CHAPTER IV
MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals and reagents

All chemicals and reagents including 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH), fluorescein sodium salt, 2,2-diphenyl-l-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-*s*-triazine (TPTZ), ferric chloride hexahydrate ($FeCl_3 \bullet 6H_2O$), Folin-Ciocalteu reagent, sodium bicarbonate (Na_2CO_3), 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), dimethyl sulfoxide (DMSO), acetylthiocholine (ATCh), butyrylthiocholine chloride (BTCh), magnesium chloride ($MgCl_2$), tertiary butylhydroquinone (tBHQ) and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA), hydrochloric acid (HCl), potassium dihydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate trihydrate ($K_2HPO_4 \bullet 3H_2O$) and sodium chloride (NaCl) were received from Merck (New Jersey, USA).

Standards including 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), gallic acid monohydrate, quercetin, kaempferol, isorhamnetin, myricetin, apigenin, luteolin, hesperetin, naringenin, caffeic acid, chlorogenic acid, gallic acid, syringic acid, vanillic acid, *p*-coumaric acid, sinapic acid, ferulic acid, *t*-cinnamic acid, 4-hydroxybenzoic acid, capsanthin, β-cryptoxanthin, lycopene, and β-carotene were received from Sigma-Aldrich (St. Louis, MO, USA). Lutein, zeaxanthin, and α-carotene were obtained from Carotenature (Lupsingen, Switzerland).

Enzymes including *Electrophorus electricus* acetylcholinesterase (AChE, 200-1000 units/mg protein) and equine serum butyrylcholinesterase (BChE, ≥10 units/mg protein) and BACE1 (β-secretase) FRET (Fluorescence resonance energy transfer) assay kit were supplied from Sigma-Aldrich (St. Louis, MO, USA).

Solvents including hexane, ethyl acetate, ethanol, acetonitrile (CH_3CN), dichloromethane (CH_2Cl_2) and methanol were received from RCI Labscan (Bangkok, Thailand).

4.1.2 Equipments

The samples were blended using a Philips 600W blender from Philips Electronics Co., Ltd. (Jakarta, Indonesia). The samples were freeze-dried using a Heto Powerdry PL9000 from Heto Lab Equipment (Allerod, Denmark). The evaporation was performed using a N-1200B series rotary evaporator with OSB-2100 bath from EYELA (Tokyo, Japan). A small volume sample (microcentrifuge scale) was centrifuged using a tabletop Spectrafuge 16M microcentrifuge from Labnet International, Inc. (New Jersey, USA). The enzymatic assay was performed on a Synergy™ HT 96-well UV-visible spectrophotometer using a Gen5 data analysis software (BioTek Instruments, Inc., Winooski, VT).

A high performance liquid chromatography (HPLC) system consisted of an Agilent 1100 series HPLC with a photodiode array detector and an Eclipase XDB-C18 guard column (4.6 mm x 12.5 mm, 5 µm) from Agilent Technologies (Santa Clara, CA, USA). The column for analyses of flavonoids and phenolic acids was a Zorbax Eclipase XDB-C18 column (4.6 mm x 150 mm, 5 µm) from Agilent Technologies (Santa Clara, CA, USA), while the column for carotenoids analysis was an isocratic reverse phase column, Vydac 201TP54-C18 (4.6 mm x 250 mm, 5 µm) from Grace Davison Discovery Science (Columbia, MD, USA). The PTFE syringe filters (0.2 µm) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The quality and quantity of chromatogram were analyzed by ChemStation (Agilent Technologies, Santa Clara, CA, USA). Water used in all HPLC experiments was Milli-Q water (18.2 MΩ-cm conductivity).

The identification of volatile compounds was performed using a gas chromatography-mass spectrometry (GC-MS) consisted of an Agilent 7890A gas chromatograph equipped with a 5975C mass spectrometry inert XL MSD with triple-axis detector (Agilent Technologies, Santa Clara, CA, USA) supplied by the Salaya Central Instrument Faculty (SCIF), Mahidol University. Volatile compounds were absorbed on a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS)

fiber (Sigma-Aldrich, St. Louis, MO, USA) and separated on a HP-5MS fused silica capillary column (30 m × 0.25 mm, 0.25μm) from Agilent Technologies (Santa Clara, CA, USA). The compounds were identified using NIST library in mass spectrometry (Scientific Instrument Services, Inc., Ringoes, NJ, USA).

4.1.3 Plant materials

Sweet peppers (*Capsicum annuum*) including unripe (green) peppers and ripe (red, orange and yellow) peppers used in this study were purchased from Baan Rai Paovaris, Nakhon Ratchasima province, Thailand (harvesting in October-November, 2013) (Figure 4.1). The peppers were cultivated in greenhouse under controlled water and fertilizer treatments.

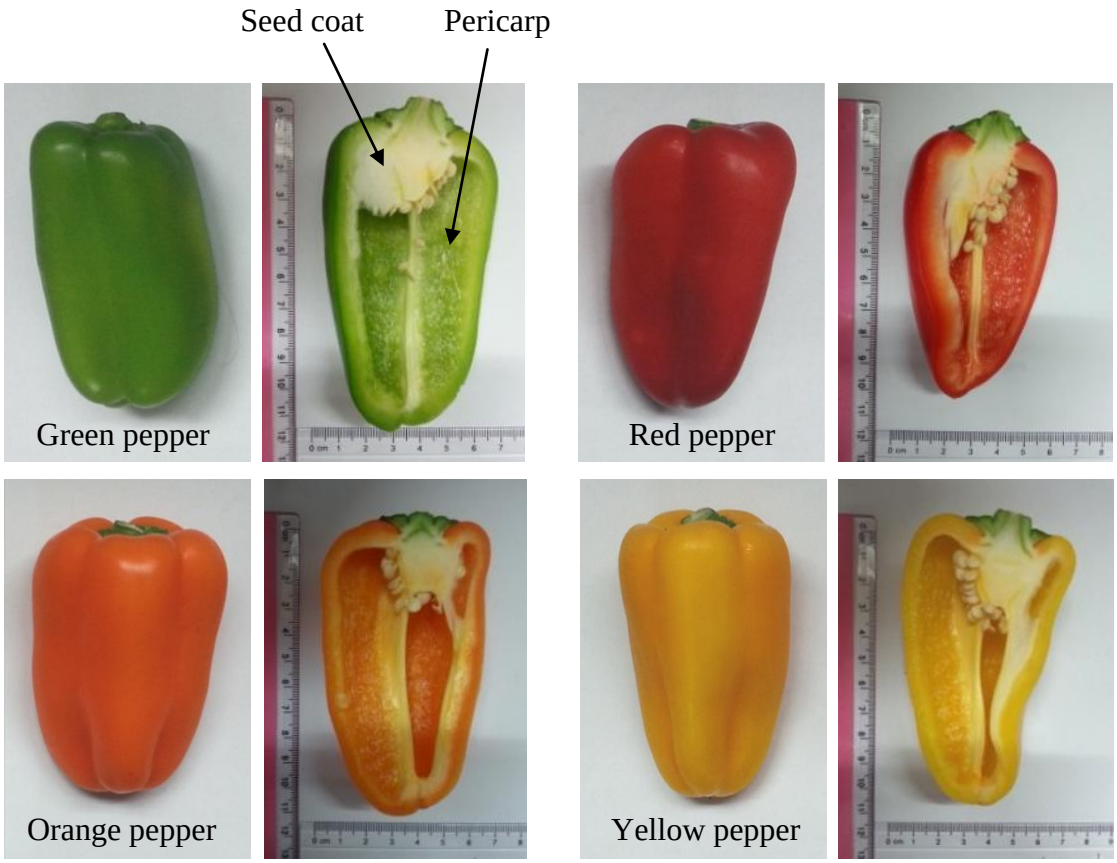

Figure 4.1 Different colored sweet peppers used in this study. The fruit colors indicate maturity stage. Green is normally the color of unripe pepper, while red, orange and yellow are representatives of ripe peppers.

4.2 Methods

4.2.1 Sample preparation and extraction

Sweet pepper fruits were cleaned with deionized (DI) water, and all seeds in the peppers were removed. Sweet pepper pericarps were cut into small pieces (about 1 cm x 1 cm). The colors of sweet peppers were analyzed by a HunterLab ColorFlex EZ spectrophotometer (Reston, Virginia, USA) using C.I.E. LAB (L*, a*, b*) system. The samples were then freeze-dried using Heto Powerdry PL9000 from Heto Lab Equipment (Allerod, Denmark) and homogenized using Philips 600W blender from Philips Electronics Co., Ltd. (Jakarta, Indonesia). The sweet pepper powder was stored in aluminum foil bag at -20 ^{o}C for further analysis.

The moisture content of fresh and freeze-dried samples was analyzed by Association of Official Analytical Chemists (AOAC) official method (930.15, AOAC, 2005). Air oven were regulated to the temperature of 135±2 ^{o}C. The samples (approx. 2 g) were spread in a low, covered aluminum dishes (≥50 mm diameter and 40 mm deep) until evenly distributed. The samples were incubated in the oven for 2 hours before being cool in a desiccator. The moisture content of the samples was then calculated as follows:

$$\%(\text{w/w})\text{moisture} = 100 \times \frac{\text{weight loss on drying (g)}}{\text{weight test portion (g)}},$$

$$\%\ \text{Dry matter} = 100 - \%(\text{w/w})\text{moisture}$$

Freeze-dried sweet pepper (11 g dry weight) was extracted by solvents (400 mL) with different polarities including non-polar hexane, semi-polar ethyl acetate and polar 70% (v/v) aqueous ethanol using a Soxhlet extractor. The extraction was continued until all colors of sweet pepper were removed (approx. 6.5 hours). The solvent of extractant was removed by a rotary vacuum evaporator at 35-40 ^{o}C. The remained residues were re-dissolved in minimum volume of DMSO and stored at -40 °C for further analysis.

The variation of extraction was investigated by extracting triplicate under all three solvents. The quality of the extraction condition was examined using macadamia nut (with known % recovery yield) extracted with petroleum ether by the same Soxhlet extractor as a control.

4.2.2 Determination of phytochemicals

Flavonoids and phenolic acids

The analyses of flavonoids and phenolic acids were determined based on Judprasong *et al*., 2013 [78] using HPLC analysis. First, freeze-dried samples (0.5 g) were extracted with 62.5% (v/v) methanol (40 mL) containing 0.5 g/L tBHQ. Additionally, 6 N HCl (10 mL) was added, and the samples were shaken at 90 °C in shaking water bath for 2 hours. After cooling the extract in ice bath for 5 minutes, 1% (v/v) ascorbic acid (100 µL) was added and mixed thoroughly. The volume of extract was made up by adding methanol in 50-mL volumetric flask. The extract was then sonicated in ultrasonic bath for 5 minutes, and then filtered through 0.2 µm PTFE syringe filter (~1-2 mL) into a vial. The HPLC analysis was utilized by the Zorbax Eclipase XDB-C18 column with a constant flow rate of 0.6 mL/min at ambient temperature. The gradient mobile phase consisted of Milli-Q water containing 0.05% (w/w) TFA (Solvent A), methanol containing 0.05% (w/w) TFA (Solvent B) and acetronitrile containing 0.05% (w/w) TFA (Solvent C). Samples were kept in the autosampler at 4^{o}C until injection (10 µL). Chromatograms were read at 325 and 338 nm and calculated by using ChemStation. Flavonoids including quercetin, kaempferol, isorhamnetin, myricetin, apigenin, luteolin, naringenin, and hesperetin, and phenolic acids, including gallic acid, 4-hydroxybenzoic acid, chlorogenic acid, syringic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid and *t*-cinnamic acid were used as standards to identify flavonoids and phenolic acids of sample by comparing retention time. Concentration of flavonoids and phenolic acids were expressed as µg/g of sample (dry weight).

Carotenoids

Carotenoids in sweet peppers were determined based on Judprasong *et al*., 2013 [78] using HPLC analysis. First, freeze-dried samples (0.2 g) were saponified by boiling in 2 N ethanolic potassium hydroxide (KOH) (50 mL) with 10% (w/v) ascorbic acid (10 mL) for 30 min in a boiling water bath. The saponified sample was then extracted by adding hexane (70 mL) and shaking for 2 min at room temperature. The solvent was removed by evaporation, and residue was re-dissolved in a mobile

phase solvent (acetronitrile:methanol:dichloromethane (80:11:9 v/v/v) with 0.01% (v/v) triethylamine and 0.01% (w/v) ammonium acetate) (3 mL). The extract was filtrated through 0.2 μm PTFE syringe filter before being analyzed by HPLC. The HPLC was utilized by the Vydac 201TP54-C18 column with a constant flow rate of 0.7 mL/min at ambient temperature. The quality and quantity of chromatogram were analyzed at 450 nm by ChemStation. Carotenoids including capsantin, lutein, zeathantin, β-cryptoxanthin, lycopene, α-carotene, *trans*-β-carotene and *cis*-β-carotene were determined by comparing retention time and UV spectrum between unknown peaks of sample and of authentic standards. The concentration of carotenoids was showed as μg/g of sample (dry weight).

Volatile compounds

Volatile compound analysis utilizing headspace solid phase microextraction (HS-SPME) and GC-MS was adapted from Azar *et al*., 2010 [79]. The small pieces (about 1 cm x 1 cm) of pericarps of fresh sweet pepper (1.5 g) in a vial were incubated in air bath at 70 ˚C for 30 minutes. Volatile compounds were then absorbed on a DVB/CAR/PDMS fiber for 20 minutes and separated by gas chromatography. The oven temperature was held at 60 ˚C for 3 minutes then set at rate of 6 °C/min until reaching 250 °C. The flow rate of helium gas, a carrier gas, was 1 mL/min. The temperature in split/splitless injector mode was set at 250 °C. Electron-impact mode (EI) was set at 70 eV. The compounds were identified by NIST library in mass spectrometry. As well, heptane was used to test the ability of instrument.

4.2.3 Determination of total phenolic content

TPC were determined according to the method of Folin-Ciocalteu method, which was adapted from Ainsworth and Gillespie, 2007 [80]. The samples (25μL) were mixed with 10% (v/v) Folin-Ciocalteu reagent (50 μL). After 5 minutes of incubation, 7.5% (w/v) saturated sodium bicarbonate (200 μL) was added. The mixture was then incubated at room temperature (25 °C) in dark room for 2 hours. The TPC was measured at a wavelength of 765 nm using a microplate reader. Gallic acid (10, 20, 40, 60, 80, 100 and 200 μg/mL) was used as a standard. The TPC was expressed in mg gallic acid equivalents (GAE)/g dry weight.

4.2.4 Determination of antioxidant activity

Antioxidant activity was determined using DPPH-radical scavenging, ferric reducing antioxidant power (FRAP) and oxygen radical antioxidant activity (ORAC) assays.

DPPH- radical scavenging assay

The DPPH assay was performed according to the method of Fukumoto and Mazza, 2000 [81] with some modifications as follows. The assay was performed in the 96-well flat-bottom microplate. DPPH was used as indicator of free radical scavenging activity. The samples (22 µL) were mixed with 150 µM DPPH in 95% (v/v) aqueous ethanol (200 µL) and incubated in the dark at room temperature (25 °C) for 30 minutes. The reaction was determined by measuring the absorbance at 520 nm using the microplate reader. The radical scavenging activity was calculated as a percentage of DPPH discoloration using the equation:

$$\% \text{ Radical scavenging activity} = 100 \times (1-(Abs_{sample}/Abs_{control}))$$

where Abs_{sample} is the absorbance at 520 nm of the sample and DPPH reagent, while $Abs_{control}$ is the absorbance at 520 nm of 95% (v/v) aqueous ethanol and DPPH reagent.

In addition, trolox solution (0.01, 0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 mM) was used as a standard. The results were determined using a standard curve of trolox solution and expressed in µmol trolox equivalence (TE)/100g dry weight.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was determined according to the method of Benzie and Strain, 1996 [82] with some modifications. The FRAP reagent containing 300 mM acetate buffer pH 3.6, 10 mM TPTZ solution in 40 mM HCL and 20 mM $FeCl_3 \bullet 6H_2O$ solution in a ratio of 10:1:1 were warmed at 37°C before use. The assay was performed in the 96-well flat-bottom microplate. The samples (20 µL) were mixed with FRAP reagent (150 µL) and incubated at room temperature (25 °C) for 8 minutes. The reaction was monitored using the plate reader at a wavelength of 595 nm. Trolox solution (7.8125, 15.625, 31.25, 62.5, 125 and 250 µM) was used as a standard. The FRAP value was determined using a standard curve of trolox solution and expressed in µmol TE/100g dry weight.

Oxygen radical antioxidant capacity (ORAC) assay

The ORAC assay was determined according to the method of Ou *et al.*, 2001 [83] with some modifications. The assay was performed in a 96-well black plate for fluorescence measurement. Sodium fluorescein was used as a fluorescent probe. The loss of fluorescence was an indication of the extent of damage from its reaction with the peroxyl radical induced by AAPH. The samples (25µL) were mixed with 40 nM fluorescein solution (150 µL) and incubated for 30 minutes at 37 °C. After the incubation, 153 nM AAPH (25 µL), a peroxyl radical generator, was added to the reaction mixture rapidly to start the reaction. The fluorescence intensity was monitored for 90 minutes in the microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Trolox (3.125, 6.25, 12.5, 25, 50 and 100 µM) was used as a standard. The results were calculated based on the differences in areas under the sodium fluorescein decay curve (AUC) and expressed in µmol TE/100g dry weight. The AUC was calculated as:

$$\text{AUC} = (0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots + f_i/f_0) \times \text{CT},$$

where f_0 is the initial fluorescence reading at 0 min, f_i is the fluorescence reading at time i minutes and CT is cycle time in minutes.

4.2.5 Determination of cholinesterase inhibitory activities

Acetylcholinesterase inhibitory activity

The analysis of AChE activity was adapted from the method of Jung *et al.*, 2009 [21]. Enzyme assay consisted of 10 ng AChE, 0.16 mM ATCh and 0.8 mM DTNB in 50 mM potassium phosphate buffer (KPB), pH 7.0. Enzyme inhibitory activity was spectrophotometrically measured at a wavelength of 412 nm using the 96-well microplate reader. The inhibitory activity of pepper extracts was calculated as percentage of inhibition using the equation:

$$\%\ \text{inhibition} = \left(1 - \frac{B-b}{A-a}\right) \text{x } 100,$$

where A is an initial velocity of the reaction with enzyme, a is an initial velocity of the reaction without enzyme, B is an initial velocity of the enzyme reaction with extract and b is an initial velocity of the reaction with extract but without enzyme.

Butyrylcholinesterase inhibitory activity

The analysis of BChE activity was adapted from the method of Jung *et al*., 2009 [21]. Enzyme assay consisted of 50 ng BChE in 50 mM KPB, pH 7.0 containing 1 mM $MgCl_2$, 0.2 mM BTCh in 50 mM KPB, pH 7.0 and 0.8 mM DTNB in 50 mM KPB, pH 7.0. Enzyme inhibitory activity was spectrophotometrically measured at a wavelength of 412 nm using the 96-well microplate reader. The inhibitory activity of pepper extracts were calculated as percentage of inhibition as above.

4.2.6 Determination of β-secretase inhibitory activity

The BACE1 inhibitory activity was measured using a β-secretase (BACE1) Activity Detection Kit (Fluorescent) (Sigma-Aldrich, St. Louis, MO). The assay kit consisted of 0.6 unit/µL BACE1 Enzyme Solution and 10 mM BACE1 Substrate Solution in Fluorescent Assay Buffer. All reactions were performed using the 96-well microplate reader and monitored at excitation wavelength of 320 nm and emission wavelength of 405 nm. The inhibitory activity was reported as percentage of inhibition as above.

4.3 Statistical analysis

All experiments were carried out in triplicate. The data were expressed as mean ± standard deviation (SD). All analyses for enzyme reactions were determined using a GraphPad Prism software version 5.1 (GraphPad Software, Inc., La Jolla, CA). Samples independent samples t-test and one way analysis of variance (ANOVA) followed by Tukey's-b multiple comparison tests using the SPSS 16.0 statistical package were performed to determine the significant differences between values. Significance of difference was defined at $p<0.05$.