APPENDICES

APPENDIX A
REAGENT PREPARATIONS

A1. Reagent preparations for Folin-Ciocalteu assay

Reagents

- Folin-Ciocalteu reagent
- Sodium bicarbonate (Na_2CO_3)
- Gallic acid monohydrate

Reagent preparations

1. 10% (v/v) Folin-Ciocalteu reagent

Dilute 500 µL of Folin-Ciocalteu reagent (stock solution) to 5 mL with deionized water. Keep the reagent at ambient temperature.

2. 7.5% (w/v) Na_2CO_3 solution

Weight 7.5 g of Na_2CO_3 in a 100-mL volumetric flask. Then, adjust the volume to 100 mL with deionized water and mix well. Keep the solution at ambient temperature.

3. 1000 µg/ml gallic acid solution (stock solution)

Weight 100 mg of gallic acid monohydrate in a 100-mL volumetric flask. Then, adjust the volume to 100 mL with deionized water. Keep the solution in a freezer.

4. 200 µg/ml gallic acid solution (working solution for serial dilution)

Dilute 80 µL of 1000 µg/mL gallic acid solution (stock solution) to 400 µL with deionized water. After that, dilute 200 µg/ml gallic acid solution by serial dilution to be 100, 80, 60, 40, 20 and 10 µg/mL, respectively. Keep the solution in an ice bath before using.

A2. Reagent preparations for antioxidant activity assays

Ferric reducing antioxidant power (FRAP) assay

Reagents

- Glacial acetic acid
- Hydrochloric acid (HCl)
- 2,4,6-tripyridyl-s-triazine (TPTZ)
- Sodium acetate trihydrate ($C_2H_3NaO_2$•$3H_2O$)
- Ferric chloride hexahydrate ($FeCl_3$•$6H_2O$)
- 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox)

Reagent preparation

1. 300 mM acetate buffer (pH 3.6)

Weight 3.1 g of sodium acetate trihydrate into a 1-L volumetric flask and add 16 mL of glacial acetic acid. Then, adjust the volume to 1 L with deionized water and mix well. Keep the solution in a refrigerator.

2. 10 mM TPTZ solution in 40 mM HCl

Weight 0.312 g of TPTZ into 100-mL volumetric flask. Then, adjust the volume to 100 mL with 40 mM HCl (0.4 mL of conc. HCL + 99.6 mL of deionized water) and mix well. Keep the solution in a refrigerator.

3. 20 mM FeCl3•6H2O solution

Weight 0.5406 g of $FeCl_3$•$6H_2O$ into 100-mL volumetric flask. Then, adjust the volume to 100 mL with deionized water and mix well. Keep the solution in a refrigerator.

4. FRAP reagent

Mixing the reagent of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM $FeCl_3$•$6H_2O$ solution in ratio of 10:1:1 respectively and warm in water bath at 37 °C before using.

5. 250 µM trolox solution (working solution for serial dilution)

Dilute 60 µL of 1000 µM trolox solution (stock solution, from ORAC assay) to 240 µL with deionized water. After that, dilute 250 µM trolox solution by serial dilution to be 125, 62.5 31.25, 15.625 and 7.8125 µM, respectively. Keep the solution in an ice bath before using.

DPPH radical scavenging assay

Reagents

- Absolute ethanol

- *2,2-diphenyl-1-picrylhydrazyl* (DPPH)
- 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox)

Reagent preparation

1. 95% (v/v) aqueous ethanol

Dilute 950 mL of absolute ethanol to 1000 *m*L with deionized water. Keep the solution at ambient temperature.

2. 150 μM DPPH in 95% (v/v) aqueous ethanol

Weight 0.00148 g of DPPH into a *25*-mL volumetric flask. Then, adjust the volume to 25 mL with 95% (v/v) aqueous ethanol and mix well**.** Keep the solution in an ice bath before using.

3. 8 mM trolox solution (stock solution)

Weigh 0.20 g of trolox into a 100-mL volumetric flask. Then, adjust the volume to 100 mL with 95% (v/v) aqueous ethanol and mix well. Keep the solution in a freezer.

4. 0.64 mM trolox solution (working solution for serial dilution)

Dilute 20 μL of 8 mM trolox solution (stock solution) to 250 μL with 95% (v/v) aqueous ethanol. After that, dilute 0.64 mM trolox solution by serial dilution to be 0.32, 0.16, 0.08, 0.04, 0.02 and 0.01 mM, respectively. Keep the solution in an ice bath before using.

Oxygen radical absorbance capacity (ORAC) assay

Reagents

- Di-potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- *2,2'-azobis(2-amidinopropane*) *dihydrochloride (AAPH)*
- Fluorescein sodium salt
- 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox)

Reagent preparation

1. ORAC buffer (stock solution)

1.1 Prepare 0.75 M KH_2PO_4 solution (stock solution)

Weight 102.07 g of KH_2PO_4 into a 1-L volumetric flask. Then, adjust the volume to 1 L with deionized water and mix well**.** Keep the solution in a refrigerator.

1.2 Prepare 0.75 M K_2HPO_4 solution (stock solution)

Weight 130.64 g of K_2HPO_4 into a 1-L volumetric flask. Then, adjust the volume to 1 L with deionized water and mix well**.** Keep the solution at 4°C.

For ORAC buffer (stock solution) preparation, mix 351 mL of 0.75 M KH_2PO_4 solution (stock solution) and 603 mL of 0.75 M K_2HPO_4 solution (stock solution), this yields 954 mL of the ORAC buffer (stock solution). Keep the solution in a refrigerator.

2. ORAC buffer (working solution)

Take 100 mL of ORAC buffer (stock solution) into a 1-L volumetric flask. Then, adjust the volume to 1 L with deionized water and mix well**.** Adjusted pH to 7.2 with a few drops of a NaOH concentrated solution. Keep the solution in a refrigerator.

3. 153 mM AAPH

Weigh 0.414 g of AAPH into a 10-mL volumetric flask. Then, adjust the volume to 10 mL with ORAC buffer (working solution) and mix well. Keep the solution in an ice bath before using.

4. 8.37×10^{-4} mM fluorescein solution (concentrated solution)

Weight 0.045 g of fluorescein sodium salt into a-100 mL volumetric flask. Then, adjust the volume to 100 mL with ORAC buffer (working solution). Keep the solution in a freezer.

5. 4.19 μM fluorescein solution (stock solution)

Dilute 100 μL of 8.37×10^{-4} mM fluorescein solution (concentrated solution) to 20 mL with ORAC buffer (working solution) and mix well. Keep the solution in a freezer.

6. Fluorescein solution (working solution)

Dilute 244 μL of 4.19 μM fluorescein solution (stock solution) to 12.5 mL with ORAC buffer (working solution) and mix well. Warm the solution in water bath at 37 °C before using.

7. 1000 μM trolox solution (stock solution)

Weigh 0.025 g of trolox into a 100-mL volumetric flask. Then, adjust the volume to 100 mL with ORAC buffer (working solution) and mix well. Keep the solution in a freezer.

8. 100 μM trolox solution (working solution for serial dilution)

Dilute 20 μL of 1000 μM trolox solution (stock solution) to 200 μL with ORAC buffer (working solution). After that, dilute 100 μM trolox solution by serial dilution to be 50, 25, 12.5, 6.25 and 3.125 μM, respectively. Keep the solution in an ice bath before using.

A3. Reagent preparations for cholinesterase inhibitory assays

AChE inhibitory assays

Reagents

- Absolute methanol
- Di-potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB)
- Acetylthiocholine (ATCh)
- *Electrophorus electricus* acetylcholinesterase (AChE)

Reagent preparation

1. 1 M K_2HPO_4 solution (stock solution)

Weight 228.23 g of K_2HPO_4 into 1-L beaker. Then, adjust the volume to 1 L with deionized water and mix well. Keep the solution in a refrigerator.

2. 1 M KH_2PO_4 solution (stock solution)

Weight 136.09 g of K_2HPO_4 into 1-L beaker. Then, adjust the volume to 1 L with deionized water and mix well. Keep the solution in a refrigerator.

3. 50 mM potassium phosphate buffer (KPB), pH 7.0

Take 30.7 mL of 1 M K_2HPO_4 solution (stock solution) into 1-L volumetric flask and add 19.2 mL of 1 M KH_2PO_4 solution (stock solution) and then add deionized water about 500 mL and mix well. Then, adjust pH to 7.0 with a few drops of a NaOH concentrated solution. After that, adjust the volume to 1 L with deionized water and mix well. Keep the solution in a refrigerator.

4. 200 mM DTNB solution in methanol (stock solution)

Weight 0.7927 g of DTNB into 10-mL volumetric flask and adjust the volume to 10 mL with absolute methanol. Keep the solution at ambient temperature.

5. 16 mM DTNB solution in 50 mM KPB pH 7.0 (working solution)

Take 966 µL of 50 mM KPB pH7.0 into 1.5-mL tube. Then, add 84 µL of 200 mM DTNB solution (stock solution) and mix well.

6. 10 mM ACTh solution (stock solution)

Weight 28.918 mg of ACTh into 10-mL volumetric flask. Then, adjust the volume to 10 mL with 50 mM KPB pH 7.0 and mix well. Keep the solution at -20^{o}C. (Molecular weight of ACTh = 289.18 g/mol)

7. 0.32 mM ACTh solution (working solution)

Dilute 184 µL of 10 mM ACTh solution (stock solution) to 5750 µL with 50 mM KPB pH 7.0. Keep the solution in an ice bath before using.

8. 0.01 mg/mL AChE solution (stock solution)

Weight 0.1 mg of AChE into 10-mL volumetric flask. Then, adjust the volume to 10 mL with 50 mM KPB pH 7.0 and mix well. Keep the solution at -20^{o}C.

9. 0.0001 mg/mL AChE solution (working solution)

Dilute 82 µL of 0.01 mg/mL AChE solution (stock solution) to 8200 µL with 50 mM KPB pH 7.0 (working solution). Keep the solution in an ice bath before using.

BChE inhibitory assays

Reagents

- Absolute methanol
- Di-potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB)
- Magnesium chloride ($MgCl_2$)
- Butyrylthiocholine chloride (BTCh)
- Equine serum butyrylcholinesterase (BChE)

Reagent preparation

1. 50 mM potassium phosphate buffer (KPB) pH 7.0 with 1mM $MgCl_2$

Weight 4.7605 mg of $MgCl_2$ and adjust the volume to 50 mL with 50 mM KPB pH 7.0 and mix well. Keep the solution in a refrigerator. (Molecular weight of $MgCl_2$ = 95.21 g/mol)

2. 16 mM DTNB solution in 50 mM KPB pH 7.0 (working solution)

Take 966 µL of 50 mM KPB pH 7.0 into 1.5-mL tube. Then, add 84 µL of 200 mM DTNB solution (stock solution) and mix well.

3. 10 mM BCTh solution (stock solution)

Weight 22.578 mg of BCTh into 10-mL volumetric flask. Then, adjust the volume to 10 mL with 50 mM KPB pH 7.0 and mix well. Keep the solution at -20°C. (Molecular weight = 225.78 g/mol)

4. 0.4 mM BCTh solution (working solution)

Dilute 232 µL of 10 mM BCTh solution (stock solution) to 5800 µL with 50 mM KPB pH 7.0. Keep the solution in an ice bath before using.

5. 0.1 mg/mL BChE solution (stock solution)

Weight 1 mg of BChE into 10-mL volumetric flask. Then, adjust the volume to 10 mL with 50 mM KPB pH 7.0 with 1mM $MgCl_2$. Mix well and keep the solution at -20°C.

6. 0.0005 mg/mL BChE solution (working solution)

Dilute 41 µL of 0.1 mg/mL BChE solution (stock solution) to 8200 µL with 50 mM KPB pH 7.0 with 1mM $MgCl_2$. Keep the solution in an ice bath before using.

A4. Reagent preparations for BACE1 inhibitory assays

Reagents

Using β-Secretase (BACE1) Activity Detection Kit (Fluorescent)

- 0.5 mg BACE1 substrate
- Dimethyl sulfoxide (DMSO)
- 3 units/µL BACE1 enzyme)
- Fluorescent assay buffer

Reagent preparation

1. 500 µM BACE1 substrate solution (stock solution)

Add 500 µL of DMSO into 0.5 mg BACE1 substrate. Mix well and keep the solution at -20^{o}C.

2. 50 µM BACE1 substrate solution (working solution)

Dilute 200 µL of 500 µM BACE1 substrate solution (stock solution) to 2000 µL with fluorescent assay buffer. Keep the solution in an ice bath before using.

3. 0.3 units/µL BACE1 enzyme

Dilute 20 µL of 3 units/µL BACE1 enzyme to 200 µL with fluorescent assay buffer. Keep the solution in an ice bath before using.

APPENDIX B
REACTIONS OF TPC AND ANTIOXIDANT ACTIVITY

B1. Principle for determination of TPC by Folin-Ciocalteu assay

The phenol is ionized in alkaline condition to become ionized phenolic acid, which is subsequencely oxidized by Folin-Ciocalteu reagent. This reagent is a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$), thus changing yellow color of Mo(VI) to a blue complex of MoO(V) in a complex of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}) under reduction process (Figure B1.1). The change of color can be measured in a spectrophotometer at 765 nm. Gallic acid is used as a control standard (Figure B1.2), and the concentration of TPC is usually reported as gallic acid equivalents (GAE) per gram dry weight.

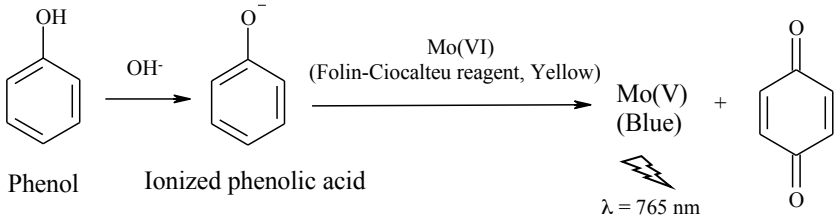
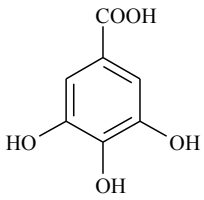


Figure B1.1 Reaction for determination of TPC by Folin-Ciocalteu assay. The yellow Mo(VI) in phosphomolybdic–phosphotungstic acid reagent is reduced to blue Mo(V) complex by ionized phenolic group. The changed color is measured in a spectrophotometer at 765 nm.



Gallic acid

Figure B1.2 Chemical structure of gallic acid monohydrate. It is a type of phenolic acid, which is a standard used in Folin-Ciocalteu Assay.

B2. Principle for determination of antioxidant activity

Ferric reducing antioxidant power (FRAP) assay

FRAP assay based on single electron transfer (SET) reactions [Ndhlala, 2010] is from the reaction between ferric 2,4,6-tripyridyl-*s*-triazine (TPTZ) and antioxidant. Ferric-TPTZ (Fe^{3+}-TPTZ) is reduced to ferrous complex (Fe^{2+}-TPTZ) by electron-donated antioxidant, which changes the color from brown to indigo. This color change can be measured at a wavelength of 595 nm as an indicator of product formation (Figure B2.1). The FRAP value is reported as TE per gram dry weight using trolox as a standard.

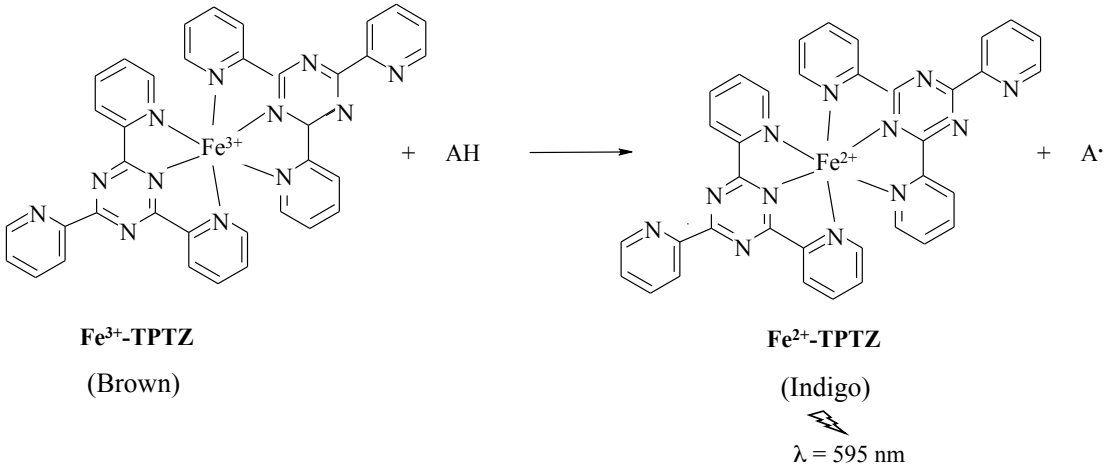


Figure B2.1 Reaction for determination of antioxidant activity by FRAP assay. The assay is measured by the reduction of brown Fe^{3+}-TPTZ to indigo Fe^{2+}-TPTZ by antioxidant (AH). The indigo color as indicator of product formation can be measured at a wavelength of 595 nm.

DPPH - radical scavenging assay

DPPH assay is based on both hydrogen transfer (HAT) reactions and single electron transfer (SET) reactions [178]. DPPH is a stable free radical, consisting of a reactive organic nitrogen atom that acts as a radical scavenger or hydrogen donor [179]. The $DPPH^{\bullet}$ radical contains an unpaired nitrogen with a deep purple color. If $DPPH^{\bullet}$ radical reacts with an antioxidant (AH) in a reduction reaction, the stable DPPH-H is generated to produce yellow product. Thus, DPPH assay is measured by a loss of deep purple color of DPPH at 520 nm after reacting with antioxidant (Figure

B2.2). The data are presented as % radical scavenging activity or trolox equivalence (TE) per gram dry weight.

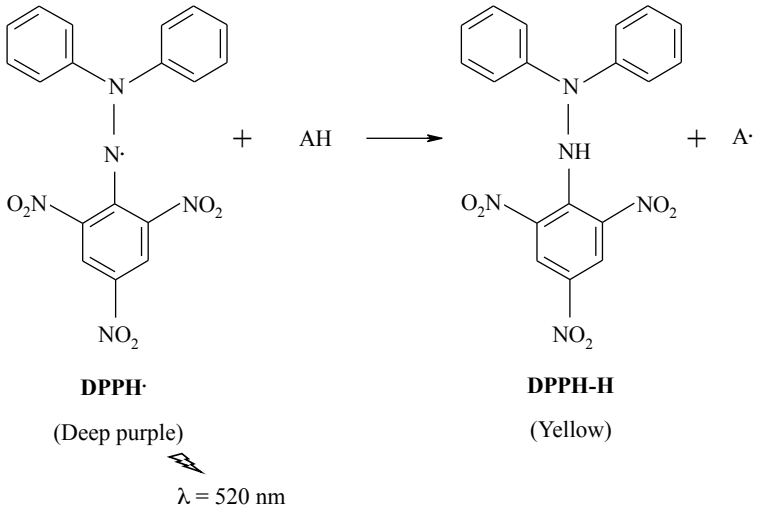


Figure B2.2 Reaction for determination of antioxidant activity by DPPH assay. The deep purple DPPH• radical reacts with antioxidants (AH) to produce a yellow DPPH-H compound. Antioxidant activity is measured with the loss of deep purple by absorbance at 520 nm.

Oxygen radical absorbance capacity (ORAC) assay

ORAC assay based on hydrogen atom transfer (HAT) reactions [178] includes the reaction between peroxyl radical of AAPH and fluorescein by oxidative damage. AAPH is oxidized to produces peroxyl radical. Then, the peroxyl radical reacts with the fluorescein to form fluoresceinyl radical, a non–fluorescent product (Figure B2.3). Antioxidant activity in ORAC assay is determined by a kinetic measuring of fluorescence intensity loss and protection of the fluorescence loss by antioxidant. The fluorescence intensity will be monitored at 37 ºC with an excitation wavelength of 485 nm and emission wavelength of 528 nm. Trolox is commonly used as a control standard (Figure B2.4), and ORAC values are usually reported as Trolox equivalents (TE) per gram dry weight.

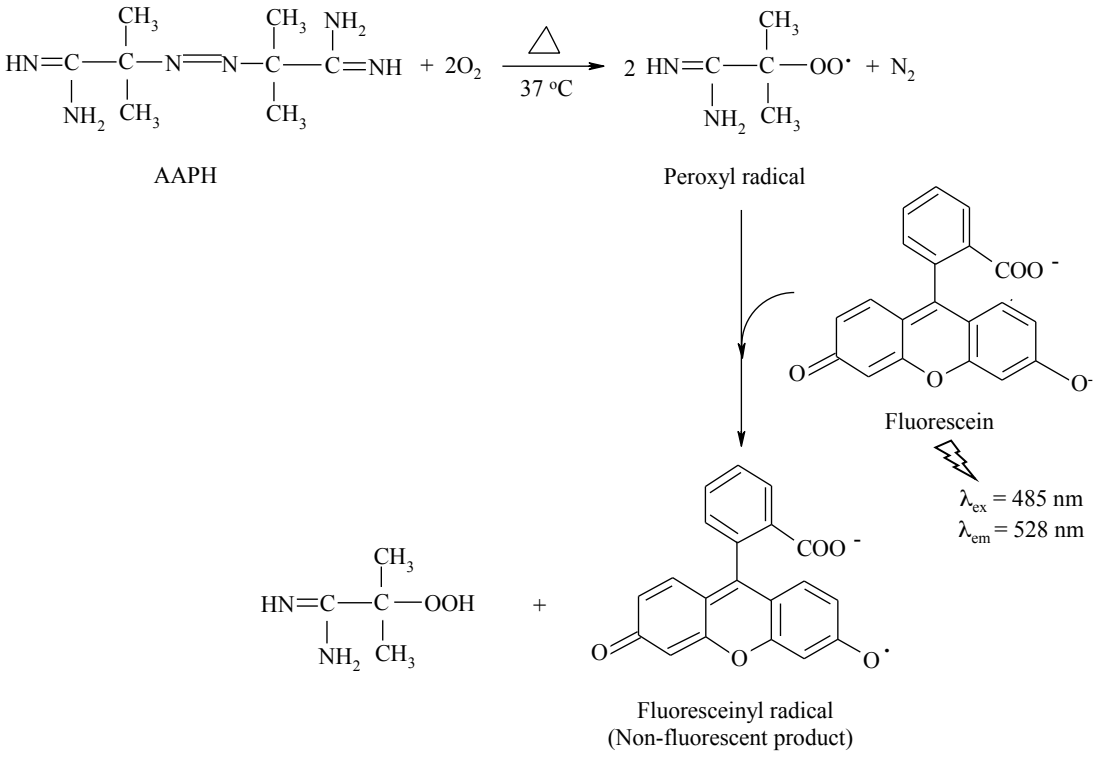


Figure B2.3 Reaction for determination of antioxidant activity by ORAC assay. AAPH is oxidized to generate nitrogen gas (N_2) and two peroxyl radicals. The peroxyl radical then reacts with the fluorescein to form fluoresceinyl radical, a non-fluorescent product. The assay measures the loss of fluorescein with an excitation wavelength of 485 nm and emission wavelength of 528 nm.

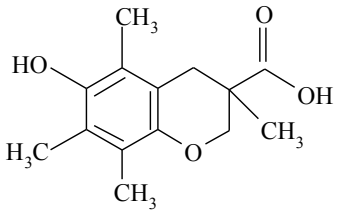


Figure B2.4 Chemical structures of trolox. It is a water soluble vitamin E analogue, which is a standard used in antioxidant assay.

APPENDIX C
REACTIONS OF ENZYME INHIBITORY ACTIVITY ASSAYS

C1. Principle of cholinesterase inhibitory assays

AChE inhibitory assay

The substrate, ATCh, is hydrolyzed by enzyme AChE to produce thiocholine, which is subsequently reacted with Ellman's reagent, DTNB, to form a yellow 2-nitro-5-mercapto-benzoic acid. This final product possesses yellow color that can be measured at a wavelength of 412 nm (Figure C1.1). However, under the presence of inhibitor, the production of thiocholine is reduced as a result of interference by inhibitor.

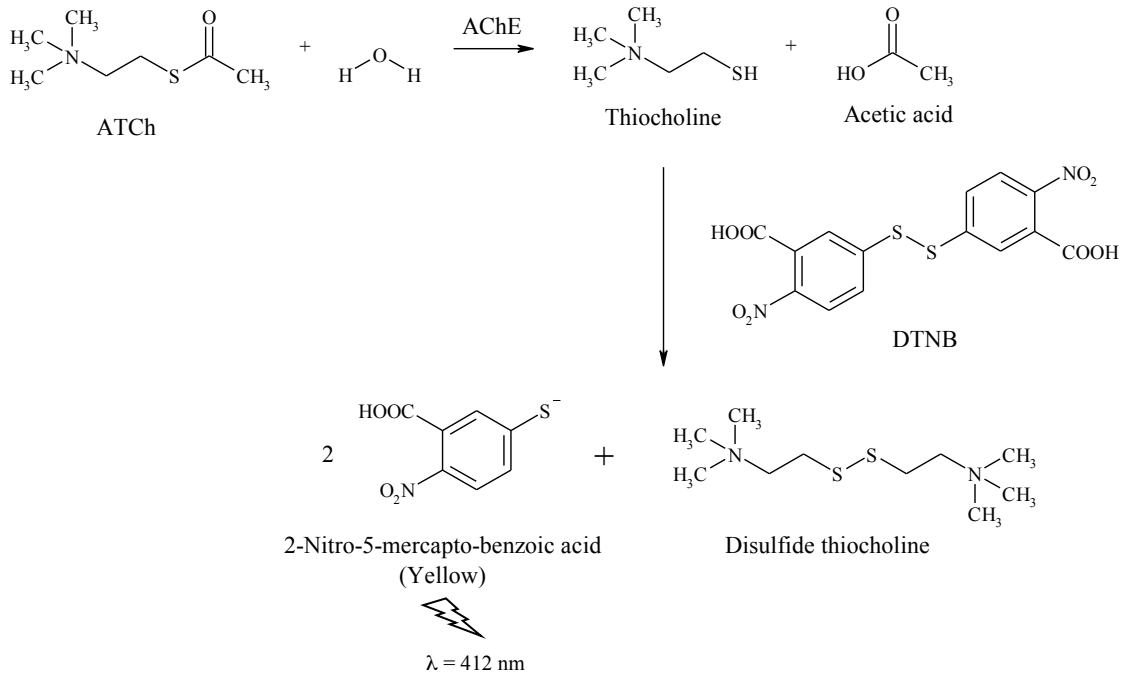


Figure C1.1 Reaction of AChE inhibitory assays. ATCh is hydrolyzed by AChE to form thiocholine and acetic acid. Thiocholine is reacted with DTNB to generate color product, which can be measured at a wavelength of 412nm.

BChE inhibitory assay

The substrate, BTCh, is hydrolyzed by enzyme BChE to produce thiocholine, which is subsequently reacted with Ellman's reagent, DTNB, to form a yellow 2-nitro-5-mercapto-benzoic acid. This final product possesses yellow color that can be measured at a wavelength of 412 nm (Figure C1.2). However, under the presence of inhibitor, the production of thiocholine is reduced as a result of interference between inhibitor and enzyme.

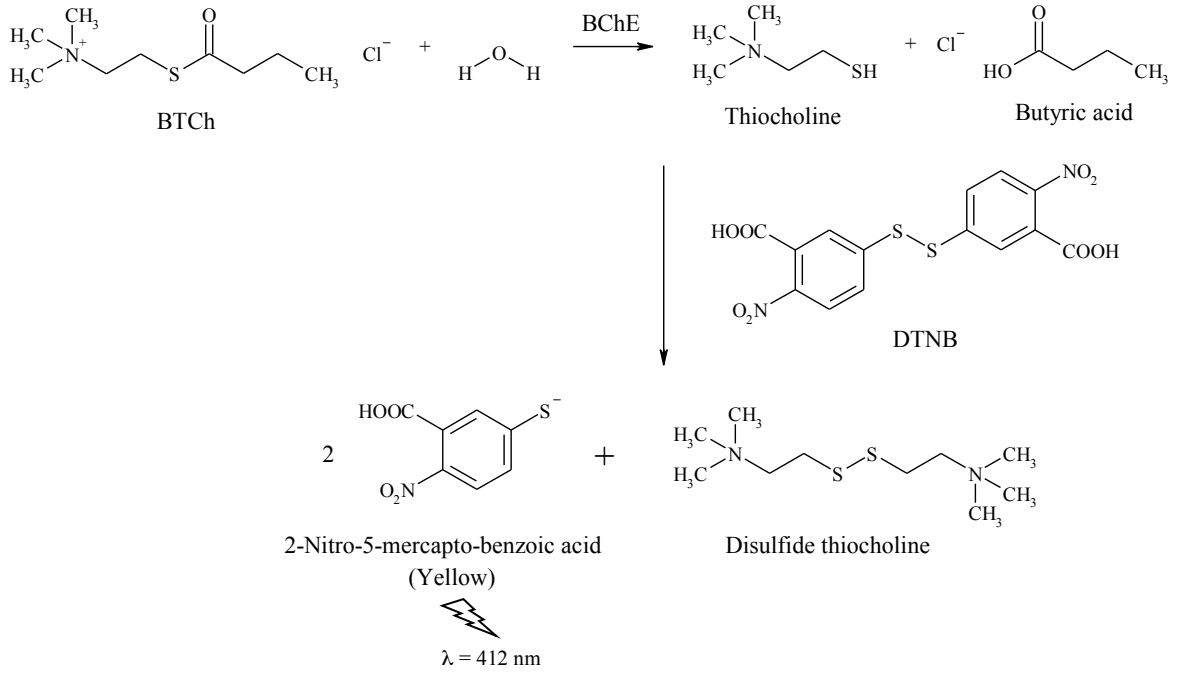


Figure C1.2 Reaction of BChE inhibitory assays. The reaction is similar to AChE inhibitory activity assay. BChE hydrolyzes BTCh to thiocholine and butyric acid. The BChE inhibitory activity is measured from the color of the product between thiocholine and DTNB at 412 nm.

C2. Principle of BACE1 inhibitory assay

The assay is based on a method of fluorescence resonance energy transfer (FRET). The FRET protease substrate, BACE1 substrate, consists of two fluorophores including a fluorescent donor (D) and a quenching acceptor (A). The energy of (D) is significantly quenched by (A) in resonance energy transfer (Figure C2), leading to dramatically reduced intrinsic fluorescence of the substrate. If BACE1 substrate is

cleavage by BACE1, the fluorophore is separated from the quenching group, restoring the full fluorescence yield of the donor. Therefore, fluorescent intensity of peptide substrate becomes increased and can be measured at an excitation wavelength of 320 nm and an emission wavelength of 405 nm. However, if the inhibitor is present in system, the cleavage of BACE1 substrate will be reduced, leading to decreased fluorescence signal [180].

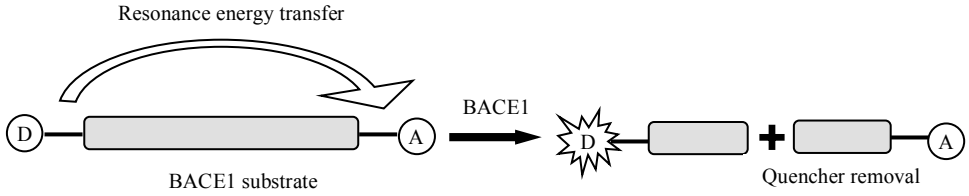


Figure C2. Reaction of BACE1 inhibitory assay. BACE1 substrate is cleavage from the quenching group (A) by BACE1 to increase fluorescence by fluorescent donor (D). The reaction can be measured at the excitation wavelength of 320 nm and the emission wavelength of 405 nm.

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APPENDIX D
PHYTOCHEMICAL CHROMATOGRAMS

Chromatogram of Volatile Compounds from GC-MS

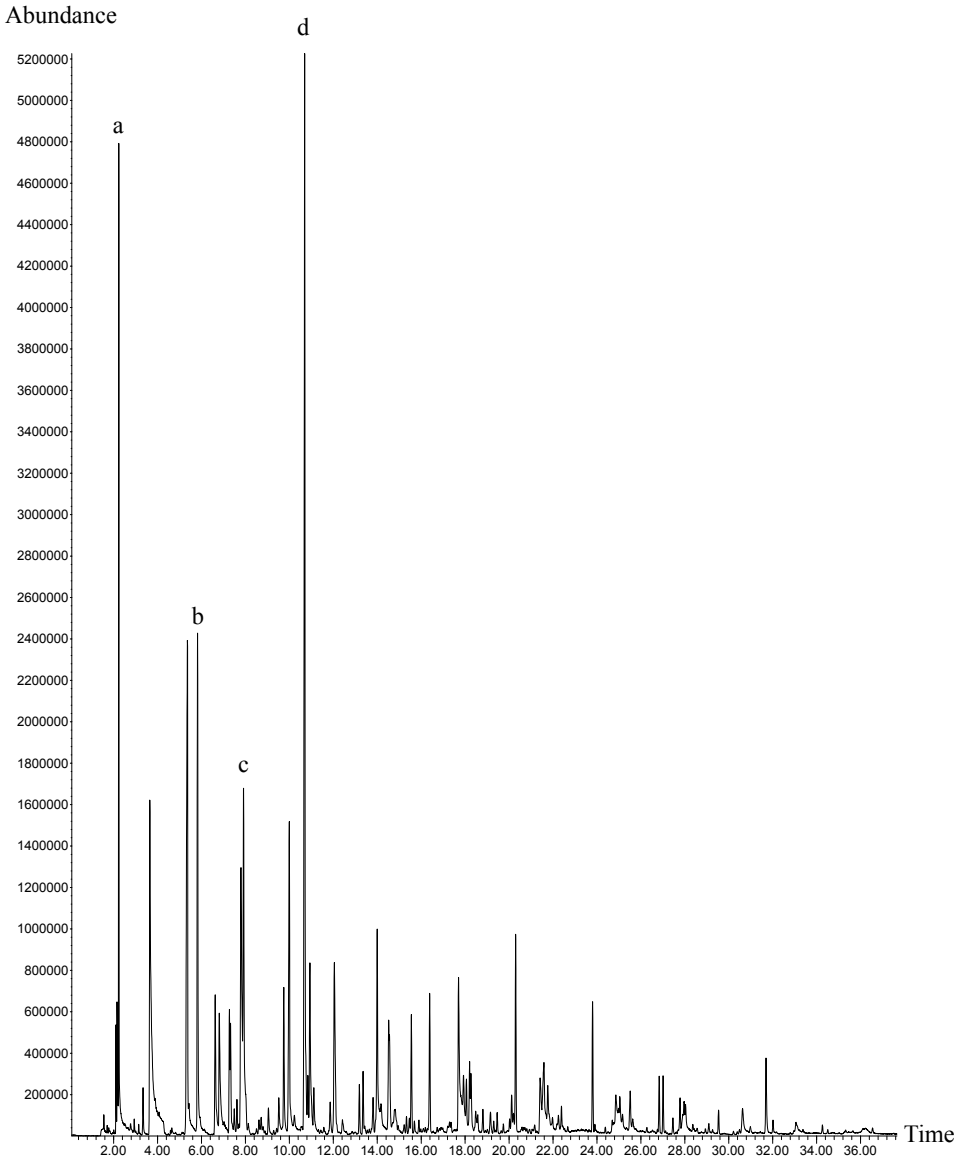

Figure D1 Chromatogram of volatile compounds from pandan leaves. The volatile compounds of pandan leaves were determined using GC-MS analysis. a, b, c and d, which high quantity of volatile compounds, were representative of fluoroacetamide, 5-amino-1-ethylpyrazole, octanal and nonanal, respectively.

Chromatograms of Phenolic Acids and Flavonoids from HPLC

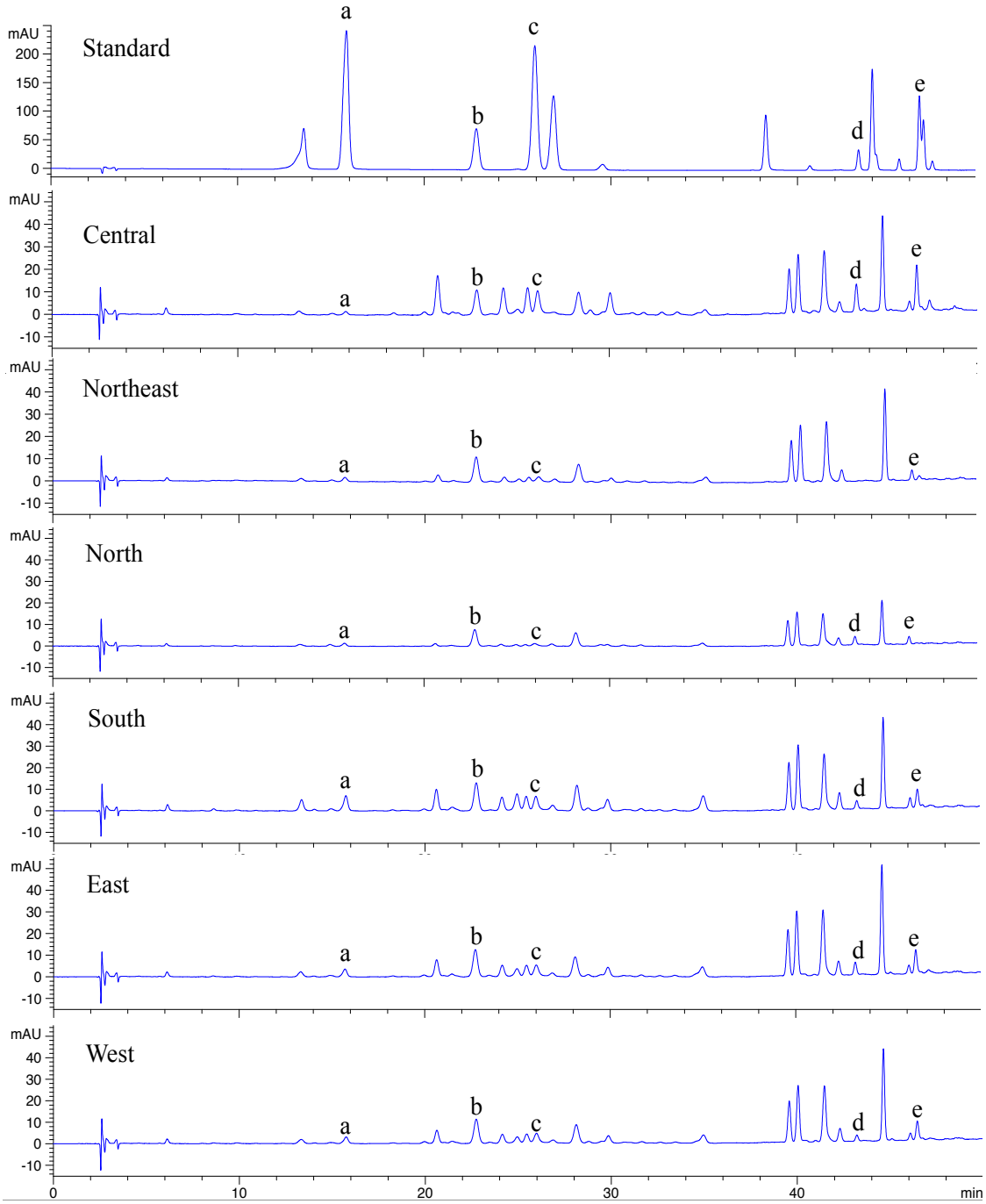

Figure D2 Chromatograms of phenolic acids and flavonoids. The phenolic acids and flavonoids of pandan leaves in different cultivated locations were determined using HPLC analysis at 338 nm. a, b, c, d and e were representative of caffeic acid, *p*-coumaric acid, sinapic acid, quercetin and keampferol, respectively.

Chromatograms of Carotenoids from HPLC

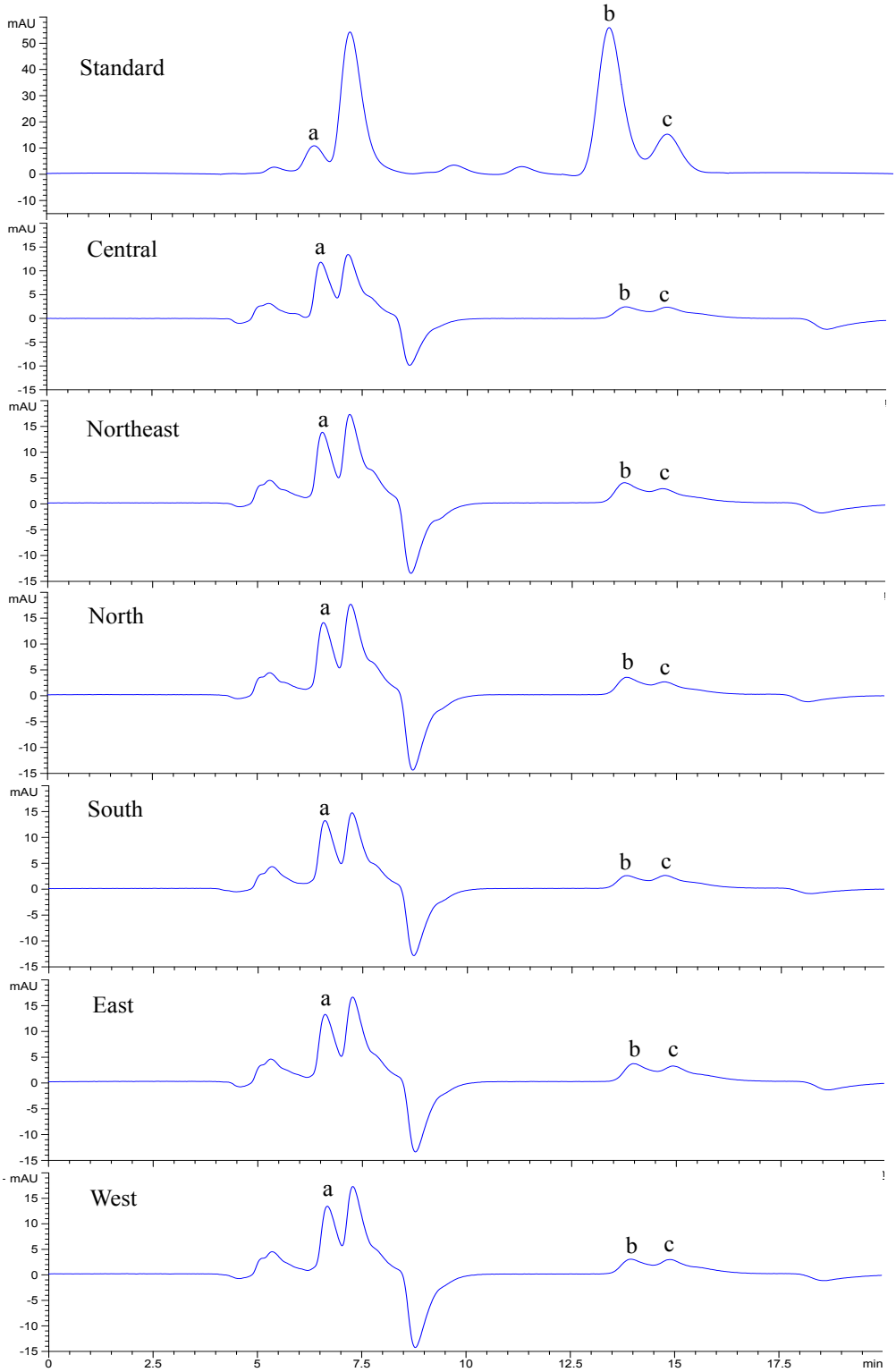

Figure D3 Chromatograms of carotenoids. The carotenoids of pandan leaves in different cultivated locations were determined using HPLC analysis at 450 nm. a, b and c were representative of lutein, α-carotene, and trans-β-carotene, respectively.