CHAPTER IV

RESULTS and DISCUSSION

Green tea extraction

The methods of green tea extraction were reported in several studies. Hot water extraction method was mostly used to extract the catechins from green tea leaves [3, 25, 27, 50]. The hot water extraction was easy to perform and gave high yield of the extract. However, the high impurity of green tea extract and the lowest reducing power among the extracts from different methods were obtained [3, 25]. Furthermore, the solvent removal was a problem of this method. In this study, the polyphenols in green tea were selectively extracted using several solvent partitioning steps. Firstly, green tea leaves were extracted with hot water because catechins compounds are the most water soluble substance. The appropriate temperature of hot water was 80 °C because it gave the highest extraction efficiency and the lowest degradation [25, 26]. Although some pigments were removed from the extract by chloroform but the final extract was still brownish. The extract should be kept in dry and cool place to prevent the degradation of active compound. The yield of this method was 17.17% of dried green tea leaves which has correlated with the result of previous report [3].

Determination of antioxidant activity

Several reports confirmed that catechins in green tea had the highest antioxidant activity comparing to vitamin C and E [5]. However, the antioxidant activity of green tea extract depended on the process of cultivation, harvesting, preparation and extraction. The determination of antioxidant activity in this study was indicated by percentage of radical scavenging activity of DPPH and IC_{50} value measured by spectrophotometric assay. Trolox was used as a positive standard control. The IC_{50} value of green tea extract (4.280 ± 0.12 ppm) was not significantly different from Trolox (4.420 ± 1.03 ppm) which means that their antioxidant activities were in the same level (*P values* < 0.05). These

values were calculated from the graph plotted between percent scavenging activity and log of concentrations (Figure 13).

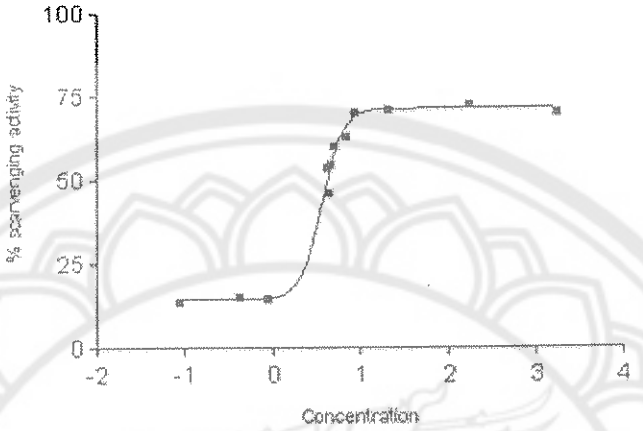


Figure 13 The representative dose-response curve of green tea extract analyzed by DPPH free radical scavenging assay.

HPLC analysis of the extract

The concentrations of catechins in green tea extract were determined by HPLC analysis method. The results are shown in Table 6. The chromatogram of catechins standard and extract are shown in Figures 14 and 15, respectively.

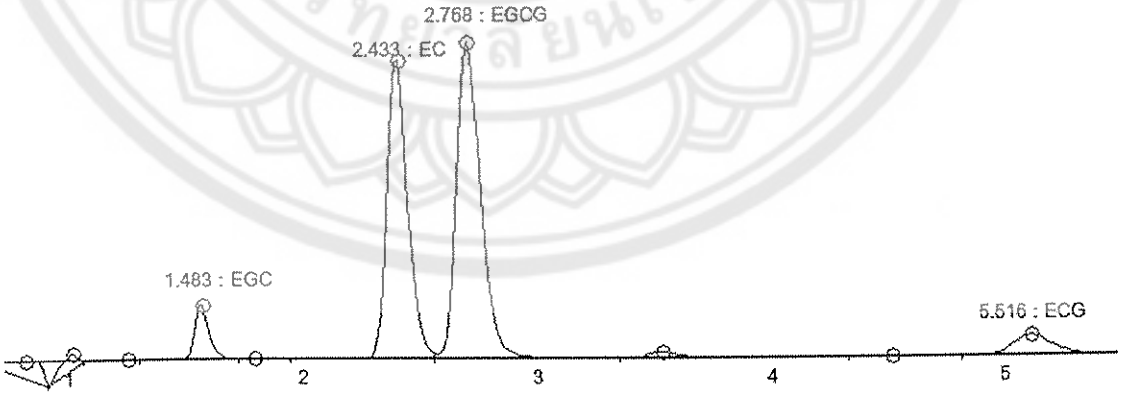


Figure 14 The HPLC chromatogram of a mixture of EGC (15.62µg/ml), EC (156.25µg/ml), EGCG (15.62µg/ml) and ECG (15.62µg/ml). The chromatogram was detected at 210 nm. Flow rate used was 2 min/ml. The injection volume was 20 µl.

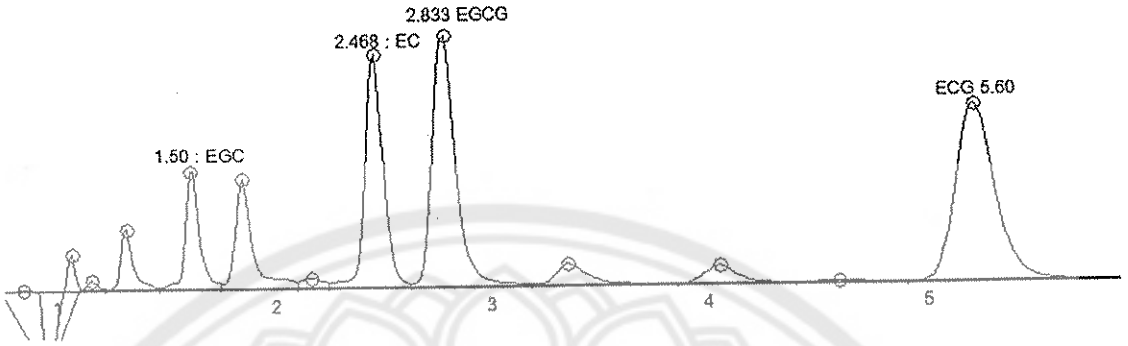


Figure 15 The HPLC chromatograms of green tea extract (0.166 mg/ml). The chromatographic conditions are described under Materials and Methods. The chromatogram was detected at 210 nm. Flow rate used was 2 min/ml. The injection volume was 20 µl.

The previous attempt to separate the catechins compounds in green tea extract by using HPLC method with isocratic elution resulted in the long retention time and peak tailing [51]. The method in this study provided much simple sample preparation and quick analysis of green tea extract.

From the chromatogram, the sequences of peaks are EGC, EC, EGCG and ECG, respectively. The separation of EC and EGCG was difficult to achieve and required a meticulous assessment of all relevant separation parameters such as column temperature and pH of mobile phase.

Method of validation

Validity of an analytical method can be verified by establishing several analytical and statistic parameters. The calibration data of the analyses of the standard polyphenols in green tea are shown in Table 3. Linearity of the detector response for all variable standards was obtained in the ranges of 62.50 – 1.95 µg/ml for EC and EGCG and 25.00 – 0.19 µg/ml for EGC and ECG. The calibration curves are shown in Figures 16, 17, 18 and 19.

The detection limit (determined at signal to noise ratio of three) of EGC, EC, EGCG and ECG were 1.50, 0.59, 0.23 and 0.38 µg/L, respectively. Furthermore, the limit of quantification (determined at signal to noise ratio of ten) of EGC, EC, EGCG and ECG were 97.00, 3.80, 1.50 and 6.10 µg/L.

Table 3 Calibration data of standard catechin compounds in the HPLC method used. The details of the HPLC conditions are described in research methodology.

Compound	Regression equation	R^2	LOD(µg/L)	LOQ(µg/L)
EGC	y = 148129x + 21.237	0.9997	1.50	97.00
EC	y = 115549x + 56.953	0.9997	0.59	3.80
EGCG	y = 142974x - 104.740	1.0000	0.23	1.50
ECG	y = 153930x + 8.0415	0.9998	0.38	6.10

y: reflect the peak area; x: the amount of compound(mg/ml); R^2: correlation coefficient; LOD: limit of detection; LOQ: limit of quatification.

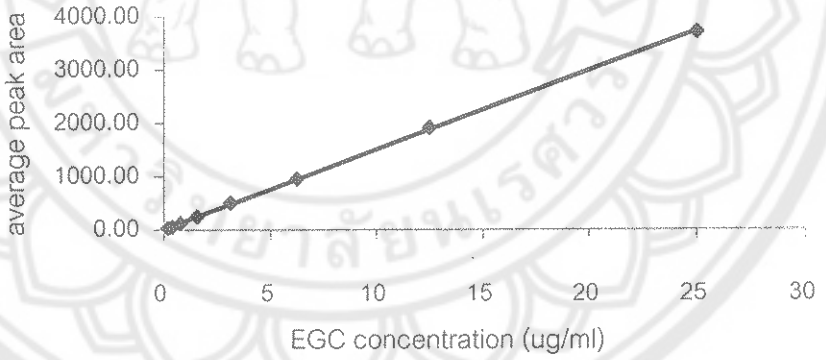


Figure 16 Calibration curve of EGC

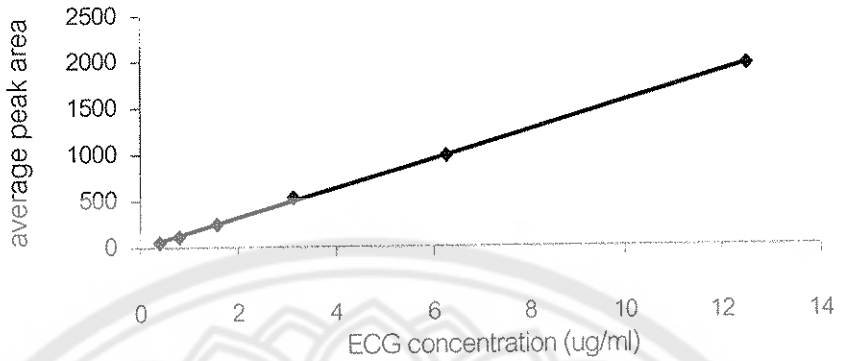


Figure 17 Calibration curve of ECG

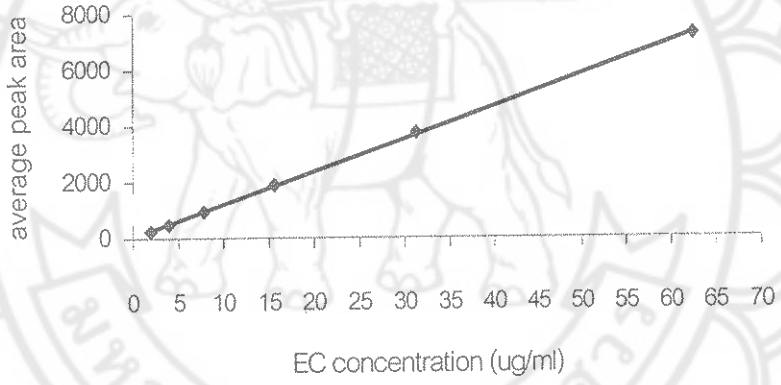


Figure 18 Calibration curve of EC

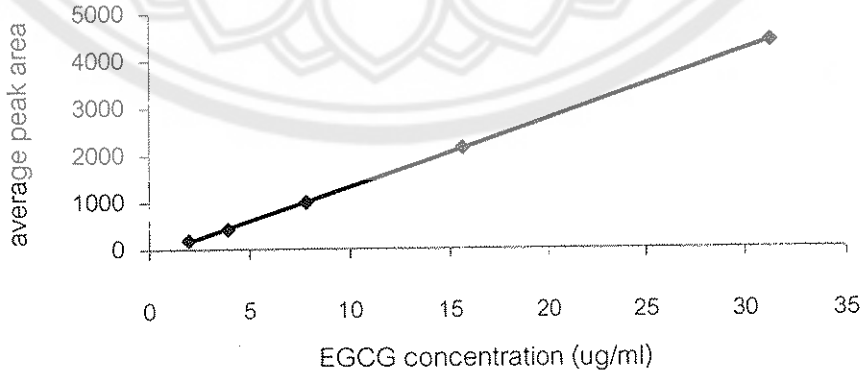


Figure 19 Calibration curve of EGCG

Accuracy of analytical method was determined by spiking sample of green tea extract with known amount of standard catechin compounds. The results are shown in Table 4. The result indicated that this method had high efficiency. The accuracy values were in the range of 85-115 % of the theoretical concentrations.

The inter-day precision and reproducibility of HPLC analysis were obtained from the determination of 5 dilutions of green tea extract for 3 consecutive days. The intra-day precision was calculated from the catechins concentration and their deviation values. The variation of intra and inter day assay were calculated as %RSD. All % RSD values obtained were less than 10 indicating that this method had high precision. The precision data were shown in Table 5.

Table 4 Accuracy data of the HPLC analyses of catechins in green tea extract. The details of the HPLC conditions are described in Research Methodology.

Sample	% Accuracy (n=3)			
	EGC	EC	EGCG	ECG
1	95.30 ± 3.33	99.58 ± 2.58	112.14 ± 4.12	99.41 ± 3.19
2	100.07 ± 0.78	106.21 ± 5.12	106.40 ± 3.36	108.84 ± 0.81
3	90.90 ± 0.49	95.91 ± 1.36	105.47 ± 4.16	97.90 ± 3.64

Concentrations of EGC and ECG in sample 1, 2 and 3 were 2.5 µg/ml, 1.25 µg/ml and 0.5 µg/ml, respectively; concentration of EGCG and EC in samples 1, 2 and 3 were 25 µg/ml, 12.5 µg/ml and 5 µg/ml, respectively

Table 5 Intra and inter-day precision of the HPLC analyses of catechins in green tea extract. The details of the HPLC conditions are described in Research.

Sample	Intra-day* (n=5)			Inter-day* (n=3)
	Day 1	Day 2	Day3	
EGC				
1	37.38 (0.98)	35.29 (1.32)	35.09 (3.55)	35.92 (3.52)
2	18.10 (1.39)	18.16 (4.60)	17.26 (4.21)	17.84 (2.82)
3	7.80 (1.08)	8.86 (2.73)	9.02 (4.32)	8.56 (7.76)
4	3.73 (2.29)	4.32 (1.83)	3.95 (6.88)	4.00 (7.47)
5	1.88 (1.96)	1.84 (3.10)	1.78 (6.37)	1.83 (2.74)
EC				
1	103.33 (0.99)	102.51 (2.98)	96.91 (4.15)	100.92 (3.46)
2	53.03 (0.63)	52.29 (3.87)	48.74 (4.79)	51.36 (4.46)
3	26.29 (1.14)	26.15 (1.86)	24.86 (5.66)	25.77 (3.07)
4	12.87 (5.00)	13.20 (6.12)	11.48 (4.85)	12.51 (7.29)
5	6.00 (1.01)	5.58 (5.03)	5.16 (1.75)	5.58 (7.47)
EGCG				
1	129.30 (1.55)	126.67 (2.00)	126.95 (3.67)	127.64 (1.13)
2	65.48 (1.14)	66.51 (3.49)	64.63 (3.96)	65.54 (1.44)
3	30.82 (7.52)	33.45 (0.44)	34.14 (6.55)	32.80 (5.35)
4	15.42 (1.27)	18.21 (3.53)	17.35 (5.01)	16.99 (8.41)
5	8.36 (6.26)	9.28 (2.41)	9.29 (3.92)	8.98 (5.93)
ECG				
1	133.58 (3.51)	131.63 (1.80)	130.40 (4.81)	131.87 (1.21)
2	67.97 (1.56)	64.60 (6.44)	66.50 (5.37)	66.36 (2.54)
3	32.75 (1.26)	33.50 (1.24)	33.92 (6.55)	33.39 (1.78)
4	15.67 (2.08)	16.97 (6.17)	15.93 (5.48)	16.19 (4.24)
5	8.35 (1.13)	8.09 (1.34)	7.79 (4.66)	8.07 (3.47)

*Values are concentration of catechins in green tea extract (mg/100 g); % relative standard deviations (% RSD) are given in parentheses.

The results of the quantitative analysis of catechins compound in green tea extract are summarized in Table 6. The results showed that the major catechins in green tea extract were ECG (13.69 ± 3.51 %), EGCG (13.10 ± 1.55 %) and EC (10.71 ± 0.99 %). The catechins contents in green tea extract depend on extraction method, temperature controlling and raw material used. Some reports showed a very high ratio of EGCG in green tea extract when using only hot water to extract green tea leaves [3]. However, using only water for extraction is not our preference because water could be removed difficultly from the extract.

Table 6 Percent content of catechins compound in green tea extract (expressed as mean ± standard deviation of triplicate analysis)

Catechins	%Content in green tea extract 100 mg
Epigallo catechin (EGC)	3.57 ± 0.98
Epicatechin (EC)	10.71 ± 0.99
Epigallo catechin gallate (EGCG)	13.10 ± 1.55
Epicatechin gallate (ECG)	13.69 ± 3.51

Preparation of chitosan microparticles

The solvent evaporation method was used to prepare microparticles because this method is an effective technique for encapsulation hydrophilic compounds into microparticles, and catechins in green tea extract are the highly water soluble compounds. The high encapsulated efficacy microparticles of diameters less than 5 μm were obtained. However, the properties of microparticles depended on the several influence parameters in preparing procedure as follow:

Table 7 Average diameter (mean ± SD) and polydispersity of chitosan microparticle produced by various conditions

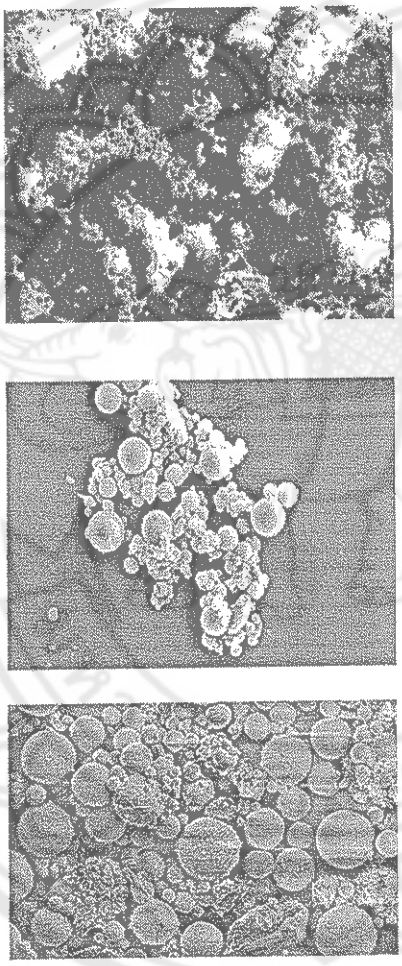
Preparing conditions				Mean diameter (μm)	Polydispersity
Chitosan (%)	TPP (%)	Speed of homogenizer (rpm)	Acetone (%)		
2	2	3000	50	NP	NP
2	6	3000	50	1.55 ± 0.05	0.344
2	12	3000	50	1.63 ± 0.05	0.324
1	12	3000	50	NP	NP
2	12	3000	50	1.63 ± 0.05	0.324
4	12	3000	50	4.18 ± 0.26	1.451
2	12	1000	50	2.54 ± 1.17	0.369
2	12	2000	50	1.90 ± 0.18	0.327
2	12	3000	50	1.63 ± 0.05	0.409
2	12	3000	50	1.61 ± 0.05	0.311
2	12	3000	75	1.66 ± 0.06	0.380

Note: NP = No Particle

The effect of chitosan concentration

Several reports indicated that the concentration of polymer affected the size and particle forming [36, 52-54]. In this study, chitosan particles produced with different chitosan concentrations had different sizes. The particles produced by using 2% and 4% chitosan concentration had mean diameter of 1.63 ± 0.05 μm and 4.18 ± 0.26 μm, respectively. There is no chitosan particle observed when using 1% chitosan concentration. This low chitosan concentration may not enough to provide a suitable viscosity for particle forming [52, 53, 55]. Furthermore, particles obtained from different chitosan concentrations had not only different in size but also in polydispersion indexes. Polydispersity value indicated the particle size distribution. The greater the polydispersity value, the broader the size distribution. The particles produced using 2% chitosan had

lower polydispersity value (0.324) than those using 4% chitosan (1.451). Therefore, particles obtained by preparing from 2% chitosan had more homogeneity in size than those from 4% chitosan. Sizes and polydispersity of particles obtained from various chitosan concentrations are shown in Table 7. Figure 20 shows electron micrographs of chitosan particles prepared from various chitosan concentration.



(a) 1% chitosan concentration

(b) 2% chitosan concentration

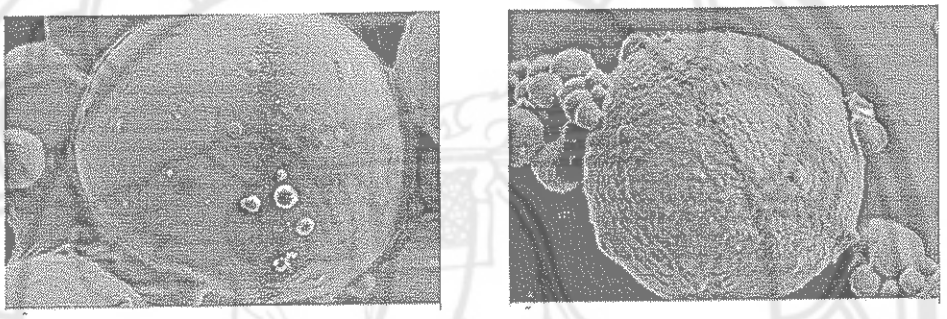
(c) 4% chitosan concentration

Figure 20 Electron micrographs of microparticles forming with various chitosan concentrations

The effect of tripolyphosphate concentration

Different concentrations of 2%, 6% and 12% tripolyphosphate (TPP) were used in this study. All other parameter in preparing procedure including chitosan concentration, homogenizer speed and acetone concentration were fixed at 2%, 3,000 rpm and 50%

respectively. Morphology of microparticles preparing with various TPP concentrations were observed using Scanning Electron Microscope and the particle sizes were measured by Brookhaven ZetaPALS® (Zeta Potential and Particle Size Analyzer). Particle was unable to be obtained with 2% TPP. The particles were detected when TPP concentrations of 6% and 12% were used. The particles morphology formed by using 6% and 12% TPP was similar in shape and size. However, the surface of particles produced using 6% TPP was smooth, but that of 12% TPP was rough. Morphologies of particle surfaces preparing with 6% TPP and 12% TPP were shown in Figure 21



(a) (b)

Figure21 Electron micrographs of chitosan particles preparing with (a) 6% TPP; (b) 12%TPP

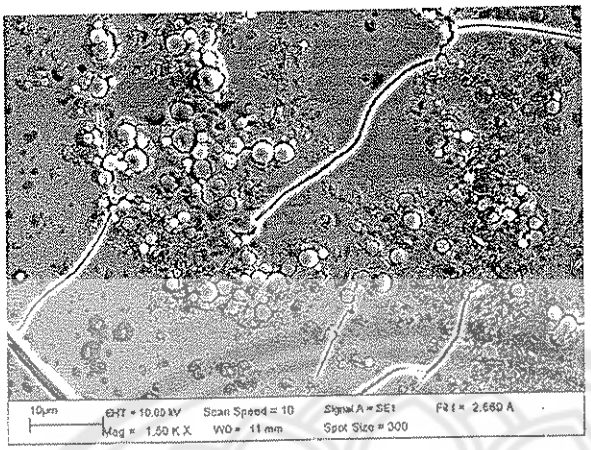
Although the different TPP concentration produced the different surface morphology of particle but the measurement of particle size of both 6% and 12% showed similar result, those particles had similar size as well as narrow size distribution. Those results were indicated by particle size measurement and polydispersity values as shown in Table 7.

The effect of homogenizer speed

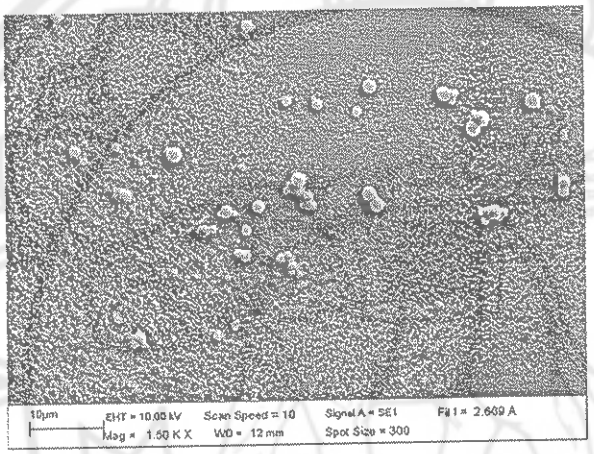
Solvent evaporation is one of many methods that produced particles by emulsification technique. Thus, the particle size depended on the speed of homogenizer or stirrer [47]. In this study, the homogenizer speed was varied at 1000, 2000 and 3000 rpm. All other parameters were fixed at the following condition: 2% chitosan concentration, 12% TPP concentration and 50% acetone concentration. Particle

morphology was observed under scanning electron microscope and particle size was determined using Brookhaven ZetaPALS®. Shape of particles produced using all homogenizing speeds was spherical. However, particles produced at 1000 rpm homogenizing speed were bigger than those obtained with 2000 and 3000 rpm. The size and polydispersity of particles prepared using various homogenizer speeds were shown in Table 7.

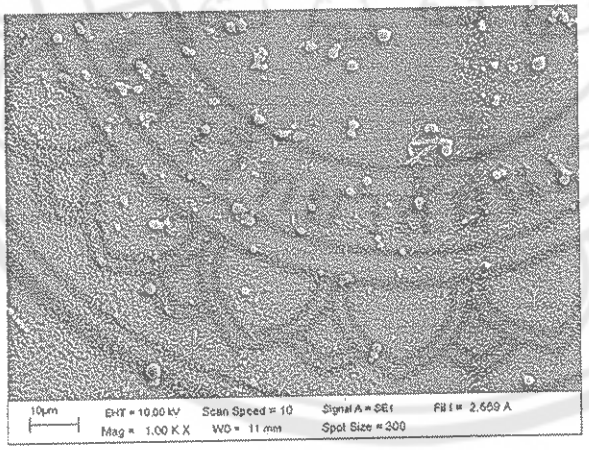
The particle produced from high speed of homogenizer had not only smaller in size but also lower in polydispersity values. Although the small size with narrow polydispersity is a desirable outcome for the particle preparation, but the higher the homogenizer speed, the hotter the temperature was generated. Thus, the high homogenizer speed was unsuitable for susceptible substances, especially catechins from green tea. Therefore, the optimum speed was 2000 rpm because at this speed smaller particles were produced with minimum heat generation. Figure 22 shows electron micrographs of chitosan particles preparing with various homogenizer speed.



a) 1000 rpm



b) 2000 rpm

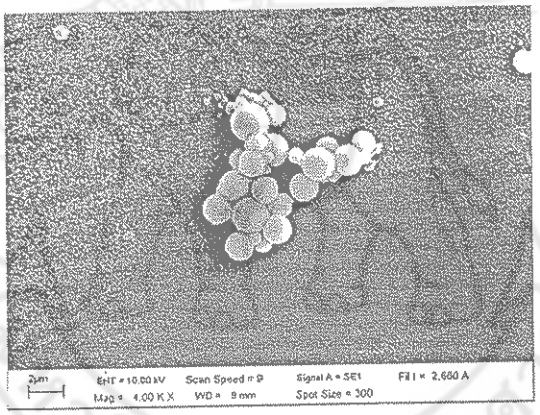


(c) 3000 rpm

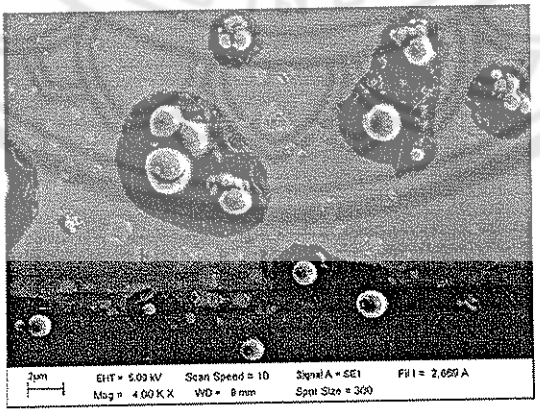
Figure 22 Electron micrographs of chitosan particles preparing with various homogenizer speed

The effect of organic solvent concentrations

Recent study indicated that size of microparticles was decreased when using high concentration of organic solvent [42, 54]. In current study, acetone was mixed with acetic acid for increasing the evaporation of water phase that lead to the particle forming. Two different acetone concentrations of 50% and 75% were used. The particles were observed for their morphology under scanning electron microscope and determined their size by Brookhaven ZetaPALS®. The particles obtained from both concentrations were spherical in shape and shown in Figure 23. The size of particles obtained with 50% and 75% acetone were 1.61 ± 0.05 µm and 1.66 ± 0.06 µm respectively. The size and polydispersity of particles prepared using various acetone concentrations were shown in Table 7.



(a) 50% of acetone



(b) 75% of acetone

Figure 23 Electron micrographs of chitosan particles preparing with (a) 50% acetone and (b) 75% acetone

The data from the studies regarding effect of preparing parameters on particle properties and forming indicated that the proper concentration of chitosan was 2%. At this chitosan concentration, smaller particles were obtained. All tested homogenizer speeds have no effect on particle size. However, homogenizer speed of 2000 rpm was appropriate for particle preparation since less heat was generated during the process. The selection of TPP concentration was 6% because the particles with smooth surface and smaller size were obtained. The last parameter was acetone concentration, 50% of acetone was suitable for particle preparation due to the toxicity consideration of organic solvent.

The results on all preparing parameters affected on particle forming indicated that the optimum condition was 2% chitosan concentration, 6% TPP concentration and 50% acetone with 2,000 rpm homogenizing speed. Figure 24 and 25 showed size distribution with polydispersity value and morphology of chitosan particles prepared at this condition.

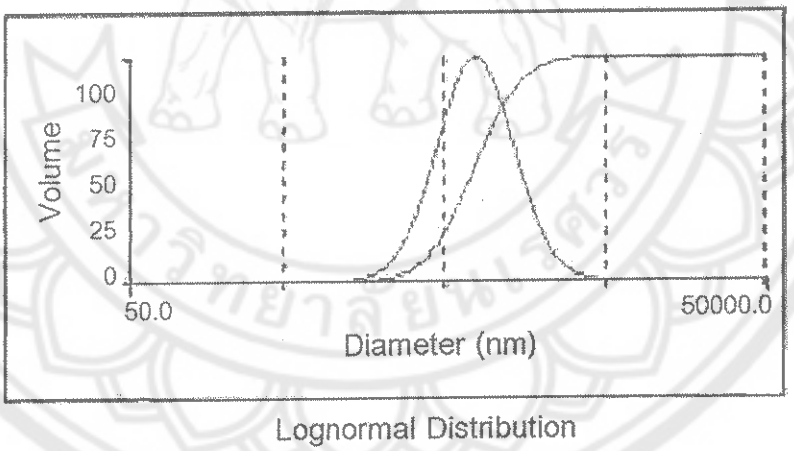


Figure 24 Size distribution and polydispersity value of particles prepared at optimum condition.

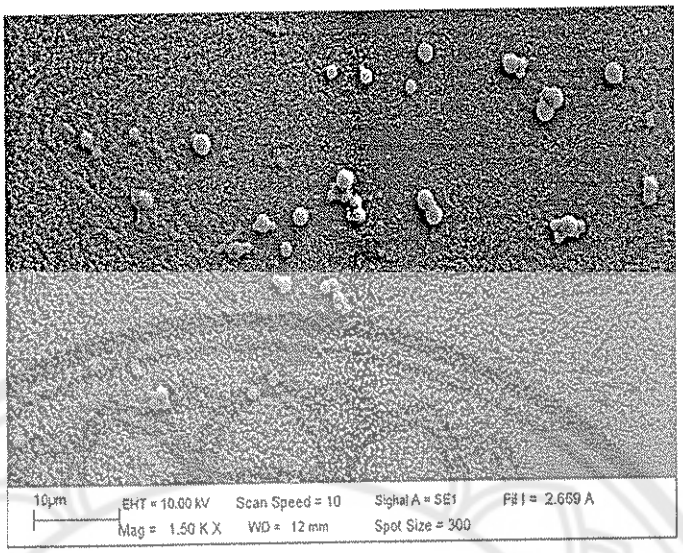


Figure 25 Electron micrograph of chitosan particles prepared at optimum condition.

The particles prepared at optimum condition had mean diameter of 1.66 ± 0.06 µm with polydispersity of 0.311. This information indicated that the particles prepared at this specific condition had small size and good homogeneity.

Entrapment efficiency

The amount of tea catechins entrapped in chitosan particles that were produced by predetermined conditions were detected using acid extraction following by HPLC analysis.

Table 8 Entrapment of tea catechins (expressed as percent of loading amount ± SD) in chitosan particles from various preparing conditions.

Loaded green tea (%)	DC5225C® (%)	Entrapment efficiency (%)			
		EGC	EC	EGCG	ECG
10	5.0	3.46 ± 0.97	17.18 ± 1.38	29.40 ± 3.49	49.41 ± 8.18
10	2.5	3.70 ± 0.55	16.13 ± 3.08	31.53 ± 5.14	40.92 ± 9.50
5	5.0	4.35 ± 0.99	28.86 ± 6.65	37.60 ± 4.39	69.69 ± 4.48
5	2.5	2.73 ± 1.57	24.30 ± 0.55	34.32 ± 2.34	61.14 ± 10.47

The particles were produced with chitosan 2%, 2000 rpm of homogenizer speed, and 50% of acetone

Table 9 Entrapped catechins to chitosan weight ratios of microparticles prepared in various conditions.

Catechins	5% of green tea		10% of green tea	
	DC5225C®		DC5225C®	
	2.5%	5.0%	2.5%	5.0%
EGC	0.00005	0.00008	0.00013	0.00010
EC	0.00130	0.00155	0.00173	0.00184
EGCG	0.00220	0.00246	0.00413	0.00385
ECG	0.00420	0.00477	0.00569	0.00676

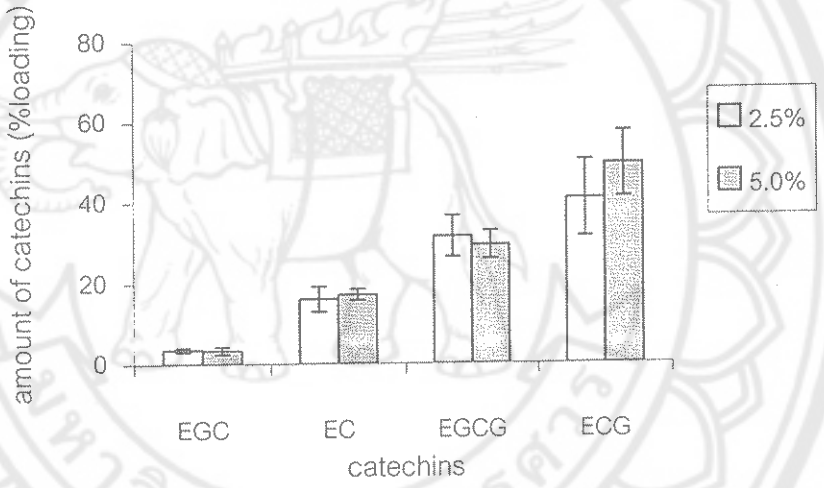


Figure 26 Comparison profiles of tea catechins entrapment (expressed as percent of loading amount) in chitosan microparticles prepared using 2.5% and 5.0% DC5225C® emulsifier with 10% green tea extract.

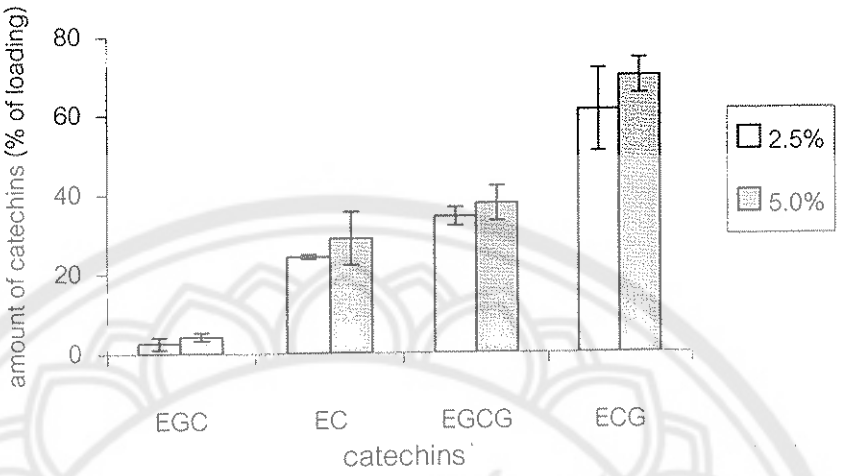


Figure 27 Comparison profiles of tea catechins entrapment (expressed as percent of loading amount) in chitosan microparticles prepared using 2.5% and 5.0% DC5225C® emulsifier with 5% green tea extract.

Entrapment of tea catechins in chitosan microparticles from various preparing conditions is shown in Table 8. The percents of tea catechins entrapped in chitosan particles to catechins loading amount decreased with increasing green tea loading. In contrary, the percents of tea catechins entrapped in chitosan particles to catechins loading amount seemed to increase with increasing emulsifier, DC5225C®. The entrapped tea catechins to chitosan ratios are shown in Table 9. The data indicated that the amount of tea catechins entrapped in chitosan particles actually increased with increasing loaded green tea extract in the preparing procedure. However, when the tea extract was loaded at 10 % which was two times higher than that at 5 %, the entrapped tea catechins were increase mere up to 40%. This was the reason why the percents of tea catechins entrapped in chitosan particles to catechins loading amount decreased with increasing green tea loading be observed. The highest entrapment was observed with ECG. All other catechins were entrapped less than that of ECG. One possible reason to explain the differences in increasing degree of each tea catechins entrapment was the different degradation kinetic among those catechins. Another influence

parameter that affected tea catechins entrapment in chitosan particles was the concentration of emulsifier [56]. This dues to the changing in catechins solubility proportion in silicone phase [56]. The solubility of catechins in silicone phase might be changed when using the different emulsifier concentration, then the changing in catechins entrapment was obtained. In this study, 2.5% of DC5225C was the optimum concentration because the particles with high entrapment were obtained. Both 2.5% and 5% of DC5225C® produced the highest entrapments with no statistically difference (*P values* < 0.05) between them.

The degradation of catechins during microparticle preparation was shown in Figure 28. EGC, EGCG and EC were less stable than ECG because their instability was induced by autoxidation, hydrolysis and epimerization reactions. these degradation reactions were accelerated by high pH and temperature [11, 57-59]. Heat was released from homogenizer during the microparticle preparation processes. The degradation of each catechins during chitosan microparticle preparation was different. In consequence, the particle encapsulation efficiency was also different. Thus, EGC, EC and EGCG as in Table 8 were lowered entrapment because of their degradation during microparticle preparation.

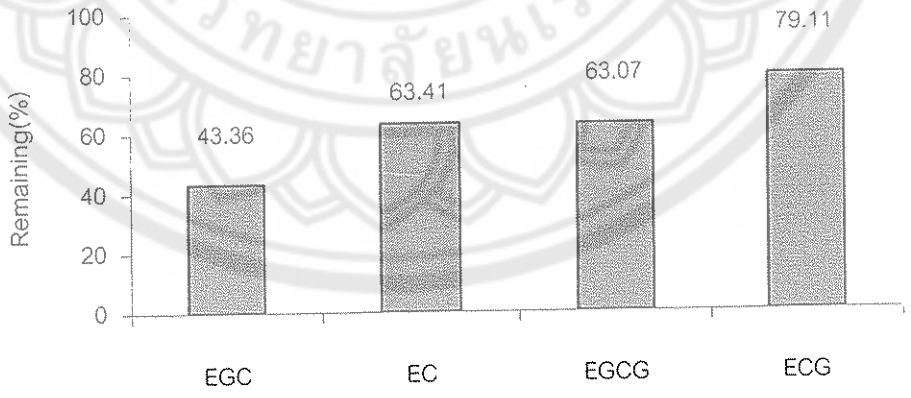


Figure 28 Percent remaining of each catechins after processing under the same condition of chitosan microparticle preparation but without DC345® and DC5225C®

In vitro release study

The release studies of chitosan particle were done in solutions with various pH. The pH of solutions was selected at 2, 5.5 and 7.4. An aqueous solution was used as dispersion medium for chitosan-green tea particles. The catechins released from particle were detected and reported as the plots between percentage of released catechins and time in Figures 29-31. The stability profiles of unentrapped catechins in solutions of tea extract were shown in Figures 32-34.

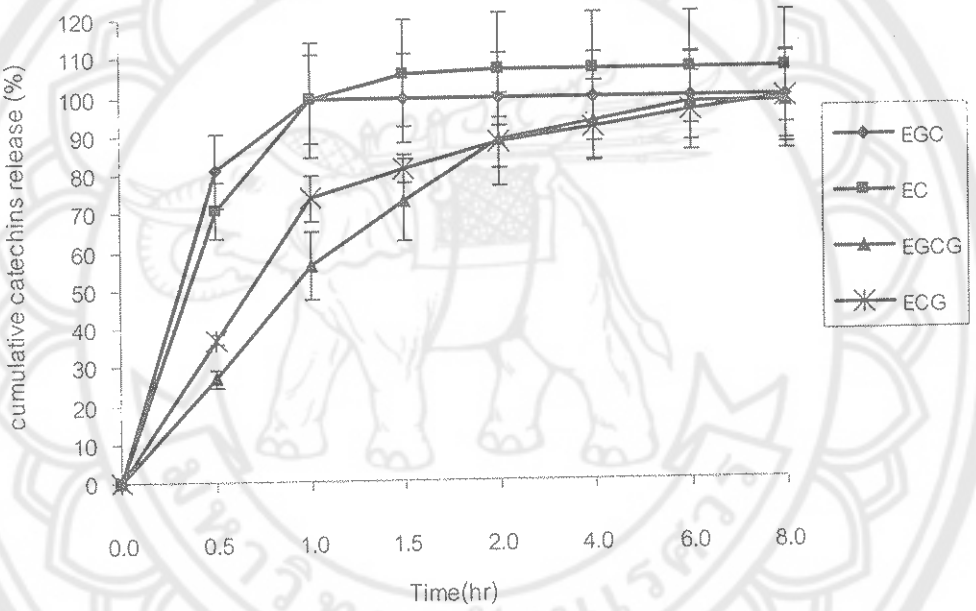


Figure 29 Release profiles of tea catechins from chitosan particles in solution pH2

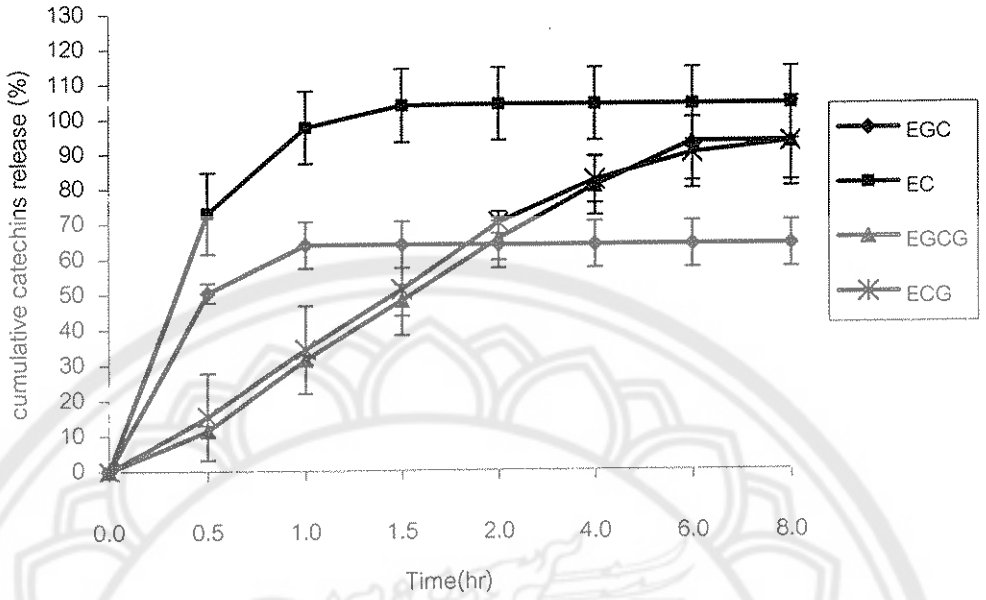


Figure 30 Release profiles of tea catechins from chitosan particles in solution pH5.5

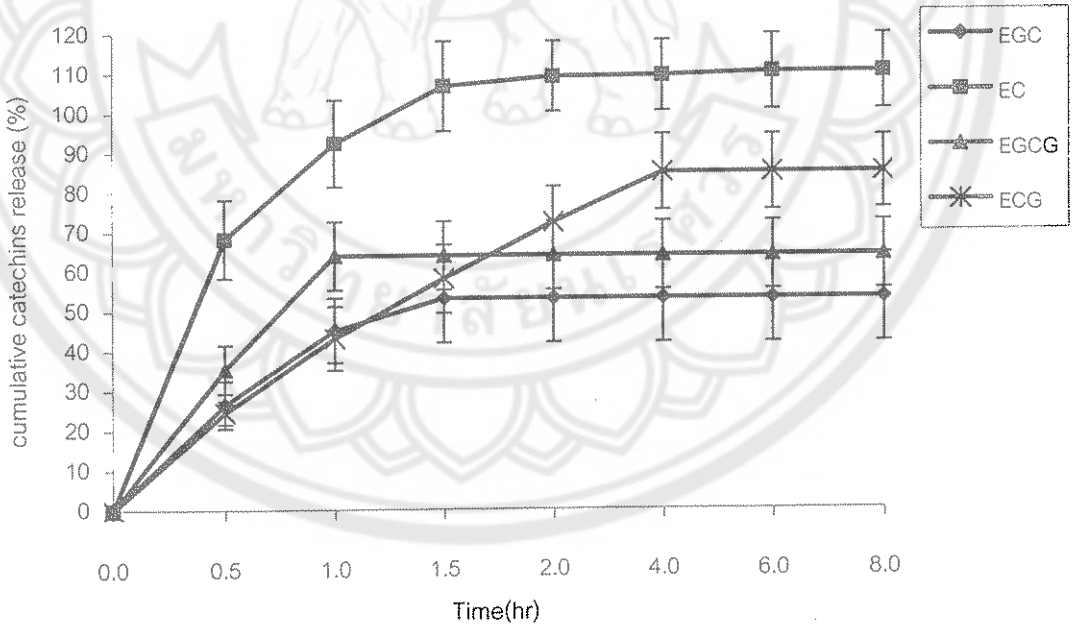


Figure 31 Release profiles of tea catechins from chitosan particles in solution pH7.4

Several reports confirmed that the releasing of substances from chitosan cross-linked with TPP particles were different in various pH solution [37, 40]. From the Figures

29-31, the catechins may be released from the chitosan particles in a wide range of pH but with different profiles. The maximum release of catechins appeared to be at pH 2 but the minimum release occurred at pH 7. These could possible be explained by their degradation kinetics and release mechanisms. In a strong acidic condition, chitosan microparticles erosion in consequence catechins releasing were happened because the chitosan particle can be dissolved and eroded in acid solution. From Figure 29, EGC and EC were released from chitosan particles rapidly with the same release pattern. They were released almost completely within 2 hours. EGCG and ECG were released from particles slowly than previous mentioned catechins. They release profile seemed to be at steady state at about 6 hours.

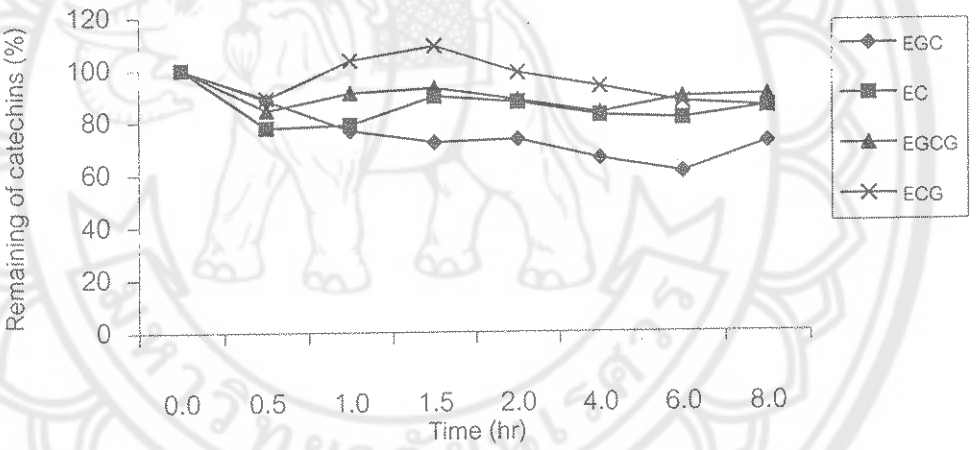


Figure 32 Stability profiles of unentrapped catechins in buffer pH 5.5

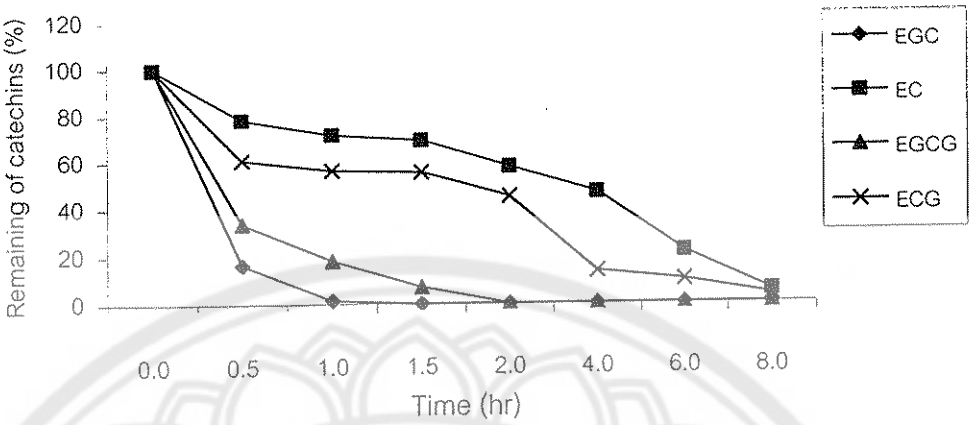


Figure 33 Stability profiles of unentrapped catechins in buffer pH 7.4

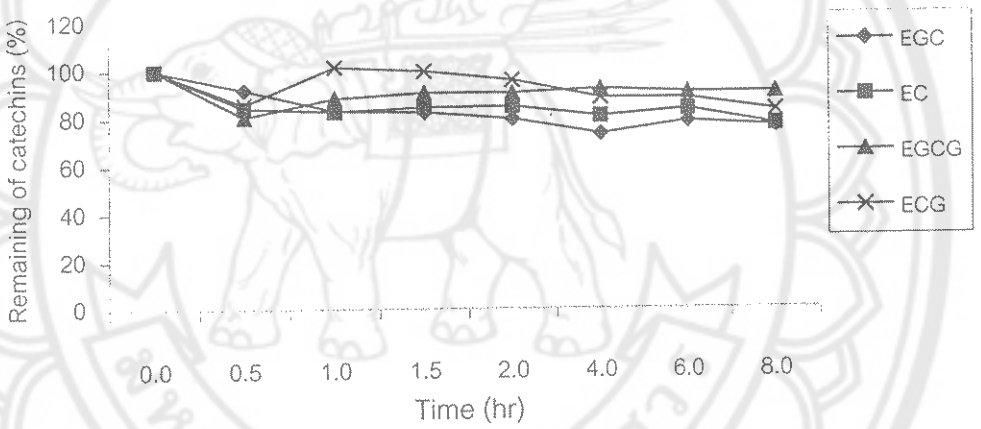


Figure 34 Stability profiles of unentrapped catechins in buffer pH 2

The releases of each catechins from chitosan microparticles in pH 5.5 were different from each other because their chemical properties and mechanism of diffusion were not similar. EGC and EC were released from chitosan particles rapidly and constantly within 1.5 hours. However EGC was released only about 60% because part of it might be degraded between samples collecting times. Some study confirmed that EGC was degraded faster than the others under the same condition [11, 58]. EGCG and ECG were released from chitosan microparticles gradually and constantly within 6 hours. Their release patterns were different from those of EC and EGC because their molecules

contained gallate group that affect their chemical property. However, most catechins were released from chitosan microparticles completely within 6 hours except EGC.

The release of EC at pH 7.4 was rapidly and completely within 1.5 hours. The release of EGC and EGCG were less than 70% at this pH. The reason for decreasing of EGC and EGCG was their degradation. Several reports confirmed that EGC and EGCG were more degraded than EC and ECG in neutral solution [11, 58, 60]. Furthermore, EC was stable than EGC under neutral condition [58] Thus, the release of EC in this study was observed more than that of ECG although its entrapment was less than ECG.

The release profiles of each catechins indicated that EC and EGC were released rapidly in neutral solution than ECG and EGCG because the catechins with gallate group in B ring less diffused out of particles in neutral solution than the ones without gallate group [26]. The gallate group causes increasing in molecular weigh that may hinder its mobility in neutral solution. Therefore, ECG and EGCG were released from chitosan microparticles gradually while EC and EGC were released more rapidly. However, the catechins release profiles also depended upon pH. The released of catechins from chitosan particulate systems involves three different mechanisms: (a) release from the surface of particle, (b) diffusion through the swollen matrix and (c) release due to polymer erosion. In majority of case, the release follows more than one type of mechanism. In case of release from the surface, adsorbed drug instantaneously dissolves when it comes in contact with the release medium. Drug entrapped in the surface layer of particles also follows this mechanism. This type of drug release leads to burst effect [36]. Drug release by diffusion involves three steps. First, water penetrates into particle system, which causes swelling of the matrix. Secondly, the conversion of solid polymer into rubbery matrix takes place, while the third step is the diffusion of drug from the swollen rubbery matrix. Hence, the release is slow initially and becomes fast later [36]. Thus, all tea catechins might be released with the diffusion mechanism at neutral pH.

Moreover, the stability of each catechins under neutral condition was different. EC and ECG were more stable than EGC and EGCG because the three vicinal hydroxyl groups at positions 3', 4' and 5' in EGCG and EGC being more vulnerable to destruction than the two vicinal hydroxyl groups at positions 3' and 4' in ECG and EC [11]. This reason affects the release of each catechins in neutral solution differently. The stability results of each catechins under various pH conditions were shown in Figures 32-34.

Stability of chitosan-green tea microparticles

The stability testing was done under various temperatures and pH conditions because the hydrolysis and autoxidation reaction were accelerated by those parameters [10]. The chitosan-green tea particles were dispersed in selected solution pH 5.5, 7.4 and 9 and incubated at 45°C, 65°C and 80°C for 24 hours. The solution of green tea extract was used as a control. The results of these studies were shown in Figures 31-33.

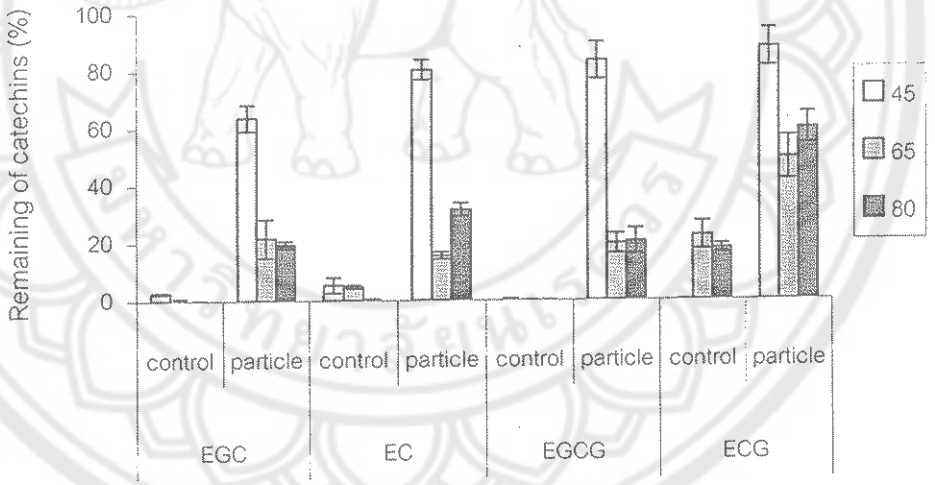


Figure 35 Comparison of stability of unentrapped and entrapped catechins in chitosan particle in various temperatures at pH 5.5

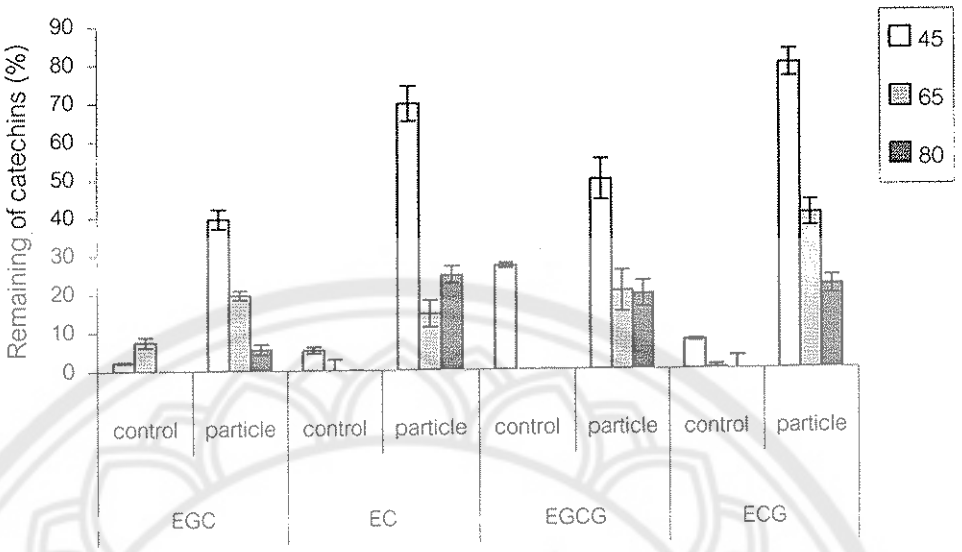


Figure 36 Comparison of stability of unentrapped and entrapped catechins in chitosan particle in various temperatures at pH 7.4

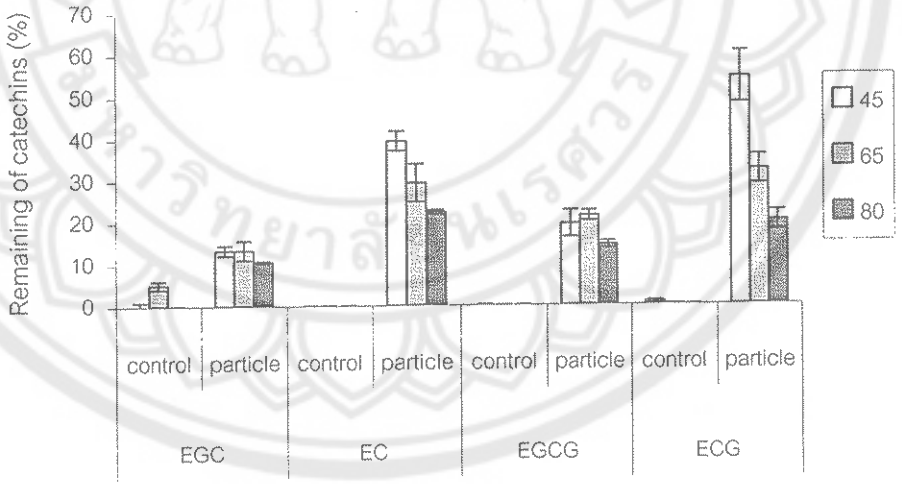


Figure 37 Comparison of stability of unentrapped and entrapped catechins in chitosan particle in various temperatures at pH 9

Generally, catechins were more degraded in high temperature and high pH. Some report shown that catechins were degraded more than 50% within 5 hour in both high pH and high temperature [11]. From the Figures 31-33, unentrapped catechins from green tea extract were degraded completely in every test temperature and pH including in acidic solution. Chitosan-green tea microparticles were more stable in pH 5 at 45 °C. However, the remaining percent was still less than 80. Furthermore, the catechins that was entrapped in particles seem to more degrade in higher temperature. The remaining of catechins in particles that were incubated in high temperature was less than 40%. However, the stability of catechins entrapped in chitosan microparticles is far better than that of unentrapped catechins.

The instability of tea catechins in chitosan microparticle caused from the water from buffer solution that can penetrate into chitosan microparticles [36] and induced the autoxidation reaction. Moreover, this reaction was accelerated by high temperature [11, 26, 60].

Photo-stability of chitosan-green tea microparticle

There were reports confirmed that autoxidation reaction of catechins in green tea was accelerated by light and heat [10]. Chitosan-green tea particles were dispersed in solution pH 5.5 and exposed to light for 24 hours. The comparative study was done between light and dark conditions. Green tea extract was used as control. The studies were tested in both powder and dispersion forms. The degradation of catechins in light exposure was occurred in both powder and dispersion forms but was lowered in chitosan-green tea microparticles. The comparison between powder and dispersion forms showed that the powder form was more stable to light exposure than dispersion form because the autoxidation reaction from water content was not occurred in powder form.

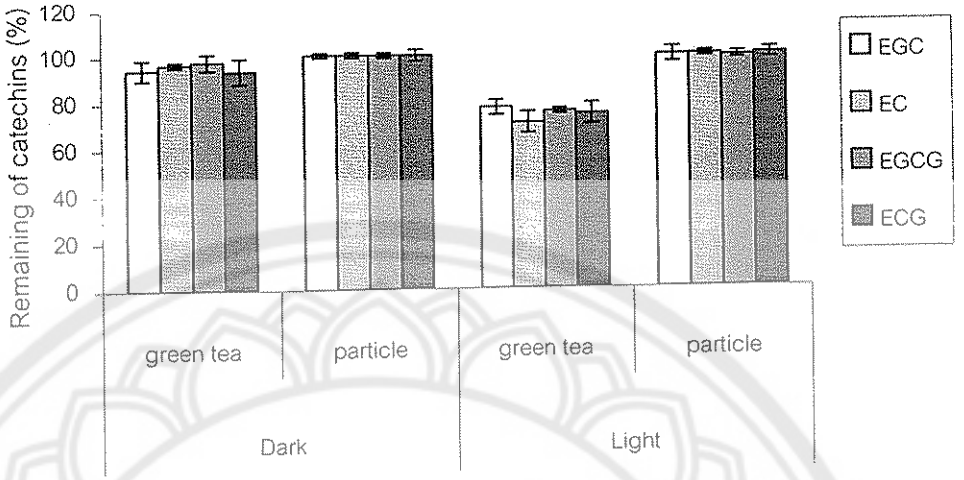


Figure 38 Comparison of photo-stability of unentrapped and entrapped catechins in chitosan microparticle in powder form.

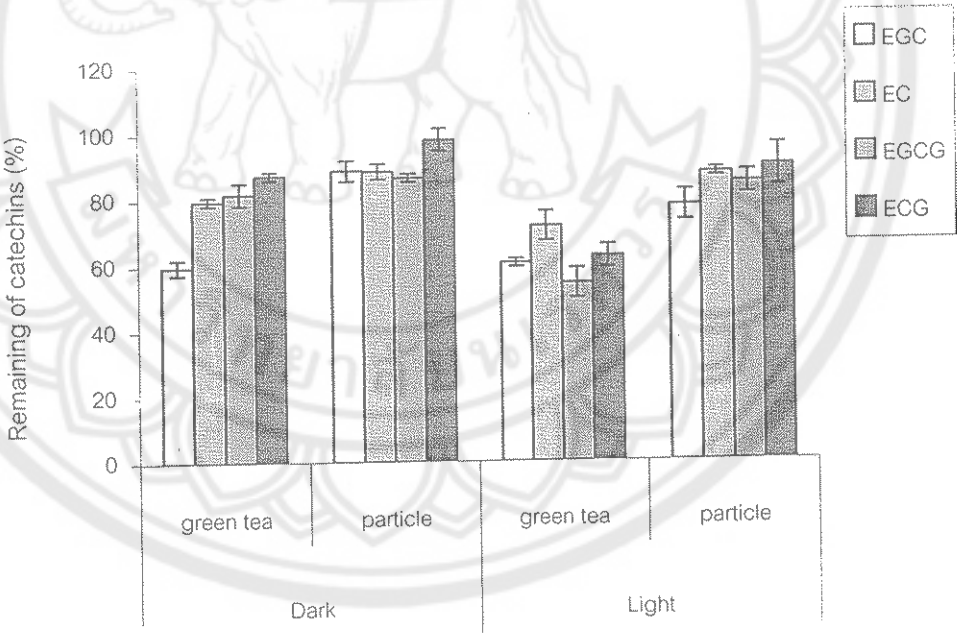


Figure 39 Comparison of photo-stability of unentrapped and entrapped catechins in chitosan microparticle in solution form

Chitosan microparticle could protect catechins from light. However, the particle stability was still susceptible when contacted with water because water could penetrate into particle and induced the autoxidation reaction.