APPENDICES

APPENDIX A
REACTION MECHANISMS OF ANTIOXIDANT CAPACITY ASSAYS AND TOTAL PHENOLS BY FOLIN-CIOCALTEU ASSAY

A1. Antioxidant Capacity Assays

Oxygen radical absorbance capacity (ORAC) assay

ORAC assay is depended on inhibitory activity of antioxidant toward peroxyl radical induced oxidations. The assay used the fluorescent *β*–phycoerythrin as a probe and peroxyl radicals as oxidizing agents that being generated through thermogenesis of 2,2'–azobis (2–amidinopropane) dihydrochloride (AAPH) [32]. The peroxyl radical then reacts with the fluorescein to form fluoresceinyl radical, a non–fluorescent product (Figure A1.1). Antioxidant capacity is determined by a loss of fluorescence intensity over time due to oxidative damage to the probe. The antioxidant quantity is derived from area under sodium fluorescein decay curve (AUC). The fluorescence intensity will be monitored at 37 ºC with an excitation wavelength of 485 nm and emission wavelength of 528 nm. Trolox, a water–soluble analogue of vitamin E is commonly used as a control standard (Figure A1.2), and ORAC values are usually reported as Trolox equivalents (TE).

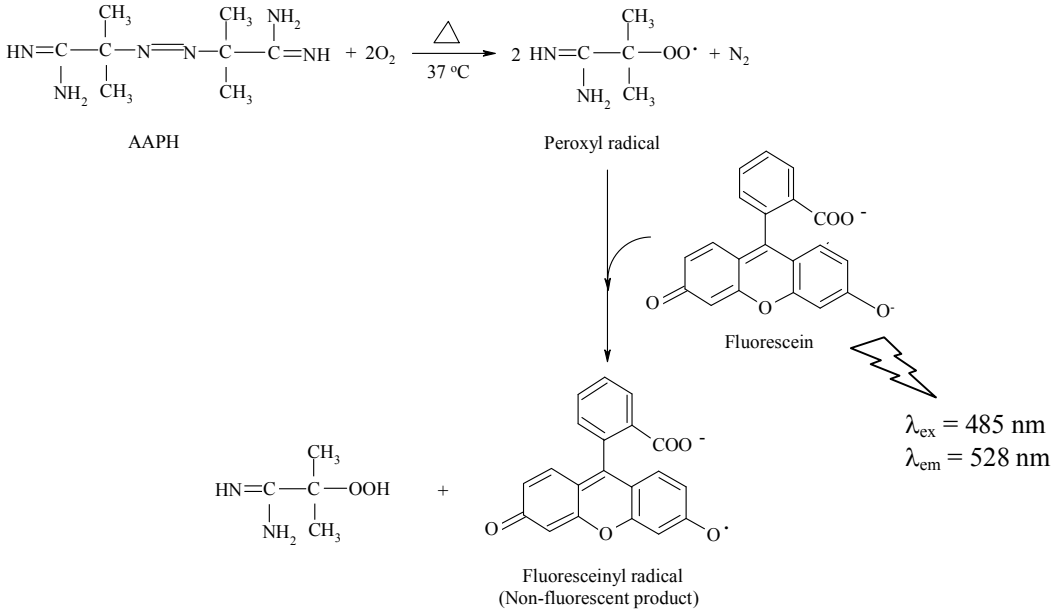

Figure A1.1 The reaction of ORAC assay. AAPH undergoes thermogenesis to generate nitrogen gas (N_2) and two alkyl radicals, which react with oxygen–forming peroxyl radicals (ROO•). The peroxyl radical reacts with the fluorescein to form fluoresceinyl radical, a non–fluorescent product. The assay measures the loss of fluorescein due to oxidative damage with an excitation wavelength of 485 nm and emission wavelength of 528 nm.

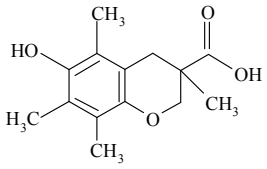

Figure A1.2 Chemical structures of Trolox®. Trolox is a water soluble vitamin E analogue, which is a standard agent in ORAC assay.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay is employed to measure antioxidant activity based on the ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions in the presence of Fe^{2+}–stabilizing ligand such as 2,4,6–tripyridyl–*s*–triazine (TPTZ) [32]. The reaction mechanism involves the reduction of brown colored Fe^{3+}–TPTZ to indigo colored Fe^{2+} analogue, which can be measured at 595 nm (Figure A1.3). As results, FRAP value is reported as TE, similarly to ORAC assay.

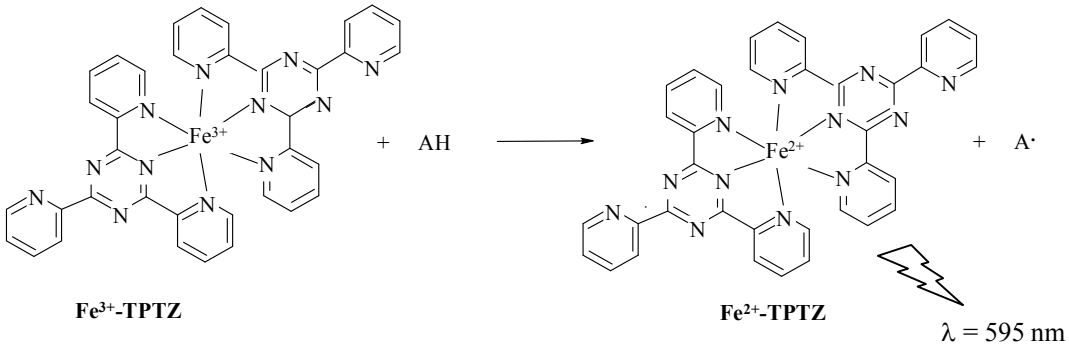

Figure A1.3 The reaction of FRAP assay. The assay measures reduction of ferric 2,4,6–tripyridyl–*s*–triazine (Fe^{3+}–TPTZ) into its ferrous analogue (Fe^{2+}–TPTZ) with an electron–donated antioxidant (AH). The assay was monitored by measuring absorbance at 595 nm.

1–Diphenyl–2–picrylhydrazyl (DPPH) radical scavenging assay

A stable free radical DPPH consists of a reactive organic nitrogen atom that acts as a radical scavenger or hydrogen donor [32]. The chemical mechanism of the DPPH assay is primarily based on SET reactions with HAT being a marginal reaction [108]. The starting material, $DPPH^{\bullet}$ radical, with a deep purple color due to an unpaired nitrogen electron reacts with an antioxidant (AH) to produce yellow DPPH–H product. Thus, the assay measurement is depended on a loss of deep purple color of DPPH at 520 nm after reacting with antioxidant (Figure A1.4). The data are presented as % radical scavenging activity.

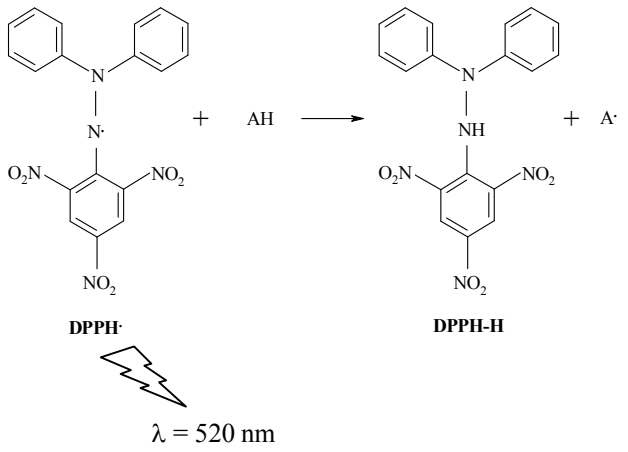

Figure A1.4 The reaction of DPPH assay. The purple DPPH$^{\bullet}$ radical forms a reaction with antioxidants (AH) to produce yellow DPPH–H. The assay was monitored by measuring absorbance at 520 nm.

A2. Total Phenols by Folin–Ciocalteu Assay

The Folin–Ciocalteu assay has been usually employed to analyze total phenolics compounds from natural products [32]. The reaction based on the reduction of phenols by phosphomolybdic–phosphotungstic acid reagent to produce a blue colored molybdenum oxide with an absorbance of 725 nm (Figure A2.1). Gallic acid is commonly used as a control standard, and the concentration of phenolic compounds is usually reported as gallic acid equivalents (GAE).

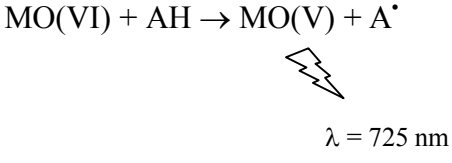

Figure A2.1 The reaction of Folin–Ciocalteu assay. Mo(VI) in phosphomolybdic–phosphotungstic acid reagent is reduced to Mo(V) with an electron being donated by an antioxidant (AH). The assay was monitored by measuring absorbance at 725 nm.

APPENDIX B
PLANT MATERIALS

All of conventional teas were manufactured by Choui Fong Tea, Chiangrai province, Thailand in exception of pu–erh tea that comes from Yunnan province in China. Otherwise, amongst fifteen Thai herbal teas, seven herbal teas were manufactured by Chao Phya Abhaibhubejhr Hospital, Prachinburi province, Thailand; including bael fruit, Indian gooseberry, rosella, safflower, Asiatic pennywort, cat's whisker and jewel vine. Beside these teas, bitter cucumber, chrysanthemum, white mulberry, pandanus, stevia, lemon grass and ginger were manufactured by Pathom Asoke Community, Nakhonpathom province, Thailand. Jiaogulan was manufactured by Mae Buran's Herb, Uthaithani, Thailand.

All of conventional teas were prepared through the process for making tea including withering, rolling, fermentation, firing or drying. White and green teas are both unfermented tea, but white tea is steamed directly after harvest. Oolong tea is a partially fermented tea, while black teas are fully fermented tea leaves. Pu–erh tea is not only fully fermented but is also aged in form of compressed teas.

All of fifteen Thai herbal teas were non–contaminated with other herbs or substances. For preparing dry herb, after harvested, the fresh herbs were placed on the large basket or the racks that exposed to direct sunlight or oven-dried at 60 °C for 1-2 day. After that, dried herbs were grinded into small size by grinding machine before packing in tea bag (1-2 g of dried herb/tea bag).

APPENDIX C
REAGENT PREPARATIONS

C1. Reagent Preparations for Antioxidant Assays

Oxygen radical absorbance capacity (ORAC) assay

Reagents

- Di–potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Trolox ((±)–6–hydroxy–2,5,7,8–tetra–methylchromane–2–carboxlic acid
- 2'–azobis (2–amidinopropane) dihydrochloride (AAPH)
- Fluorescein sodium salt

Reagent preparation

1. ORAC buffer stock solution

- Prepare 0.75 M KH_2PO_4 stock solution

Weight 102.07 g of KH_2PO_4 in a 1 L volumetric flask then diluted with deionized water to the volume in order to yield 1 L (store in a refrigerator).

- Prepare 0.75 M K_2HPO_4 stock solution

Weight 130.64 g of K_2HPO_4 in a 1 L volumetric flask then diluted with deionized water to the volume in order to yield 1 L (store in a refrigerator).

Take 603 mL of 0.75 M K_2HPO_4 stock solution and add 351 mL of 0.75 M KH_2PO_4 stock solution, this yields 954 mL of the ORAC buffer stock solution (store 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 to7.2 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, mix well and store in an ice bath before used.

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

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. Fluorescein stock solution (4.19 µM)

Take 20 mL of ORAC buffer working solution and remove 100 µL. Then, take 100 µL of fluorescein concentrated solution and mix well. Keep the solution in a freezer.

6. Fluorescein working solution

Take 25 mL of ORAC buffer working solution into a 25 mL volumetric flask and remove 488 µL. Then, take 488 µL of fluorescein stock solution, mix well and warm in water bath at 37 °C before use.

7. 1000 µM Trolox stock solution

Weigh 0.025 g of trolox into a 100 mL volumetric flask. Then, adjust the volume to 100 mL with deionized water and mix well. Keep the solution in a freezer.

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

Take 900 µL of the ORAC buffer working solution into a 1 mL tube. After that, add 100 µL of 1000 µM Trolox stock solution to yield 100 µM Trolox standard. Make serial dilution to 50, 25, 12.5, 6.25 and 3.125 µM Trolox standard.

Ferric reducing antioxidant power (FRAP) assay

Reagents

- Glacial acetic acid
- Hydrochloric acid (HCl), 36.46%
- 2, 4, 6–tripyridyl–s–triazine (TPTZ)
- Sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$)
- Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$)

- Trolox (±)–6–hydroxy–2,5,7,8–tetra–methylchromane–2–carboxlic acid

Reagent preparation

1. 300 mM acetate buffer

Weight 3.1 g of sodium acetate trihydrate into a 1 L volumetric flask. Then, add 16 mL of glacial acetic acid. After that, 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 (conc. 0.4 mL + deionized water 99.6 mL) and mix well. Keep the solution in a refrigerator.

3. 20 mM $FeCl_3 \cdot 6H_2O$

Weight 0.5406 g of $FeCl_3 \cdot 6H_2O$ 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 from 1–3 before use in ratio of 10: 1: 1 respectively and warm in water bath at 37 °C before use.

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

Take 750 µL of the deionized water into a 1 mL tube. After that, add 250 µL of 1000 µM Trolox stock solution to yield 250 µM Trolox standard. Make serial dilution to 125, 62.5, 31.25, 15.625 and 7.8125 µM Trolox standard.

1–Diphenyl–2–picrylhydrazyl (DPPH) radical scavenging assay

Reagents

- Absolute ethanol
- 1,1–Diphenyl–2–picrylhydrazyl (DPPH)
- Trolox ((±)–6–hydroxy–2,5,7,8–tetra–methylchromane–2–carboxlic acid

Reagent preparation

1. 95% Ethanol

Take 950 mL of absolute ethanol into a 1000 mL cylinder. Adjust the volume to 1000 mL with deionized water.

2. 150 µM DPPH in 95% Ethanol

Weight 5.91 mg of DPPH into a 100 mL volumetric flask. Then, adjust the volume to 100 mL with 95% ethanol and mix well**.**

3. 8 mM Trolox stock solution

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

4. 1.28 mM Trolox working solution (for serial dilution)

Take 840 µL of the 95% ethanol into a 1 mL tube. After that, add 160 µL of 8 mM Trolox stock solution to yield 1.28 mM Trolox standard. Make serial dilution to 0.64, 0.32, 0.16 and 0.08 mM Trolox standard.

C2. Reagent Preparations for Folin–Ciocalteu Assay

Reagents

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

Reagent preparation

1. 10% Folin–Ciocalteu's reagent (FCR)

Dilute 10 ml of FCR stock solution to 100 ml with deionized water in a volumetric flask. Keep the reagent at ambient temperature.

2. 7.5% 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. 1 mg/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. 100 µg/ml Gallic acid working solution (for serial dilution)

Take 900 µL of the deionized water into a 1 mL tube. After that, add 100 µL of 1 mg/mL Gallic acid stock solution to yield 100 mg/mL Gallic acid standard. After that, make a Gallic acid standard dilution to 80, 60, 40, 20 and 10 mg/mL respectively.

C3. Reagent Preparations for Anti–glycation Assays

Reagents

- Di–potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Bovine serum albumin (BSA)
- D–glucose
- Methyglyoxal
- Sodium azide
- Gallic acid monohydrate

Reagent preparation

1. 1 M K_2HPO_4 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 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. 100 mM potassium phosphate buffer (KPB) with 0.02% sodium azide, pH 7.4

Take 60.2 mL of 1 M K_2HPO_4 stock solution into 1 L volumetric flask and add 19.8 mL of 1 M KH_2PO_4 stock solution and then add deionized water about 500 mL and mix well. Then, add 0.2 g of sodium azide, mix well and adjust pH to 7.4 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. 30 mg/mL BSA

Weight 3 g of BSA into 150 mL beaker. Then fill up to 100 mL with 100 mM KPB with 0.02% sodium azide (pH 7.4), mix well and store in an ice bath before used.

5. 750 mM D–glucose

Weight 13.545 g of D–glucose into 150 mL beaker. Then fill up to 100 mL with 100 mM KPB with 0.02% sodium azide (pH 7.4), mix well and store in an ice bath before used.

6. 3 mM methylglyoxal

Take 100 mM KPB with 0.02% sodium azide (pH 7.4) into a 100 mL volumetric flask and remove 18.47 µL. Then, add 18.47 µL of 16.236 M methylglyoxal solution, mix well and store in an ice bath before used.

7. 10 mM Gallic acid stock solution

Weight 17.012 mg of gallic acid monohydrate in a 10 mL volumetric flask. Then, adjust the volume to 10 mL with 100 mM KPB with 0.02% sodium azide (pH 7.4). Keep the solution in a refrigerator.

8. 800 µM Gallic acid working solution (for serial dilution)

Take 13.8 mL of 100 mM KPB with 0.02% sodium azide (pH 7.4) into a 15 mL tube. After that, add 1.2 mL of 10 mM Gallic acid stock solution to yield 800 µM gallic acid standard. After that, make a Gallic acid standard dilution to 400, 200, 100, 80, 60, 40 and 20 µM, respectively.

C4. Reagent Preparations for Lipase Inhibitory Assays

Reagents

- Absolute methanol
- Tris(hydroxymethyl) aminomethane ($NH_2C(CH_2OH)_3$)
- Di–potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Potassium Chloride (KCl)
- Triton X–100
- Ethylenediaminetetraacetic acid (EDTA)
- 5,5'– dithiobis (2–nitro benzoic acid) (DTNB)
- 2,3–dimercapto–1–propanol tributyrate (DMPTB)
- Bovine serum albumin (BSA)
- *Candida rugosa* lipase

Reagent preparation

1. 50 mM Tris, 10 mM KCl, 1 mM EDTA, pH 7.2 (Assay buffer)

Weight 6.057 g of $NH_2C(CH_2OH)_3$, 0.7455 g of KCL and 0.292 g of EDTA into 1 L beaker. Then, add deionized water about 500 mL, mix well and adjust pH to 7.2. Then, adjust the volume to 1 L with deionized water and mix well. Keep the solution in a refrigerator.

2. 10% (v/v) Triton X–100 in 50 mM Tris, 10 mM KCl, 1 mM EDTA, pH 7.2 (Substrate buffer)

Take 90 mL of assay buffer into 150 mL beaker. Then, add 10 mL of Triton X–100 and mix well. Keep the solution in a refrigerator.

3. 50 mM Tris, 10 mM KCl, 1 mM EDTA, pH 7.2 with 0.1% BSA, pH 8.0 (Enzyme buffer)

Take 250 mL of assay buffer into 250 mL beaker. Then, add 0.5 g of BSA and mix well. Adjust pH to 8.0 with a few drops of a NaOH concentrated solution. Keep the solution in a refrigerator.

4. 50 mM potassium phosphate buffer (KPB), pH 7.4

Take 30.1 mL of 1 M K_2HPO_4 stock solution into 1 L volumetric flask and add 9.9 mL of 1 M KH_2PO_4 stock solution and then add deionized water about 500 mL and mix well. Then, adjust pH to 7.4 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.

5. 200 mM DTNB stock solution in Methanol

Weight 0.7927 g of DTNB into 10 mL volumetric flask and adjust the volume to 10 mL with absolute methanol.

6. 16 mM DTNB in 50 mM KPB

Take 920 µL of 50 mM KPB into 1.5 mL tube. Then, add 80 µL of 200 mM DTNB stock solution and mix well.

7. 10 mM DMPTB stock solution

Take 10 mL of substrate buffer into 15 mL tube and remove 33.45 µL. Then, add 33.45 µL of DMPTB solution and mix well. Keep the solution in a refrigerator.

8. 0.2 mM DMPTB

Take 10 mL of assay buffer into 15 mL tube and remove 200 µL. Then, add 200 µL of 10 mM DMPTB stock solution, mix well and store in an ice bath before used.

9. 1 mg/mL *Candida rugosa* lipase stock solution

Weight 1 mg of *Candida rugosa* lipase into 1.5 mL tube. Then, adjust the volume to 1 mL with enzyme buffer. Mix well and keep the solution in a refrigerator.

10. 0.001 mg/mL *Candida rugosa* lipase

Take 10 mL of enzyme buffer into 15 mL tube and remove 10 µL. Then, add 10 µL of 1 mg/mL *Candida rugosa* lipase stock solution, mix well and store in an ice bath before used.

C5. Reagent Preparations for ACE Inhibitory Assays

Reagents

- Di–potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Sodium hydroxide (NaOH)
- Sodium chloride (NaCl)
- Hydrochloric acid (HCl), 36.46%
- *o*–phthaldialdehyde
- Hippuryl–histidyl–leucine (HHL)
- Rabbit lung angiotensin–converting enzyme (≥2.0 units/mg, modified Warburg–Christian)

Reagent preparation

1. 100 mM potassium phosphate buffer (KPB), pH 8.3

Take 47 mL of 1 M K_2HPO_4 stock solution and 3 mL of 1 M KH_2PO_4 stock solution into 500 mL beaker. Then, add 400 mL of deionized water and adjust the pH to 8.3 with a few drop of NaOH concentrated solution. Then, adjust the volume to 500 mL with deionized water and mix well. Keep the solution in a refrigerator.

2. 0.025 M NaOH

Weight 0.1 g of NaOH into 100 mL beaker and adjust the volume to 100 mL with deionized water.

3. 0.28 M NaOH

Weight 5.6 g of NaOH into 500 mL beaker and adjust the volume to 500 mL with deionized water.

4. 3 M NaCl

Weight 17.53 g of NaCl into 100 mL beaker and adjust the volume to 100 mL with deionized water.

5. 3 M HCL

Take 24.87 mL of 12.06 M HCL into 100 mL volumetric flask and adjust the volume to 100 mL with deionized water.

6. 20 mg/mL *o*–phthaldialdehyde

Weight 40 mg of *o*–phthaldialdehyde into 2 mL tube. Then, add 2 mL of absolute methanol and mix well.

7. 3 M hippuryl–histidyl–leucine (HHL)

Weight 25.77 mg of HHL (429.27 g/mol) into 50 mL tube and add 4.165 mL of 0.025 NaOH. Then, add 13.835 mL of 100 mM KPB, pH 8.3 and add 2 mL of 3 M NaCl. Mix well and keep the solution in a refrigerator.