Applied Chemistry Project

Project title Preparation of Proanthocyanidin Nanoparticles from Grape Seed Extract

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Faculty of Science, Chulalongkorn University

Preparation of Proanthocyanidin Nanoparticles from Grape Seed Extract

by
Mr. Phanop Phatararuji
Mr. Purin Jittatham

In Partial Fulfillment for the Degree of Bachelor of Science
Program in Applied Chemistry (International Program)
Department of Chemistry, Faculty of Science
Chulalongkorn University
Academic Year 2020

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Accepted by Department of Chemistry, Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science Program in Applied Chemistry (International Program)

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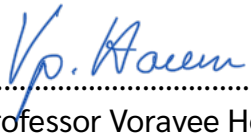

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Abstract

In this experiment, we aimed to synthesize the proanthocyanidin nanoparticles from grape seed extract (GSE) with and without encapsulating calcium citrate by coprecipitation method. The concentrations of GSE, calcium ion, and citrate ion were varied to specify the suitable proportion that nanoparticles can be formed. UV-Vis spectrophotometer and scanning electron microscope were used to characterize the shape and size of nanoparticles. However, the size of the particles was not in the nanoscale. Therefore, further development is needed to successfully synthesize the proanthocyanidin nanoparticles.

Keywords: Grape seed extract, Proanthocyanidin, Nanoparticles, Calcium Citrate

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Phanop Phatararuji
Purin Jittatham

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Chapter 1
Introduction

1.1 Research problem and significance

Nowadays, the development of antibiotics from natural products to the trend of the modern world is plentifully increasing. The grape seed extract is one of the natural products that received abundant interest because it has a low cost when compared to the benefits gained. GSE was known as a powerful antioxidant based on the capacity of a sample to scavenge the ABTS radical cation as compared to a standard antioxidant (Trolox).[1] Moreover, drug carrier for the human body and skin has also been raised as an important issue with various experiments being conducted to achieve satisfying and appropriate outcomes in many aspects. Nanoparticles are currently used to deliver effective drugs due to the improving effectiveness of biological drug storage at sites within target cells. Calcium carbonate is the main compound used to synthesize the nanoparticles. But many experiments found that the use of $CaCO_3$ cannot be administered orally and makes it difficult to control the nanoparticles because of their high sensitivity toward acidic conditions.[2] Moreover, it also affects the preservation of the drug and makes it difficult to preserve the nanoparticles in solid form. Furthermore, recent works show that using novel calcium and citrate ions through the coprecipitation method obtained calcium citrate nanoparticles (CaCit NPs) can get rid of those restrictions.[3] Hence, it is very interesting to navigate through the process to become a proanthocyanidin nanoparticle from grape seed extract. In this experiment, the grape seed extracts (GSE) containing 95% proanthocyanidin were used as the active ingredient reacting with calcium ions. Then, citrate ion was used to facilitate the formation of nanoparticles.

1.2 Objectives

1.2.1 Synthesis and characterisation of proanthocyanidin nanoparticles from grape seed extract.

1.3 Literature review

1.3.1 Grape seed Extract (GSE)

Grape seed extract or GSE from *Vitis vinifera* contains polyphenol compounds which are broadly distributed in plants, fruits, and vegetables.[4,5] The polyphenols in GSE contained flavan-3-ols (catechin, epicatechin, proanthocyanidin (condensed tannin), and procyanidin.[6] Procyanidin dimer (C_4-C_8) are mostly found in grape seed extract as a B-type procyanidin.[7,8] The B-type procyanidin including epicatechin-(4$\beta \rightarrow$8)-catechin (B1), epicatechin-(4$\beta \rightarrow$8) -epicatechin (B2), catechin-(4$\alpha \rightarrow$8)-catechin (B3), and catechin-(4$\alpha \rightarrow$8)-epicatechin (B4).[9] The chemical structures are shown in **Figure 1-1**. The grape seed extract was composed of 89% proanthocyanidin as a B type proanthocyanidin identified by using high pressure liquid chromatography (HPLC).[10] This was strongly suggested that the increased peak in human blood is procyanidin B type. Also, about 60-70% of polyphenolic are present in seed more than in the pulp and skin of *Vitis vinifera* that present only 10-35%.[11] Moreover, grape seed

extract is considered as a powerful antioxidant and has many other biological effects such as antiviral, antibacterial and radical-scavenging properties. The proanthocyanidin efficacy test illustrated that it can help to increase antioxidants makes the human skin cleaner.[1] Moreover, GSE also has a protective effect on foods from UV light-induced degradation.[12]

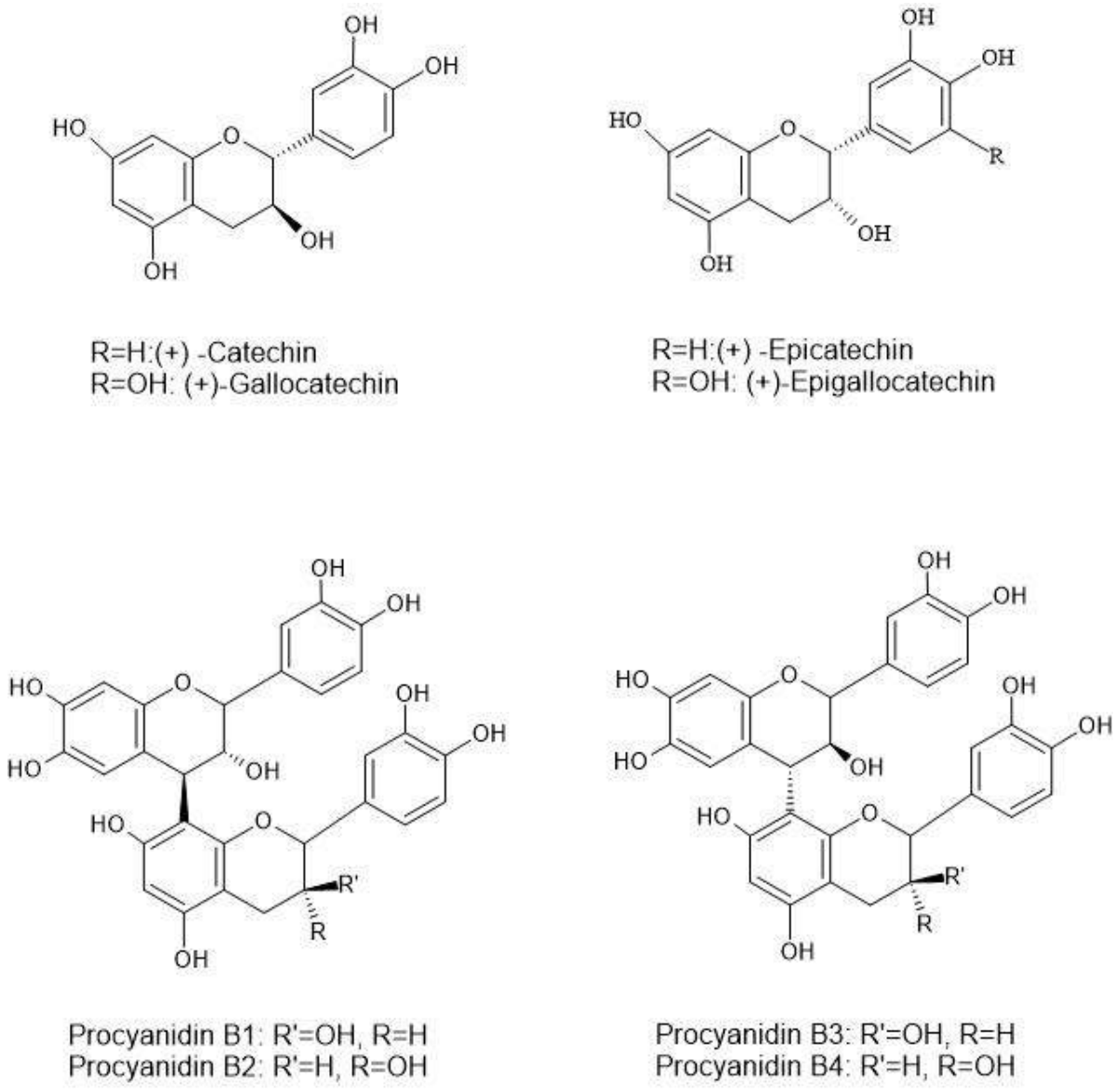

Figure 1-1 Chemical structures of polyphenols in grape seed extract

1.3.2 Calcium Citrate Nanoparticles (CaCit NPs)

Nowadays, the use of nanoparticles to deliver drugs to specific cells and tissues together with the controlled release therapy can help decrease the toxicity from drugs and increase patient safety by providing less medication.[13] Calcium Citrate Nanoparticles arising from coprecipitation method between calcium and citrate ions. Calcium citrate nanoparticles drug carriers activities were tested by incubating the synthesized fluorescent-tagged NPs with human keratinocytes using a confocal microscope.[3] The results of the experiment showed that CaCit NPs could be used as a Trojan carrier to release drugs into specific cells. The chemical structure of the citrate ion is shown in **Figure 1-2**.

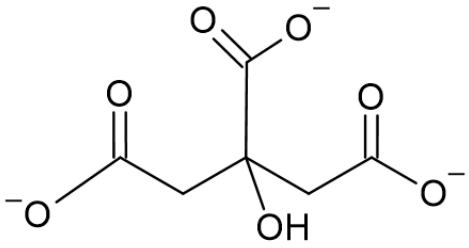

Figure 1-2 Chemical structure of citrate ion

1.3.3 Characterization Method

1.3.3.2 Scanning Electron Microscope (SEM)

A Scanning electron microscope or SEM uses high energy electrons beam to generate signals from the surface of solid specimens. This technique provides images of high resolution, meaning that closely spaced features can be examined at high magnification.[14] SEM instruments and some images of CaCit in different concentrations are shown in **Figure 1-3**.

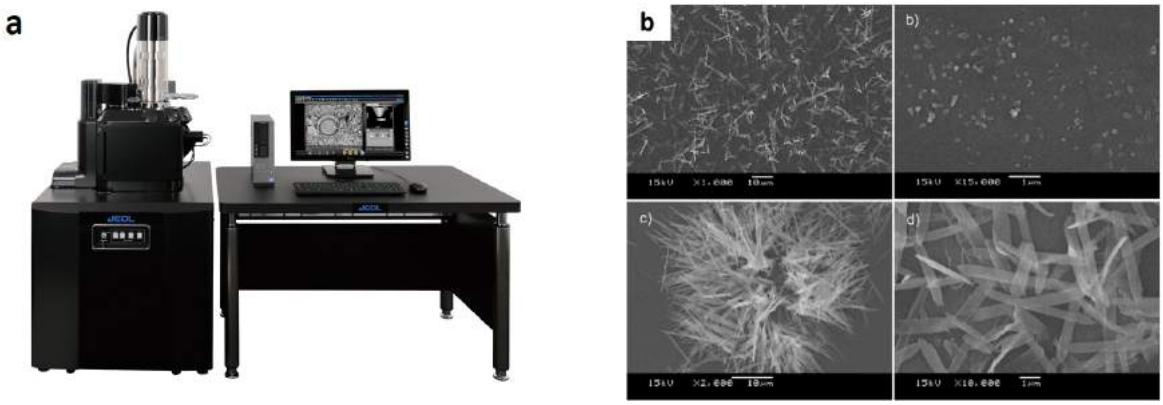

Figure 1-3 a) Scanning electron microscope (Jeol JSM-IT300)
b) SEM images of Ca-Cit in different ratio concentration[3]

Chapter 2
Experimental

2.1 List of equipment and instrument

2.1.1 Thermo Scientific Genesys 10S UV-Vis Spectrophotometer
2.1.2 Jeol JSM-IT300 Scanning Electron Microscope

2.2 List of chemicals and materials

2.2.1 Grape seed extract powder 95% OPC (Asianbioplex, Thailand)
2.2.2 Calcium chloride dihydrate (Merck, Germany)
2.2.3 Trisodium citrate dihydrate (Merck, Germany)
2.2.4 Sodium Hydroxide (Merck, Germany)
2.2.5 Deionized Water

2.3 Experimental procedure

2.3.1 Chemical Preparation

The sodium hydroxide (1.00 M) stock solutions were prepared by dissolving 1.200 g of NaOH (M=40.00 g/mol) in 30.00 mL of DI water.

The various concentrations of calcium ion stock solution were prepared by dissolving $CaCl_2$ (M=147.01 g/mol) in 30.00 mL of DI water as shown in Table **2-1**.

Table 2-1 The various concentrations of calcium ion with mass.

Concentration of Calcium ion (M)	$CaCl_2$ Weight (g)
0.020	0.088
0.040	0.176
0.080	0.353
0.130	0.573

The various concentrations of citrate ion stock solutions were prepared by dissolving Trisodium citrate (Na_3Cit) (M=294.1 g/mol) in 30.00 mL of DI water as shown in the **Table 2-2**.

Table 2-2 The various concentrations of citrate ion with mass.

Concentration of Citrate ion (M)	Na_3Cit Weight (g)
0.010	0.088
0.020	0.176
0.030	0.265
0.040	0.353
0.050	0.441

2.3.2 Preparation of Grape Seed Extract Stock Solution

The various concentrations of Grape seed extract (GSE) stock solutions were prepared by dissolving the grape seed extract powder (M=290 g/mol) in 3.00 mL of DI water as shown in table 2-3 and then vortexed continuously for 5 minutes. The pH of GSE solutions were adjusted to be in the range of 7.30-7.50 using NaOH stock solution.

Table 2-3 The various concentrations of GSE solution with mass.

Concentration of GSE (M)	GSE weight (g)
0.050	0.044
0.060	0.052
0.070	0.061
0.080	0.070
0.090	0.078
0.100	0.087

2.3.3 Synthesis of Proanthocyanidin Nanoparticles (Pure Drug Assembly)

First, 3-mL of various concentrations of calcium ion solution as shown in **table 2-4** were added into the GSE solution (0.100 M). The mixtures were vortexed continuously for 5 minutes. The mixtures were rocked at various times. After that, the mixtures were centrifuged at 5000 rpm / 25 minutes at 25℃. The precipitates were washed with DI water and frozen at -80℃ overnight. The solid was freeze-dried for further characterization.

Table 2-4 The concentrations of mixture. (GSE + Ca^{2+})

Concentration of GSE (M)	Concentration of Calcium ion (M)
0.100	0.130
	0.080
	0.040
	0.020

2.3.4 Synthesis of CaCit-Based Proanthocyanidin Nanoparticles

First, 3-mL of various concentrations of citrate ion solution was added into the varied concentration of GSE solution as shown in **table 2-5**. The mixtures were vortexed continuously for 5 minutes. Then a 3-mL of varied calcium ion solutions were added into the mixture and then vortexed again for 5 minutes. The mixture was rocked at different amounts of times. After completing rocking, centrifuged the mixture at 5000rpm / 25 minutes at 25℃. The precipitates were washed with DI water and frozen at -80℃ overnight. The precipitates were freeze-dried for further characterization.

Table 2-5 The concentrations of mixture. (GSE + CaCit)

Concentration of GSE (M)	Concentration of Citrate ion (M)	Concentration of Calcium ion (M)
0.090	0.010	0.020 / 0.030
0.080	0.020	0.020 / 0.030
0.070	0.030	0.020 / 0.030
0.060	0.040	0.020 / 0.030
0.050	0.050	0.020 / 0.030

2.3.5 Characterization of Nanoparticles

2.3.5.1 UV-Visible Absorption Analysis

UV-Vis absorption was observed using UV-Vis Spectrophotometer (Thermo Scientific Genesys 10S, USA) at 400-800 nm. The samples were analyzed against the DI water as a blank.

2.3.5.2 Shape and Size Analysis

Shape and size of nanoparticles were characterized using Scanning Electron Microscopes at 20.0 kV (Jeol JSM-IT300). The samples were prepared by redisperse the solid with DI water until the solution was perfectly dissolved. Then drop the solution onto the stubs with carbon tape to prevent all the volatile objects and import the moisture proof cabinet overnight and wait until the aqueous to evaporate and until the solid substance is placed on the carbon tape as shown in **Figure 2-1**.

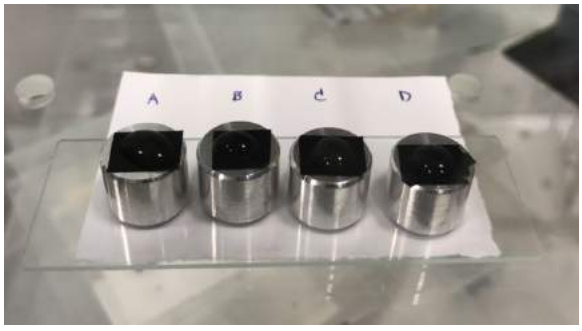

Figure 2-1 Aqueous substance sample A-D on stubs

Chapter 3
Results and discussion

3.1 Synthesis of Proanthocyanidin Nanoparticles (Pure Drug Assembly)

3.1.1 Effects of calcium ion concentration to grape seed solution (Pure Drug Assembly)

First, the proanthocyanidin nanoparticles were synthesized from grape seed extract (GSE) using coprecipitation method between GSE and calcium ions. Due to the grape seed substance being phenolic which is insoluble in water, we attempted to adjust the pH first by adding NaOH and found that the mixture was perfectly dissolving. The calcium ion was added to the grape seed solution to precipitates the molecules and to obtain the pure drug assembly. The concentration of the GSE was fixed at 0.010 M and the concentration of calcium ions were varied from 0.130 M to 0.020 M to observe the possibility of forming nanoparticles. From the result **(Table 3-1)**, after the completion of the rocking time and leave it for a while all of the mixture were precipitated within 30 minutes. This indicated that the size of a particle was large and more likely to be macroscale than nanoscale.

Table 3-1 The result of the effect of calcium ion conc. to grape seed solution (pure drug assembly)

$[Ca^{2+}]$ / Time	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs
0.020					
0.040					
0.080					
0.130					

3.2 Synthesis of CaCit-Based Proanthocyanidin Nanoparticles

3.2.1 Effects of citrate ion and calcium ion concentration to grape seed solution

Since we tried to synthesize a pure drug assembly (GSE + Ca^{2+}) but the result was negative. Due to the past works of our research group indicated that the use of citrate ion can successfully synthesize the nanoparticles. Therefore, we tried the second method by using the citrate ion with GSE. The citrate ion was involved in the reaction to attempt to form the nanoparticles. The result in **Table 3-2** shows that at 48 hours, the samples of 0.0700 M GSE with 0.0300 M citrate ion and 0.0200/0.0300 M calcium ion were turbid and not precipitated out after leaving at room temperature for hours. This indicated that the size of a particle had the possibility to be a nanoscale.[2,15]

Table 3-2 The result of the effect of citrate and calcium ion conc. to grape seed solution

[GSE]	[$C_6H_5O_7^{3-}$]	[Ca^{2+}]	12 hrs	24 hrs	48 hrs
0.050	0.050	0.020			
		0.030			
0.060	0.040	0.020			
		0.030			

Table 3-2 (continued)

The result of the effect of citrate and calcium ion conc. to grape seed solution

[GSE]	$[C_6H_5O_7^{3-}]$	$[Ca^{2+}]$	12 hrs	24 hrs	48 hrs
0.070	0.030	0.020			
		0.030			
0.080	0.020	0.020			
		0.030			
0.090	0.010	0.020			
		0.030			

3.3 Characterization of Nanoparticles

3.3.1 UV-Vis Absorption Analysis

The mixture of 0.070 M GSE with 1.00 M NaOH (pH=7.45) was chosen to analyze to observe the characteristic absorption and to be used as a comparison with the colloidal GSE solutions. The mixture was varying the concentration by diluting 4, 6, 8, 10 times the final concentration respectively. The wavelength (λ_{max}) is about 502-506 nm which is related to the characteristic wavelength of red-violet color at the wavelength (λ_{max}) = 510nm. However, diluting more than 10 times the final concentration caused the line to be out of the linear range.

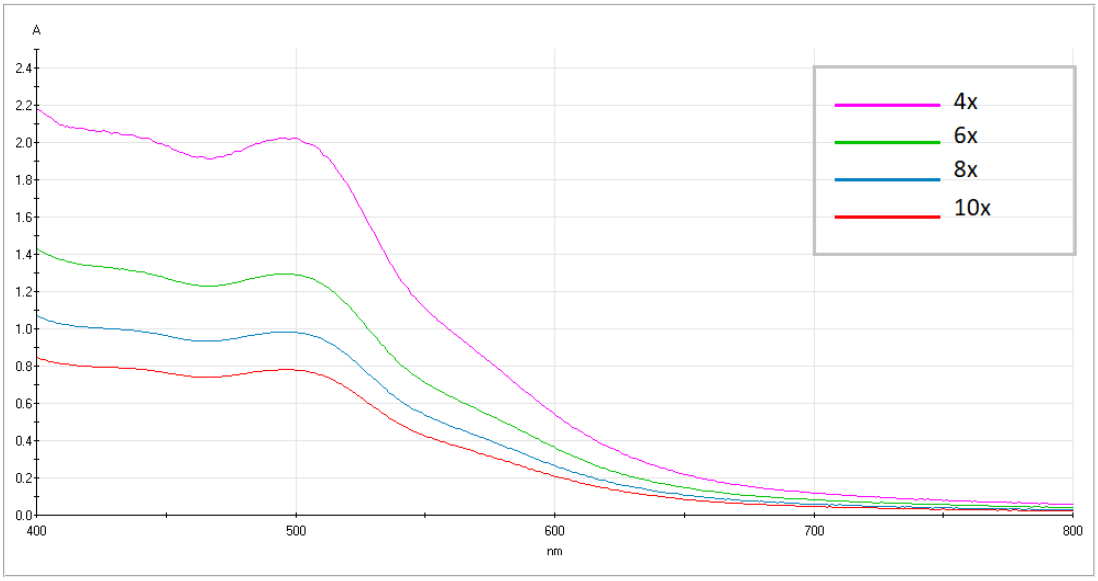

Figure 3-1 UV-Vis spectra of 0.070 M GSE at pH=7.45 (4, 6, 8, 10 times dilution)

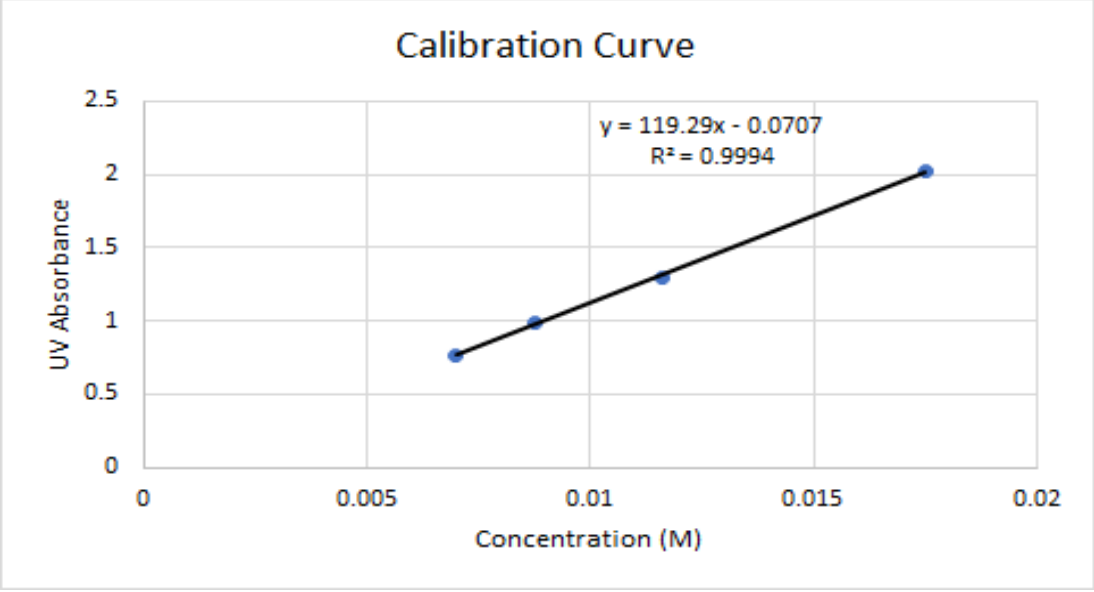

Figure 3-2 Calibration curve of 0.070 M GSE at pH=7.45 (4, 6, 8, 10 times dilution)

Next, the colloidal GSE solution contained 0.070 M GSE in 1.00 M NaOH (pH=7.45) with 0.030 M citrate ion and 0.020/0.030 M calcium ion at 48 hours rocking time was chosen to analyze by varying the concentration of the mixture 4, 6, 8, 10 times of the final concentration respectively. The graph shows the characteristic absorption spectrum at 550 nm with extra new absorption band at 430 nm **(Figure 3-3 and Figure 3-4)**. This is not resulted from the absorption of citrate ion because the characteristic absorption of citrate ion was at 210 nm.[16] Therefore, this new absorption band may be possibly caused by the unknown interaction of GSE molecules. Further study is required.

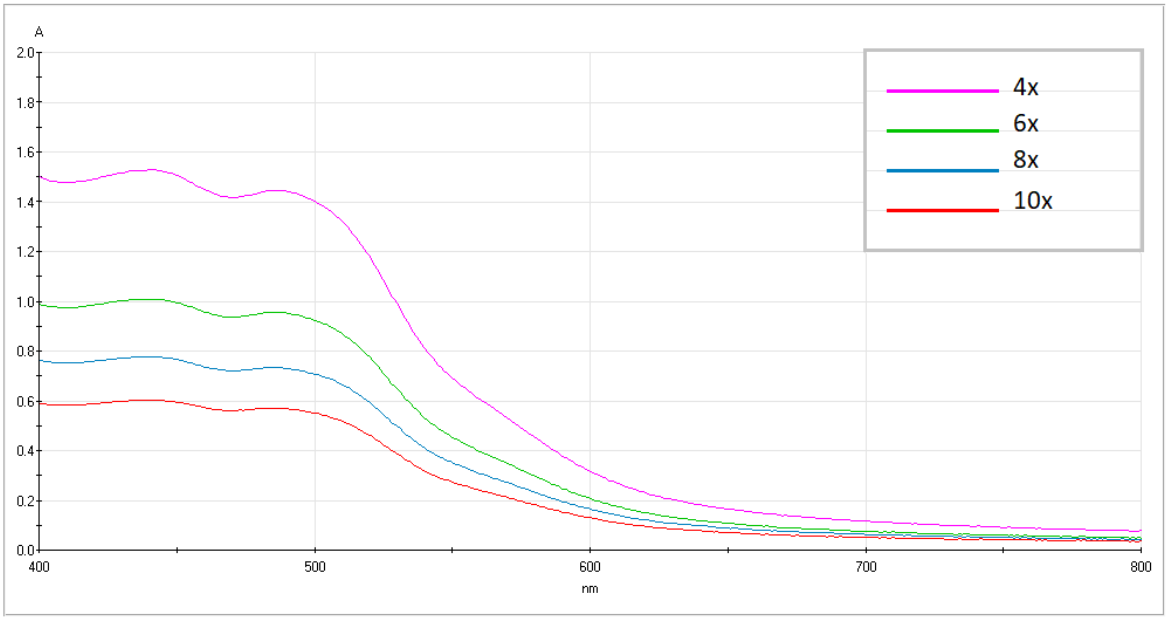

Figure 3-3 UV-Vis spectra of 0.070 M GSE at pH=7 with 0.030 M citrate ion and 0.020 M calcium ion (4, 6, 8, 10 times dilution)

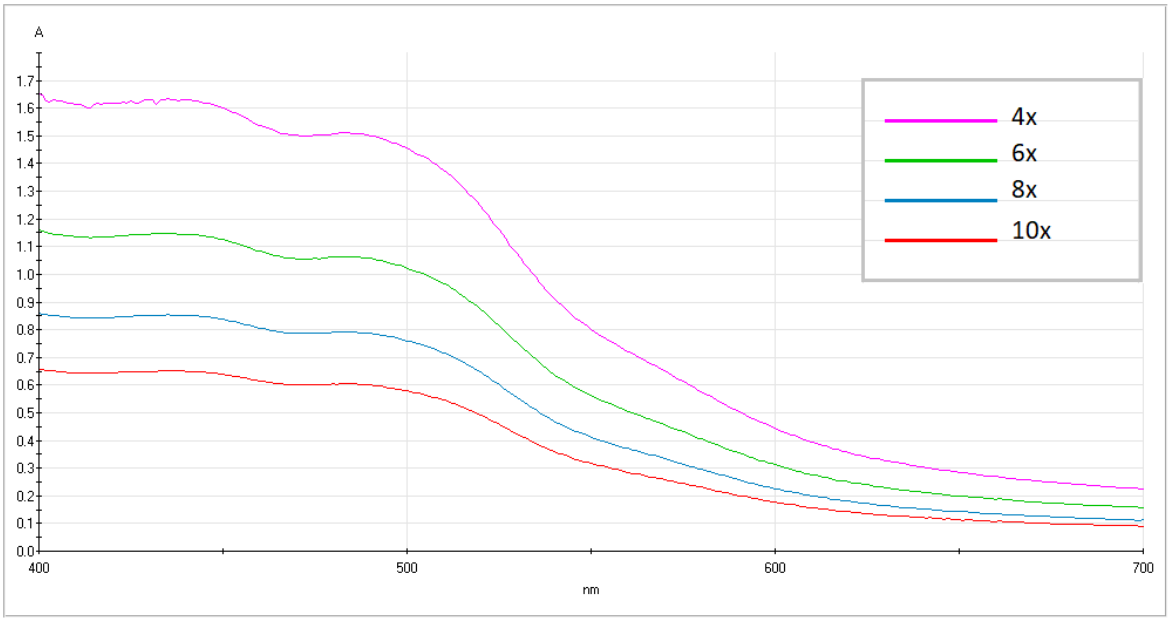

Figure 3-4 UV-Vis spectra of 0.070 M GSE at pH=7 with 0.030 M citrate ion and 0.030 M calcium ion (4, 6, 8, 10 times dilution)

3.3.2 Shape and Size Analysis

The 48 hours rocking sample was labeled into A, B, C, and D with the difference of condition and concentration as shown in Table **3-3**.

Table 3-3 Sample conditions. (for SEM)

Sample	[GSE]	$[C_6H_5O_7^{3-}]$	$[Ca^{2+}]$	Conditions
A	0.070	0.030	0.020	No washing
B	0.070	0.030	0.020	Washed
C	0.070	0.030	0.030	No washing
D	0.070	0.030	0.030	Washed

Even though the mixture was turbid colloid without precipitation, the result from the SEM image of samples A, B, C and D illustrated that the particles were in inflation shape and the dispersion is quite far apart. Image J software was used to measure the particle size. The result from the software was shown in **Table 3-4**. The result showed that the particle size is not nanoscale within the range of 100-500 nm.[13]

Table 3-4 Particle size from Image J software.

Sample	Smallest Size (μm)	Average (μm)	SD
A	3.3784	24.6180	21.6543
B	3.5662	25.0053	20.1224
C	3.2684	22.4535	19.1984
D	3.3286	23.0732	21.6489

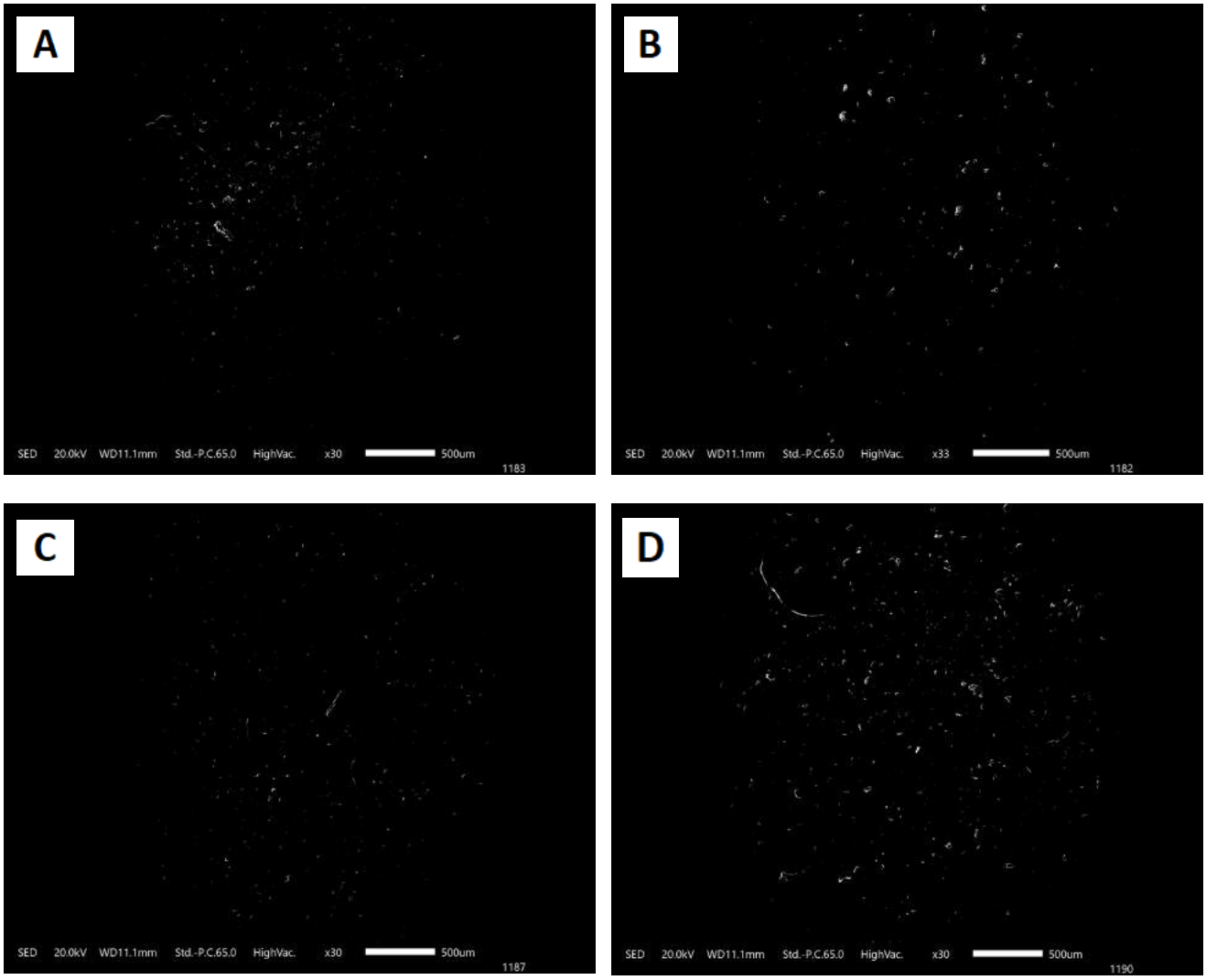

Figure 3-5 SEM image of proanthocyanidin particles, Samples A-D at 500 µm

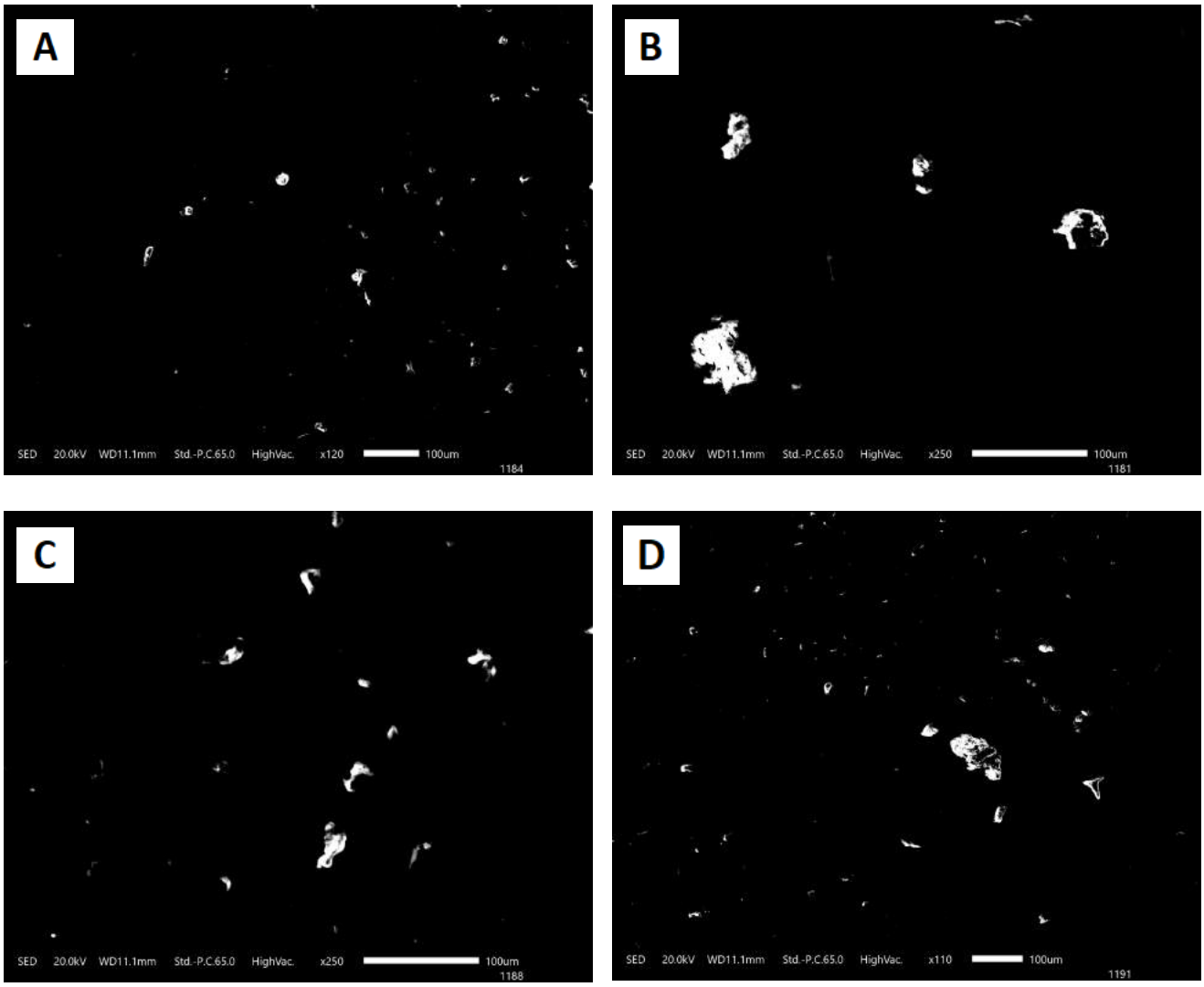

Figure 3-6 SEM image of proanthocyanidin particles, Samples A-D at 100 µm

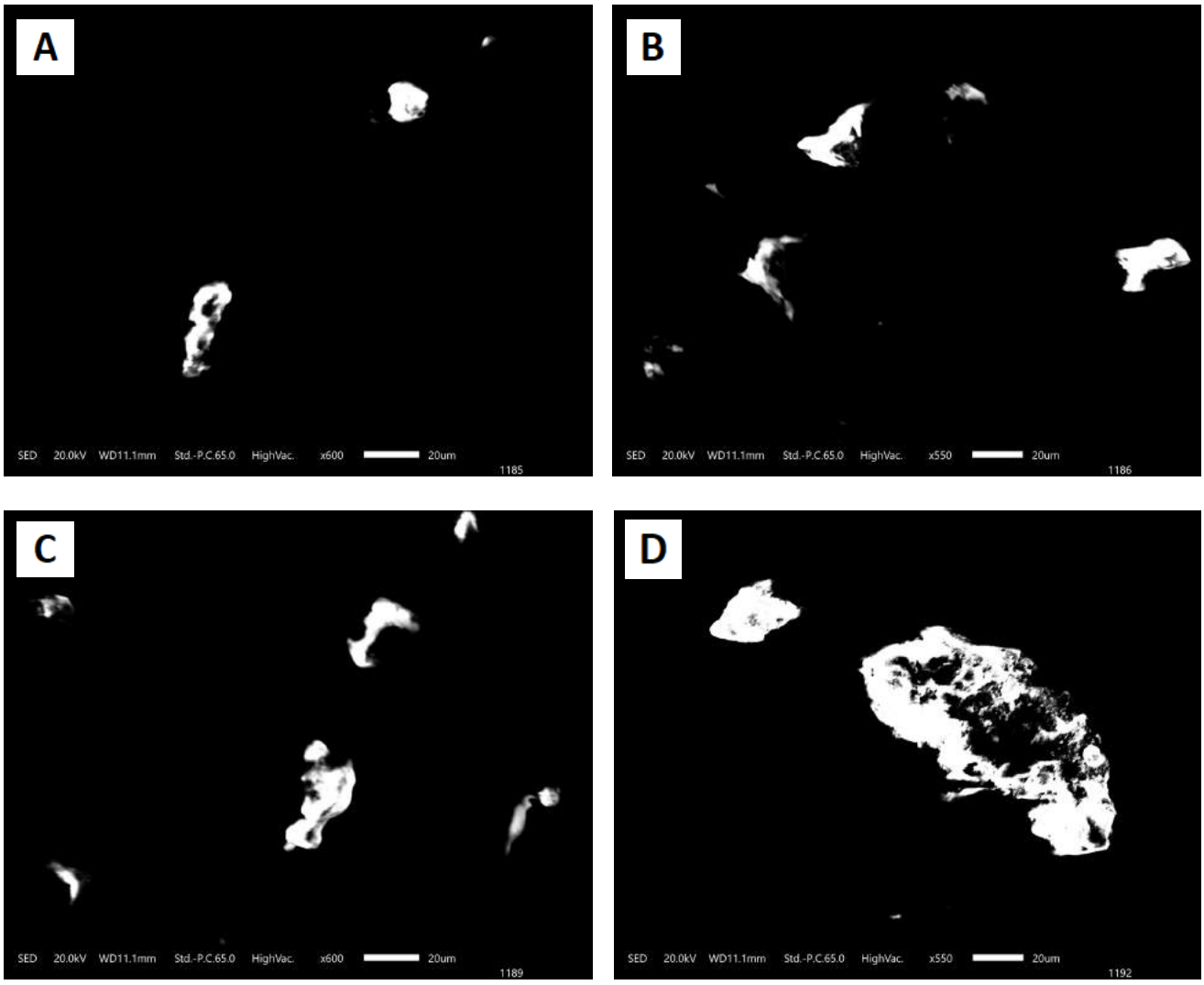

Figure 3-7 SEM image of proanthocyanidin particles, Samples A-D at 20 μm

Chapter 4
Conclusion

In this research, proanthocyanidin nanoparticles were synthesized *via* coprecipitation methods. The synthesis of proanthocyanidin nanoparticles as a pure drug assembly showed a negative result, none of the samples had shown the trend to be nanoparticles. Citrate ion was attempting to form the nanoparticles. The result showed a positive 48 hours of rocking of samples with 0.0700 M GSE in pH=7.45 with 0.030 M citrate ion and 0.020/0.030M calcium ion. The samples were colloid, no visible substance present in the mixture, and can redisperse the freeze-dry solid. However, the SEM picture did not show a positive result as the size of the particles is around 3-25 μm which is not as small as nanoparticles. In addition, the substance is quite scattered away from each other and the solid has an unconscious shape. Even though the methods used in this experiment were following from the reference[3] but the results were different compared to our reference results where the size is stable at 100-200 nm and the shape is spherical. However, the finest condition was 0.070 M GSE with 0.030 M citrate ion and 0.030 M calcium ion with the smallest particle size in all conditions at 3.2684 μm. A further experiment is needed in order to solve the problem of the size of particles.

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Biography

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