CHAPTER IV
RESULTS AND DISCUSSION

1. Preliminary study of rosmarinic acid encapsulation

1.1 HPLC analysis and method validation of rosmarinic acid

The chromatographic separation was performed on a Cosmosil® C18 column using the mobile phase consisted of methanol and water at the ratio 35:65 in 0.1% v/v of acetic acid. Under the chromatographic conditions described, the rosmarinic acid was well dissolved in the solution and eluted at 29 min (see appendices). No interfering peaks of the polymer DG, AG and mannitol were found at the retention time of standard rosmarinic acid.

1.1.1 Linearity

Calibration curves were constructed using six series of rosmarinic acid spiked at concentration levels in the range of 5-200 µg/mL. A linearity was obtained between the peak area versus concentration added, as shown in calibration curve plotted in Figure 19. The linearity of the calibration curve is validated by the high value of the correlation coefficient (see appendices).

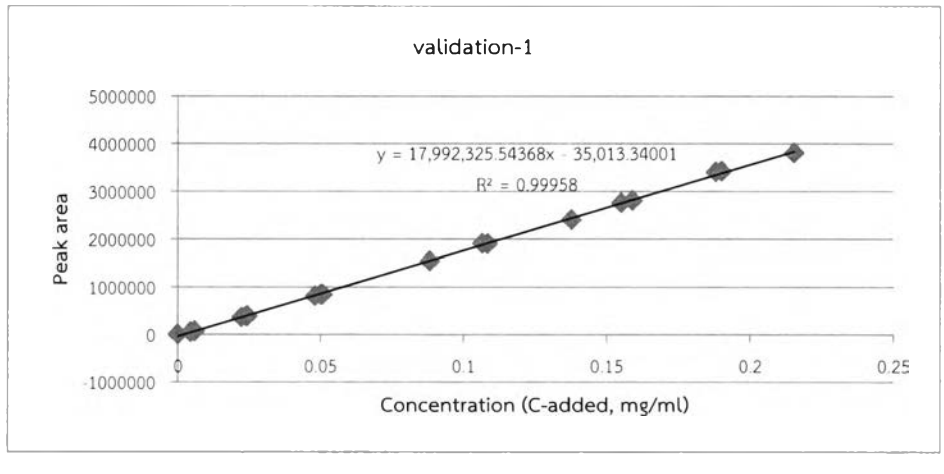


Figure 19 Calibration curve of rosmarinic acid

1.1.2 Accuracy and precision

The accuracy and precision were determined by triplicately analysis of rosmarinic acid at the concentrations of 25, 50 and 100 µg /mL on three separate

days. Concentrations of RA were determined using calibration standard curve prepared for rosmarinic acid in the range of 5-200 µg/mL for each day. %Recovery were found within range 95-105%. Within-day and between-day data given in appendices, indicated CV values were found to be less than 1.4%.

1.1.3 Sensitivity

The limit of quantification was found to be 1.826 µg/mL calculated from 10 times of standard deviation of the concentration found by spiking small amount of rosmarinic acid. The limit of detection was found to be 0.548 µg/mL, calculated from 3 times of standard deviation of the concentration found by spiking small amount of rosmarinic acid.

1.2 Stress test of rosmarinic acid

Stress test of rosmarinic acid was determined in order to study a stability of rosmarinic acid and used for testing a specificity of method validation. The test of photostability, temperature and humidity, the remaining rosmarinic acid was found more than 80% without degradation peak in 18 hours. Rosmarinic acid amount was decreased about 20% in 96 hours at temperature 60°C and 144 hours in photostability and humidity testing. For oxidation testing, rosmarinic acid was decreased by 7% within 1 hour and rapidly decreased about 47% within 9 hours, so the experiment for oxidation was ended at 9 hours (see appendices). The decreasing of peak area in stress testing were observed, however, no degradation product peak was founded at 320 nm and the peak purity index were still high in range between 0.998139-0.999997. The degradation product might occurred and absorbed at other wavelength. From the stress test results, the major instability of RA were caused by oxidation and temperature.

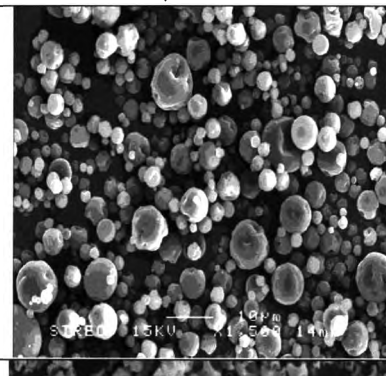
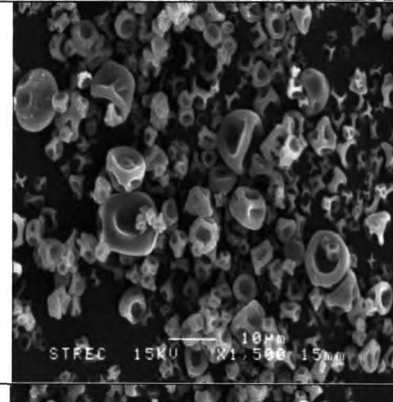
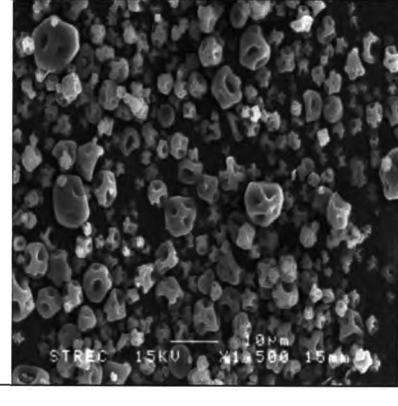
1.3 Characterization of rosmarinic acid encapsulation

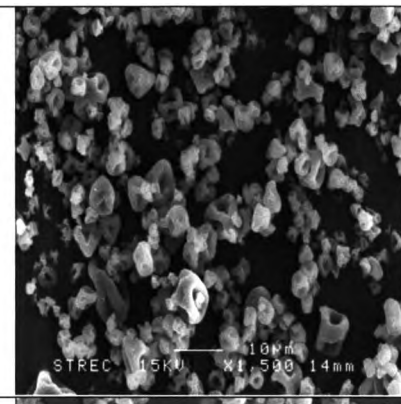
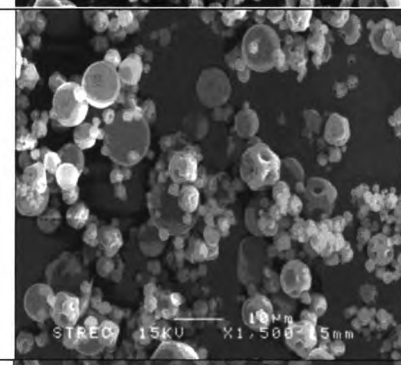
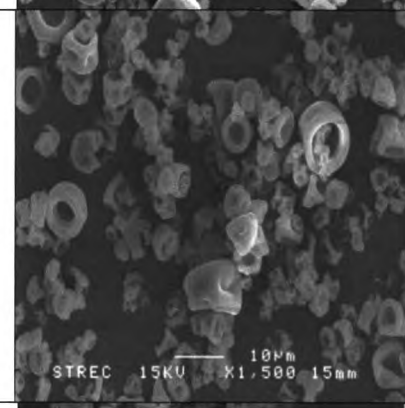
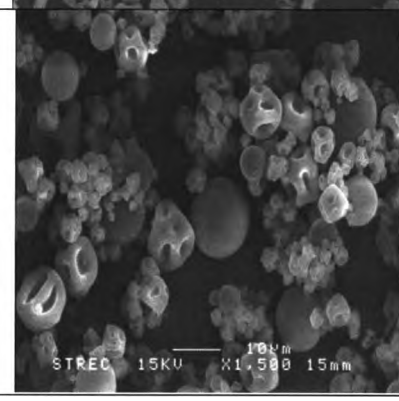
1.3.1 Morphological characterization

1.3.1.1 Particle morphology from scanning electron microscope

The particle morphology from SEM was shown in Figure 20. It was found that spray drying process created spherical shape microparticles where as freeze drying process, after cake matrix sieving, gave irregular flatted shape particles. The DG polymer might cause the shrink of the particles obtained from SD process

more than AG polymer as clearly seen in Figure 20. Figure 21 showed the surface morphology of FD powders. The surface of FC-1AG was smoother than FC-1DG which showed more roughness than those of AG, while the mixture was smoother than those using pure DG at 1%. However the formulations prepared by using 2% polymer were not significantly different and found to be smoother than those using 1%.

Formulaticns	Encapsulated RA microparticles (1,500X), SD process
SN-1AG	
SN-1DG	
SN-1DGAG1:1	

SN-1DGAG2:1			
SN-2AG			
SN-2DG			
SN-2DGAG1:1			

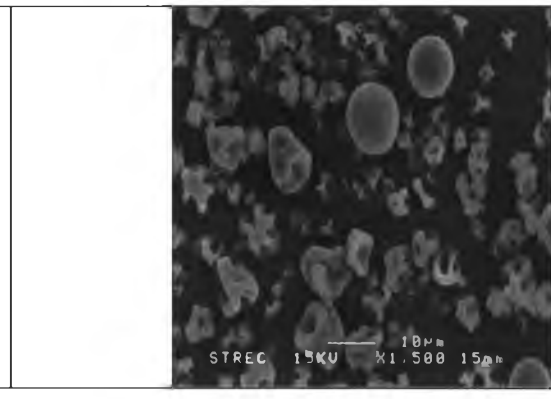
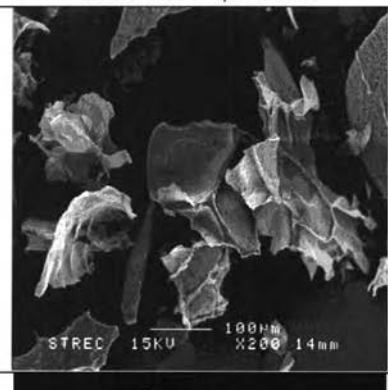
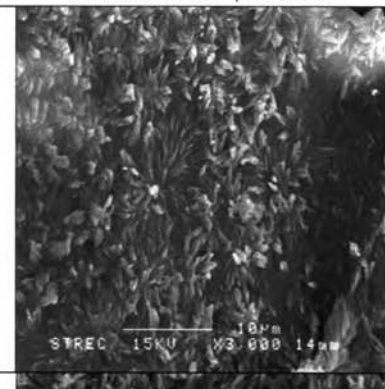
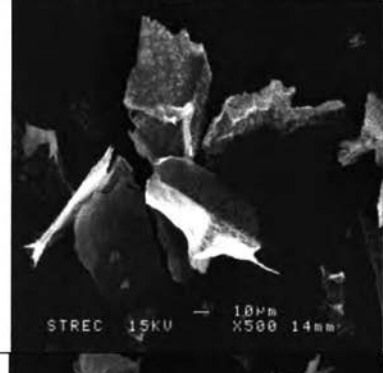
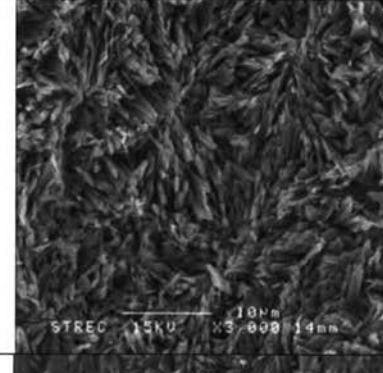
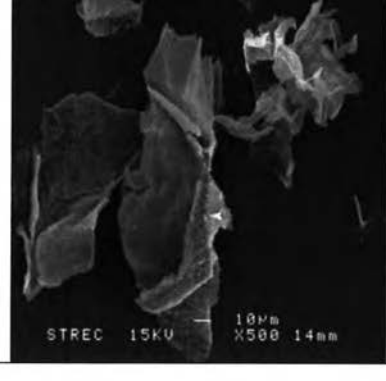
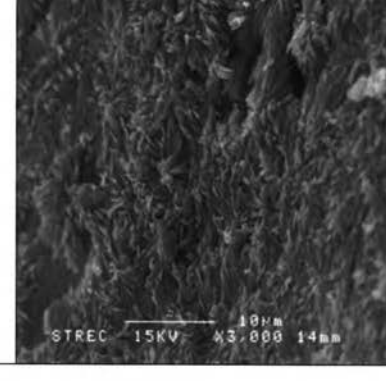
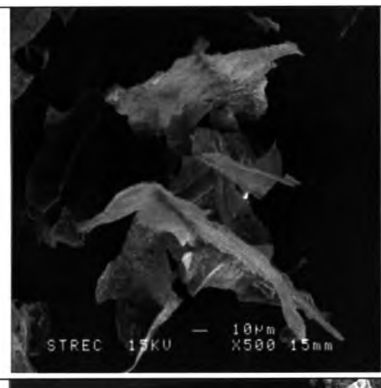
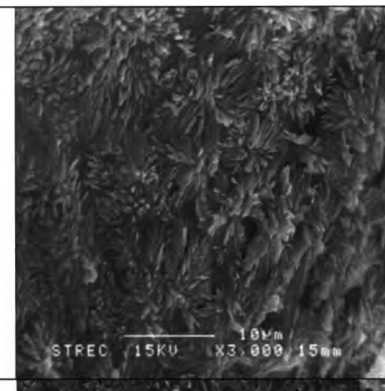
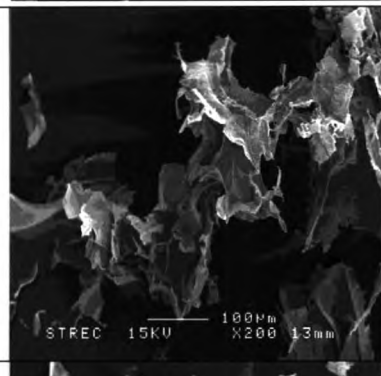
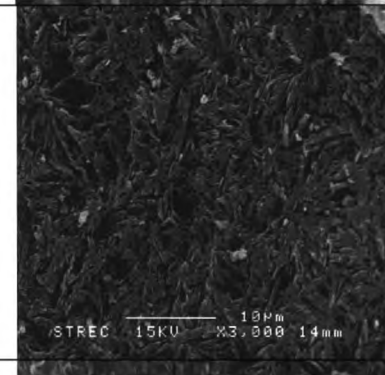
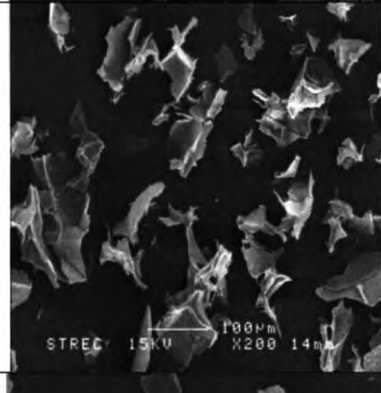
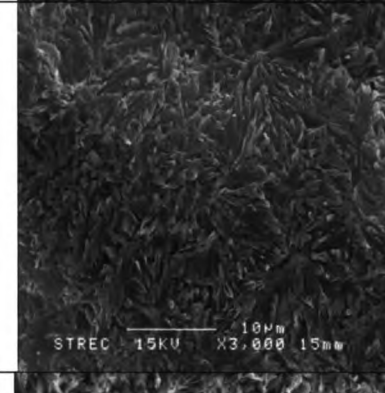
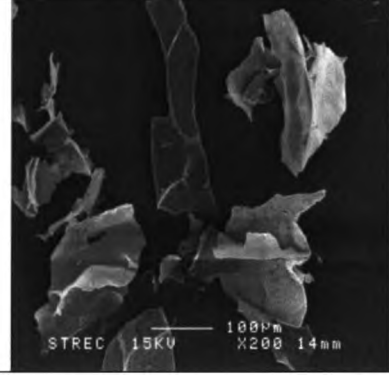
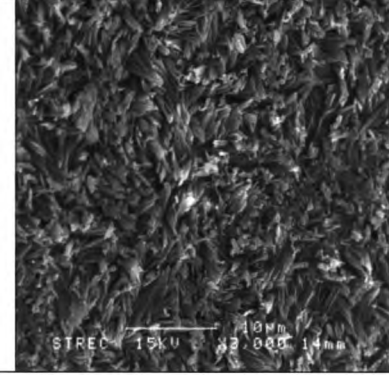
SN-2DGAG2:1	

Figure 20 SEM image of noncrosslinked-spray dried RA microparticles

Formulations	Encapsulated RA microparticles (500X), FD process	Encapsulated RA microparticles (3,000X), FD process
FC-1AG		
FC-1DG		
FC-1DGAG1:1		

FC-1DGAG2:1		
FC-2AG		
FC-2DG		
FC-2DGAG1:1		

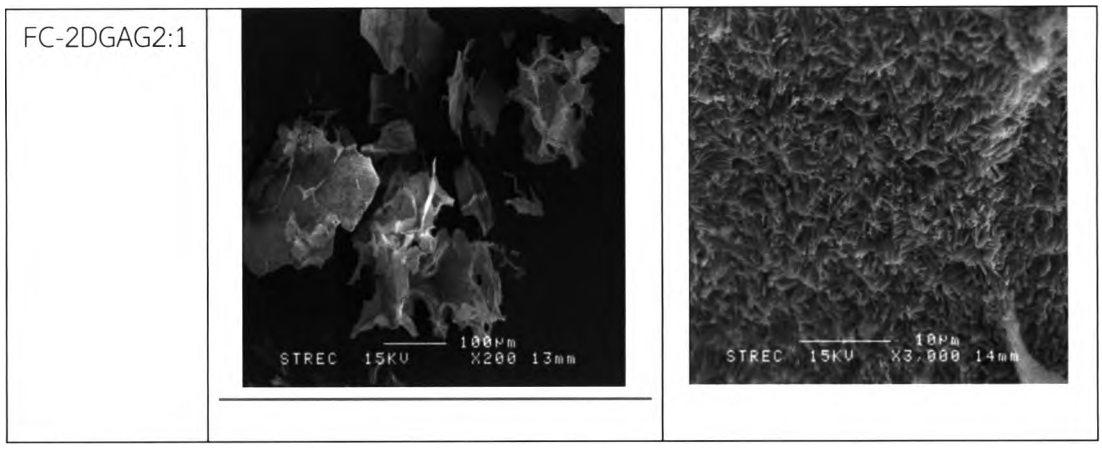


Figure 21 SEM image of freeze dried RA microparticles

1.3.1.2 Particle size of encapsulated rosmarinic acid microparticles

The particle size results from SD and FD processes are shown in Figure 22. From SD process, the sizes of crosslinked-spray dried microparticles were found to be larger than those of noncrosslinked-spray dried microparticles because of agglomeration of microparticles in crosslinking bath. The sizes of microparticles were ranged within 11 to 52 µm and the particles from SD process were very small compared to the powders obtained from FD process. The mixed polymer of AG and DG which processed through spray dry caused the particle size to decrease when compared to the formulations using either DG or AG one polymer. DG polymer caused the microparticles to be larger than those of AG polymer seen all in using only pure polymer and the mixed polymer. The particle size trended to be increased with the increasing of polymer concentrations except SN-1AG which using AG 1% compared to SN-2AG which using AG 2% where the size decreased with increasing of polymer concentrations.

In FD process, the particle size in the formulations using pure polymer either DG or AG trended to be increased with the increasing of polymer concentrations. The using of DG alone showed high effect in particle size in FD that larger size founded comparing to those of AG. The mixed polymer of AG and DG which processed through freeze dry caused the particle size to be smaller as compared to formulations those using DG alone but still larger than those of AG.

The particle sizes of FD microparticles which were produced by sieving the cake matrix were found to be larger than SD microparticles.

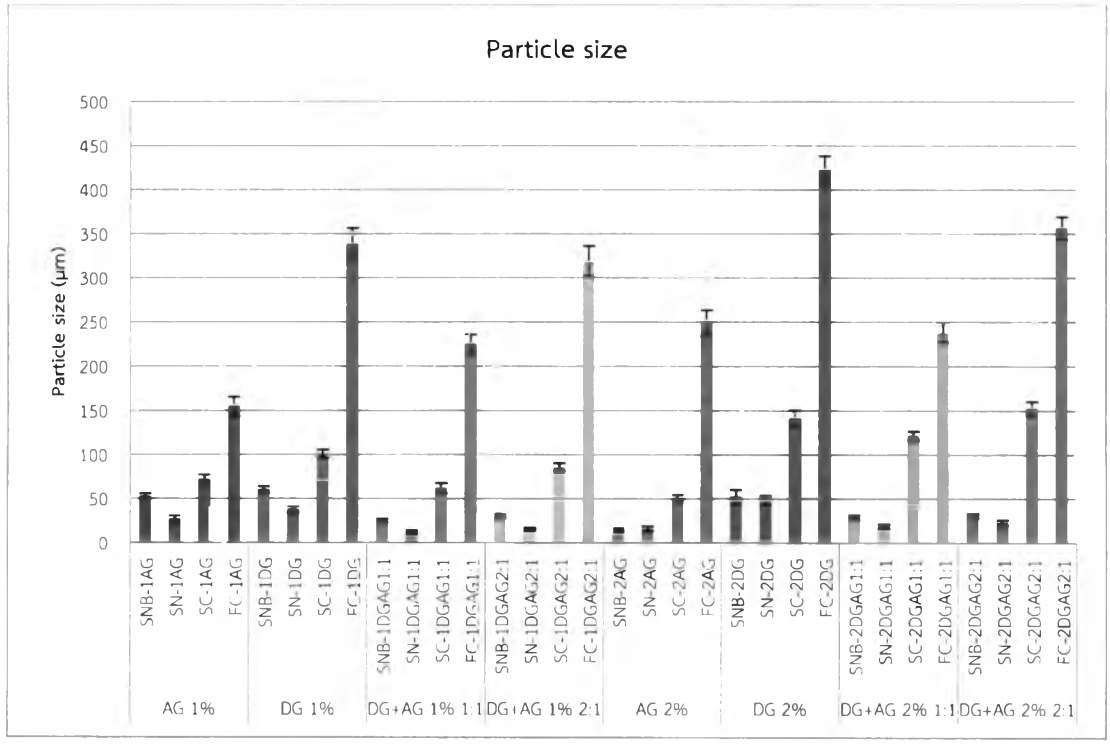


Figure 22 Particle size of noncrosslinked and crosslinked spray dried and freeze dried microparticles

1.3.2 Solid state characterization of encapsulated RA microparticles

1.3.2.1 Differential scanning calorimetry analysis

The DSC thermogram showed broad peaks of DG and AG polymer which might be the dehydration peak of the polymer and the physical mixture of polymer showed the same result as pure polymer. In rosmarinic acid thermogram, the melting peak at 170°C was seen. Figures 23-27 showed that the melting endothermic peak of rosmarinic acid at 170°C was seen in physical mixture with polymer while the peak was disappeared in all formulations through spray drying and freeze drying process. So rosmarinic acid might be incorporated into the structure of polymer as disappearance of its peak. In FD process, mannitol was used as bulking agent and showed the melting peak at the same temperature region as rosmarinic acid. So the crosslinked blank FD particles were prepared and also analyzed by DSC. The thermogram in Figure 26 showed the peak shifted from 170°C to 150 °C which meant that mannitol melting peak shifted as passed the crosslink FD process. As shown in Figure 27, the thermogram of encapsulated RA though FD process showed that the endothermic peak were not seen at 170°C.

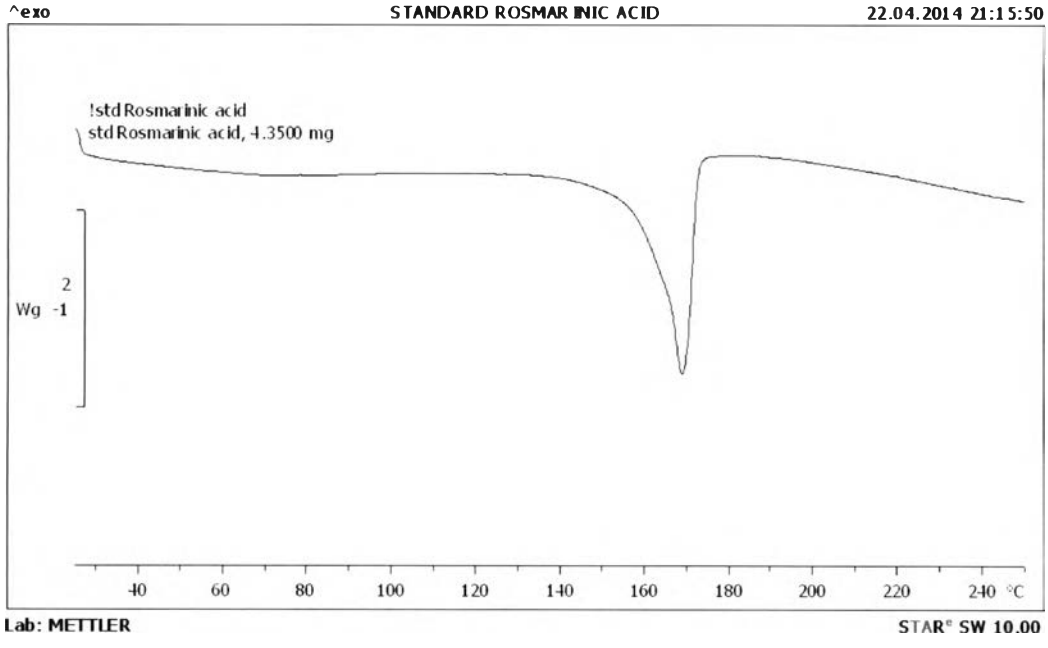


Figure 23 DSC thermogram of rosmarinic acid

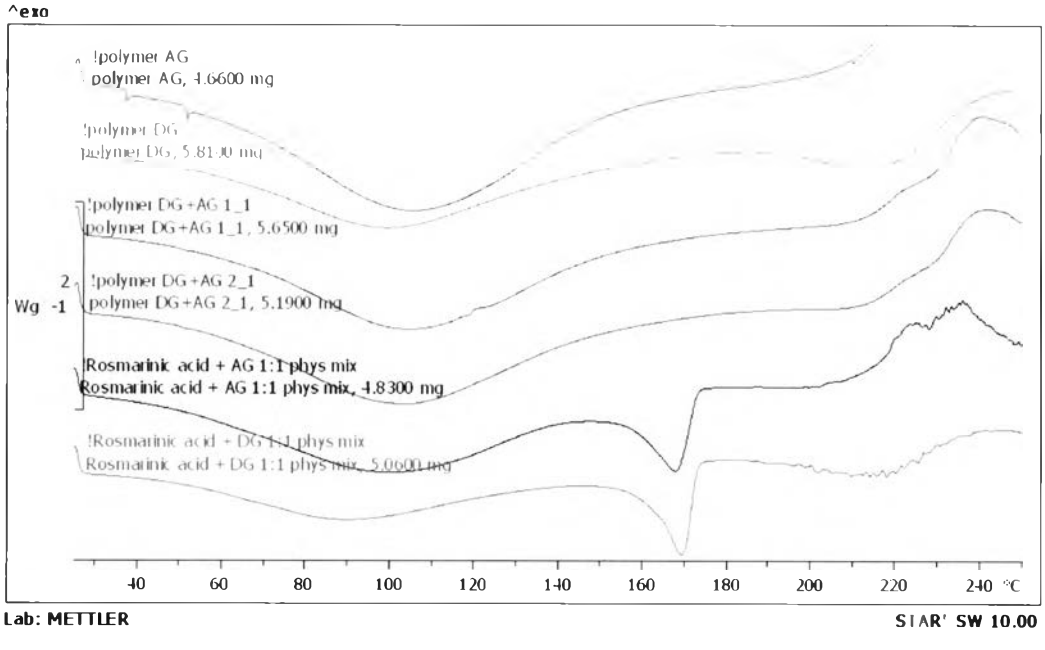


Figure 24 DSC thermogram of polymers, physical mixture of polymers and physical mixture of rosmarinic acid with polymer

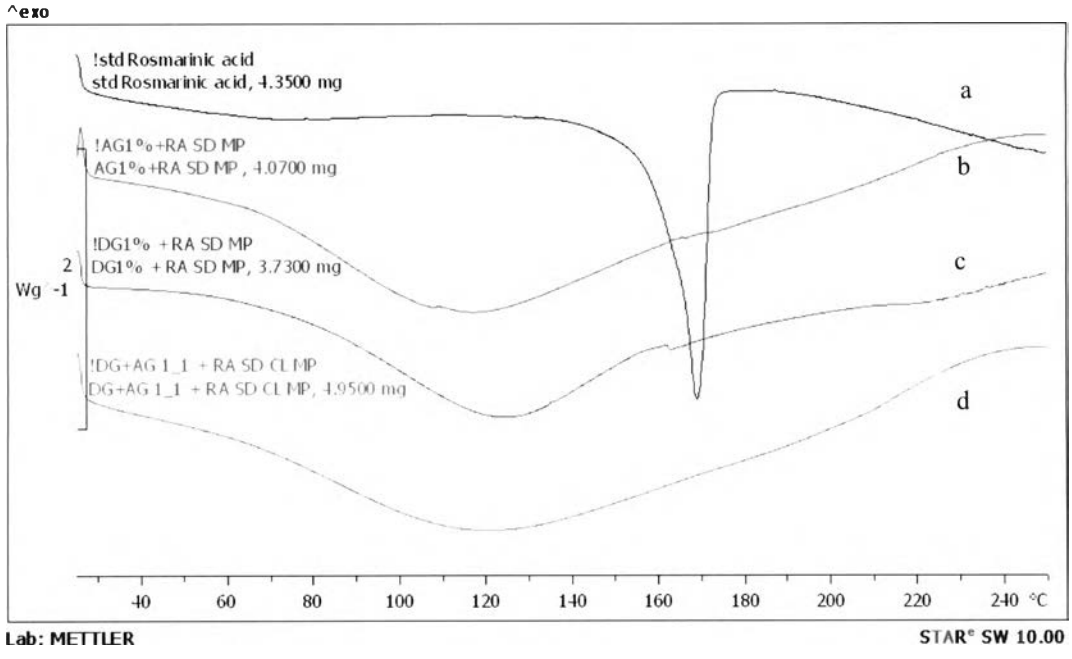


Figure 25 DSC thermogram of spray dried RA microparticles

a: standard rosmarinic acid, b: SN-1AG, c: SN-1DG, d: SC-1DGAG1:1

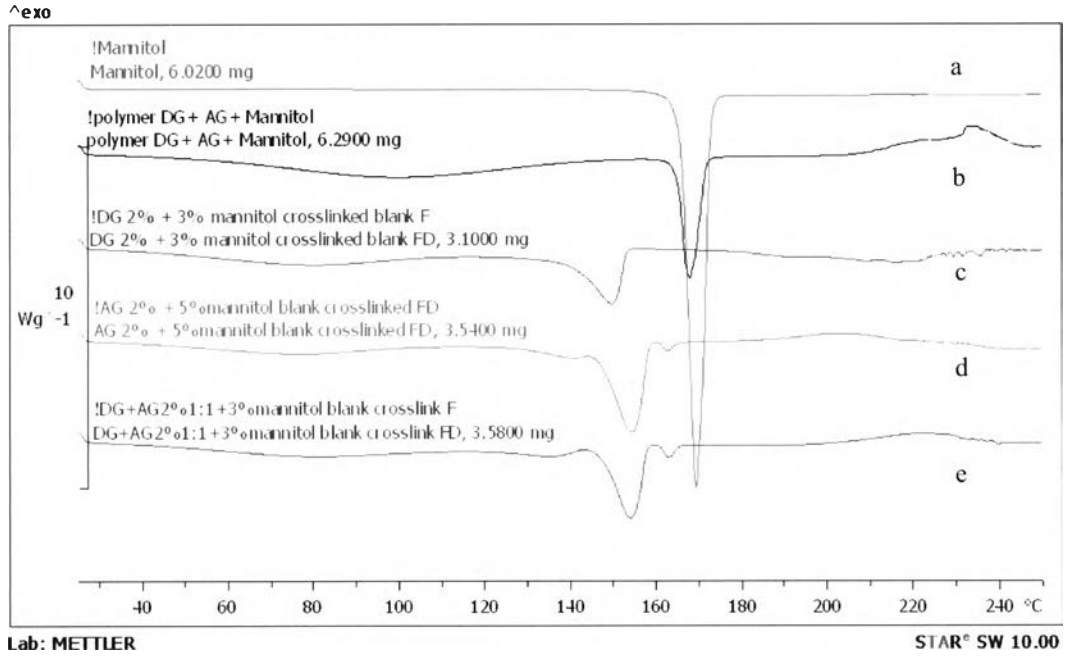


Figure 26 DSC thermogram of polymers, mannitol and blank crosslinked freeze-dried powders

a: mannitol, b: physical mixture of DG, AG, mannitol, c: FC-2DG blank

d: FC-2AG blank, e: FC-2DGAG1:1 blank

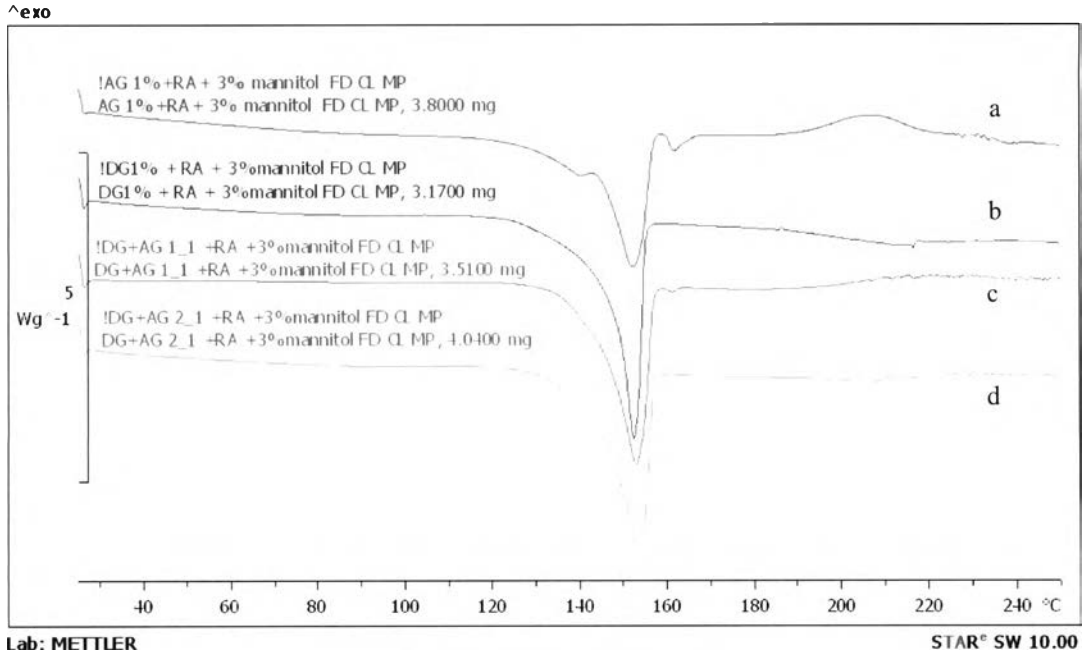


Figure 27 DSC thermogram of crosslinked-freeze dried RA particles

a: FC-1AG, b: FC-1DG, c: FC-1DG1:1, d: FC-1DGAG2:1

1.3.2.2 X-Ray powder diffraction analysis

The results from Figure 28-29 showed that the sharp peak of rosmarinic acid was seen in the diffractogram of standard rosmarinic acid while the peak was disappeared after passing through spray drying process both noncrosslinked and crosslinked. The powders obtained from FD process showed some peaks as seen in Figure 30. This caused by the bulk forming as mannitol was used in the FD formulations. However the sharp peak in the same 2θ as standard rosmarinic acid was all disappeared. These results showed the change of the solid state before and after the process of rosmarinic acid which rosmarinic acid turned into amorphous state and incorporated into the polymer.

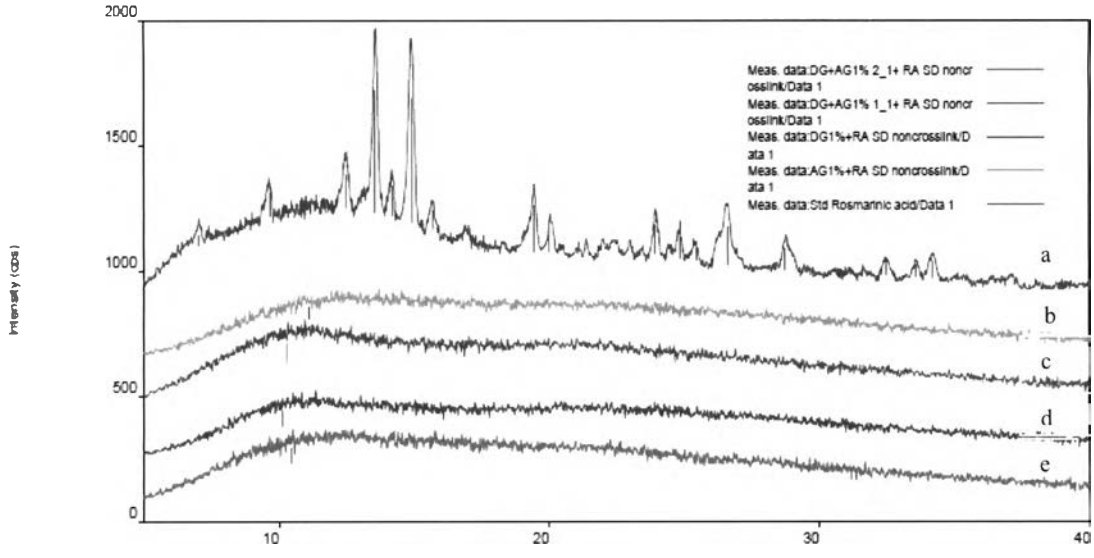


Figure 28 XRD diffractogram of noncrosslinked-spray dried RA microparticles

a: standard rosmarinic acid, b: SN-1AG, c: SN-1DG

d: SN-1DGAG1:1, e: SN-1DGAG2:1

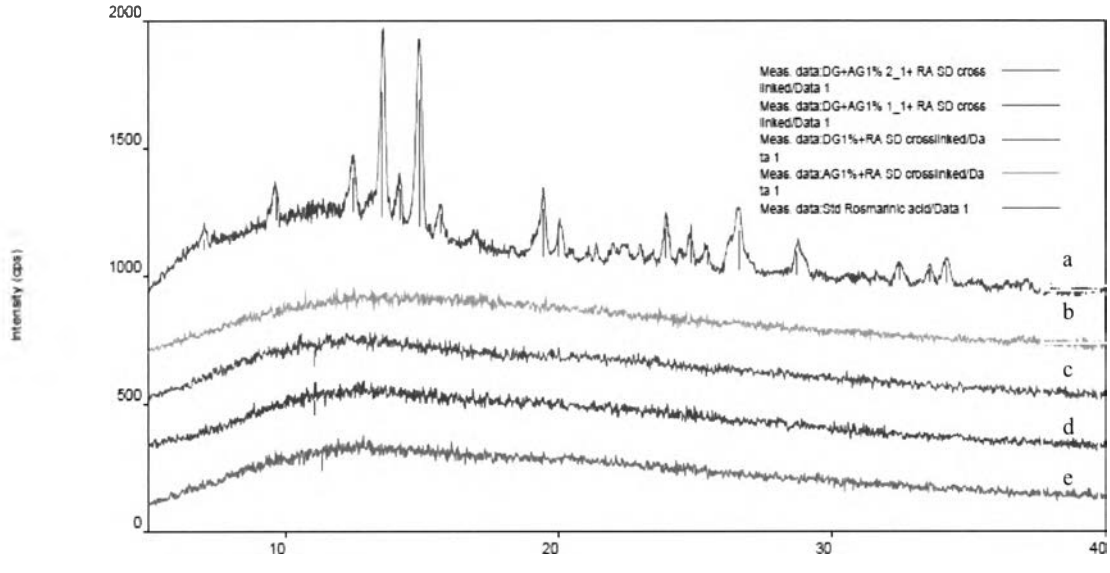


Figure 29 XRD diffractogram of crosslinked-spray dried RA microparticles

a: standard rosmarinic acid, b: SC-1AG, c: SC-1DG

d: SC-1DGAG1:1, e: SC-1DGAG2:1

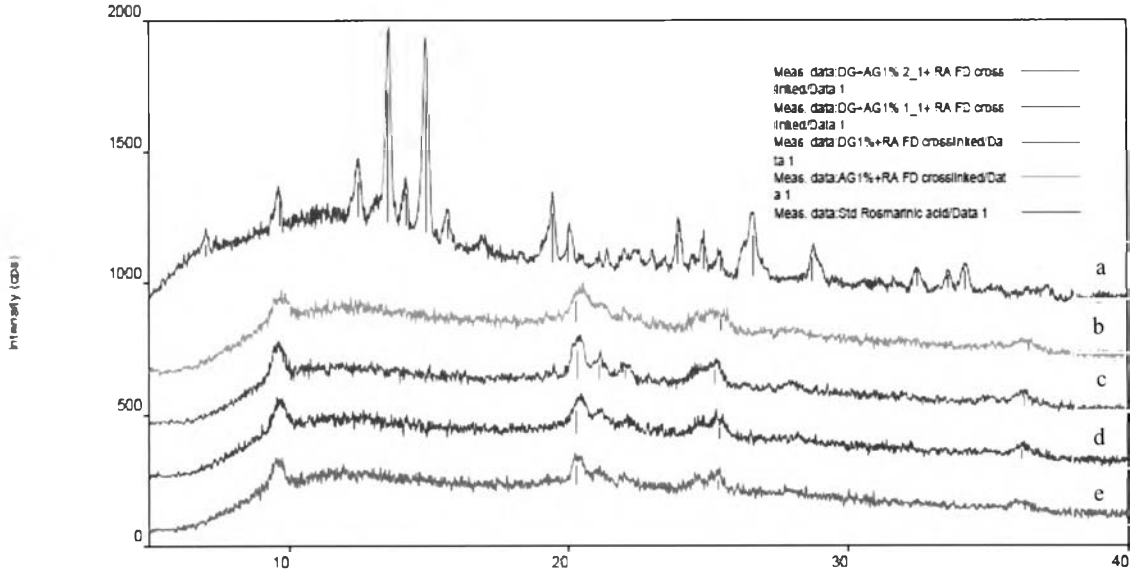


Figure 30 XRD diffractogram of crosslinked-freeze dried RA particles

a: standard rosmarinic acid, b: FC-1AG, c: FC-1DG

d: FC-1DGAG1:1, e: FC-1DGAG2:1

1.3.2.3 Fourier-Transform Infrared Spectrometry

FTIR was used to characterize the presence of specific chemical groups in RA, DG and AG. The functional groups exhibited important absorption bands from FTIR measurements as shown in Figure 31. Characteristic O-H stretching in phenols peaks appeared at around ν = 3,200-3,500cm^{-1} and 1,000-1,300 cm^{-1} region attributed to C-O stretching of ester was shown in standard rosmarinic acid. The C=O stretching carbonyls was also shown at ν = 1,706 cm^{-1} of standard rosmarinic acid spectra with sharp peak as rosmarinic acid has carbonyl group in both ester bond and carboxylic bond. The peak between ν =1,500-1,600 cm^{-1} was shown as arcmatic hydrocarbon peak which rosmarinic acid has two aromatic groups and peak at ν = 817 cm^{-1} was attributed to =C-H bending of alkene. IR spectra of DG and AG were quite the same as the strong peak appeared at 1013 cm^{-1} of DG and at ν =1,027 cm^{-1} of AG attributed to the characteristic of C-O stretching ether but the differences were at ν = 1,731 cm^{-1} which C=O stretch carbonyls contribution of DG and ν =1,407, 1,596 cm^{-1} of sodium alginate and the physical mixture with polymer spectra while board spectra around ν =3,000-3,600 cm^{-1} were shown in spectra of DG and AG as polysaccharide contains many hydroxyl groups. The physical mixture of DG and AG displayed all peaks in those three area so there was no interaction between the two polymers. With the same physical mixture rosmarinic acid and two polymers, the

result was shown the same peak as rosmarinic acid and peak at ν =1,027 cm^{-1} of DG and AG was still shown but at lower intensity.

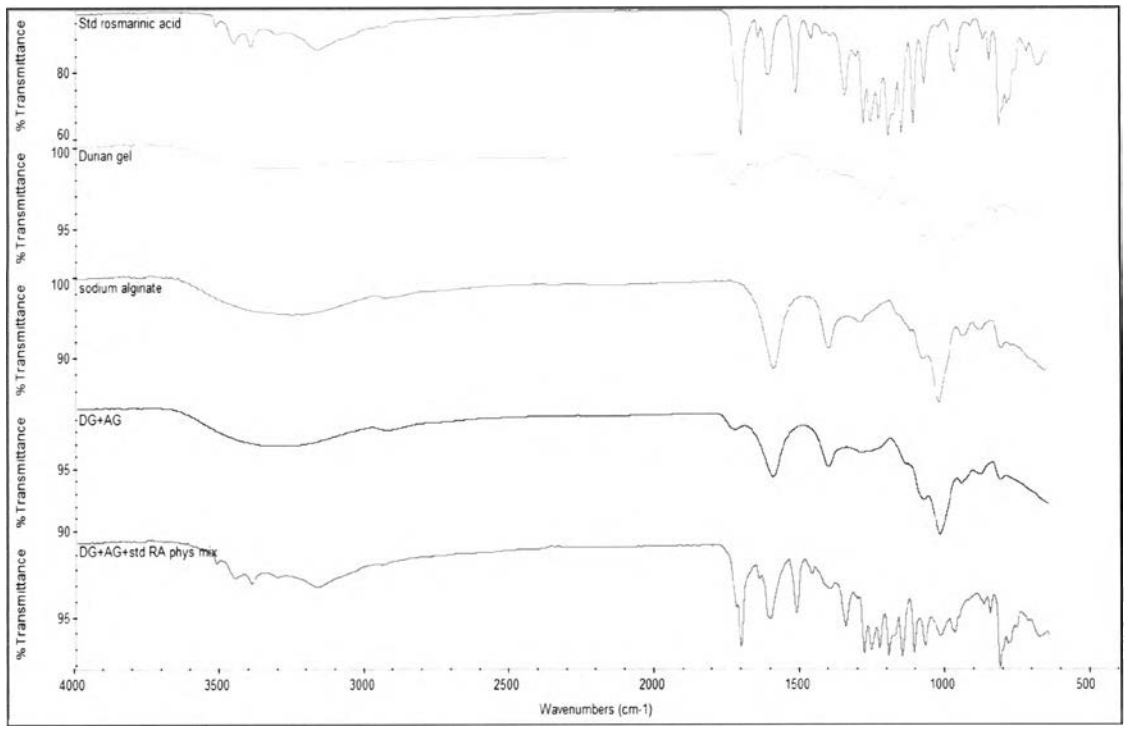


Figure 31 FTIR spectra of rosmarinic acid, physical mixture of polymer DG and AG and physical mixture of rosmarinic acid with polymers

From Figure 32-33, FTIR spectra of noncrosslinked-spray dried RA microparticles. All formulations showed disappearance some peaks of rosmarinic acid at 817 cm^{-1} which assigned to =C-H bending alkene, the sharp peak at 1,706 cm^{-1} C=O stretching carbonyls 1,000-1,300- cm^{-1} region attributed to C-O stretching of ester bond and O-H stretching in phenols at around ν =3,200-3,500 cm^{-1} also disappeared while the sharp peaks of sodium alginate and DG were still shown. Sodium alginate and DG might affect rosmarinic acid and interacted in ester and alkene group of rosmarinic acid.

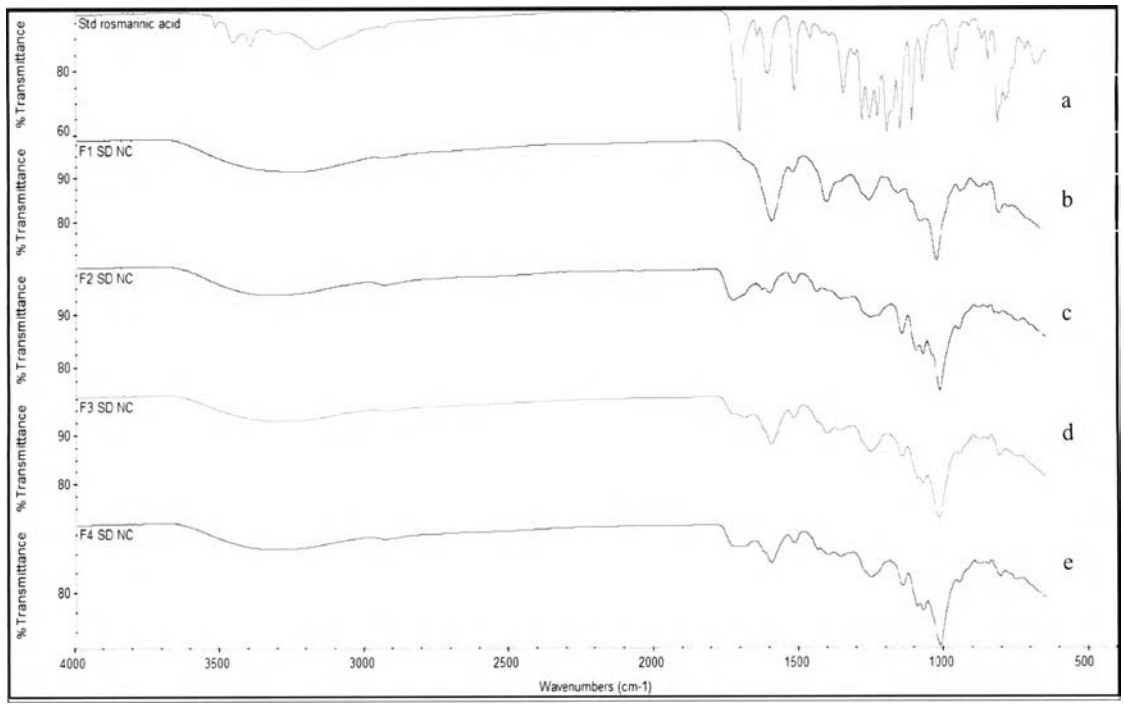


Figure 32 FTIR spectra of noncrosslinked-spray dried RA microparticles

a: standard rosmarinic acid, b: SN-1AG, c: SN-1DG

d: SN-1DGAG1:1, e: SN-1DGAG2:1

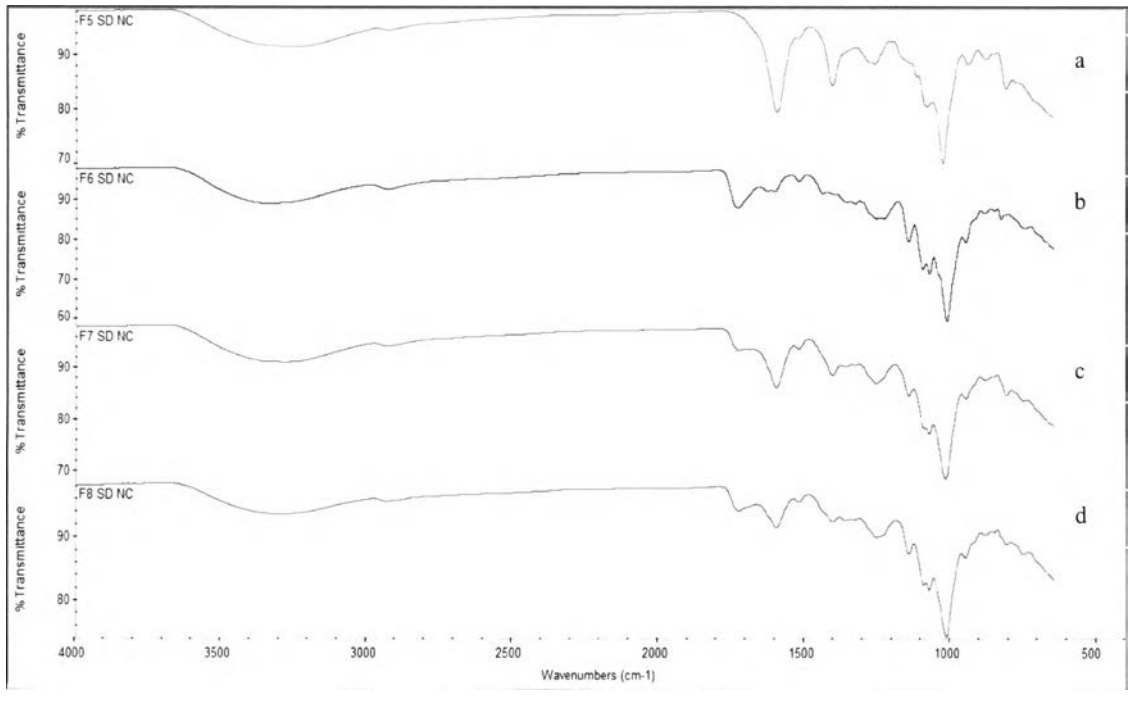


Figure 33 FTIR spectra of noncrosslinked-spray dried RA microparticles

a: SN-2AG, b: SN-2DG, c: SN-2DGAG1:1, d: SN-2DGAG2:1

FTIR spectra of crosslinked-spray dried RA microparticles were shown in Figures 34 and 37. The results showed that the spectra of SC-1DG were similar to SC-2DG. However when comparing between noncrosslinked and crosslinked-spray dried microparticles as Figure 37, SN-1DG and SC-1DG showed slightly different at ν =1,605 cm^{-1}. So the interaction from the crosslinking process might occurred. The crosslinked spray dried microparticles were test for only two formulations since rosmarinic acid was heavily lost after crosslinking of other formulations.

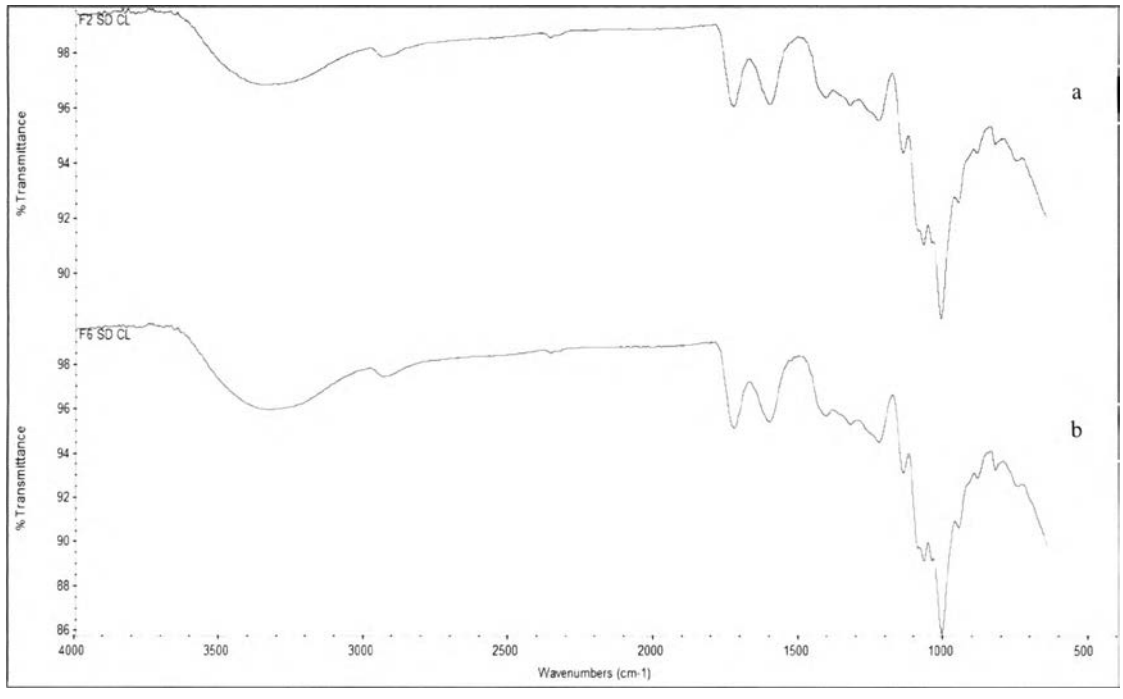


Figure 34 FTIR spectra of crosslinked-spray dried RA microparticles

a: SC-1DG, b: SC-2DG

In the case of encapsulated freeze dried RA microparticles, all formulations were observed the same FTIR spectra results as seen in Figure 35-36. These spectra showed disappearance of peaks of rosmarinic acid at 817 cm^{-1}, 1,706 cm^{-1}, 1,000-1,300 cm^{-1} region and O-H stretching in phenols peaks appeared at around ν = 3,200- 3,500cm^{-1}. The new sharp peaks were observed at 1,022, 1,085 cm^{-1} assigned to C-O stretching of alcohol and strong broad peak at 3,191 cm^{-1} to O-H stretching of alcohol as mannitol was used as bulking agent and has a lot of hydroxyl group. The sharp peak of AG at 1,407 cm^{-1} was disappeared in all formulations which using sodium alginate. So sodium alginate might have an physical interaction with rosmarinic acid through freeze drying process.

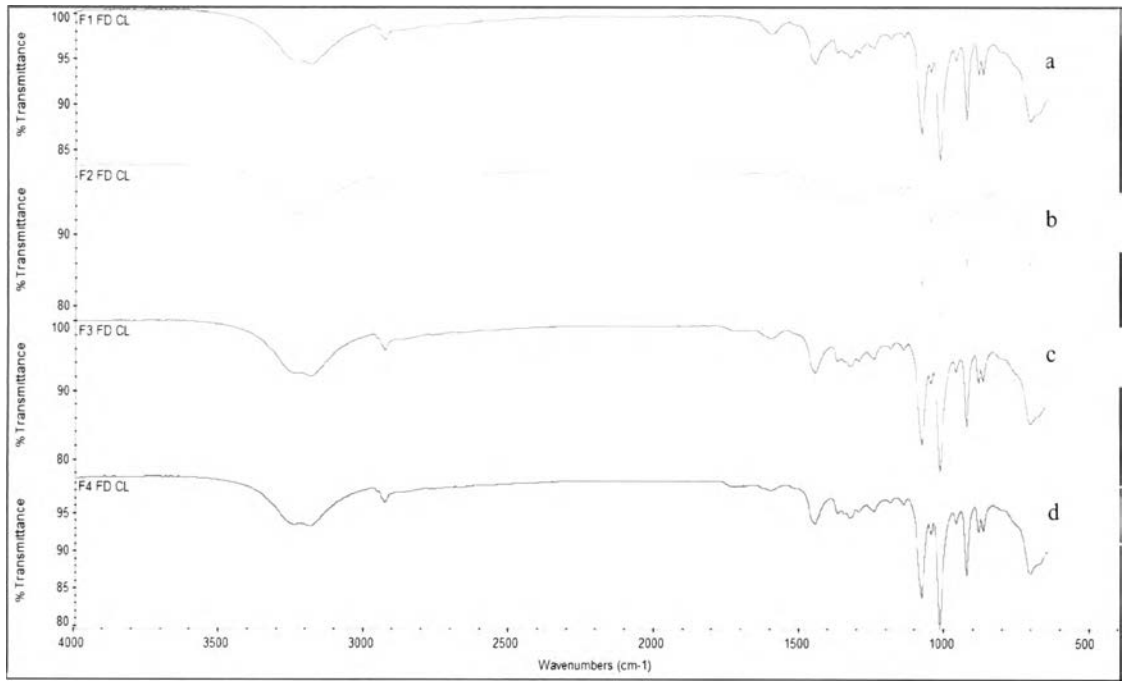


Figure 35 FTIR spectra of crosslinked-freeze dried RA microparticles

a: FC-1AG, b: FC-1DG, c: FC-1DGAG1:1, d: FC-1DGAG2:1

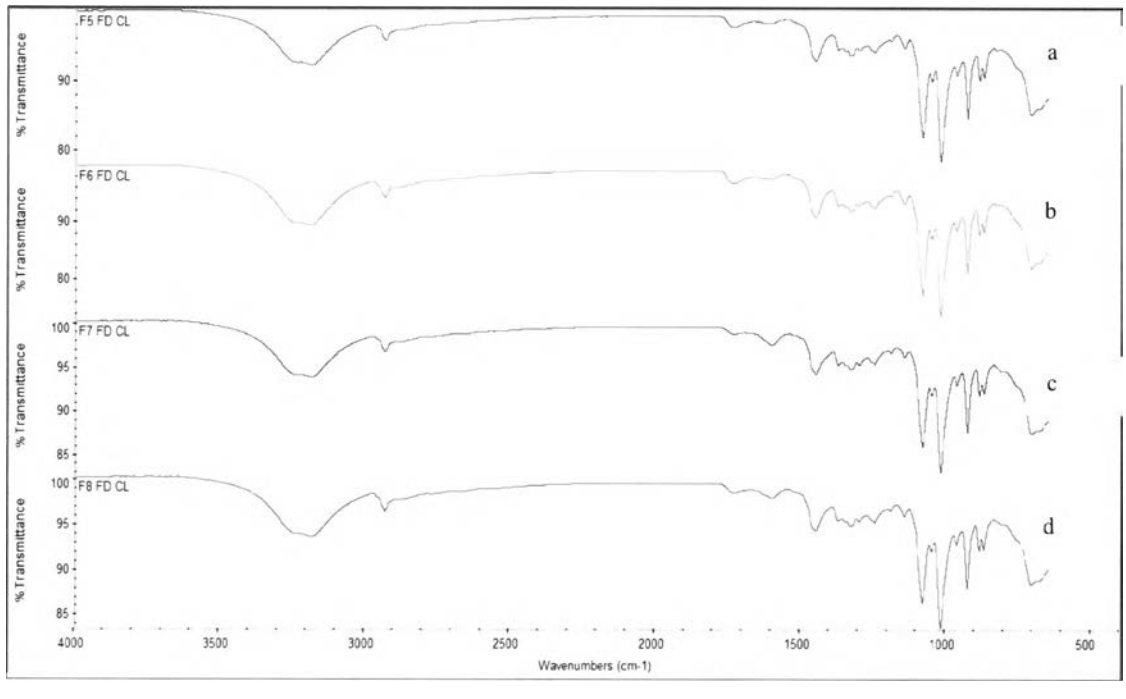


Figure 36 FTIR spectra of crosslinked-freeze dried RA microparticles

a: FC-2AG, b: FC-2DG, c: FC-2DGAG1:1, d: FC-2DGAG2:1

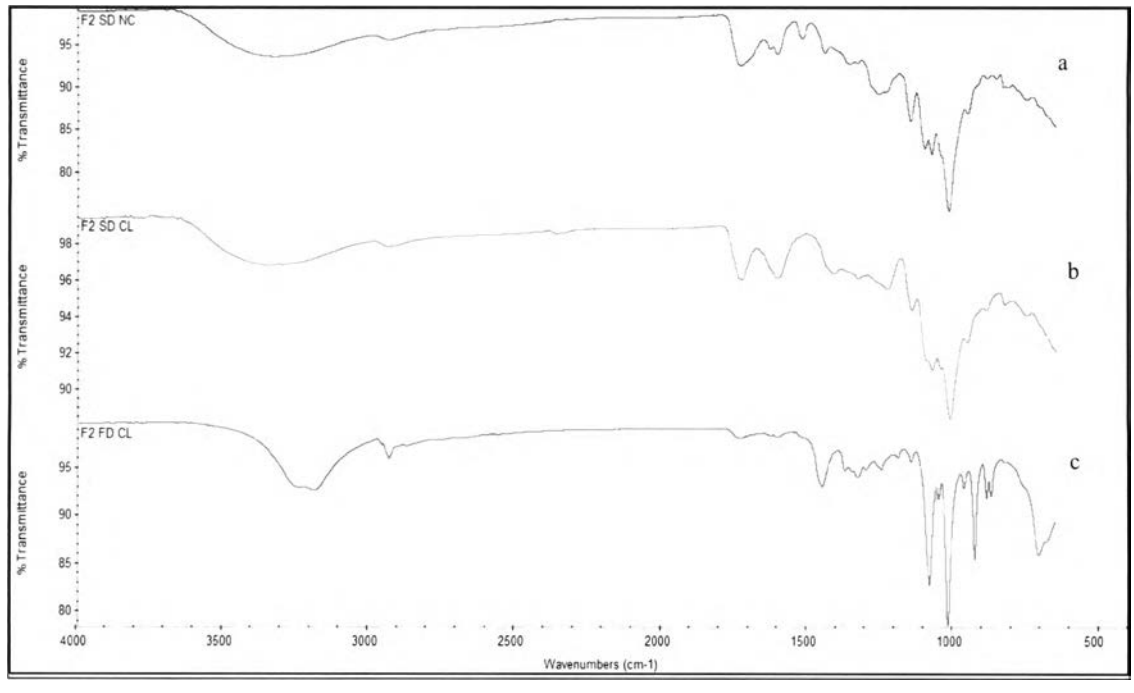


Figure 37 FTIR spectra of microparticles made of DG1% by SD and FD process

a: SN-1DG, b: SC-1DG, c: FC-1DG

1.3.3 Determination of entrapment efficiency

Entrapment efficiency (% EE) results are shown in Figure 38. It was found that %EE of crosslinked-spray dried formulations provided the drastically lower of rosmarinic acid amount than those of noncrosslinked ($p<0.05$). So the crosslinking process caused the reducing of %EE of spray dried crosslinked microparticles significantly to lower than 5%. Rosmarinic acid might be highly lost in the crosslinking process prepared in the calcium chloride bath and washed with methanol. The formulations using AG alone yielded the lower entrapment efficiency than the rest.

Entrapment efficiency of the formulations processed from freeze dry were higher than spray dry as rosmarinic acid did not lost during the process. So the %EE of crosslinked FD formulations were approximately 100%.

As well, the % EE of the freeze drying process (FC) was found to be higher than those of noncrosslinked spray drying process (SN). There were some formulations from SN where %EE founded around 70-90% which might be caused by the spray drying process that affected to the lost of active substance. So in entrapment efficiency, the FD process provided better than SD process. However in case of morphological aspect, SD provided more spherical shape.

The entrapment efficiency of spray dried RA microparticles using DG was found to be higher than those using AG ($p<0.05$), possibly due to the polymer structure, since DG is a pectic polysaccharide which has a complex branched chain heteropolysaccharide and AG is a linear chain polysaccharide. The branched structure of DG might help rosmarinic acid to be well entrapped into the structure. This effect also occurred in the study of alginate-pectin microcapsules which entrapped folic acid, the folic acid was increasingly entrapped in the formulation containing pectin as polymer [42].

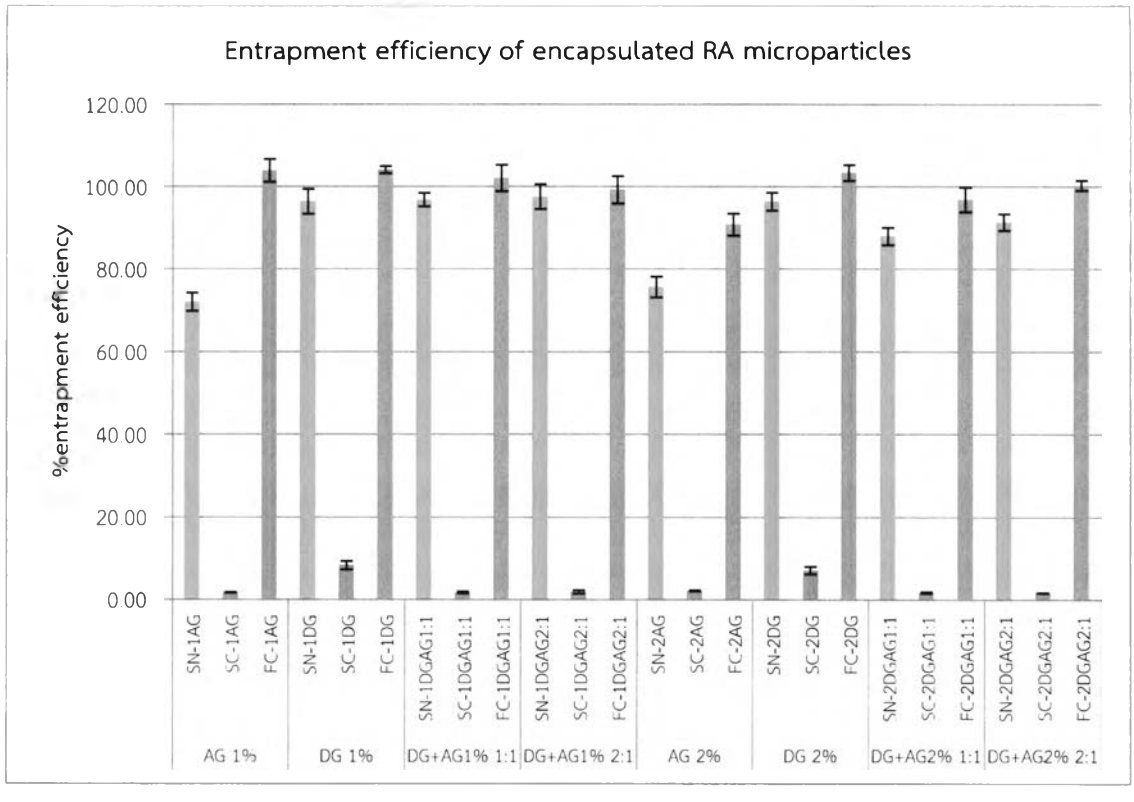


Figure 38 Entrapment efficiency of encapsulated RA microparticles

1.3.4 *In vitro* release study

The release profiles of rosmarinic acid from the encapsulated particles are shown in Figure 39-40. From Figure 39 SN-2DGAG1:1 showed the fastest release and reached 100% at 6 hours and some formulations showed that the release could not be reached 100% as seen in SN-1DG, SN-1DGAG1:1, SN-2DG and SN-2DGAG2:1 but the rest reached up to 100% after 12 hours. So in SD process, the increasing of DG polymer caused the release to be lower and the increasing of polymer concentration caused the lower release as well. In the case of polymer type, DG yielded slower release than AG.

From Figure 40 the powders from formulation FC-1DG and FC-2DG using DG 1% and DG 2% respectively showed slower release when compared to the rest with the same result as SD, DG caused the slower and lower release. FC-1DG showed the slowest release and did not reach 80% of rosmarinic acid release. This might caused by the structure of DG has more branched chains than AG as DG has a pectic polysaccharide structure but AG is a linear chain polysaccharide so DG affected slower gelling and the active substance was longer retained in the microparticles, even though DG gave a rougher surface to the particles that could make the faster release, DG exhibited the slower gelling and slower release of RA from the particles. Since AG showed the faster release profiles. The mixture of DG and AG as SN-2DGAG1:1 and FC-2DGAG2:1 helped faster release when compared to pure DG polymer in both SD and FD processes.

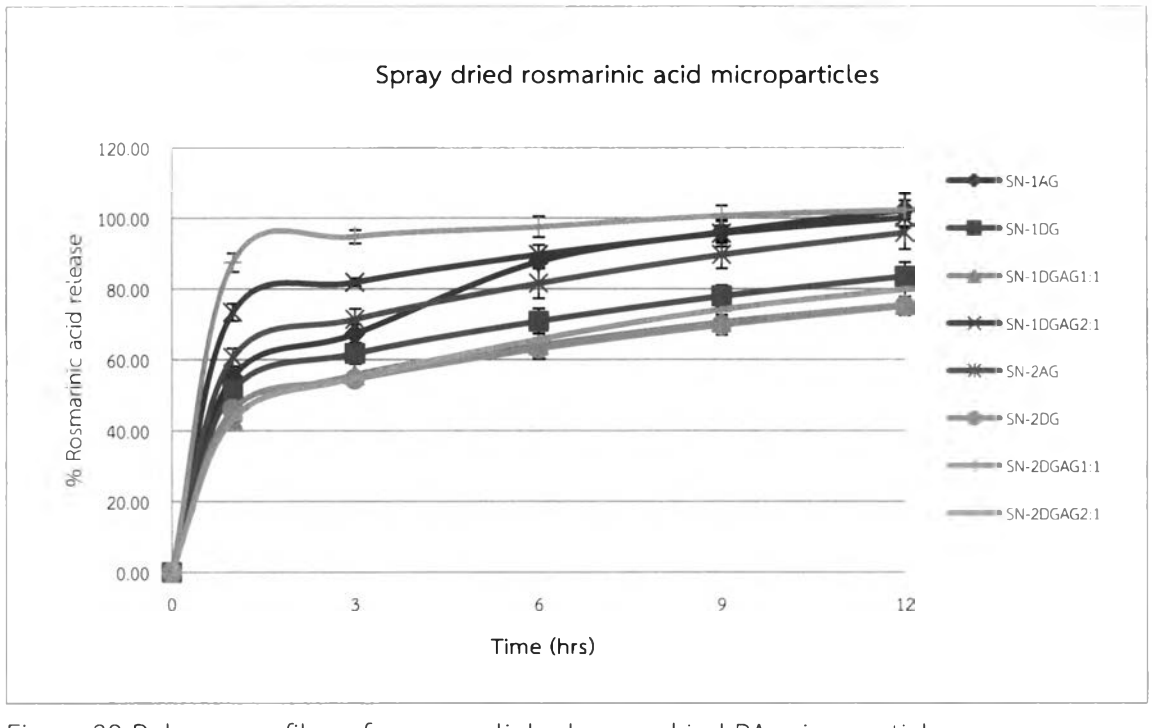


Figure 39 Release profiles of noncrosslinked-spray dried RA microparticles

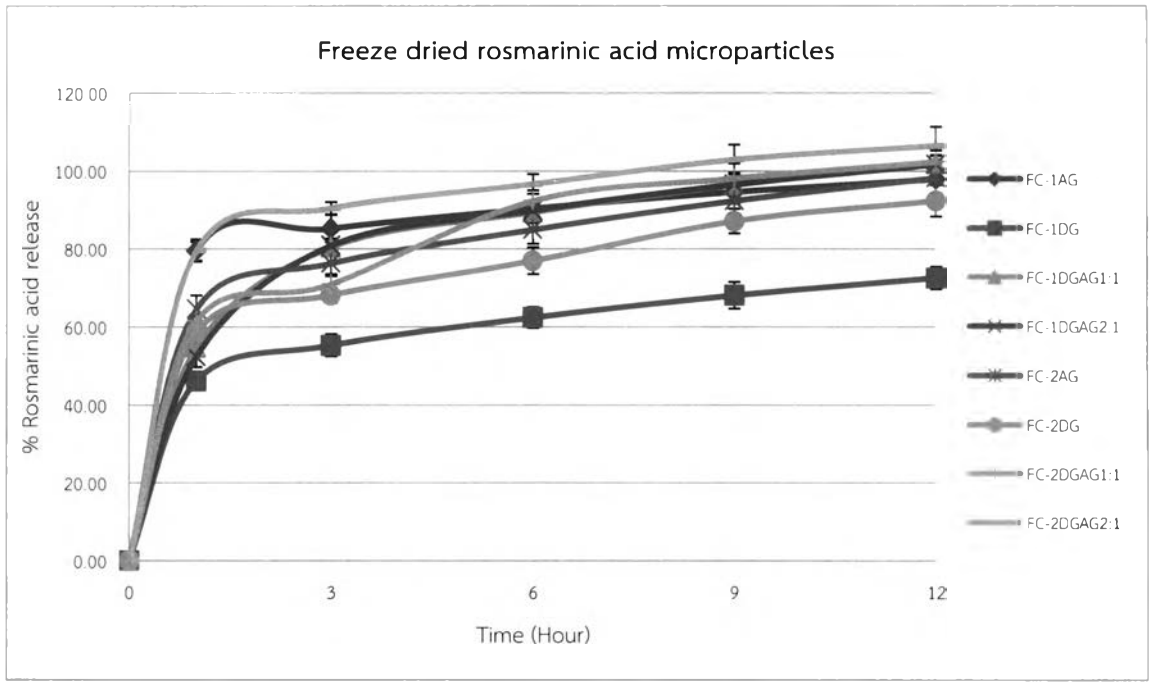


Figure 40 Release profiles of freeze dried RA microparticles

1.3.5 Stability study

In this stability study, encapsulated RA particles through SD and FD process were compared to standard rosmarinic acid which was not passed any process and stored at the same conditions as the formulations. The standard rosmarinic acid showed stability decrease 26.44% after 3 months so the encapsulated formulations that showed significantly increased stability were chosen for the further experiment. As the results shown in Figure 41 SN-1AG showed better remaining of rosmarinic acid when compared to the start of itself which decreased less than 15% of rosmarinic acid. In Figure 42 through FD process showed better stability than in SD process and FC-2DG showed the best stabilty of rosmarinic acid. SN-1AG, FC-1DG, FC-2AG, FC-2DG and FC-2DGAG1:1 were chosen due to the stability increased compared to other fomulations. In case of the type of polymer, AG seemed to be able to increase stability better through spray drying process while DG through freeze drying process.

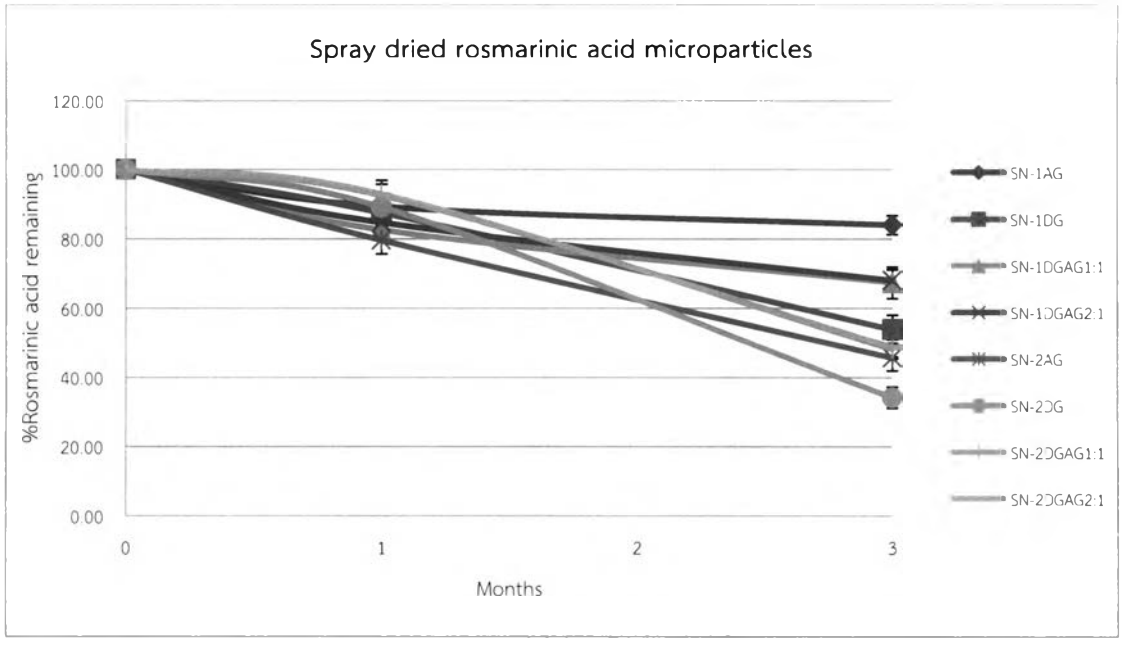


Figure 41 Stability study of noncrosslinked-spray dried RA microparticles

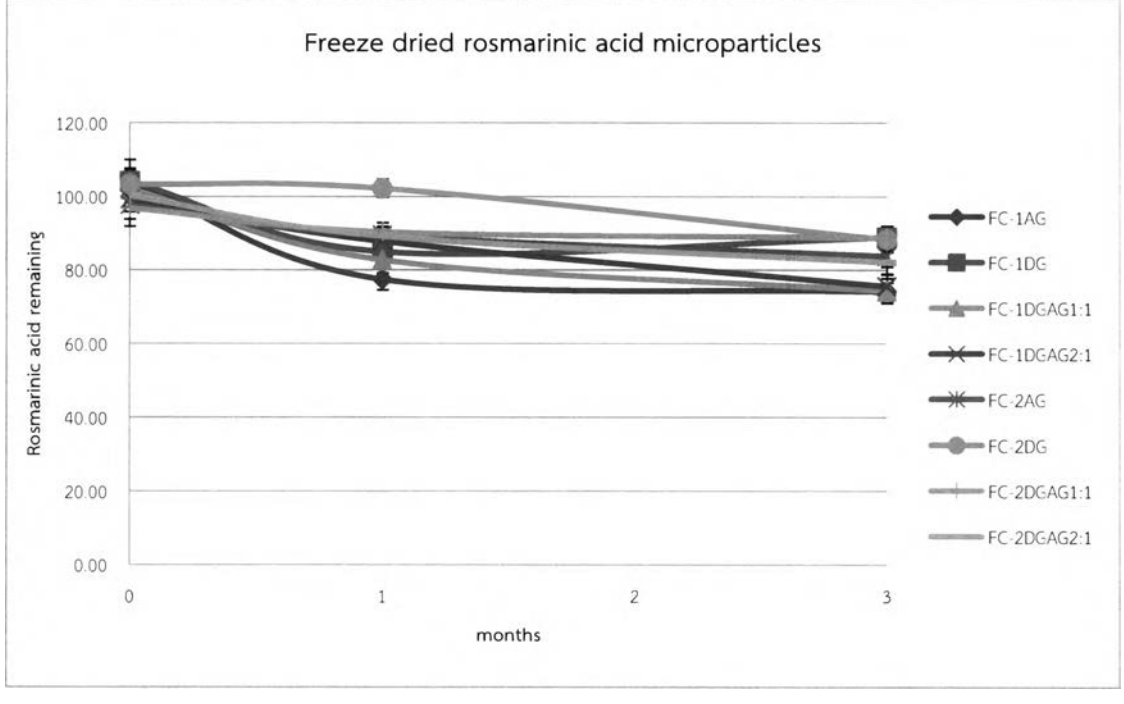


Figure 42 Stability study of crosslinked-freeze dried RA microparticles

2.*Thunbergia laurifolia* callus extract microparticles

2.1 Callus induction and proliferation from TL leaves

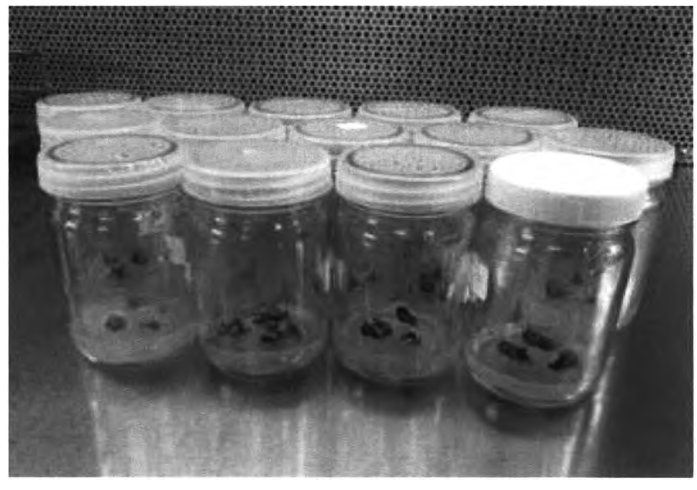


Figure 43 *T. laurifolia* callus before harvesting and freeze drying process

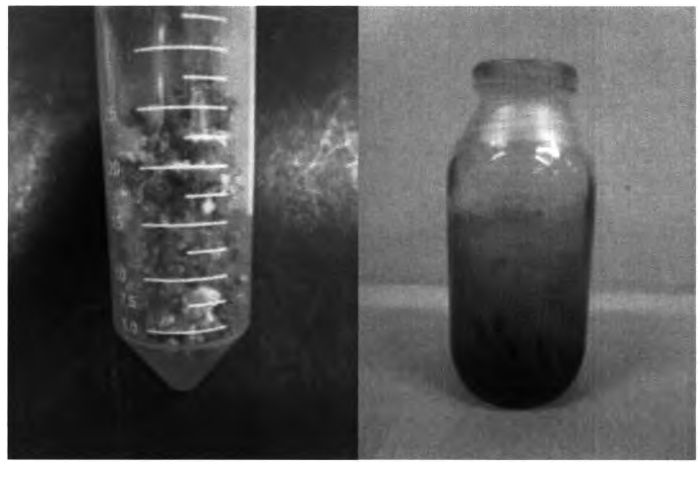


Figure 44 *T. laurifolia* freeze dried callus (left) and *T. laurifolia* callus extract (right)

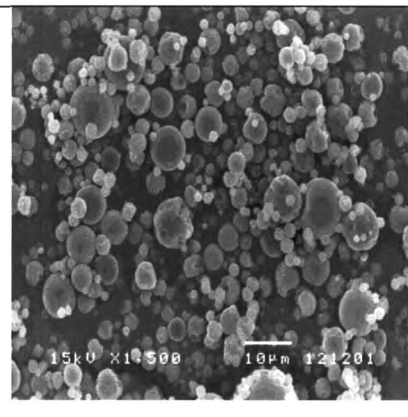
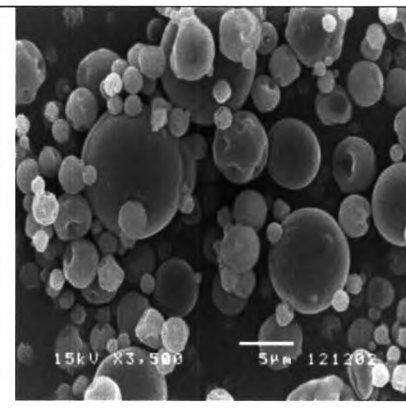
T. laurifolia callus was dark-brown compact callus before harvest as seen in Figure 43. After removed from agar medium and dried by freeze drying process, dried callus was obtained as shown in Figure 44 which was dark-brown dried callus. The extract from callus was brown viscous semisolid extract which was also shown in Figure 44. The RA amount analyzed using HPLC founded that 1 mg of callus extract contains 22.05 mcg of RA and % yield of TL callus extract was 4.72%. In case of

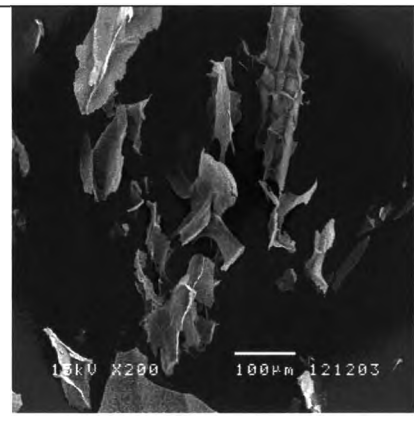
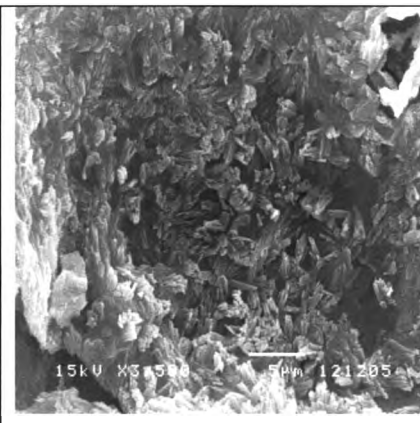
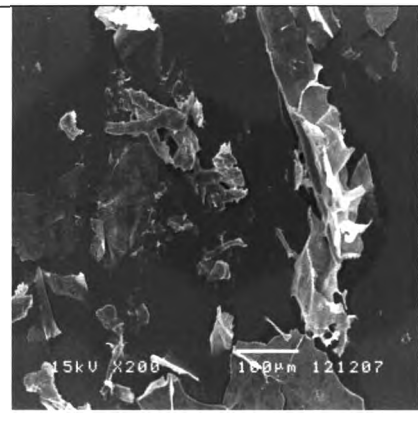
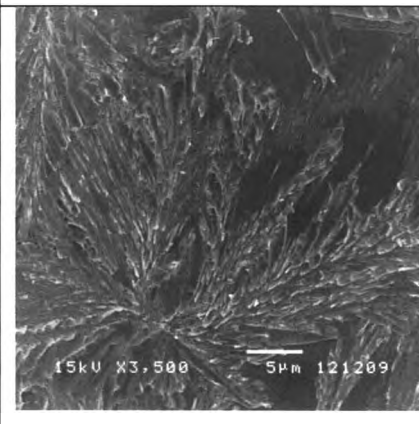
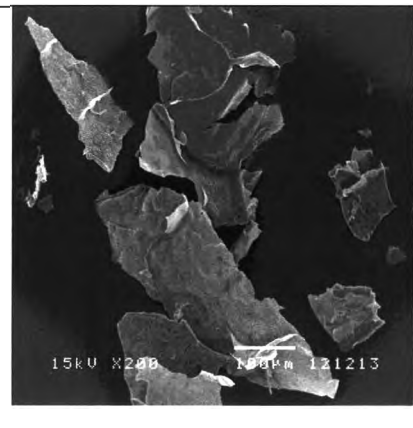
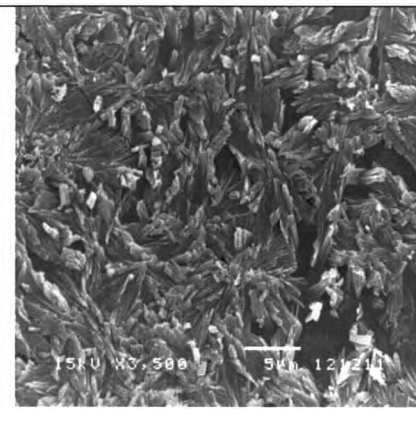
water extraction, there was no peak of rosmarinic acid appeared in the chromatogram (see appendices).

2.2 Morphological characterization of encapsulated TC microparticles

2.2.1 Particle morphology from scanning electron microscope

The particle morphology of encapsulated TC particles from SEM are shown in Figure 45. Similar to previous study, it was found that spray drying process created spherical shape microparticles where as freeze drying process, after cake matrix sieving, created flatted shape powders. The AG spray dried microparticles was still shown the shrink in some particles. The surface morphology of TC-FC-1DG and TC-FC-2DG showed more roughness than that of TC-FC-2AG while the surface of TC-FC-2DGAG1:1 which consisted of the mixed polymer was smoother than the particles from pure DG.

Formulations	Encapsulated TC microparticles (200x, 1,500x), SD process	Encapsulated TC microparticles (3,500x), SD process
TC-SN-1AG	 1,500x	

TC-FC-1DG	 200x	
TC-FC-2AG	 200x	
TC-FC-2DG	 200x	

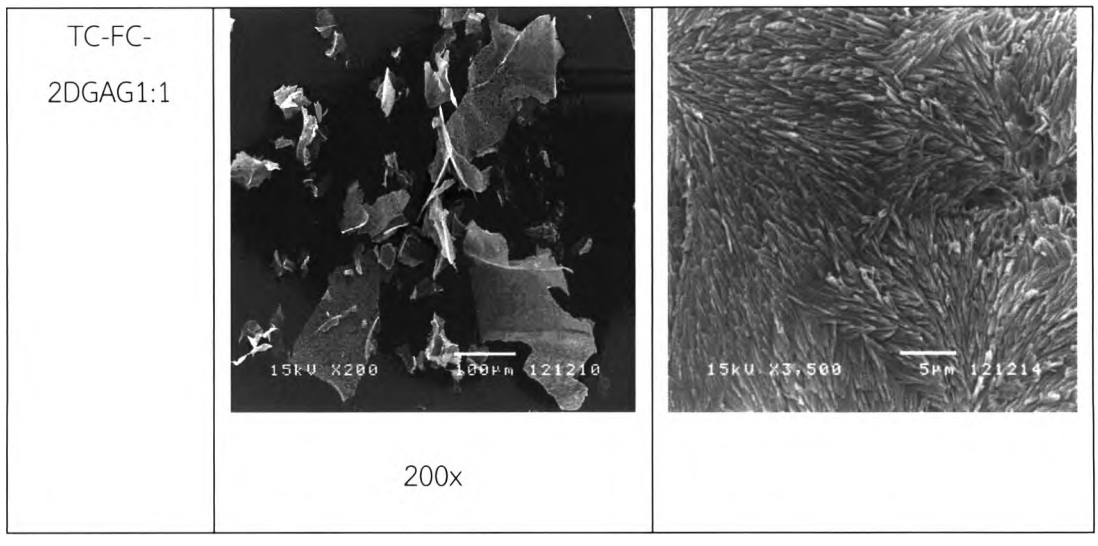


Figure 45 SEM image of encapsulated TC microparticles

2.2.2 Particle size of encapsulated TC microparticles

The particle size results from SD and FD processes are shown in Table 8 and Figure 46. Similar to previous experimental results, the particles from SD process were very small when compared to the powders obtained from FD process. The higher polymer concentration provided the larger size of the particles. In the case of using pure polymer, DG formulation showed the larger particle size than AG formulation as seen in formulation TC-FC-2AG, TC-FC-2DG ($p<0.05$). The mixed polymer of AG and DG in formulation TC-FC-2DGAG1:1 which processed through freeze dry showed the smaller particle size than that of using the pure polymer at the same concentration ($p<0.05$).

Table 8 Particle size of encapsulated TC microparticles

Formulation	Polymer	Particle size (µm)
TC-SN-1AG	AG1% SD-NC	16.51±0.75
TC-FC-1DG	DG1% FD-C	153.39±3.61
TC-FC-2AG	AG2% FD-C	230.71±5.52
TC-FC-2DG	DG2% FD-C	351.93±5.23
TC-FC-2DGAG1:1	DG+AG 2% 1:1 FD-C	256.42±4.97

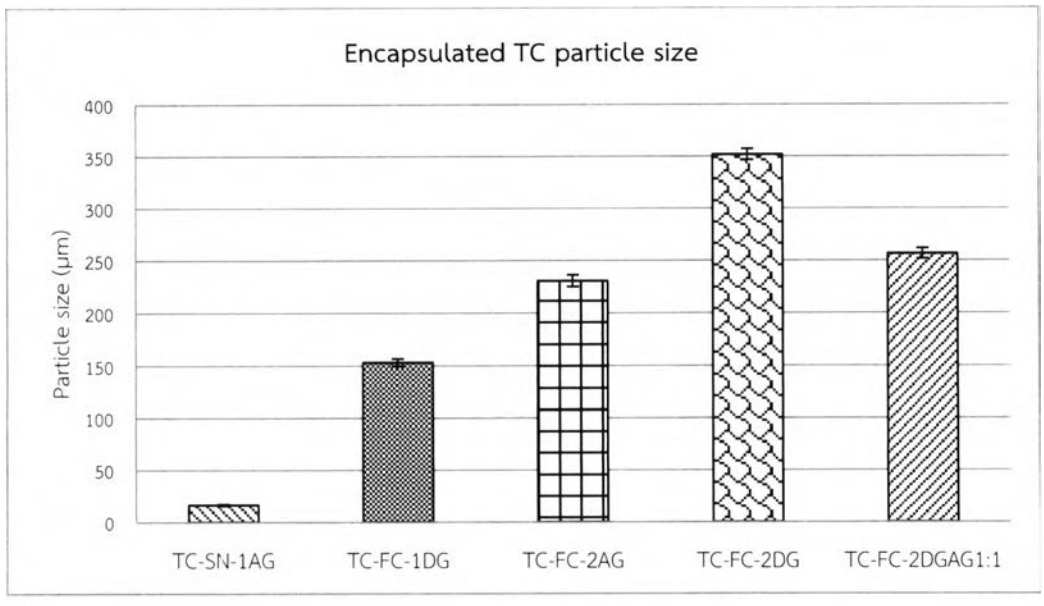


Figure 46 Particle size of encapsulated TC microparticles

2.3 Solid state characterization of encapsulated TC microparticles

2.3.1 Differential scanning calorimetry analysis of TC-microparticles

The results from Figure 47 showed that the broad peak at 110-140 °C which might be dehydration peak and the endothermic peak between 155-195°C was seen in TL callus crude extract while all the peak were disappeared in TC-SN-1AG, TC-FC-1DG and TC-FC-2DG which made of AG1% through spray drying and DG1% and 2% respectively through freeze drying process. Thus the callus extract was incorporated into the polymer. However TC-SN-1AG showed peak at 124 °C which was exothermic peak so recrystallization might occurred at this temperature. So in case of freeze drying, DG polymer exhibited better incorporation of the extract into polymer structure than AG.

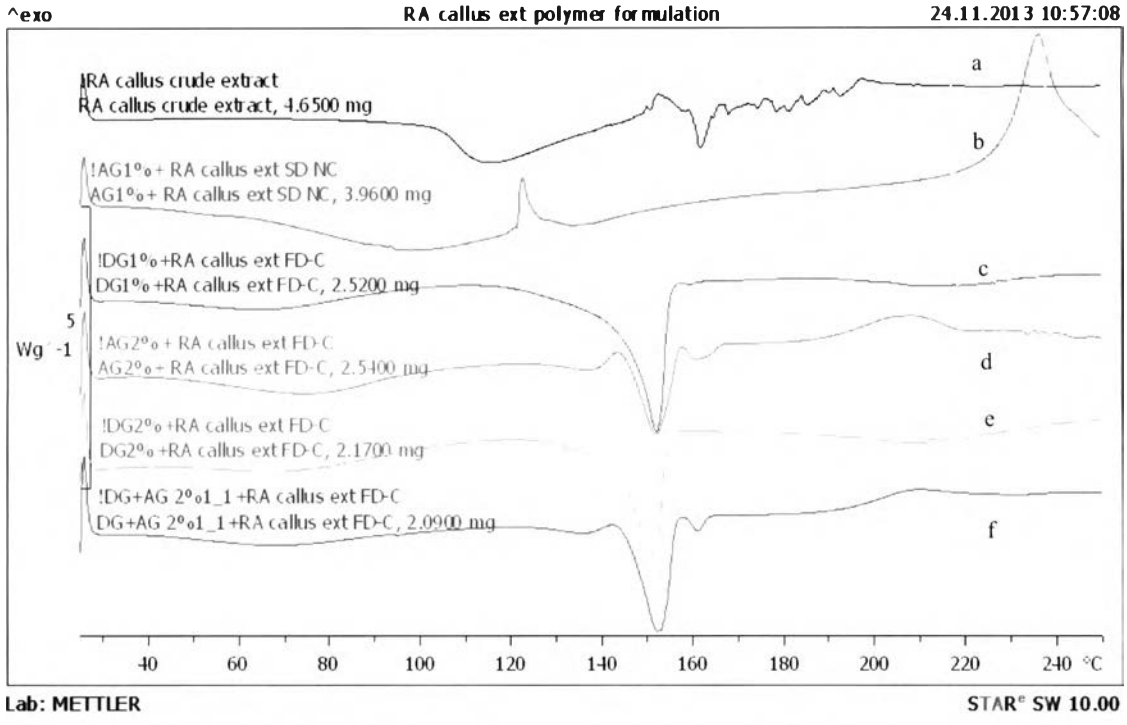


Figure 47 DSC thermogram of encapsulated TC microparticles

a: TL callus extract (TC), b: TC-SN-1AG, c: TC-FC-1DG

d: TC-FC-2AG, e: TC-FC-2DG, f: TC-FC-2DGAG1:1

2.3.2 X-Ray powder diffraction analysis of encapsulated TC microparticles

The results from Figure 48 showed the halo pattern in the diffractogram of TL callus extract due to the semisolid structure of the crude extract. The extract did not appeared in the solid state but in the viscous semisolid state. The pattern observed from the formulation showed tiny peaks, this might cause by the bulk forming excipients of the formulation through freeze drying process.

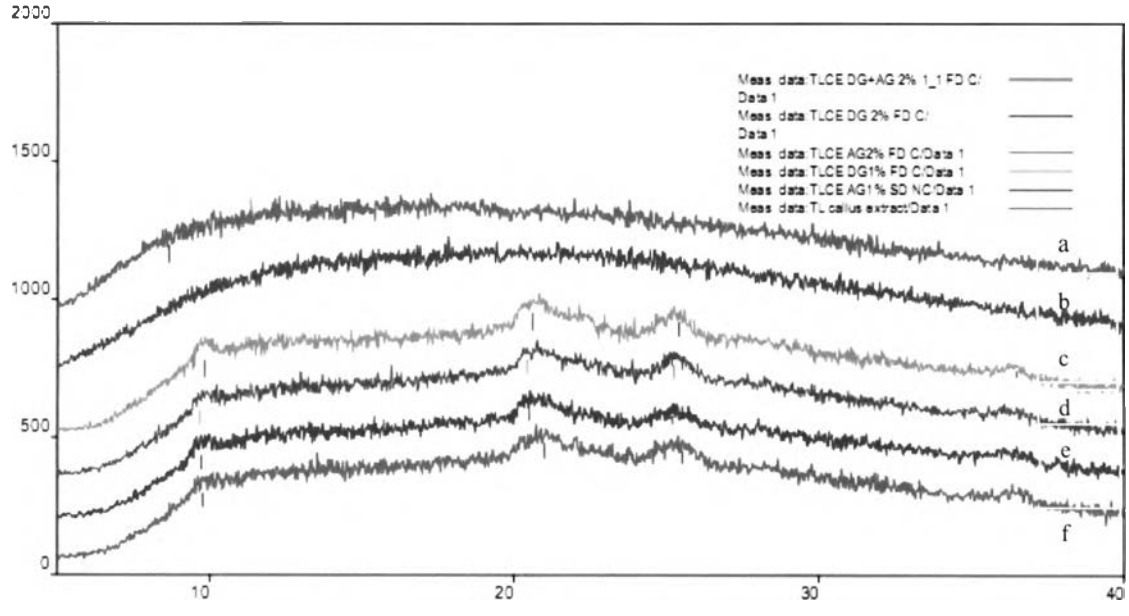


Figure 48 XRD diffractogram of encapsulated TC microparticles

a: TL callus extract (TC), b: TC-SN-1AG, c: TC-FC-1DG, d: TC-FC-2AG

e: TC-FC-2DG, f: TC-FC-2DGAG1:1

2.3.3 Fourier-Transform Infrared Spectrometry

From figure 49, TL callus extract and TL callus extract after freeze drying process showed the same IR spectra. TC-SN-1AG showed slightly different IR spectra comparing with TL callus extract, so there was no significant interaction between TL callus extract and sodium alginate (see appendices for clearer spectra). When comparing to the freeze dried TC microparticles, TC-FC-2DG and TC-FD-2DGAG1:1 showed the peak of mannitol which also occurred in the previous study.

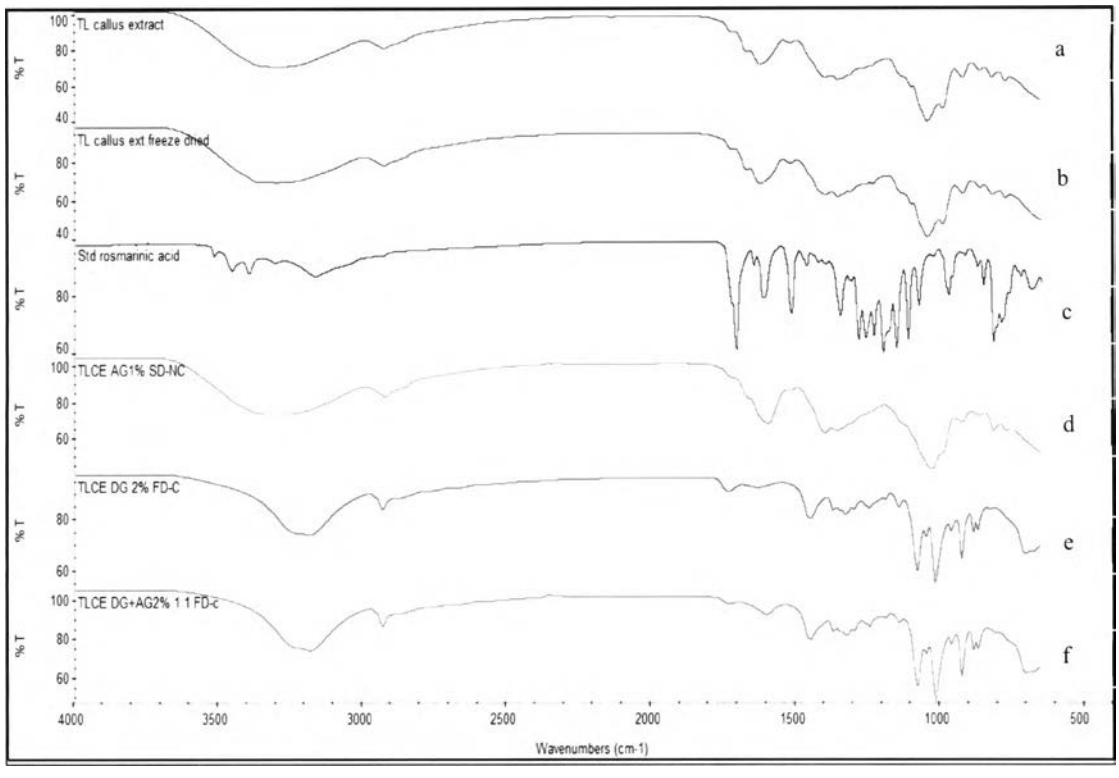


Figure 49 FTIR spectra of TL callus extract, TL callus extract freeze dried, spray dried and freeze dried TC microparticles

a: TL callus extract (TC), b: TL callus extract freeze-dried (TC),

c: Rosmarinic acid d: TC-SN-1AG, e: TC-FC-2DG, f: TC-FC-2DGAG1:1

2.4 Determination of entrapment efficiency of TC microparticles

Entrapment efficiency (% EE) results are shown in Table 9 and Figure 50. The % EE of the freeze drying process (TC-FC-1DG, TC-FC-2AG, TC-FC-2DG, TC-FC-2DGAG1:1) were found to be significantly higher than that of spray drying process (TC-SN-1AG) ($p<0.05$). All TC-FC-1DG, TC-FC-2AG, TC-FC-2DG, TC-FC-2DGAG1:1 which were monitored through freeze drying process did not shown much significantly different.

Table 9 Entrapment efficiency of encapsulated TC microparticles

Formulation	Entrapment efficiency (%EE) (mean±SD)
TC-SN-1AG	79.51±1.67
TC-FC-1DG	98.39±2.85
TC-FC-2AG	100.71±2.14

TC-FC-2DG	101.93±1.18
TC-FC-2DGAG1:1	97.42±3.03

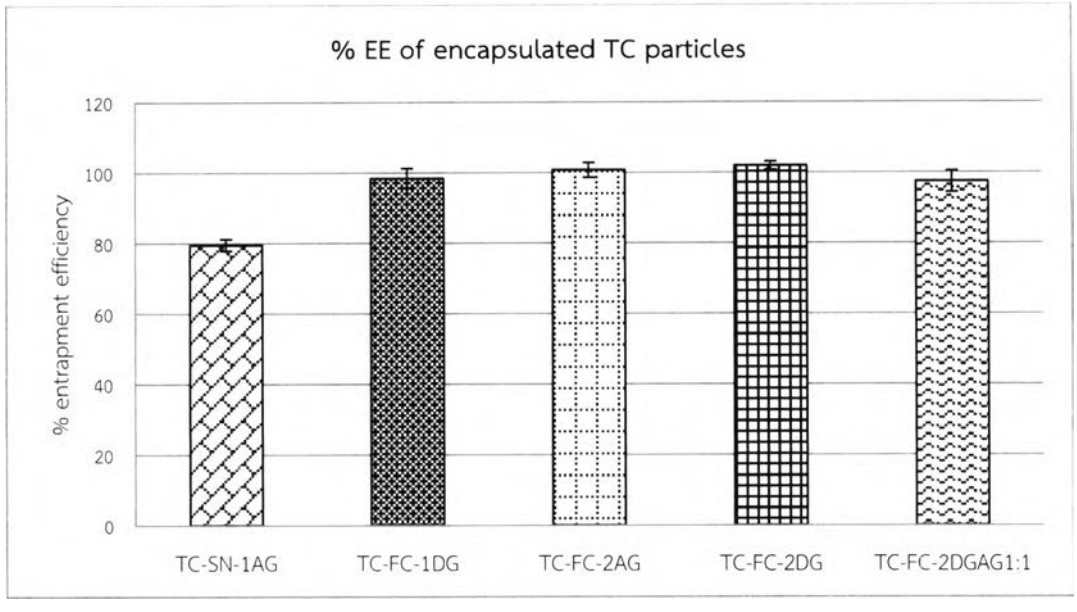


Figure 50 Entrapment efficiency compared between noncrosslinked-spray dried TC microparticles with crosslinked-freeze dried TC microparticles

2.5 In vitro release study of encapsulated TC microparticles

The release profiles of rosmarinic acid from the encapsulated TC particles were shown in Figure 51. TC-FC-1DG showed the fastest release in the first to third hour of release profile and the rest were similar in the initial phase. After three hours TC-FC-2DGAG1:1 released at fastest rate and % RA release was up to 100% at the ninth hour and the rest showed pretty similar release profiles from 6 to 9 hours which released near to 100% at 12 hours. In this case AG might affect slower release more than DG as seen in TC-FC-1DG which showed the fastest rate at the beginning which made of DG pure polymer and TC-SN-1AG showed the slowest release rate. These results were different compared to encapsulated RA microparticles release study as DG showed more retaining of the release in that study. The reason might be TL callus extract composed of a lot of active substances with a small amount of rosmarinic acid and other substances were not clarified in this study so the release profiles and interaction with the polymer were different comparing to rosmarinic acid. The encapsulated TC microparticles also obtained the slower release of

rosmarinic acid compared to the encapsulated RA microparticles in the previous study.

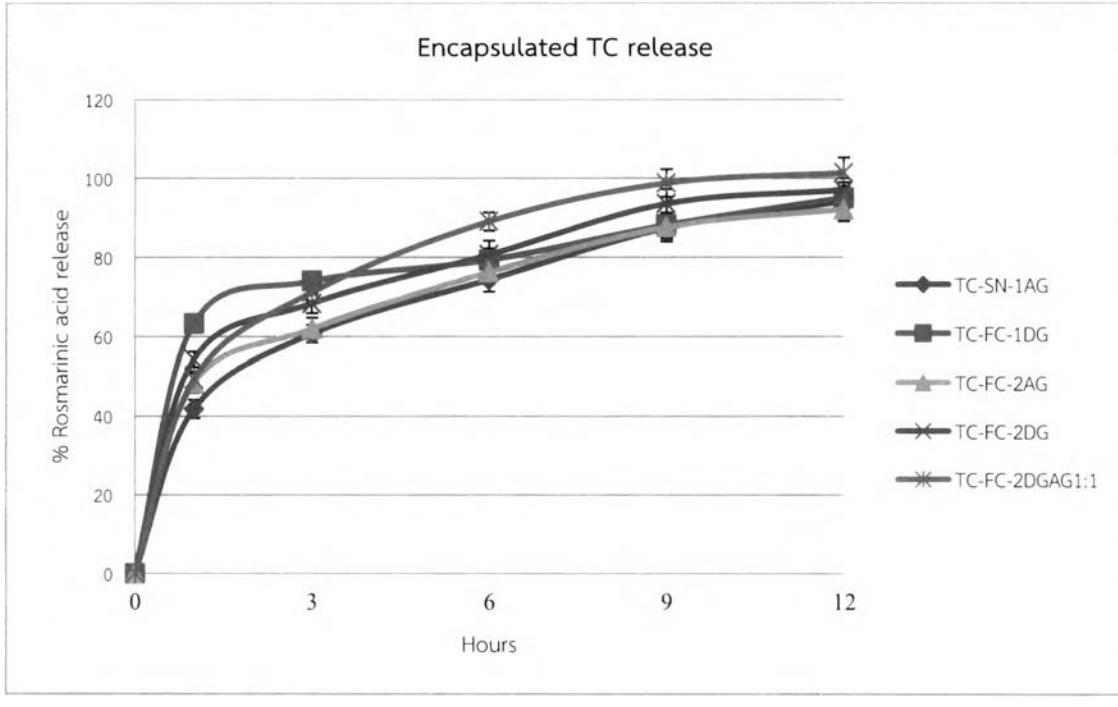


Figure 51 ***In vitro*** **release profiles of encapsulated TC microparticles**

2.6 Stability of encapsulated TC microparticles

Stability study of encapsulated TC particles through SD and FD process were compared to TL callus crude extract which was not passed any process and stored at the three conditions and sampling at time interval for three months. As seen in Figures 52-54, the results showed that the TL callus extract displayed the rosmarinic acid stability decrease much more than those of all formulations in all conditions as expected. All formulations in 4°C, %rosmarinic acid remaining decreased less than 10% from the beginning while TC decreased around 17% after 3 months. As for the results from the condition 30°C/75%RH, the decreasing stability profiles of the formulation were quite similar and TC showed significantly degraded compared to all formulations. The most extreme condition of storage as 40°C/75%RH showed the fastest rate of degradation and rosmarinic acid remained in TC decreased more than 30% after 3 months. Meanwhile all formulations decreased much more than other conditions. TC-FC-1DG showed the most dramatically decrease in stability which more than 20% of rosmarinic acid decreased as observed. In the case of all conditions TC-FC-2DGAG1:1 showed the best result of rosmarinic acid stability. However, at 30°C/75%RH TC-FC-2DGAG1:1 still showed the decrease more than 10%

but significantly increased the stability when compared to TC ($p<0.05$). So this encapsulated formulation showed improvement in stability.

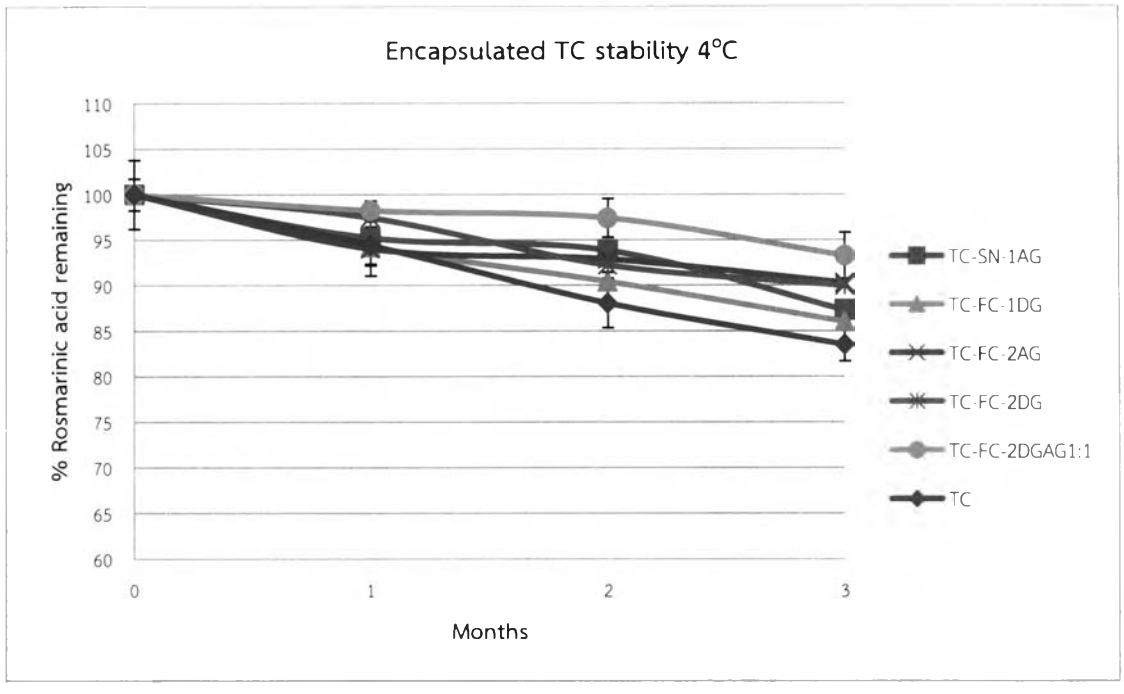


Figure 52 Stability study of encapsulated TC microparticles stored in 4°C

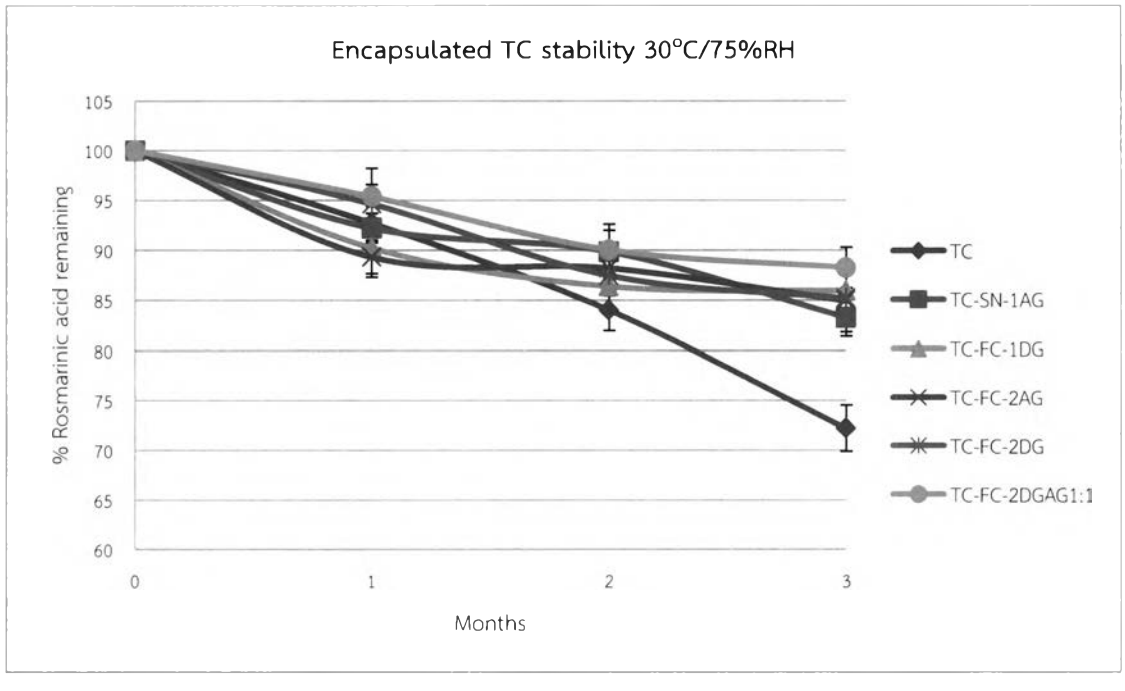


Figure 53 Stability study of encapsulated TC microparticles stored in 30°C/75%RH

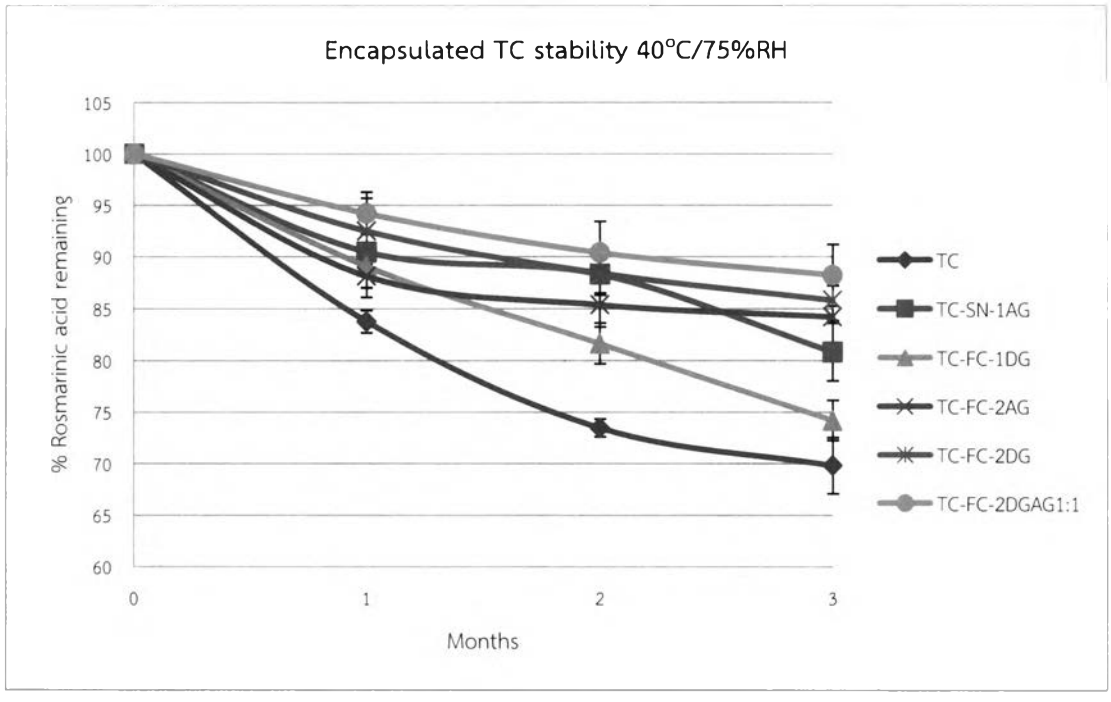


Figure 54 Stability study of encapsulated TC microparticles stored in 40°C/75%RH

3. TC MPs-loaded freeze dried patch

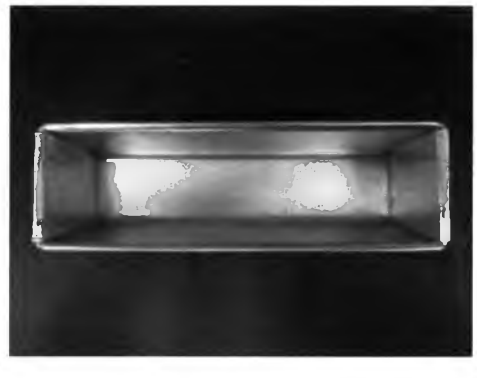


Figure 55 TC MPs-loaded solution in aluminium mold before freeze drying process

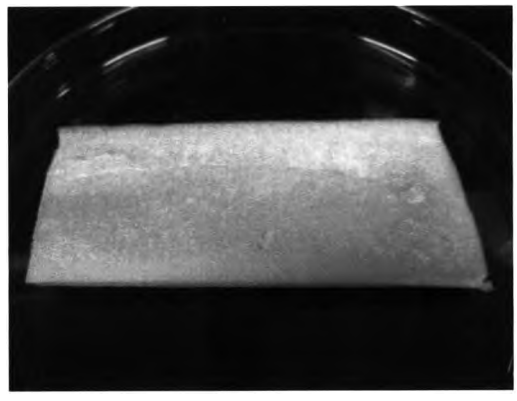


Figure 56 TC MPs-loaded freeze dried patch

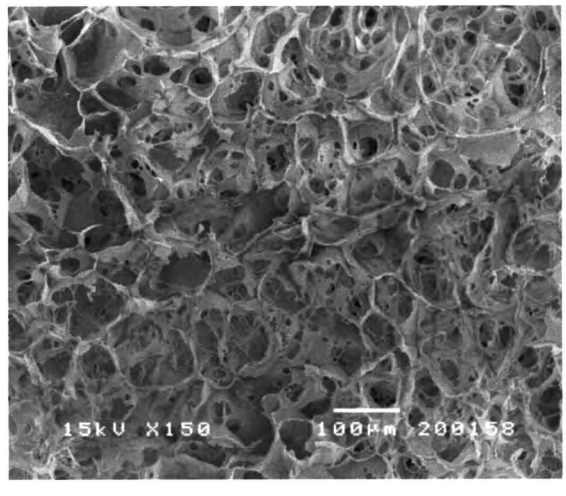


Figure 57 SEM image TC MPs-loaded freeze dried patch (x150)

The TC MPs-loaded solution was pale-orange colour viscous solution as seen in Figure 55. The DG solution was prepared for forming a patch, when adding the TC microparticles, the solution was more viscous since the microparticles were swelled. After freeze drying, the freeze dried patch obtained from 1% DG solution was off-white flexible patch seen as Figure 56. As these patches were processed through freeze drying process so they have high porosity as seen in Figure 57 and quite highly moisture absorbed, however the microparticles which were placed in the patch were not clearly seen. The freeze dried products were kept in aluminium bag and placed in desiccator immediately after passing through the process.

3.1 In vitro release study of TC MPs -loaded freeze dried patch

The release study of TC MPs-loaded freeze dried patch using DG 1% as patch polymer were performed by modified franz diffusion cell. The DG freeze dried patch added with TC (PTC) was prepared to use as control group. TC MPs -loaded freeze

dried patches showed release profiles slower than the PTC. Furthermore TC MPs -loaded freeze dried patches also performed the slower release when compared with the encapsulated TC microparticles in previous study as rosmarinic acid needed to release through the patch layer.

The *in vitro* release results were compared in Figure 58, the release of the control group was faster than the patch those added TC-loaded particles. At the initial phase of release, PTC was significantly seen the burst release at the first hour and up to 80% at the third hour while the rest those contained the formulations slowly released to 80% for 6 hours. Freeze dried DG patch which contained TC-FC-1DG, TC-FC-2AG, TC-FC-2DG, TC-FC-2DGAG1:1 were observed the same release profiles while that contained TC-SN-1AG which particles processed through spray drying process was observed to be the slowest release.

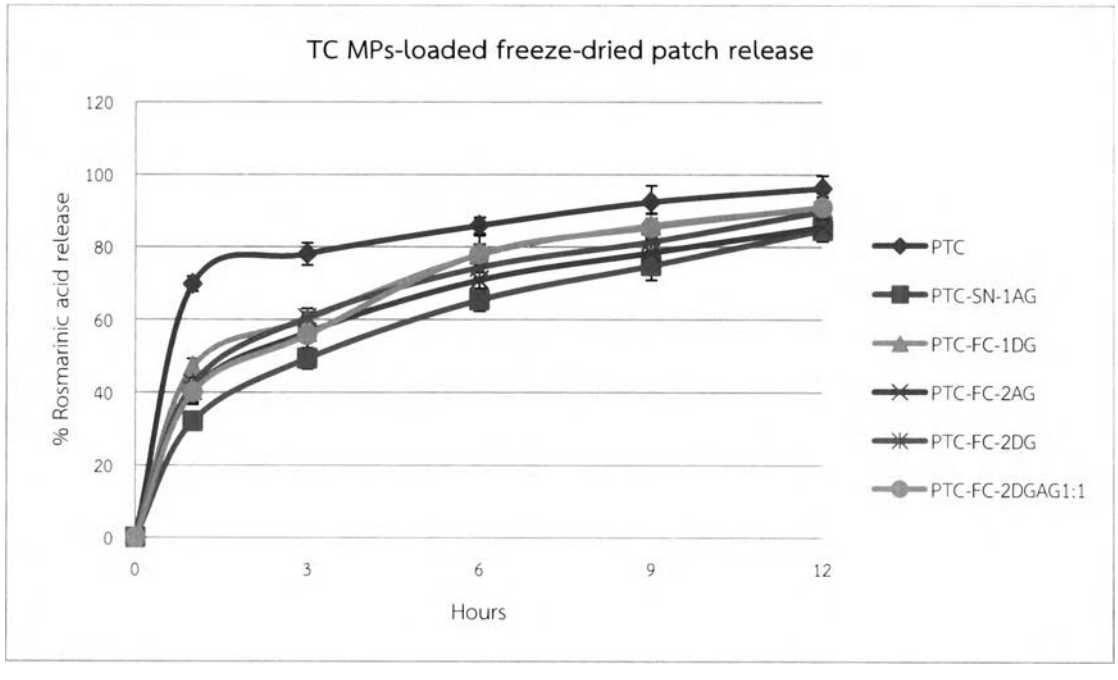


Figure 58 ***In vitro*** **release profiles of TC MPs -loaded freeze dried patch**

3.2 Stability study of TC MPs -loaded freeze dried patch

The stability study of TC MPs -loaded freeze dried patch were investigated by storage at three conditions: 4°C, 30°C/75%RH and 40°C/75%RH. PTC was performed as control group along with PTCexc which TC and the excipients (exc) used in microparticle formulation was added as powder form. So there were two control groups in this stability study to compare with PTC-SN-1AG, PTC- FC-1DG, PTC-FC-2AG, PTC-FC-2DG, PTC-FC-2DGAG1:1. The results from 4°C storage condition were shown in

Figure 59. All the formulations and also the control groups were trended to have the same stability profiles with not significantly different which % rosmarinic acid reduced around 10% after 3 months. However the PTC-FC-2DG showed the fastest reduced of % rosmarinic acid and the lowest remained amount of rosmarinic acid after 3 months.

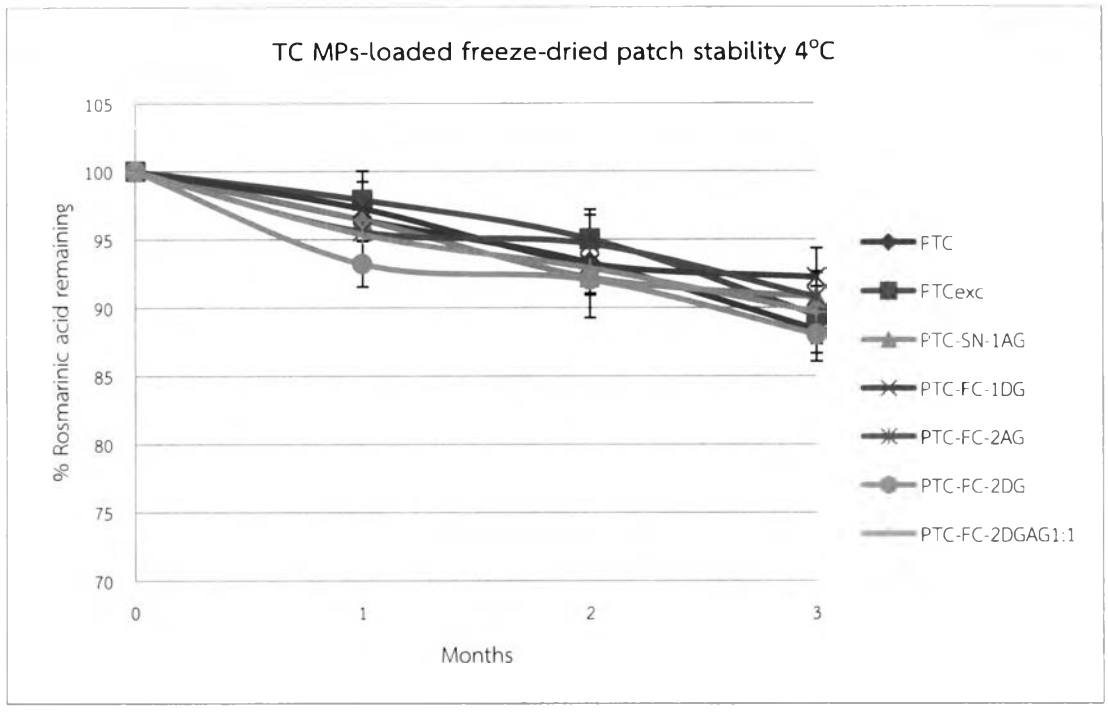


Figure 59 Stability study of TC MPs -loaded freeze dried patch stored at 4°C

Stability profiles of TC MPs -loaded freeze dried patch stored at 30°C/75%RH were shown in Figure 60. It was observed that the patch color turned into darkened color after 3 months storage. It was founded that rosmarinic acid reduced by 10-15% in PTC-SN-1AG, PTC-FC-1DG, PTC-FC-2AG, PTC-FC-2DG, PTC-FC-2DGAG1:1 profiles and reduced by 20-25% in the two control groups after 3 months. The lowest rosmarinic acid remaining was shown in PTC-FC-1DG while PTC-FC-2AG provided the best stability. As the results compared from Figure 58 and 59, the higher temperature affected to the two control groups more than the patches those contained microparticles so the decreasing of remained rosmarinic acid were clearly shown in stability profiles at 30°C/75%RH.

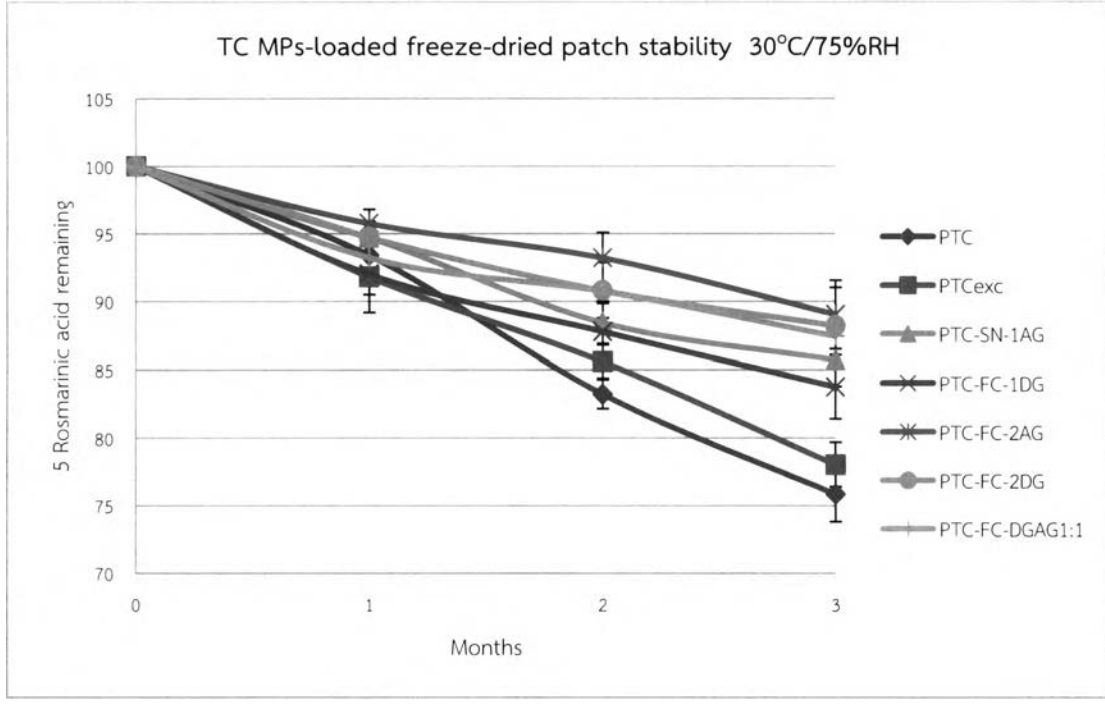


Figure 60 Stability study of TC MPs -loaded freeze dried patch stored at 30°C/75%RH

After stored at 40°C/75%RH for 3 months, the observed results from Figure 61 showed that PTC, one of control group, had the fastest rate and the worst stability result which rosmarinic acid reduced approximately 30%. While another group showed 20% rosmarinic acid decreased after 3 months. The patches those contained formulations displayed the better stability results which rosmarinic acid reducing around 13-18% when comparing with PTC and PTCexc ($p<0.05$). PTC-FC-2DGAG1:1 observed the best stability result at the end of storage but it was not significantly different among all formulations.

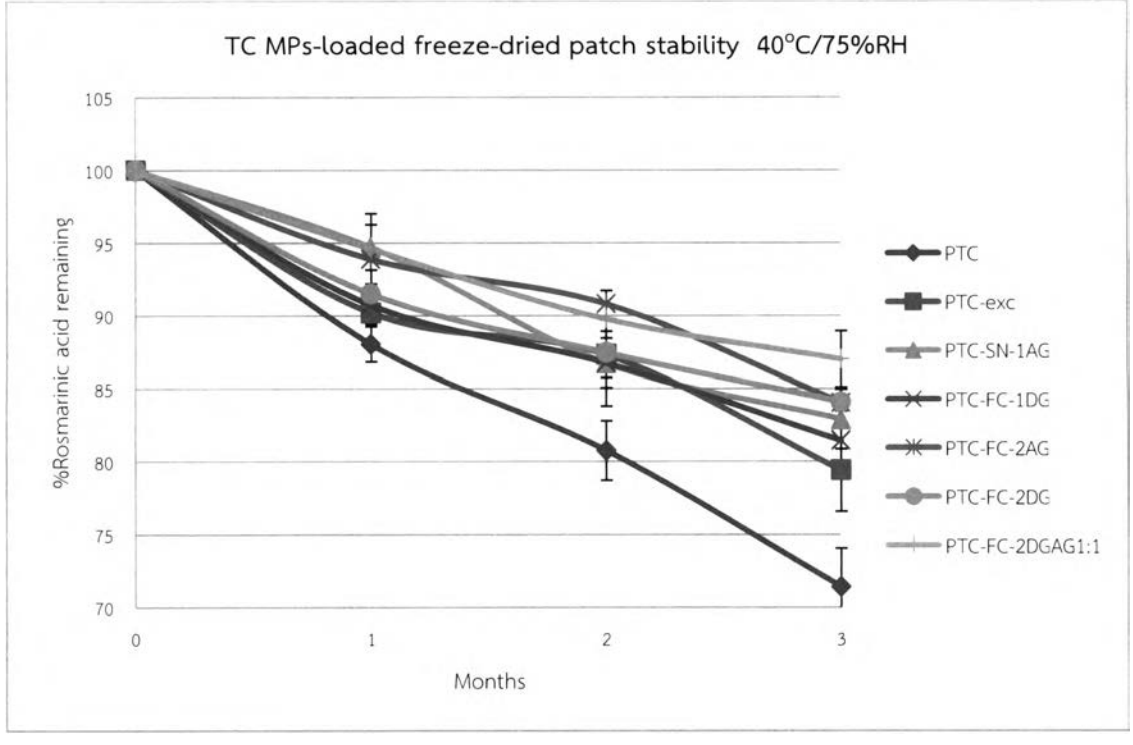


Figure 61 Stability study of TC MPs -loaded freeze dried patch stored at 40°C/75%RH

When comparing all the conditions above, PTC-FC-2AG and PTC-FC-2DGAG1:1 showed the better stability in all conditions compared to the rest and PTC-FC-2DGAG1:1 was observed for the best result at the end of accelerated condition. So the mixed polymer of DG and AG at the ratio 1:1 prepared by FD process created the most protective property for protecting rosmarinic acid which included in TC. So DG can improve the stability of the callus extract and be able to use as a mixture with sodium alginate which exhibits proper release profile and stability.