CHAPTER III
METHODOLOGY

3.1 Analytical procedure

This research is analytical research of the antioxidant activity of three different variety of mango seed kernel and grape seed extract which extracted by four different extraction solvents to comparison the appropriate extraction method that usable in local industry.

3.2 Research facility

Environmental laboratory in Faculty of Environment and Resource Studies, Mahidol University Salaya Campus, Thailand.

3.3 Materials and instruments

1) Oven
2) UV/Vis spectrophotometer
3) Blender brand SHARP Model EM-171JG 600 watt
4) Sharp knifes
5) Chopping boards
6) Stainless steel trays
7) Aluminum foils
8) Plastic bags
9) Water bath
10) Vortex mixer
11) Shaker
12) Centrifuges
13) Filter paper No. 4

14) Pipette in various volumes
15) Micropipette 100 µl and 1 ml

3.4 Standards and reagents

1) 95% Ethanol
2) Rice whisky(contain 40% ethanol) in brand "Ruangkhao"
3) DPPH (2,3-diphenyl-1-picrylhydrazyl)
4) Phosphate buffer pH 6.6
5) Potassium ferricyanide
6) Trichloroacetic acid
7) Ferric chloride
8) Gallic acid
9) L-ascorbic acid
10) Folin – Ciocalteu's phenol reagent
11) 35% Na_2CO_3(w/v)
12) Distilled water

3.5 Source of mango seed kernels and grape seeds

This study will use three varieties of mango seed kernels which are Kaew, Keaw Morakot and Mahachanok. These mango seeds were collected from June 2012. When Kaew and KeawMorakot were collected from Udonthani province, Mahachanok collected from the assist of The Royal Chitralada Projects; Fruit Juice Pasteurisation Plant. And grape seeds that used in this study were The Black Queen variety, collected by the assist of The Village Farm and Winery limited company.

3.6 Mango seed kernels and grape seeds preparation

3.6.1 Mango seed kernels preparation

Separated seed from the mango fruits, clean the seeds by water and then separated the mango seed kernels out of the seeds and chopped into small pieces. Dried the small pieces of mango seed kernels in the oven at 60^{o}C until completely dried. Finally, grinded the small pieces of mango seed kernels until fine texture of 100 mesh sieve (Abdalla, 2006) and stored in a desiccator.

3.6.2 Grape seeds preparation

Washed the Black Queen grapes seeds and aerated to dry. Dried the grape seeds in the oven at 60^{o}C until completely dried, then grinded the grape seeds into 100 mesh sieve and stored in a desiccator.

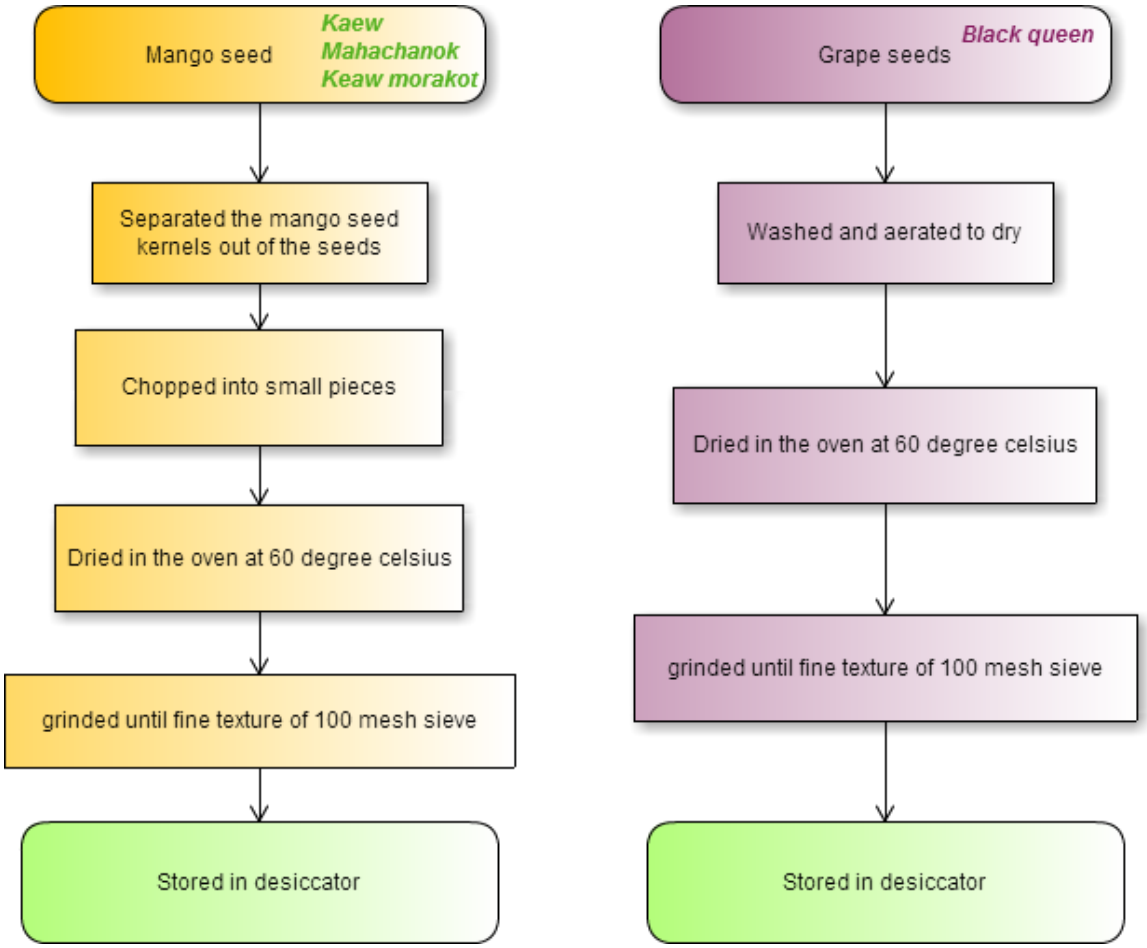

Figure 3.1 Mango seed kernels and grape seeds preparation

Figure 3.2 Mango seed kernel powder sample in Kaew variety

Figure 3.3 Mango seed kernel powder sample in Keaw morakot variety

Figure 3.4 Mango seed kernel powder sample in Mahachanok variety

Figure 3.5 Grape seed powder sample in Black Queen variety

3.7 The extraction methods

In this research will compare four extraction solvents: 95% ethanol, rice whisky (contained 40% ethanol), water and hot water to determine the effectiveness of each extraction solvent.

3.7.1 Extraction method applied from Maisuthisakul (2009)

Added 300 ml of 95% ethanol to 100 g samples powder. Mixed together and shake 200 rpm at 20 ^{o}C within 4.5 hrs. Then Filtered through muslin cloth and filtered again with No. 4 filter paper. Evaporated ethanol in the supernatant using rotary evaporator at 50 ^{o}C after that take the sample in an oven at 70 ^{o}C for completely dried, stored in a dessicator (three replicated experiments). Do the same with 95% ethanol by changes extraction solvent to be rice whisky (contained 40% ethanol), water and hot water.

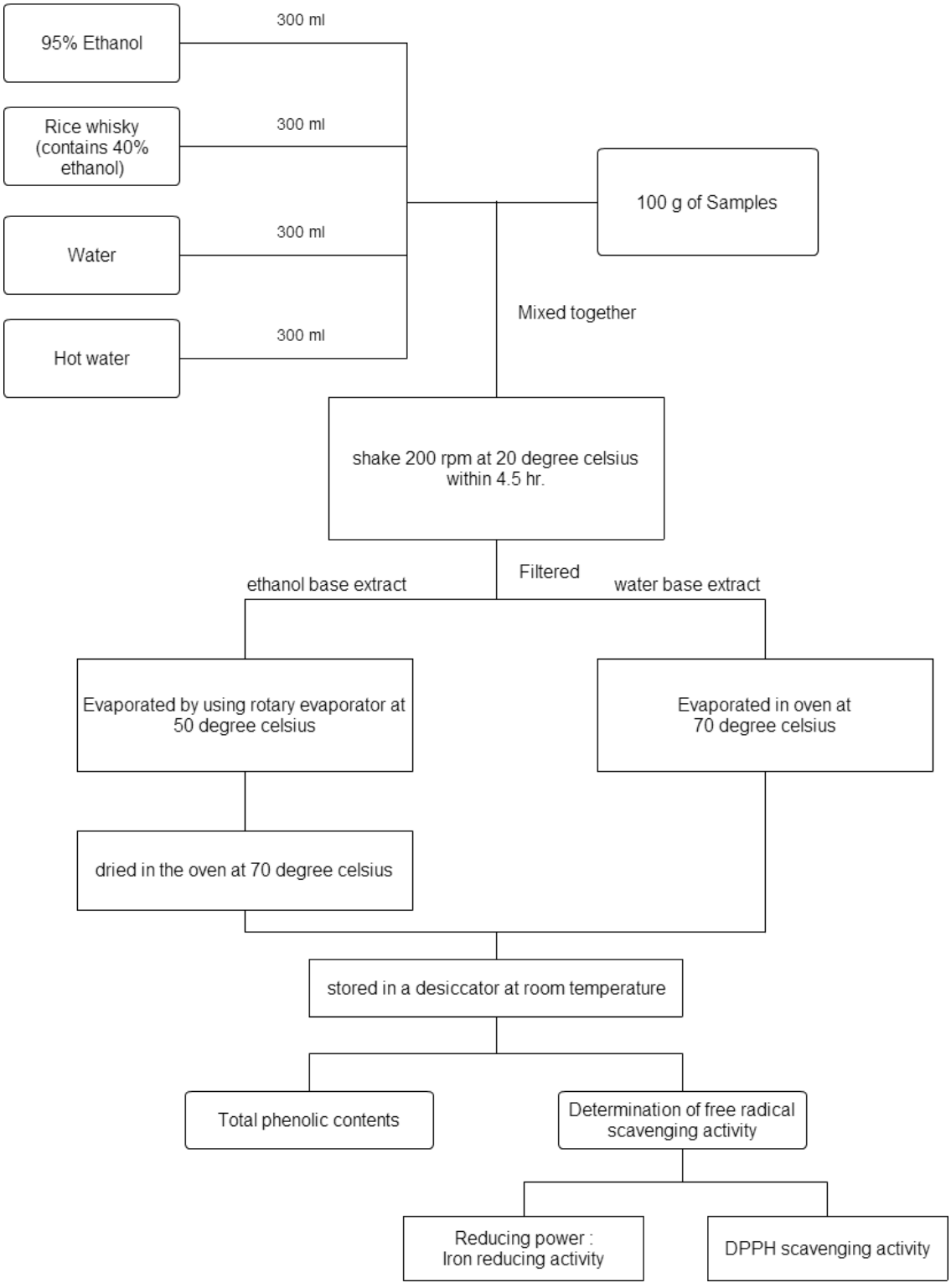

Figure 3.6 Flow steps of extraction method

3.8 Determination of total phenolic content

The total phenolic content was determined by the method of Khammuang (2011) that applied from Singleton (1965) using the Folin-Ciocalteu colorimetric method. The method of measurement technique was modified by use the spectrophotometry at 725 nm. The extract 0.5 ml (100 ppm) mixed with 0.5 ml Folin–Ciocalteu's phenol reagent and incubated at room temperature in 3 minutes. Then added 0.5 ml. of 35% Na_2CO_3 (w/v) and the reaction was incubated at room temperature in the dark place for 90 minutes. Added 5 ml distilled water and mixed together by vortex mixer after that which the absorbance was read at 725 nm by UV/VIS spectrophotometer. Gallic acid was used for making calibration curve and the results were calculated for total phenolic content by comparison with gallic acids standard curve and express as mg of gallic acid equivalents / g of weight extract (mg.GAE/g extract). (five replicate experiments)

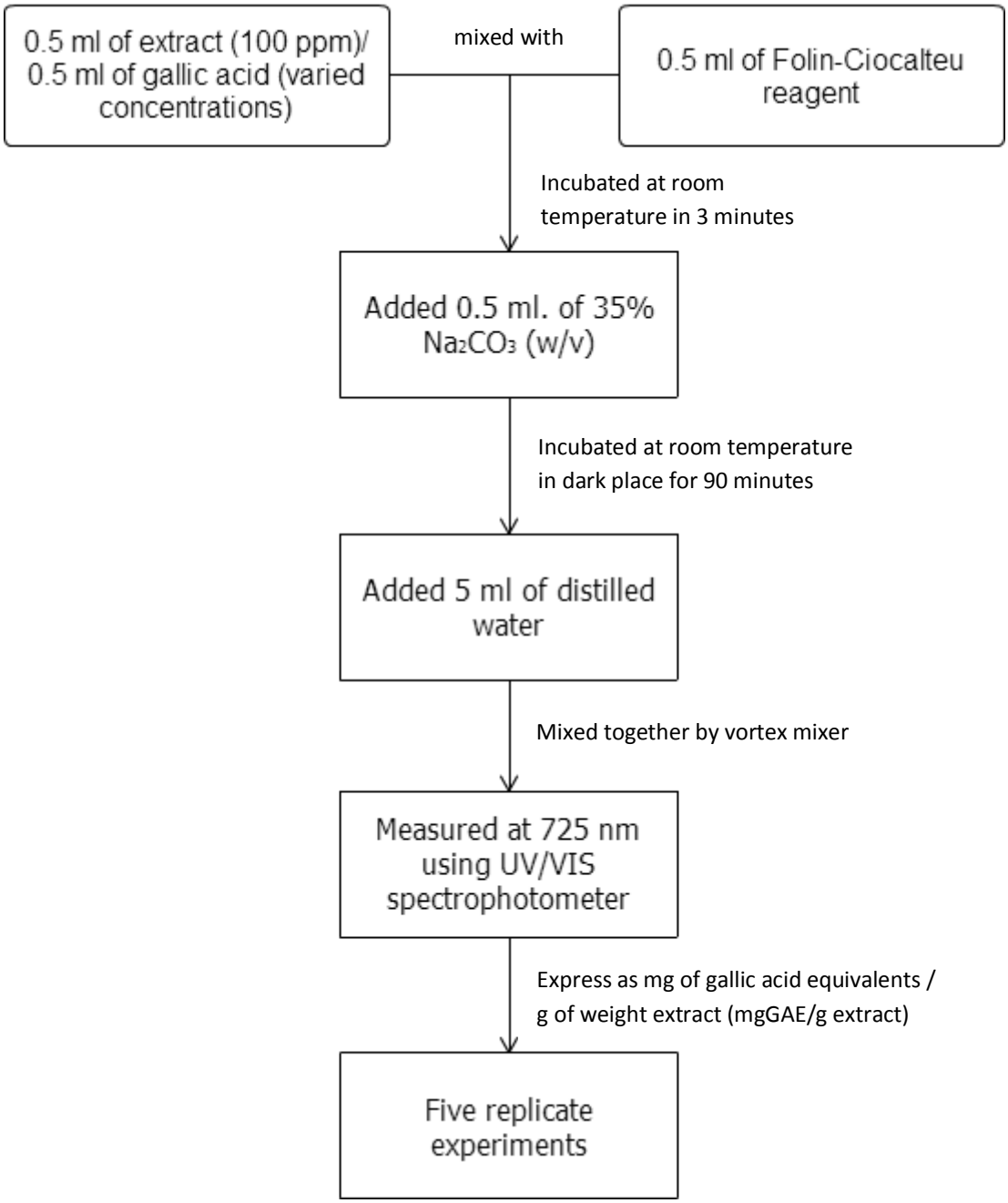

Figure 3.7 Flow steps of determination of total phenolic content

3.9 Determination of antioxidant activity by DPPH radical scavenging assay

The measurement of the DPPH radical scavenging activity was determined according to the method of Ribeiro (2008) which applied from Blois (1958). This method used DPPH (2,2-Diphenyl-1-picrylhydrazyl) as reagent that was a stable radical which is a purple colour and absorbs the light at wavelength of 517 nm. If the samples had high antioxidant capabilities, the intensity of purple solution was reduced. The results were reported as % inhibition of DPPH activity.

The extract (100 ppm) 100 µl was mixed with 5 ml of DPPH (2,2-diphenyl-1-picrylhydrazyl) 0.1 mM in ethanol. After 30 minutes incubation at room temperature in the dark, the optical density of the mixture was measured at 517 nm using UV/Vis spectrophotometer. % inhibition of DPPH activity was calculated using the formula below;

% scavenging		=	[(Acontrol – A sample) / Acontrol] x 100
by	A sample	=	the absorbance of samples
	A control	=	the absorbance of controls

L-ascorbic acid 100 ppm was used as positive control and ethanol with DPPH was used as blank. (five replicate experiments)

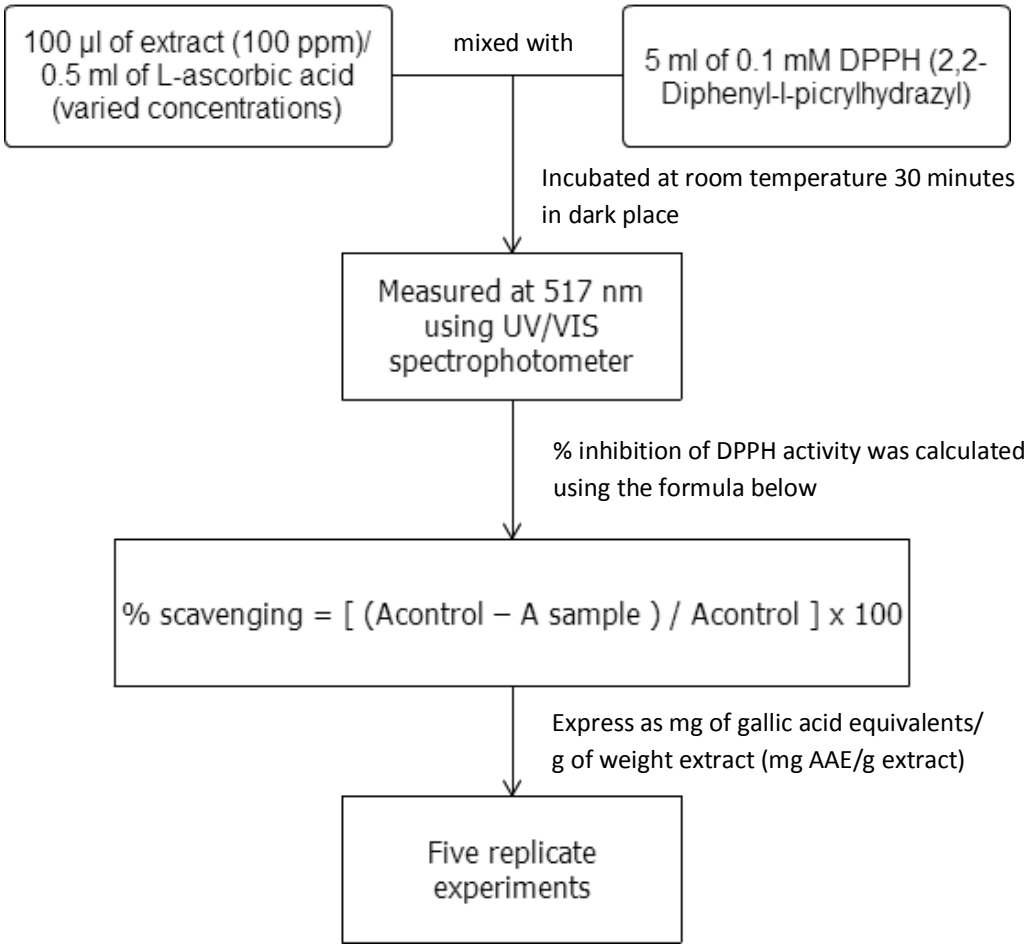

Figure 3.8 Flow steps of determination of antioxidant activity by DPPH radical scavenging assay

3.10 Determination of Reducing power: Iron reducing activity

The reducing power was determined according to the method of Ribeiro (2008) which applied from Oyaizu (1986). The extract 2.5 ml (100 ppm) mixed with 2.5 ml phosphate buffer in concentration 0.2 M (pH6.6) and added 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] (1% w/v in distilled water). Then the mixture was mixed by vortex mixer and incubated in water bath at 50^oC in 30 minutes. To stop the reaction, added 2.5 ml trichloroacetic acid (10% w/v in distilled water) and centrifuged at 980 rpm. After that the supernatant 5 ml was mixed with 5ml distilled water and 1 ml ferric chloride (0.1% w/v in distilled water). The absorbance was read at 700 nm by UV/VIS spectrophotometer. L-ascorbic acid was used for making calibration curve and the results were calculated for reducing power by comparison with ascorbic acids standard curve and express as mg of ascorbic acid equivalents/ g of weight extract (mg AAE/g extract). Blank of the samples was followed by the method above but did not take the extract. (five replicate experiments)

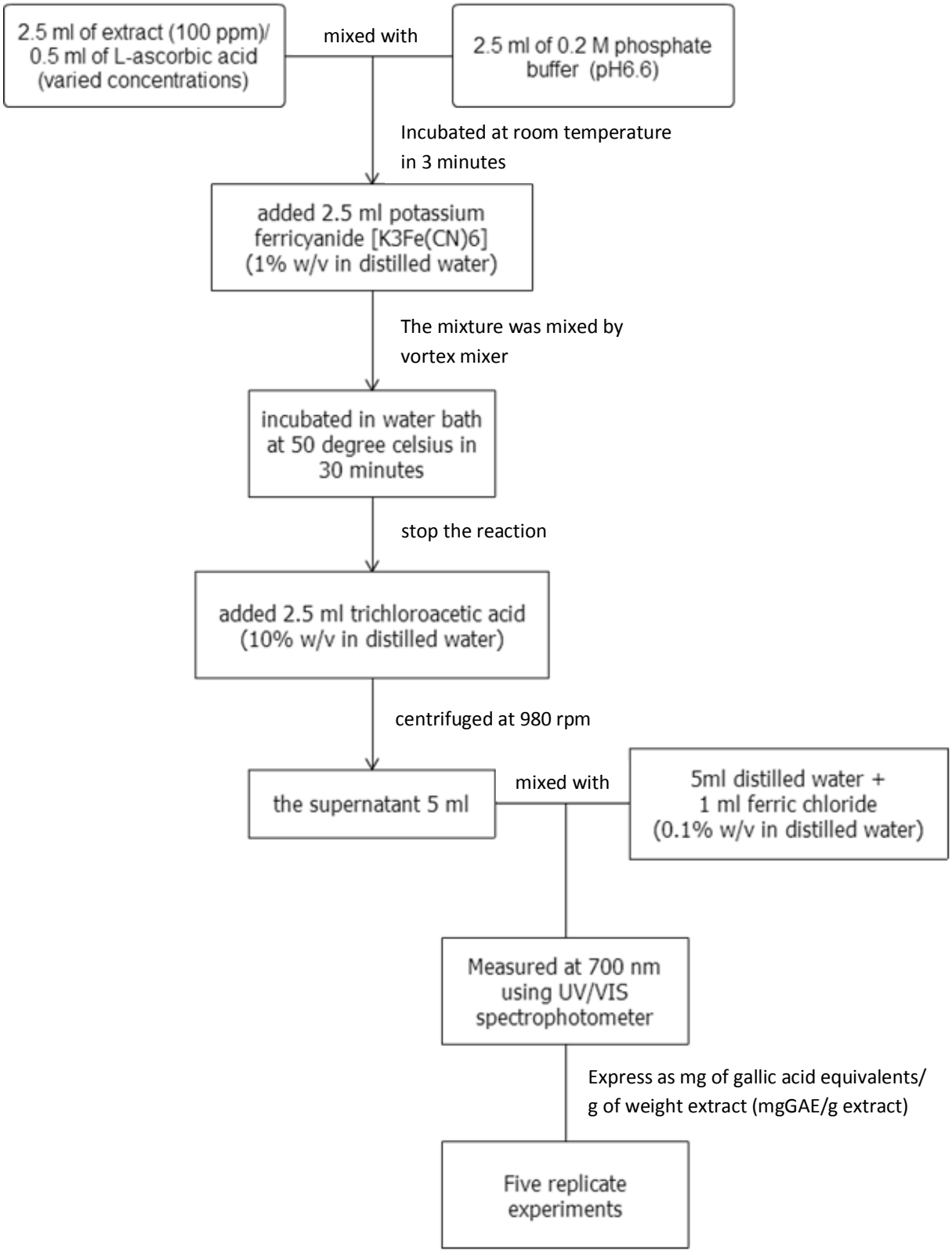

Figure 3.9 Flow steps of determination of Reducing power

3.11 Statistical analysis

The results were presented as mean ± standard deviation (the extraction was repeated 3 times and all experiments were repeated 5 times). Analysis of variance using SPSS (Version 16, SPSS Inc., Chicago, USA.) and significant difference was statistically considered at the level of $p < 0.05$ including

- Comparative analysis of each mango seed kernel varieties extraction will use two way ANOVA analysis which is the comparison of the difference between the two representative samples above. The control variables were 3 varieties of mango seed kernel and 4 various analysis.

- Comparative analysis of 3 varieties of mango seed kernel and Black Queen variety of grape seed with each analysis will use one way ANOVA analysis which is the comparison of the difference between two representative samples above without control variables.