CHAPTER II

EXPERIMENTAL

2.1 Apparatus and Chemicals

2.1.1 Apparatus

1) High performance liquid chromatographic system, Agilent Technologies, U.S.A., consists of;

a) Degasser, Model G1322A, Agilent Technologies, USA

b) Autosampler, Model G 1329A, Agilent Technologies, USA

c) Binary gradient pump, Model G 1312 A, Agilent Technologies, USA

d) Diode array detector, Model G1315A, Agilent Technologies, USA

e) Mass spectrometry detector, G 1946 A, Agilent Technologies, USA

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

3) Analytical balance, Model BA 210S, Sartorius AG, Germany

4) Membrane filter, 0.45 µm, Whatman Litmited Maidstone, England

5) Ultrasonicator, Model 8891, Cole Parmer, USA

6) Filter apparatus, Model 11250, Millipore, USA

7) Vacuum rotary evaporator, Model R-124, Buchi AG, Switzerland

8) Shaker, Model SK-101, HL Instrument, Thailand

9) Water purification system, Model Milli-Q system, Millipore, US

10) LC column, Zorbax Eclipse Plus C_{18} (4 × 100 mm, 3µm), Agilent Technologies, USA

11) LC column, Hypersil BDS-C_{18} (4 × 100 mm, 3.5 µm), Agilent Technologies,USA

2.1.2 Chemicals

1) Methanol, AR grade, Merck, Germany
2) Dichloromethane, AR grade, Merck, Germany
3) Isopropanol, AR grade, Merck, Germany
4) Formic acid, AR grade, Fluka
5) Methanol, HPLC grade, Merck, Germany
6) Acetic acid, HPLC grade, Merck, Germany
7) Acetonitrile, HPLC grade, Merck, Germany
8) Cyanidin-3-*O*-glucoside, HPLC grade, Apin Chemicals, England

2.2 Black rice samples

Samples used in this work were two black rice varieties, namely, BGMSN 11 and Khumdoisakhet, which were glutinous. Leaves of BGMSN 11 are green whereas those of Khumdoisakhet are rather dark black purple color (see **Figure 2.1**). Fresh black rice leaves and seed at seven growth stages were collected. The rice samples were grown in the experimental filed of the department of Agronomy Faculty of Agriculture, Chiang Mai University during October-February, 2006-2007.

(A) BGMSN 11 cultivar (B) Khumdoisakhet cultivar

Figure 2.1 Two Thai black rice cultivars used in the experiment: (A) BGMSN 11 (B) Khumdoisakhet.

2.3 Extraction of anthocyanins from Thai black rice samples

2.3.1 Selection of solvents

Four organic solvents were used for comparison of extraction efficiency: (1) a mixture of dichloromethane and methanol (1:4 v/v) (2) methanol, (3) methanol containing 0.5% formic acid (4) isopropanol. The rice bran sample (10 g) was put in a 250 ml Duran button, and then it was added 50 ml of each solvent. The mixture was shaken for 60 min using SK 101 shaker. After that the rice bran extracts were filtered through the filter paper (Whatman No.1) and concentrated under reduced pressure and then filtered through a membrane filter having a pore size of 0.45 μm before analysis.

2.3.2 Cleaning up of black rice sample extracts

2.3.2.1 Solid phase extraction using cartridge C_{18} (SPE C_{18})

Approximately 10 g of each the rice bran was subjected to extractions previously described. The methanolic extract obtained was taken to dryness under reduced pressure (40 ºC) and redissolved in 10 ml of methanol containing 0.5 % formic acid. The extracts was then passed through an SPE C_{18} column, previously conditioned with 20 ml methanol. The loaded cartridge which retained anthocyanins was then eluted with methanol containing 0.5 % formic acid (20 ml). The methanolic extract was concentrated to dryness under reduced pressure (40 ºC) and redissolved again in methanol (4 ml). Each extract was then filtered through a membrane filter having a pore size of 0.45 μm analysis.

2.3.2.2 Partition with hexane

The black rice bran was weighed 10 g into a 250 ml Duran bottom and them mixed with 50 ml of methanol containing 0.5% formic acid. The mixture was shaken for 60 min using SK 101 shaker. After that the extracts were filtered through the filter paper (Whatman No.1), then an aliquot of 50 ml of the solutions were submitted to a partition with 50 ml of hexane with continuous shaking. The mixture was let to stand until two phases were clearly observed. The methanolic phase was then collected and evaporated to dryness under reduced pressure (40 ºC). The dry crude was redissolved in methanol containing 0.5% acetic acid (4 ml). The extract was then filtered through a membrane filter having a pore size of 0.45 μm before being subjected to further analysis.

2.4 Separation of anthocyanins from black rice bran extracts by LC-DAD and LC-ESI-MS

2.4.1 Optimization of separation conditions

Rice bran extracts obtained from **experimental 2.3.1** were used for optimization of LC-DAD and LC-MS conditions for separation of the components in the rice bran extract. These conditions were mobile phase composition and flow rate, column dimension**,** and gradient profile of mobile phase.

2.4.1.1 Optimization of mobile phase composition

Methanol, acetonitrile and water were used for optimization of mobile phase composition. The conditions of HPLC used in the experiment were as follows;

HPLC conditions:

Column	Zorbax eclipse plus C_{18} (4×100 mm, 3 µm)			
Gradient profile:	1-10	min	10	% A
	10-60	min	10-25	% A
Flow rate	0.4 ml/min			
Detector	DAD (wavelength 520 nm)			
Inject volume	20 µl			

Mobile phase varying mobile phase composition in series as follows:

Series 1:

A: Methanol	10 %
B: Water	90 %

Series 2:

A: Methanol	10 %
B: 0.5% Acetic acid in water	90%

Series 3:

A: Acetonitrile	10 %
B: Water	90 %

2.4.1. 2 Optimization of mobile phase flow rate

The values of mobile phase flow rate were varied at 0.3 and 0.4 ml/min. The conditions of HPLC used in the experiment were as follows;

HPLC conditions:

Column	Zorbax eclipse plus C_{18} (4×100 mm, 3 µm)		
Mobile phase	A: Methanol		
	B: 0.5% Acetic acid in water		
Gradient profile:	1-10 min	10	% A
	10-60 min	10-25	% A
Detector	DAD (wavelength 520 nm)		
Injection volume	20 µl		
Flow rate	varying between 0.3-0.5 ml/min		

2.4.1. 3 Optimization of column dimension

For the chromatographic system, the reverse phase was used for analysis anthocyanin components in plants. Zorbax eclipse plus C_{18} and Hypersil BDS C_{18} were used for optimization of column dimension. The conditions of HPLC used in the experiment were as follows;

HPLC conditions:

Column	1) Zorbax eclipse plus C_{18} (4×100 mm, 3 µm)	
	2) Hypersil BDS C_{18} (4×100 mm, 3.5 µm)	
Mobile phase	A: Methanol	
	B: 0.5% Acetic acid in water	
Gradient profile:	1-10 min	10 % A
	10-160 min	10-100 % A
	160-180min	100 % A
Detector	DAD (wavelength 520 nm)	
Injection volume	20 µl	
Flow rate	0.4 ml/min	

2.4.1. 4 Optimization of gradient profile of mobile phase

The gradient profiles of mobile phase were optimized for component separation. The conditions of HPLC used in the experiment were as follows;

HPLC conditions:

Column	Zorbax eclipse plus C_{18} (4×100 mm, 3 µm)
Mobile phase	A: Methanol
	B: 0.5% Acetic acid in water
Flow rate	0.4 ml/min
Detector	DAD, (wavelength 520 nm)
Inject volume	20 µl
Gradient profile:	varying compositions (%) in series as follows:

Series 1:

1-10	min	10	% A
10-150	min	10-25	% A

Series 2:

1-10	min	10	% A
10-150	min	10-45	% A

Series 3:

1-10	min	10	% A
10-180	min	10-100	% A

2.5 Identification of anthocyanins in the Thai black rice extracts by LC-ESI-MS and LC-ESI-MS/MS

Mass spectrometry detector used in this work has a type of ionization source which was electrospray ionization. For analysis of anthocyanins 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 spectrum useful for the identification. Thus the values of these parameters were varied individually to obtain the optimal values. The type setting of electrospray spray chamber used in this experiment is presented in **Figure 2.2**.

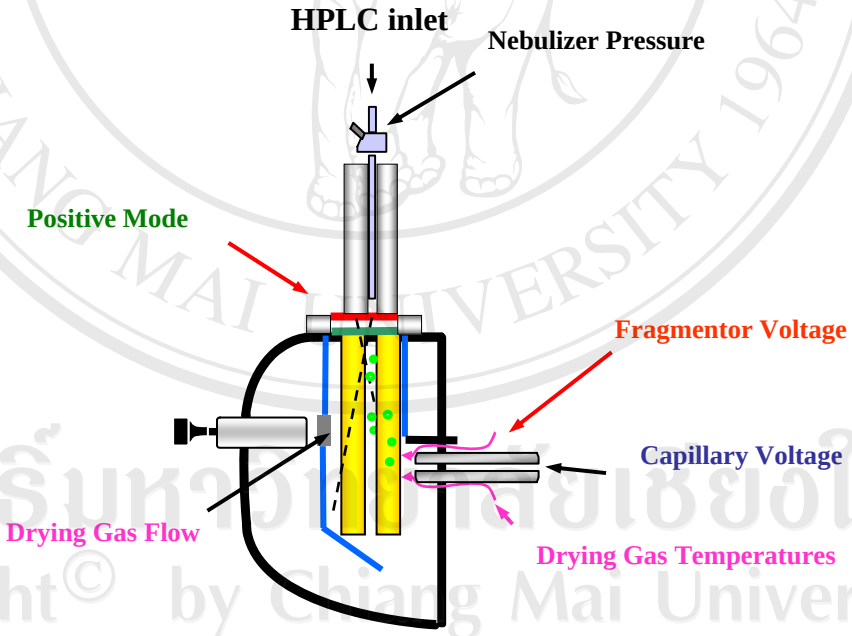

Figure 2.2 Schematic diagram of the Agilent LC-MS electrospray spray chamber setting.[67]

2.5.1 Optimization of electrospray ionization conditions

2.5.1.1 Optimization of fragmentor voltage in positive ionization mode

A solution of standard cyanidin-3-*O*-glucoside in methanol having concentration of 5 µg/ml was used to optimize the electrospray ionization conditions. Twenty microlites 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 110, 120, 130, 140, and 150 V at a time and the other conditions were as follows.

HPLC conditions:

Mobile phase	Methanol
Detector	DAD (at wavelength 520 nm)
Injection volume	20 µl
Flow rate	0.4 ml/min

MS conditions:

Ionization mode	Positive ion mode
Data acquisition mode	Scan mode (200-800 amu)
Quadrupole temperature	100 °C
Capillary voltage	3500 V
Drying gas temperature	330 °C
Drying gas flow rate	12 l/min
Nebulizer pressure	30 psi

2.5.1.2 Optimization of capillary voltage

The FI-ESI-MS operation was the same as in **experimental 2.5.1.1** where as the values of capillary voltage were varied at 3000, 3500, 4000, 4500 and 5000 V and the fragmentor voltage was 110 V.

2.5.1.3 Optimization of drying gas temperature

The FI-ESI-MS operation was the same as in **Experimental 2.5.1.1** where as the values of drying gas temperature were varied at 300, 310, 320, 330, 340, and 350 °C. The fragmentor voltage and capillary voltage were set at 110 and 3500 V, respectively.

2.5.1.4 Optimization of drying gas flow rate

The FI-ESI-MS operation was the same as in **experimental 2.5.1.1** where as the values of drying gas flow rate were varied at 8, 9, 10, 11, and 12 l/min. The fragmentor voltage, capillary voltage and drying gas temperature were set at 110 V, 3500 V and 350 °C, respectively.

2.5.1.5 Optimization of nebulizer pressure

The FI-ESI-MS operation was the same as **experimental 2.5.1.1** where as the values of nebulizer pressure was varied at 26, 28, 30, 32, and 34 psi. The fragmentor voltage, capillary voltage, drying gas temperature, and drying gas flow were set at 110 V, 3500 V, 330 °C, and 12 l/min, respectively.

2.5.2 Optimization of MS/MS conditions

In this work, hybrid quadrupole time-of-flight mass spectrometer (Q-TOF-MS/MS) was used for identification of anthocyanins in the black rice sample extracts. The black rice extracts 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)	3.0
Cone (eV)	30
Extractor (V)	10
RF Lens (V)	0.5
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 anthocyanins of interest
Ion energy (V)	2.0
Steering (V)	2.0
Entrance (eV)	65.0
Pre-filter (V)	5.0

MS 2 (Time-of-Fight)

Transport (V)	4.0
Aperture 2 (V)	14.6
Acceleration (V)	200
Tube Lens	75
Offset 1	-0.1
Offset 2	0.0
Pusher (V)	980
TOF (kV)	9.1
Reflectron	35.25
Pusher cycle time (μs)	Auto
Pusher frequency (Hz)	16129.03
Multiplier (V)	650
MCP (V)	2000

Dissociation patterns of the molecular ion of anthocyanins in the black rice extracts were recorded as MS/MS or product ion mass spectra, which are useful for structural identification of the anthocyanins. This technique provides the product ion mass spectra (MS/MS) having structural information based on the characteristic fragmentation pattern of acylate and one, two or three glycoside linkage with anthocyanidin. The different composition and type of glycoside linkage with anthocyanidins were provided the characteristic mass spectrum pattern.

2.6 Determination of the relative contents of anthocyanins in extracts of leaves and seed of the two cultivars of Thai black rice at seven growth stages

Relative contents of anthocyanins in the extracts of leaves or seed of the two Thai black rice cultivars at different growth stages were obtained by using LC-ESI-MS technique. Neutral red was used as the internal standard that could be added to the black rice sample extracts at concentration 10 µg/ml. Peak areas normalization was utilized to determine the relative contents of the individual anthocyanin components. Three replicates of each sample extract were analysed by LC-ESI-MS. The quantities of each anthocyanin in leaf or seed extracts of the two cultivars of Thai black rice at seven different growth stages were determined as the average values with standard deviations (SD.). The conditions of LC-ESI-MS used in this experiment were as follows:

HPLC:

Instrument	Agilent HP 1100 Series LC-MSD
Column	Zorbax eclipse plus C_{18} (4 × 100 mm, 3 µm)
Mobile phase	A: Methanol
	B: 0.5% Acetic acid in water
Gradient profile:	1-10 min 10 % A
	10-150 min 10-45 % A
Detector	DAD (wavelength 520 nm)
Injection volume	20 µl
Flow rate	0.4 ml/min

MS:

Ionization mode	Positive ion mode
Data acquisition mode	Scan mode (200-800 amu)
Quadrupole temperature	100 °C
Fragmentor voltage	110 V
Capillary voltage	3500 V
Drying gas temperature	330 °C
Drying gas flow rate	12 l/min
Nebulizer pressure	30 psi