintain ferulic acid when pH increased up to 5.5, but gradually unleashed ferulic acid in higher pH. Figure 4.6 displayed the % leakage of ferulic acid from the two vesicles in various pHs.

Table 4.6 Ferulic acid content changes of liposomes and chitosomes in different pHs

pH	External ferulic acid (mg/mL)		Ferulic acid leakage (mg/mL)	
	Liposomes	Chitosomes	Liposomes	Chitosomes
4.5	34.50	36.00	0.00	0.00
5.5	42.40	36.59	7.90	0.59
6.5	47.85	40.41	13.35	4.41
7.5	52.16	45.71	17.66	10.71
8.5	76.67	51.19	42.17	15.19

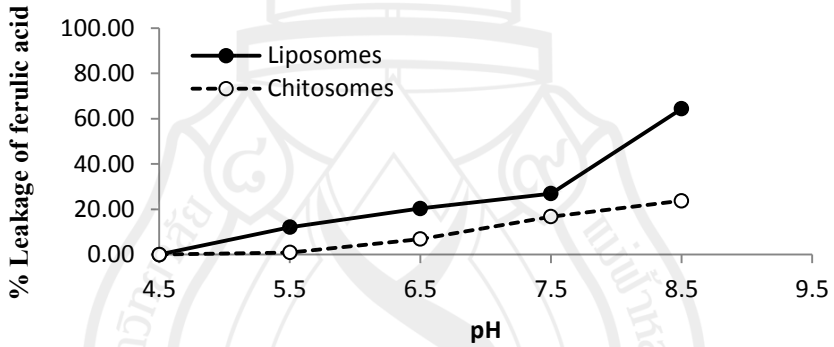


Figure 4.6 The % leakage of ferulic acid from the vesicles in various pHs

CHAPTER 5

CONCLUSION AND DISCUSSION

5.1 Preparation of Liposome and Chitosome

The varied concentration of ferulic acid loaded into the liposomal system affected the % entrapment efficiency and % recovery. Increasing of ferulic acid amount showed the augmentation of the trapped amount, yet was inconsistent with the % entrapment efficiency and % recovery. The suitable concentration with optimal entrapment efficiency of 57.90% and 98.82% recovery was 0.1% or 100mg/100ml. of ferulic acid. Sonication plays an important role in size reduction, consistency and distribution of liposome (Manosroi & Manosroi, 2007; Cho et al., 2013). Longer sonication time makes the liposome smaller, however prolongation of time also raises the system temperature and may affect the denaturation of heat labile active agent (Manosroi & Manosroi, 2007). Since the bath sonicator used had no cooling system, during the experiment of sonication time variation, the heat developed by the expanded time and reach to 65^{o}C in 60 minute model corresponding to the previous comment. The adapted technique of deionized ice addition in the sonicator tank was applied to avoid heat generation by ultrasonic wave vibration and helped controlling the system temperature (20-22^{o}C). The 20 minute sonication model with applied cooling system had 34.86% higher entrapment efficiency and also 28.51% higher recovery than the earlier system (without ice) of equal sonication time. So the optimal condition selected to use along this study was 0.1% ferulic acid loading and 30 minutes sonication under applied cooling system. This final condition increased entrapment efficiency for 10.28% comparing to original method (10 minute model with absent of ice) at the same loading concentration.

The positive charge chitosan naturally adheres to the anionic liposome surface forming soft armour wrapped around the vesicle (Sonvico et al., 2006; Bang et al., 2011). Coating with chitosan had no change in loading capacity of the liposome vesicle. The result is similar to several previous studies (Karn et al., 2011; Gibis et al., 2013).

5.2 Physical Characters of Liposome and Chitosome

The appearance of empty liposome solution was almost transparent and the cloudiness increased in liposome entrapped ferulic acid while chitosome apparently became opaque. The results were in consonance with several researches about liposomes and chitosan coated liposomes, since loaded particles were bigger than blank particles while the chitosan wrapped around liposomes and the excess chitosan were bulky, then induced light scattering so they had more turbidity (Gibis et al., 2013; Limvongsuwan, 2005).

Though liposomes and chitosomes in this study were observed by simple binocular microscope, their features at 1000X magnification (eyepiece = 10X and objective lens = 100X) were sketchily identified as spherical shape with some lamellar patterns of liposomes and some were approximately round to ellipsoidal shape. This was not distinct from other studies with TEM results (Gibis et al., 2013; Leelapornpisid, 2010; Limvongsuwan, 2005), however the accurate size could not be identified with simple microscope.

The estimated diameter of particles by micrometer in the microscope roughly displayed as nanometer, however their accurate sizes were confirmed by measurement with Zeta nanosizer. The association between sonication time and the size of liposome mentioned earlier (Manosroi & Manosroi, 2007; Cho et al., 2013) was the explanation why 30 minute sonication liposome (840.9±151.7 nm.) had 32.9% smaller than the 10 minute sonication liposome (1253.7±311.1 nm). According to the study of Gibis et al. (2013), chitosan coated around loading liposome shrink the size of liposome, but did not affect the entrapment content. The chitosome of this study was 469.6±48.4 nm in size and there was no significant different in entrapment efficiency.

5.3 Stability of Liposome and Chitosome in Different Conditions

Ferulic acid is unstable to high temperature (Fiddler et al., 1967; Wang et al., 2011), liposome in this study was able to preserve the ingredient at 4^{o}C and also ambient temperature and fairly control the content at 45^{o}C for a several days. The longer storage time reduced its capability to continue ferulic acid maintenance, especially high temperature exposure at 45^{o}C since liposome has limitation to deal with high temperature (Sulkowski et al., 2005; Sabin et al., 2006). Coating with chitosan enhanced ability to prolong the loss of active ingredient content in liposomes under high temperature and long retention period, this result was similar to several previous studies (Ngo et al., 2012; Gibis et al., 2013). On the first week ferulic acid in chitosome at all conditions were hardly changed and remaining percentage were around 96% (ambient and 45^{o}C) to 98% (4^{o}C). The chitosome in this study postponed the leakage and denaturation of ferulic acid for about a month, a week after that 4^{o}C and ambient chitosome showed an additional minor leakage for around 4-6% while the 45^{o}C ones clearly released more ferulic acid from the particle at 17.85%. Two weeks after a month the remaining ferulic acid content of 4^{o}C chitosome was 85.33% while ambient and 45^{o}C chitosomes were seriously dropped to 34.03% and 20.47%, respectively. The ferulic acid content result was complied with the average particle size that expanded in higher temperature and longer storage duration. The 2 factors represented the superiority of chitosome over liposome to prolong the stability of ferulic acid.

Liposome was unstable when pH changed. Chitosome did not change until pH was 6.5. The solubility of chitosan dropped at pH higher than 6.5 was the explanation (Leelapornpisid et al., 2010).

5.4 Conclusion

Liposome had short stability with limitation of heat and pH. Increased sonication time under cooling system improved % entrapment efficiency, % recovery and reduced its particle size. Chitosan prolonged the stability of ferulic acid in

liposome and delayed the loss of ferulic acid in high temperature. It retarded the leakage of ferulic acid in the solution with pH less than 6.5 due to the solubility property.

Some antioxidants were reported to provide skin protection via oral administration as dietary supplement and some via topical administration as drug or cosmetics. Ferulic acid is one with limitation in oral bioavailability, skin delivery is an interesting alternative route for this antioxidant (Zhang et al., 2010). The topical photoprotective efficacy and safety of ferulic acid was confirmed by several studies (Saija et al., 2000, Carlotti et al., 2008; Lin et al., 2005, Oresajo et al., 2008). The recommended dosage of ferulic acid at 0.7% in aqueous solution as topical administration of in vivo study showed a significant protection against UVB-induced erythrema (Saija et al., 2000; Tsuno Rice Fine Chemicals Co., Ltd., n.d.). Incorporation of this active ingredient in cosmetic formula was also obstructed with its unstability by pH and temperature (Wang et al., 2011). The co-project research between L'Oreal Research and Fudan University of Shanghai suggested that low pH, low temperature and using high percent of dipropylene glycol as solvent were the effective factors to ensure a better stability of ferulic acid in a cosmetic medium (Wang et al., 2011).

The key success for topical application is skin delivery, lipophilicity is a key for ferulic acid to permeate through the stratum corneum, however the permeated amount may be insufficient to trigger a pharmacological effect (Zhang et al., 2010). Liposome is a delivery system with high safety and compatibility to human skin (Sharma & Sharma, 1997). Its lipid bilayer structure imitating human skin barrier ensures effectiveness of permeation through skin and delivery of active ingredient (Torchilin, 2005). Ferulic acid in liposome with chitosan coating for better stability may be an attractive choice for topical administration. The transdermal ability, formulation application and skin tolerance to the chitosomal ferulic acid should be explored in the further study.

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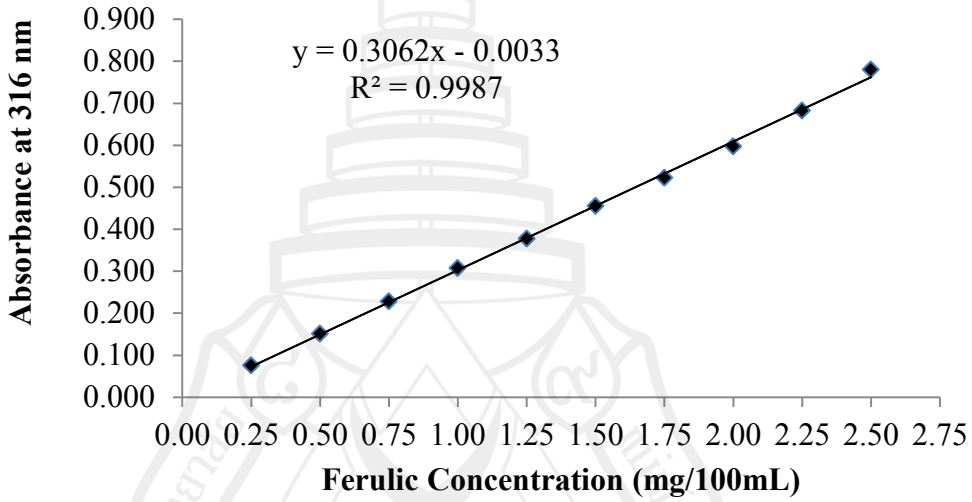
APPENDICES

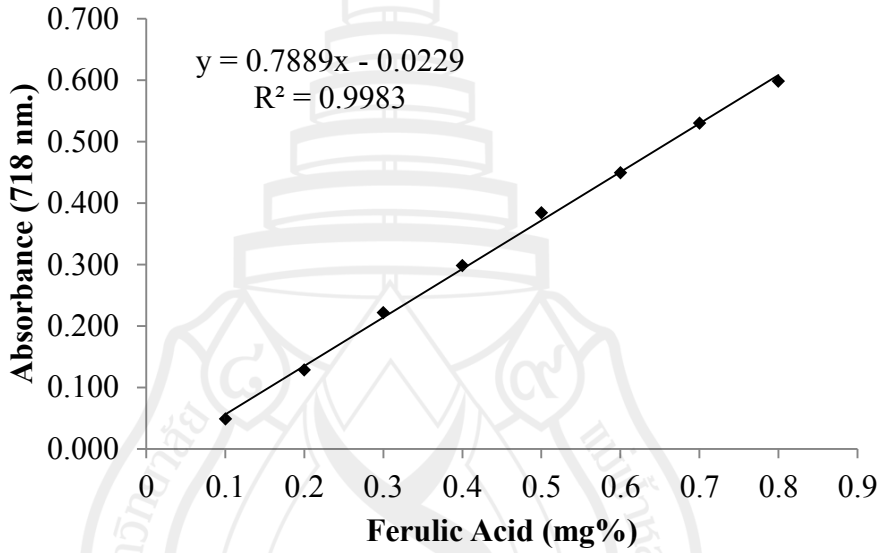
APPENDIX A

STANDARD GRAPH OF FERULIC ACID AT MAXIMUM WAVE LENGTH (316 NM)



APPENDIX B

STANDARD GRAPH OF FERULIC ACID DETERMINATION BY FOLIN-CIOCALTEU METHOD



APPENDIX C

PROCEEDING

EFFECT OF CHITOSAN ON THE STABILITY OF FERULIC ACID ENTRAPPED LIPOSOME

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ABSTRACT

This study intended to prolong the stability of liposome with chitosan. Containing positive charges from ammonium groups of its structure, chitosan can easily self-assemble with negatively charge liposome. Ferulic acid, a heat and light sensitive ingredient with several benefits for aesthetic purposes, e.g. sunscreening agent, whitening and antioxidant was chosen to be entrapped. Applied simple hand-shaking method was established with 65.52% entrapment efficiency and 94.38% yield recovery determined by spectrophotometry at maximum wavelength of 316 nm. Temperature controlling sonication system improved entrapment efficiency for 10.28%. The diameter of normal liposome significantly expanded when the temperature and storage time increased as well as the leakage of ferulic acid. Coating with chitosan prolonged the stability of ferulic acid in liposome and delayed the loss of ferulic acid in high temperature. Chitosan coated liposome had capability to maintain vesicle size and internal active ingredient content for a month.

Keywords: Chitosan, Ferulic acid, Liposome, Stability

INTRODUCTION

The emerging trend of functional products from naturally occurring plants becomes stronger and stronger with eco friendly perceptions and multifunctional benefits applied in food, drug and cosmetic industries. The worldwide growth of natural and organic cosmetic market has a rosy future (Matthews, 2013; Nichol, 2012). Phytochemicals stay in the limelight, tremendous scientific researches study about those plant derived substances and their advantages for health, wellness and beauty purposes. Ferulic acid is a ubiquitous phenolic phytochemical found in fruits, vegetables and cereal crops (Rybka, Sitarski & Raczynska-Bojanowska, 1993). It demonstrates a huge dimension of

the valuable functional effects such as antioxidant, anti-inflammatory, antimicrobial and photoprotective properties. Most of researches have been studied about its therapeutic effects against diseases (Srinivasan, Sudheer & Menon; 2007; Lin et al, 2005). Experiments applied ferulic acid for cosmetic aspect gradually increased, particularly its benefits as a sunscreen and sunscreen enhancer (Lin et al, 2005). In vitro and in vivo transdermal studies support the topical application and safety of ferulic acid however the further experiment for enhancement of its absorption is needed (Zhang et al, 2010). Ferulic acid decomposition induced by light, air, pH and temperature affected the appearance and efficacy of this substance in cosmetic formulations (Ouimet et al, 2013; Wang et al, 2011). Several experiments have been explored to stabilize the ingredient such as adding of photosensitizer like linoleic acid (Carlotti et al, 2008), insertion in vehicles like cyclodextrin (Anselmi et al, 2008), solid-lipid nanoparticle (Trombino et al, 2013) and liposome (De-Ling et al, 2007). Liposome is a remarkable delivery system with high attentions from scientists and clinicians due to its safety and compatibility with human body, but short stability (Sharma & Sharma, 1997). Chitosan is a naturally harmless compound with spontaneous ability to bind with liposome so several researchers tried to strengthen liposome and expanded its lifespan with chitosan (Gibis, Rahn & Weiss; 2013; Karn et al, 2011; Ngo et al, 2012). The effect of chitosan, a nontoxic cationic polymer, on the stability of liposome-entrapped ferulic acid is observed in this study. The loaded liposomes were coated with chitosan and monitored in different conditions compared with uncoated liposome. The encapsulation efficiency, active ingredient content, morphology, and size of liposomes are also reported.

MATERIALS

Ferulic acid was obtained from Tsuno Rice Fine Chemicals Co., Ltd, Japan. Hydrogenated soya lecithin was purchased from Nikko Chemicals, Japan. Cholesterol was ordered from Sigma-Aldrich (Thailand) Co., Ltd. Chitosan was a gift from Marine Bio Resource Co., Ltd., Thailand. Triton X-100 was from Panreac, Dow Chemical. Folin-Ciocalteu reagent, chloroform, acetic acid and other reagents and chemical substances were supplied by Merk, Ltd. (Thailand). All chemicals and reagents were used as received.

METHODOLOGY

Determination of Ferulic Acid by Spectrophotometry

Ferulic acid solution in deionized water was scanned between 200-400 nm at every single nm to identify the maximum wavelength (λ_{max}). 10 concentrations of standard ferulic acid solution (0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25 and 2.50 mg%) were prepared in volumetric flask and the absorbance was measured at the acquired λmax. The standard graph was plotted using the retrieved absorbance and concentrations.

Preparation of Liposome

Simple hand shaking technique using reduced pressured vacuum rotary evaporator to form film of hydrogenated soya lecithin and cholesterol in chloroform was adapted (Manosroi, 2013). The

concentration of ferulic acid loading solution was varied to be 0.05, 0.10, 0.20, 0.30, 0.40 and 0.50% (w/v). The sonication alternate periods were ten minute interval from 10 to 60 minutes. New sonication system with deionized ice adding in the sonicator tank was developed to avoid overheating during longer period of sonication. The best condition for entrapment will be adapted and applied along the study. Empty liposome was assembled with the identical method at the chosen sonication pace, yet using deionized water in place of ferulic acid solution.

Preparation of Chitosan Coated Liposome (Chitosome)

0.1% (w/w) chitosan in 1% (v/v) acetic acid was processed and adjusted pH to 5.5 with 1N NaOH and kept at 4°C. Primary liposome (5 ml) was continuously dropped to chitosan solution (5 ml) while gently agitated with magnetic stirrer (Leelapornpisid et al, 2010; Karn et al, 2011).

Entrapment Efficiency and Recovery Calculation

The absorbance of liposome was measured at the selected λmax against empty liposomes to determine the external (untrapped) ferulic acid reckoning from standard graph. Triton-X 100 (1%) was added ahead to each sample at the same amount then sonicated for 2 minutes to open vesicles for total bulk of internal and external ferulic acid mixture before identification of total (trapped + untrapped) ferulic acid by spectrophotometry. Internal (trapped) ferulic acid content was calculated by subtraction of external amount from total bulk (Qin et al, 2008). Repeated the same method was applied for chitosomes and empty chitosome was used as blank.

$$\%\boldsymbol{Entrapment\ Efficiency} = \frac{(\boldsymbol{A_{actual} - A_{exterior)}})}{\boldsymbol{A_{initial}}}\ \boldsymbol{X\ 100}$$

$$\%\ \boldsymbol{Recovery} = \frac{\boldsymbol{A_{actual}}}{\boldsymbol{A_{initial}}}\ \boldsymbol{X\ 100}$$

$A_{exterior}$: the content of untrapped ferulic acid remained in the external phase

A_{actual}: the sum of untrapped ferulic acid and the trapped ferulic acid obtained by breaking of liposomal system

$A_{initial}$: the initial content of ferulic acid loaded in the system

Physical Characterization of Liposome and Chitosome

The appearance of the prepared vesicles was investigated by naked eyes. Morphology was examined under simple binocular microscope. Zetasizer (Nano ZS90, Malvern Instruments, England) was used to measure diameter of each sample to identified average particle size.

Stability Study

Liposomes and chitosomes were kept in various temperatures (4±2°C, ambient temperature and 45±2°C); their particle size and ferulic acid contents were monitored on different periods. Stability of liposomes was also checked at discriminative pH values, i.e., 5.5, 6.5, 7.5, and 8.5 by comparing of their remained ferulic acid content with Folin-Ciocalteu method at 718 nm (Jadhav et al, 2012).

RESULTS AND DISCUSSION

Determination of Ferulic Acid by Spectrophotometry

The ferulic acid scanning pattern was a broad spectrum curve covering especially from 280-340 nm (Figure1) which conforms to sun screening ability of the ingredient against UVA and UVB (Carlotti, 2008; Oresajo et al, 2008). The λ_{max} is 316 nm. The standard graph is showed in Figure 2.

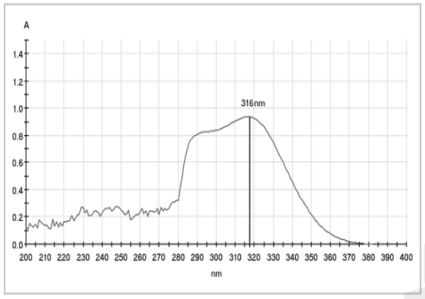


Figure 1 Absorption chromatogram of ferulic acid

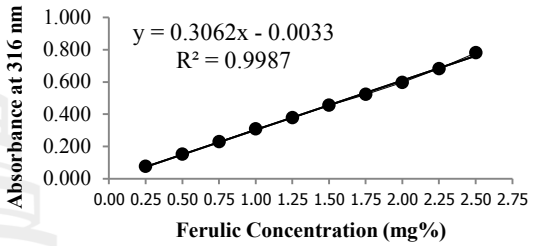


Figure 2 Standard curve of ferulic acid.

Liposome Preparation

The varied concentration of ferulic acid loaded into the liposomal system affected the % entrapment efficiency and % recovery. Increasing of ferulic acid amount showed the augmentation of the trapped amount, yet was inconsistent with the % entrapment efficiency and % recovery. The suitable concentration with optimal entrapment efficiency of 57.90% and 98.82% recovery was 0.1% or 100mg/100ml of ferulic acid (Table1). Sonication plays an important role in size reduction, consistency and distribution of liposome (Manosroi & Manosroi, 2007; Cho et al, 2013). Longer sonication time makes the liposome smaller; however prolongation of time also raises the system temperature and may affect the denaturation of heat labile active agent (Manosroi & Manosroi, 2007). In order to minimize the particle size, the sonication time was varied from the original one of Manosroi (2013). Temperature of the sonication tank after 10 minute sonication period was 25^{o}C, but it climbed up to be 32 ^{o}C, 40 ^{o}C , 49 ^{o}C, 59 ^{o}C and 65 ^{o}C when the period was extended to 20, 30, 40, 50 and 60 minutes, respectively. Ice bath used to control temperature at 20-22^{o}C. The final protocol of 0.1% ferulic acid loading amount and 30 minute sonication time with cooling bath was selected after varied loading amount and sonicated duration. This developed procedure provided 65.52% entrapment efficiency and 94.38% yield recovery which was 10.28% higher than the original method (Table 2).

To prove the benefit of cooling sonication system, liposome preparations (20 minute sonication) with ice and without ice during sonication were compared. The cooling system had 57.33% entrapment efficiency and 97.08% recovery while % entrapment and recovery of that without ice were 22.47% and 68.57%, respectively.

Table 1 Entrapment efficiency and recovery of liposomes with different ferulic acid loading amount

Ferulic acid content (mg/100mL)				% Entrapment Efficiency	% Recovery
Initial Load	Untrapped	Untrapped + Trapped)	Trapped		
50.00	23.05	34.28	11.23	22.47	68.57
100.00	40.92	98.82	57.90	57.90	98.82
200.00	48.14	154.90	106.75	53.38	77.45
300.00	14.28	260.34	117.54	39.18	86.78
400.00	21.26	356.82	144.19	36.05	89.20
500.00	14.75	396.00	248.51	49.70	79.20

Table 2 Comparison of liposomes with various sonication times

Sonication time (min)	Ferulic acid content (mg/100ml)			% Entrapment Efficiency	% Recovery
	Initial Load	Untrapped	Untrapped +Trapped		
10	100.00	44.45	99.69	55.24	99.69
20	100.00	39.75	97.08	57.33	97.08
30	100.00	28.86	94.38	65.52	94.38
40	100.00	40.27	93.25	52.98	93.25
50	100.00	57.12	93.25	36.13%	93.34
60	100.00	52.24	93.60	41.36%	93.60

Chitosan Coated Liposome (Chitosome)

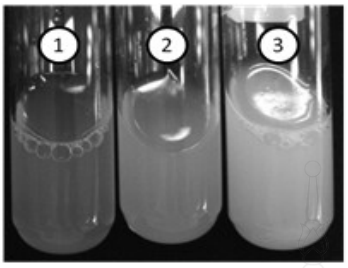
Chitosan was soluble in acid condition, 0.1% acetic acid was used as solvent before adjusted to proper pH with 1N NaOH (Leelapornpisid et al, 2010). The chitosan: liposome ratio was 1:1 (v/v) (Karn et al, 2011). The chitosome had 64.12% entrapment efficiency and 93.42% recovery. Coating with chitosan showed non-relative change in loading capacity of the liposomal vesicle, the result is similar to several previous studies (Karn et al, 2011; Gibis et al, 2013).

Physical Appearance and Morphology

The appearance of empty liposome solution was almost transparent and the cloudiness increased in liposome entrapped ferulic acid while chitosome apparently became opaque (Figure 3). The results were in consonance with several researches about liposomes and chitosan coated liposomes, since loaded particles were bigger than blank particles while the chitosan wrapped around liposomes and the excess chitosan were bulky, then induced light scattering so they had more turbidity (Gibis et al, 2013; Limvongsuwan, 2005).

Figure 4 demonstrated the spherical or ellipsoidal shape of multi-vesicular liposome loaded ferulic acid and its chitosan coated nanoparticles (chitosomes) using 100X objective lens. The differentiation of each particle in various temperatures and times by microscopic inspection was

difficult to examine empirically. The results of particle size shift and change in ferulic acid content in the next part were done in parallel showed clearer evidences.



① = Empty Liposome
② = Ferulic Acid Loaded Liposome
③ = Ferulic Acid Loaded Chitosome

Figure 3 Appearance of empty liposome, loaded liposome and chitosome

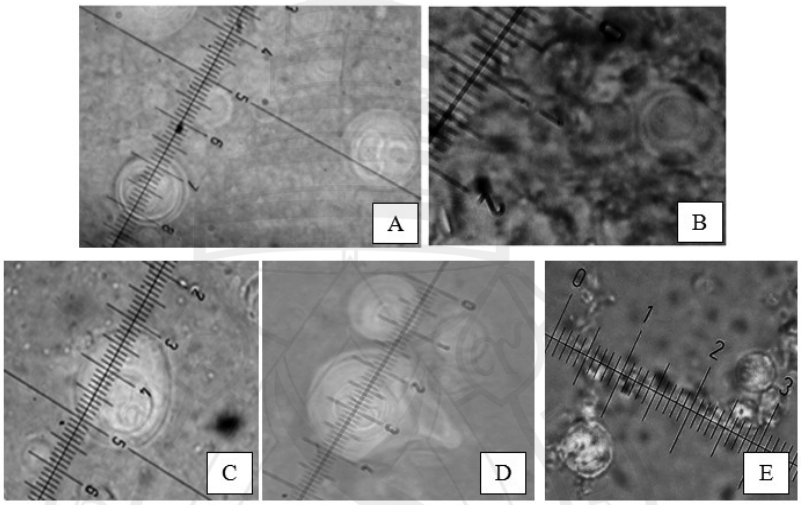


Figure 4 Microscopic images of liposomes and chitosomes (x1000)
A: Fresh liposomes B: Fresh chitosomes C: Wk3 liposomes, ambient
D: Wk3 liposomes; 45^{o}C E: Wk3 chitosomes; ambient

The estimated diameter of particles by micrometer in the microscope roughly displayed as nanometer, however their accurate sizes were confirmed by measurement with Zetasizer (Nano ZS90, Malvern Instruments, England). The association between sonication time and the size of liposome mentioned earlier (Manosroi & Manosroi, 2007; Cho et al, 2013) was the explanation why 30 minute sonication liposome (840.9±151.7 nm) had 32.9% smaller than the 10 minute sonication liposome (1253.7±311.1 nm). According to the study of Gibis et al (2013), chitosan coated liposome has smaller size than primary liposome, but did not affect the entrapment content. The chitosome of this study was 469.6± 48.4 nm in size and there was no significant different in entrapment efficiency.

Stability Study

Ferulic acid is unstable to high temperature (Fiddler, Parker, Wasserman, & Doerr; 1967), liposome in this study was able to preserve the ingredient at 4^{o}C and also ambient temperature (24±3^{o}C; winter season) and fairly control the content at 45^{o}C for a several days (Figure5). The longer

storage time reduced its capability to continue ferulic acid maintenance, especially high temperature exposure at 45°C since liposome has limitation to deal with high temperature (Sulkowski, Pentak, Korus & Sulkowska, 2005). Coating with chitosan enhanced ability to prolong the loss of active ingredient content in liposomes under high temperature and long retention period (Ngo et al, 2012; Gibis et al, 2013). On the first week ferulic acid in chitosomes at all conditions were hardly changed (Figure 6) and remaining percentage were around 96% - 98%. The chitosome in this study postponed the leakage and denaturation of ferulic acid for about a month, a week after that 4°C and ambient chitosome showed an additional minor leakage for around 4-6% while the 45°C ones clearly released more ferulic acid from the particle at 17.85%. Two weeks after a month the remaining ferulic acid content of 4°C chitosome was 85.33% while ambient and 45°C chitosomes were seriously dropped to 34.03% and 20.47%, respectively. The ferulic acid content result was complied with the average particle size that expanded in higher temperature and longer storage duration.

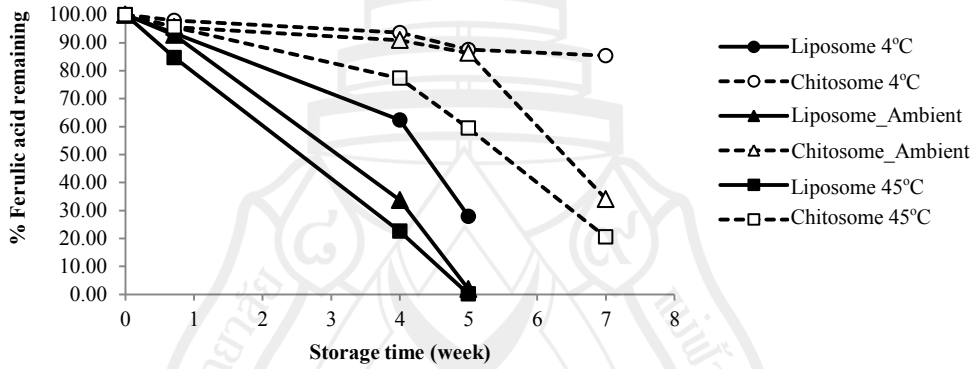


Figure 5 The % remaining of liposomal and chitosomal ferulic acid kept in various temperatures

There was a minor change in particle sizes among primary liposomes on day 5 with a tendency to be larger when the temperature rose. 4°C liposome had not change within 4weeks, while the size of the other two conditions expanded. The particle proportion of all temperature chitosomes were the same at day5 and slightly transformed at week 4, but not as much as liposomes. Their particle sizes of different temperatures on day 5 and week 4 were compared in Figure6. The result complied with the outcome of ferulic acid content remaining in the vesicles.

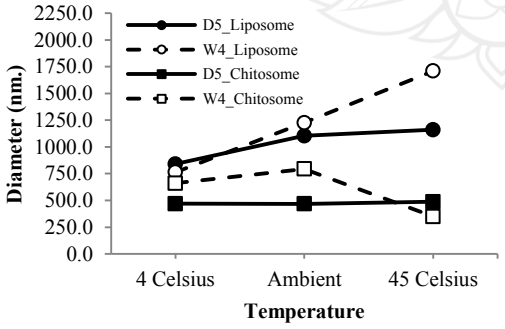


Figure 6 Particle size of liposomes and chitosomes kept at different temperature on day 5 and week 4

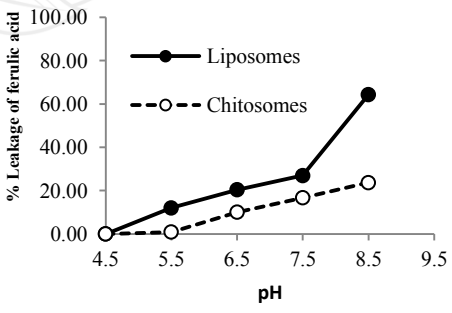


Figure 7 The % leakage of ferulic acid from the vesicles in various pHs

Since ferulic acid spectrum was changed by pH (Friedman & Jürgens, 2000), Folin-Ciocalteu method (Jadhav, Kareparamban, Nikam & Kadam, 2012) was used to determine the external ferulic acid content in variety of pH. Both liposomes and chitosomes solutions had average pH around 4.5, their remaining ferulic acid content under various pH conditions (5.5, 6.5, 7.5, and 8.5) were compared (Figure 7). Liposome was unable to maintain ferulic acid within the particle when pH rose. The higher the pH, the more leakage it had. Chitosomes had better ability to maintain ferulic acid when pH increased up to 5.5, but gradually unleashed ferulic acid in higher pH. The solubility of chitosan dropped at pH higher than 6.5 may be the explanation (Leelapornpisid et al, 2010).

CONCLUSION

Liposome had short life stability with limitation of heat and pH. Increased sonication time under cooling system improve % entrapment efficiency and reduced its particle size. Chitosan prolonged the stability of ferulic acid in liposome and delayed the loss of ferulic acid in high temperature. It retarded the leakage of ferulic acid in the solution with pH less than 6.5 due to the solubility property.

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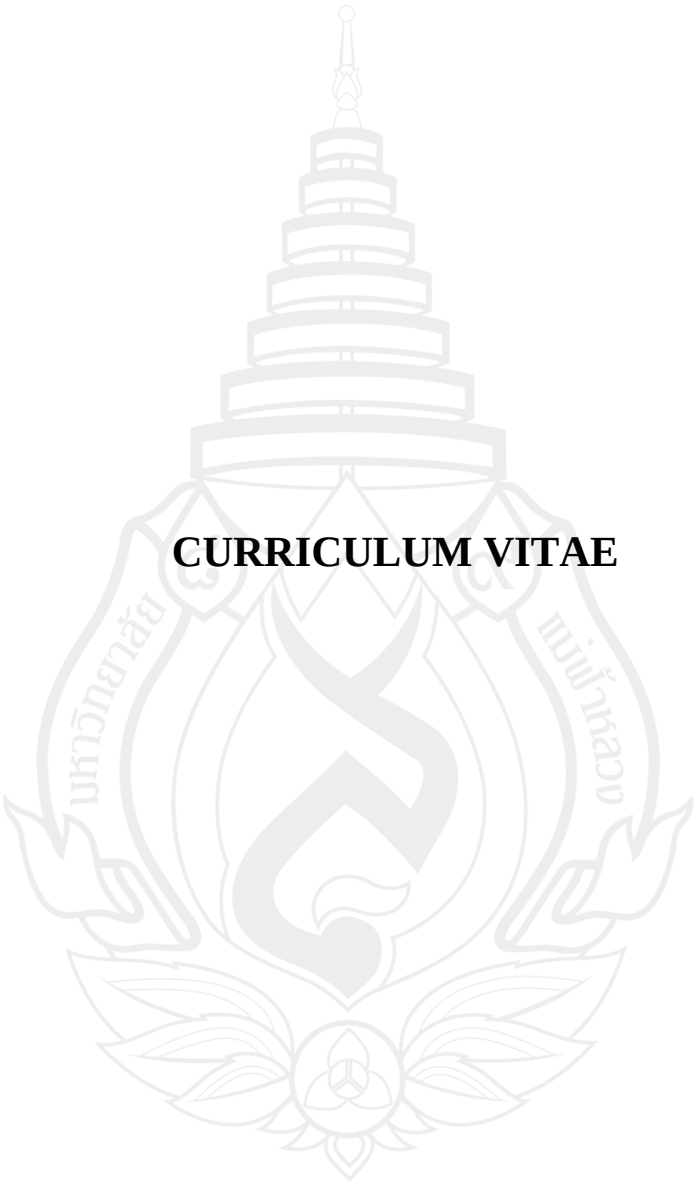

CURRICULUM VITAE

CURRICULUM VITAE

NAME	Mrs. Tuangthip Pongprayoon
DATE OF BIRTH	28 February 1970
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EDUCATIONAL BACKGROUND

1997	Master of Sciences Biotechnology, Faculty of Sciences Mahidol University, Thailand
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WORK EXPERIENCE

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