CHAPTER 2

EXPERIMENTAL

2.1 Apparatus and Chemicals

2.1.1 Apparatus

1) High performance liquid chromatographic system (HP1100) manufactured by Agilent Technologies, U.S.A., consisting of quaternary pump, vacuum degasser, Rheodyne manual injector valve (20 µl loop), UV-VIS detector and data processing system (HP chemstation)

2) High performance liquid chromatography – mass spectrometric system manufactured by Agilent Technologies, U.S.A., consisting of binary pump, vacuum degasser, auto injector, mass spectrometer detector and data processing system (HP chemstation)

3) Q-TOF 2 hybrid quadrupole time-of-flight mass spectrometer, Micromass, England

4) UV-VIS spectrophotometer (Lamda-25), Perkin-Elmer, U.S.A.

5) Analytical column, a Luna C_{18} column, 250 x 4.6 mm I.D., 5 µm, Phenomenex, U.S.A.

6) Glass syringe, 100 µl, Hamilton, U.S.A.

7) Filter unit, Millipore, U.S.A.

8) Filter membrane, 0.45 µm, Sartorius, Germany

9) Ultrasonicator model 8891, Cole-Parmer, U.S.A.

10) Vacuum pump, Gast, U.S.A.

11) Rotary evaporator, Buchi Rotavapor FF-124, Buchi Laortechnik AG, Switzerland, consisting of

a) Water bath, B-480

b) Air pump, KNF Laboport

c) Cooling device NESLAB U.S.A.

2.1.2 Chemicals

The chemicals used in this work with their purity grade and suppliers are listed in **Table 2.1**.

Table 2.1 List of chemicals used, their purity grade and suppliers

Chemical	Purity grade	Supplier
Methanol	A.R.	Lab Scan, Ireland
Methanol	HPLC	Merck, Germany
Acetonitrile	HPLC	Merck, Germany
Hexane	A.R.	Fisher, England
Acetic acid	A.R.	Merck, Germany
Phosphoric acid	A.R.	BDH, England
Formic acid	A.R.	Fisher, England
Hydrochloric acid	A.R.	Lab Scan, Ireland
Acetone	A.R.	Burdick & Jackson, Korea
Gallic acid	HPLC	Fluka, Switzerland
Catechin Hydrate	HPLC	Fluka, Switzerland
Epicatechin green tea	HPLC	Sigma, U.S.A.

Caffeic acid	HPLC	Sigma, U.S.A.
Rutin Trihydrate	HPLC	Fluka, Switzerland
Quercetin dihydrate	HPLC	Sigma, U.S.A.
2,2-Diphenyl-1-picrylhyrazyl	≥ 85%	Fluka, Switzerland
Folin-Ciocalteus reagent	A.R.	Merck, Germany
Sodium carbonate	≥ 99.5%	Carlo Erba, Spain

2.1.3 Materials

Samples used in this work were green, brown, and red seaweeds. All the seaweeds were obtained from various places in Thailand.

2.2 Preparation of the Solutions

2.2.1 Preparation of phenolic compounds standard stock solutions

Each stock solution of phenolic compound standards (1000 ppm) was prepared by dissolving 10 mg of each of phenolic compounds, including gallic acid, catechin, epicatechin, caffeic acid, rutin and quercetin in 10 ml of methanol. Before use, the stock solution was further diluted to 100 ppm and desired concentrations.

2.2.2 Preparation of mobile phase for analysis of phenolic compounds

The investigation for suitable mobile phase was initiated using four types of binary solvent systems where methanol and acetonitrile were mixed with two common acid solutions, i.e. phosphoric acid and acetic acid. Common acid solutions were prepared by dissolving the appropriate volume of each acid in Milli-Q water.

For example, the preparation of 0.1% (v/v) acetic acid solution was done by adding 1 ml of concentrate acetic acid in 1000 ml of Milli-Q water. In the case of 0.1% (v/v) phosphoric acid, it was prepared in a similar manner to the acetic acid solution.

All mobile phase solutions were filtered using a filter unit with 0.45 μm filter membrane and vacuum pump. Finally, the prepared solutions were degassed for 20 minutes using ultrasonicator.

2.3 Extraction by Solvent Extraction

2.3.1 Optimization of solvent

Literature suggests limitations on reporting antioxidants capacity in plants based on the various extraction methods [46]. The procedure used to extract antioxidants may be incomplete. In most of these reports, solvent extraction has been the most commonly used method for extraction of phenolic compounds. The extraction could be improved by more polar solvents such as methanol [47]. Binary mixture of methanol: water has been used as an extraction solvent [48], which has been shown to improve the extraction upon solvent acidification [49].

Four solvents were used for comparison of extraction efficiency, namely (1) methanol 100%, (2) methanol: water (75:25), (3) methanol: water: formic acid (75:20:5), (4) methanol: water: hydrochloric acid (75:20:5). Five grams of seaweeds sample were crushed in liquid nitrogen and mixed with 100 ml of extraction solvent. The mixture was shaken for 60 min. The homogenate was centrifuged at 6,000 rpm for 10 min and the supernatant was filtered through a filter paper (Whatman No.1). The residue was extracted twice with extraction solvent and then centrifuged. After

that the combined supernatants were partitioned with hexane and evaporated to remove both methanol and water. The dried residue was redissolved in 100% methanol and then filtered through a membrane filter having a pore size of 0.45 μm before being subjected to further analysis.

2.4 Determination of Total Phenolic Compound Content

The total phenolic content of seaweeds extract was determined using Folin-Ciocalteau reagent [46]. In brief, the reaction mixture contained 50 μl of this seaweed extract solution was mixed with 250 μl of freshly prepared Folin-Ciocalteau reagent. The reaction solution was left at room temperature for 5 min. Then 0.75 ml of 20% sodium bicarbonate solution and 3 ml of milli-Q water were added. The mixture was incubated at room temperature for 120 min and filtered through a 0.45 μm membrane filter. The absorbance of the solution was determined at 760 nm using a UV-Visible spectrophotometer. The test for each extract was triplicated. The averaged absorbance was used in calculation. Gallic was used as a standard to prepare a standard curve. The total phenolic compound content was expressed as μg gallic acid equivalent/ gram of dry seaweeds (μgGAE/g DW).

2.5 Determination of Antioxidant Activity Using DPPH Radical Scavenging Method

Free radical scavenging capacity of seaweeds extracts was evaluated according to the previously reported procedure using the stable 2, 2-diphenyl-1-picryhydracyl radical (DPPH) [46]. The seaweeds extract solution for DPPH test was prepared by re-dissolving in methanol. The final concentration of DPPH solution was 100 μM in

methanol. A 2 ml aliquot of the DPPH solution was mixed with 10, 20, 30, 40 and 50 µl of the seaweeds extract/ methanol solution and transferred to a cuvette. The absorbance at 517 nm was measured against a blank of pure methanol, after an incubation period of 40 min at room temperature in the dark, using a UV-Visible spectrophotometer. The DPPH radical scavenging activities of the samples were compared with that of standard gallic acid. The radical scavenging effect was calculated by the following equation:

$$\text{Scavenging effect (\%)} = [(A_c - A_s)/A_c] \times 100 \qquad (2.1)$$

where

A_c is the absorbance of the control at 517 nm, and

A_s is the absorbance of the extract/standard at 517 nm.

This experiment was repeated in triplicate, and the results were averaged for each extract of seaweeds sample. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

2.6 Liquid Chromatographic Analysis

The determination of some phenolic compounds, including gallic acid, catechin, epicatechin, caffeic acid, rutin and quercetin in various seaweeds samples by high performance liquid chromatography has been investigated. The experimental procedure for this technique is shown in **Fig. 2.1**.

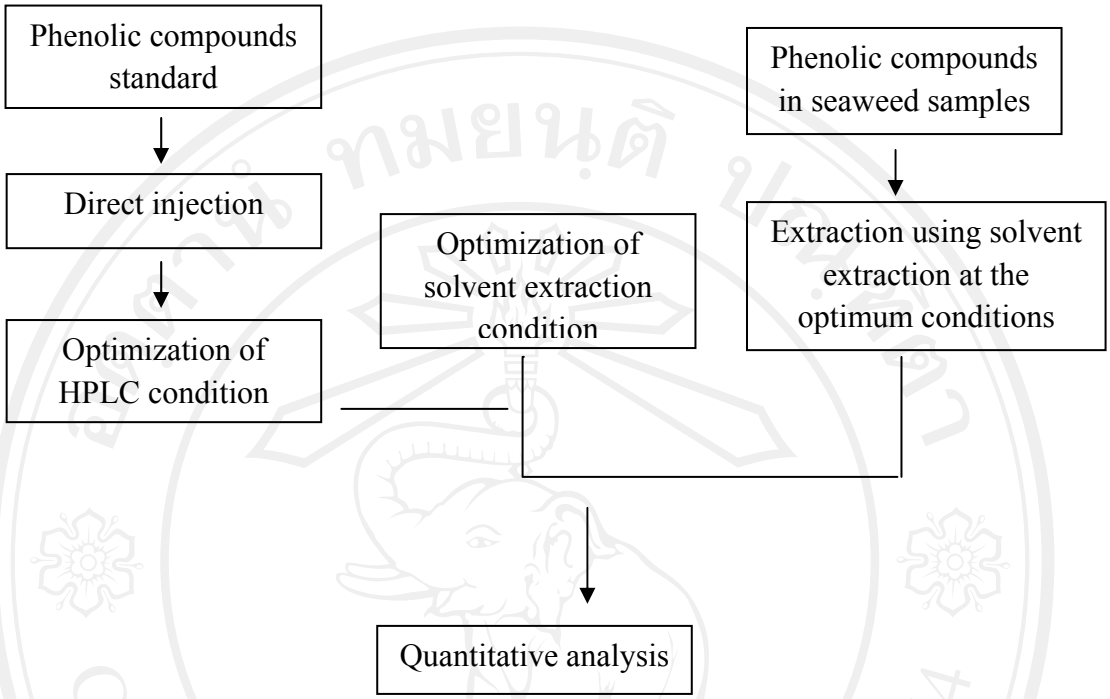

Fig. 2.1 Summary of experimental procedure for the determination of phenolic compounds by HPLC.

In this work, the experiment was divided into two parts. Firstly, the separation of phenolic compounds was investigated using binary solvent systems in RP-HPLC. Secondly, the conditions of HPLC and extraction procedure were optimized for the simultaneous determination phenolic compounds. Moreover, precision, detection limit and accuracy of these proposed methods were carried out under the optimum conditions.

2.6.1 Separation of phenolic compounds

Optimum separation conditions of phenolic compounds are achieved by adjusting the compositions of mobile phase, types and the concentrations of common acid (acetic acid and phosphoric acid). A 20 µl of standard mixture containing six phenolic compounds including 3 ppm gallic acid, 10 ppm catechin, 10 ppm epicatechin, 5 ppm caffeic acid, 5 ppm rutin and 3 ppm quercetin was injected onto the column Luna C_{18} column at the constant flow rate of mobile phase (1.0 ml min^{-1}). The confirmation of these compounds was carried out using mass spectrometry.

2.6.1.1 Optimization of HPLC conditions

a) Detection wavelength

The absorption spectra of 5 ppm of each phenolic compound solution, including gallic acid, catechin, epicatechin, caffeic acid, rutin and quercetin were examined in the wavelength range between 200 to 600 nm using a UV-VIS spectrophotometer. Maximum absorbance values of phenolic compounds were determined by injecting mixed standard phenolic compounds into HPLC at three different wavelengths (255, 275 and 295 nm).

b) Type and mobile phase composition

The binary solvent systems employing methanol and acetonitrile investigated in this work were mixed with 0.1% (v/v) phosphoric acid in water. A 20 µl volume of mixed phenolic compounds standard was injected onto the column at the flow rate of 1.0 ml min^{-1} and at the detection wavelength of 280 nm. The three series of mobile phase compositions are shown in **Table 2.2**.

Table 2.2 The gradient elution program of three series of mobile phase compositions

Series 1					
Gradient System	Time of Mobile Phase Changed (min)				
	0	2.5	7	10	17
Methanol	20	20	35	35	60
Acidic solution*	80	80	65	65	40
Series 2					
Gradient System	Time of Mobile Phase Changed (min)				
	0	2.5	7	10	17
Methanol	20	20	40	60	60
Acidic solution*	80	80	60	40	40
Series 3					
Gradient System	Time of Mobile Phase Changed (min)				
	0	4	7	10	17
Acetonitrile	10	10	30	50	60
Acidic solution*	90	90	70	50	40

* = 0.1% (v/v) phosphoric acid in water

c) Type and concentration of acid

Two types of common acid, i.e. acetic acid and phosphoric acid were employed at the concentration range 0.1-0.5 % (v/v). A 20 µl volume of mixed phenolic compound standards was injected onto the column at the flow rate of 1.0 ml min^{-1} in gradient elution program is shown in **Table 2.3** and at the detection wavelength of 275 nm.

Table 2.3 The gradient elution program of mobile phase compositions

Gradient System	Time of Mobile Phase Changed (min)					
	0	6	15	20	25	35
Acetonotrile	15	15	20	40	50	15
Acidic solution*	85	85	80	60	50	85

* = 0.1% (v/v) acetic acid in water or 0.1% (v/v) phosphoric acid in water

d) Mobile phase flow rate

The optimum mobile phase flow rate was determined by injecting a 20 µl volume of phenolic compound standard mixture onto the column utilizing the mixture of 0.1% (v/v) acetic acid in water and acetonitrile as mobile phase in gradient elution program (**Table 2.3**) at the flow rates 0.4-1.2 ml min^{-1} and the detection wavelength of 275 nm.

2.6.1.2 Detection limit

The standard solutions were prepared in the range of 0.2-1.0 ppm for gallic acid, 0.5-2.5 ppm for catechin, 0.5-2.5 ppm for epicatechin, 0.5-2.5 ppm for caffeic acid, 0.1-0.5 ppm for rutin and 0.4-2.0 ppm for quercetin. A 20 µl volume of each standard mixture in **Table 2.4** was injected onto the column under the optimum conditions.

Table 2.4 Concentration of phenolic compounds in standard mixtures for determination of detection limit

Compound	Concentration (ppm)				
	1	2	3	4	5
Gallic acid	0.2	0.4	0.6	0.8	1.0
Catechin	0.5	1.0	1.5	2.0	2.5
Epicatechin	0.5	1.0	1.5	2.0	2.5
Caffeic acid	0.5	1.0	1.5	2.0	2.5
Rutin	0.1	0.2	0.3	0.4	0.5
Quercetin	0.4	0.8	1.2	1.6	2.0

2.6.1.3 Precision

The precision of a method is the degree of closeness of the results which is usually reported as a percent of relative standard deviation. The precision can be expressed as the standard deviation (SD) and the relative standard deviation (RSD). The smaller the value of the relative standard deviation, the greater precision of an analysis [50].

The standard mixture solutions were prepared in the ranges listed in **Table 2.5**. The repeatability was investigated using five injections of the standard mixture in the same day and the reproducibility was determined in different days for seven injections under the optimum conditions.

Table 2.5 Concentration of phenolic compounds in standard mixtures for determination of repeatability, reproducibility and recovery test

Compound	Concentration (ppm)	
	1	2
Gallic acid	0.6	1.2
Catechin	1.5	2.5
Epicatechin	1.5	2.5
Caffeic acid	3.0	5.0
Rutin	0.3	0.5
Quercetin	2.0	3.0

2.6.1.4 Recovery test

The efficiency of solvent extraction for extracting phenolic compounds in seaweed samples and efficiency of technique were investigated by spiking mixed phenolic compound standards in the ranges listed in **Table 2.5** into seaweed sample before extraction.

2.7 Confirmation of Phenolic Compounds by LC-ESI-MS and LC-ESI-MS/MS

The type of ionization source of the mass spectrometer used in this work was electrospray ionization. For analysis of phenolic compounds of interest, the electrospray ionization parameters such as fragmentor voltage, capillary voltage, drying gas flow rate, drying gas temperature, and nebulizer pressure need to be optimized to achieve high sensitivity of detection and appropriate mass spectra which are useful for the identification. Thus, the values of these parameters were varied individually to obtain the optimal values. A schematic diagram of the electrospray spray chamber used in this work is presented in **Fig. 2.2**.

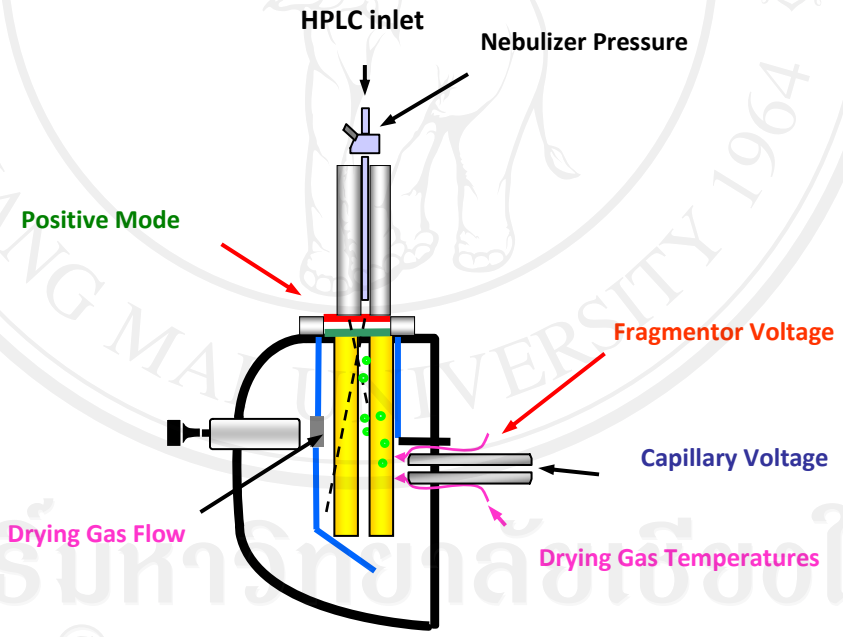

Fig. 2.2 Schematic diagram of the Agilent LC-MS electrospray spray chamber setting [51]

2.7.1 Optimization of electrospray ionization conditions

2.7.1.1 Optimization of fragmentor voltage in negative ionization mode

A solution of standard quercetin in methanol having concentration of 5 ppm was used to optimize the electrospray ionization conditions. A 20 µl of this solution was injected through the flow injection (FI) system of the electrospray ionization mass spectrometer (ESI-MS) without passing through a column. The fragmentor voltage was varied at 60, 80, 100, 120, 140 and 160 V at a time and the other conditions are as follows.

HPLC conditions:

Mobile phase	Acetonitrile
Detector	DAD (at wavelength 275 nm)
Injection volume	20 µl
Flow rate	0.5 ml min^{-1}

MS conditions:

Ionization mode	Negative ion mode
Data acquisition mode	Scan mode (50-400 amu)
Quadrupole temperature	100 $^{\circ}C$
Capillary voltage	3500 V
Drying gas temperature	320 $^{\circ}C$
Drying gas flow rate	10 l min^{-1}
Nebulizer pressure	30 psi

2.7.1.2 Optimization of capillary voltage

The FI-ESI-MS operation was carried out in the same manner as which is described in **Section 2.7.1.1** whereas the values of capillary voltage were varied at 2000, 2500, 3000, 3500, 4000 and 4500 V and the fragmentor voltage was 140 V.

2.7.1.3 Optimization of drying gas temperature

The FI-ESI-MS operation was carried out in the same manner as which is described in **Section 2.7.1.1** whereas the values of drying gas temperature were varied at 300, 310, 320, 330 and 340 $^{\circ}$C. The fragmentor voltage and capillary voltage were set at 140 and 4000 V, respectively.

2.7.1.4 Optimization of drying gas flow rate

The FI-ESI-MS operation was carried out in the same manner as which is described in **Section 2.7.1.1** whereas the values of drying gas flow rate were varied at 8, 9, 10 and 11 l min^{-1}. The fragmentor voltage, capillary voltage and drying gas temperature were set at 140 V, 4000 V and 320 $^{\circ}$C, respectively.

2.7.1.5 Optimization of nebulizer pressure

The FI-ESI-MS operation was carried out in the same manner as which is described in **Section 2.7.1.1** whereas the values of nebulizer pressure was varied at 20, 22, 24, 26, 28 and 30 psi. The fragmentor voltage, capillary voltage, drying gas temperature, and drying gas flow were set at 140 V, 4000 V, 320 $^{\circ}$C, and 10 l min^{-1}, respectively.

2.7.2 Optimization of MS/MS conditions

In this work, hybrid quadrupole time-of-flight mass spectrometer (Q-TOF-MS/MS) was used for identification of some phenolic compounds in the sample extracts. Standard phenolic compounds were introduced into electrospray ionization chamber, where the ion source and other mass spectrometer parameters were set as follows.

Ion source (Electrospray ionization)

Capillary (kV)	2.40
Cone (eV)	30
Extractor (V)	1
RF Lens (V)	0.78
Source temperature (°C)	100
Desolvation temperature (°C)	200

MS 1 (Quadrupole)

LM resolution	5.0
HM resolution	5.0
Collision energy (eV)	Varied according to the molecular ion of phenolic compounds of interest
Ion energy (V)	2.0
Steering (V)	0.42
Entrance (eV)	65.0
Pre-filter (V)	5.0

MS 2 (Time-of-Fight)

Transport (V)	3.6
Aperture 2 (V)	14.8
Acceleration (V)	200
Tube Lens	80
Offset 1	-0.1
Offset 2	0.0
Pusher (V)	980
TOF (kV)	9.1
Reflectron	35.69
Pusher cycle time (μs)	Auto
Pusher frequency (Hz)	16129.03
Multiplier (V)	530
MCP (V)	2000

Dissociation patterns of the molecular ion of the standard compounds, i.e. gallic acid, catechin, epicatechin, caffeic acid, rutin and quercetin were recorded as MS/MS or product ion mass spectra, which are useful for structural identification of the phenolic compounds.

2.8 Determination of Phenolic Compounds in Seaweed Samples

2.8.1 Quantitative analysis

Seaweed sample extracts were analyzed under the optimum HPLC conditions.

2.8.2 Identification of phenolic compounds in seaweed samples

The negative-ion electrospray (ES) – mass spectrometry (MS) was used for the confirmation of phenolic compounds in seaweed samples. The extracted seaweed samples were injected onto the column using 0.1% (v/v) acetic acid in water and acetonitrile as mobile phase in gradient elution program (**Table 2.3**) at the flow rate of 0.5 ml min^{-1}. The MS conditions used were fragmentor voltage, capillary voltage, drying gas temperature, drying gas flow and nebulizer pressure set at 140 V, 4000 V, 320 $^{\circ}$C, 10 l min^{-1} and 22 psi, respectively.