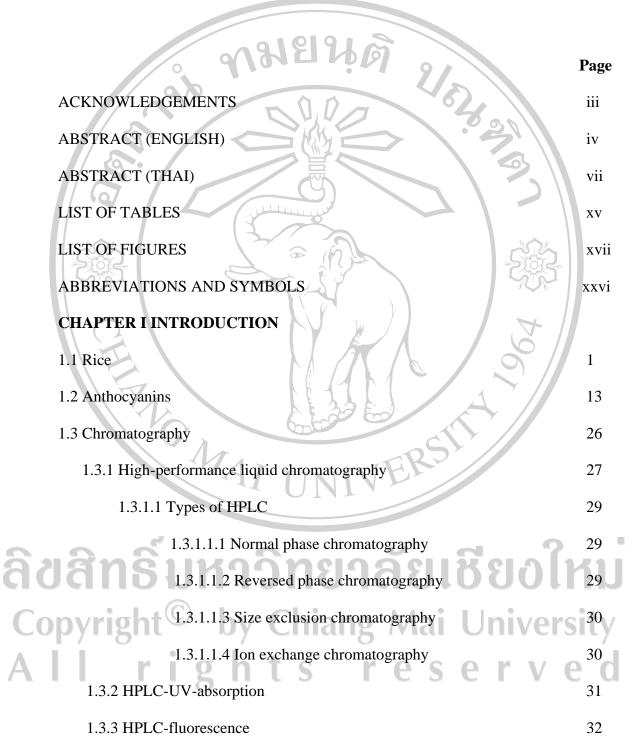
TABLE OF CONTENTS



Х

| | Page |
|---|------|
| 1.3.4 HPLC-photo diode array | 32 |
| 1.3.5 HPLC-electrochemical detection | 34 |
| 1.3.6 HPLC-mass spectrometry | 34 |
| Fast atom bombardment (FAB) | 37 |
| MALDI mass spectrometry | 38 |
| Almospheric pressure ionization | 40 |
| Electrospray ionization (ESI) | 41 |
| Atmospheric pressure chemical ionization (APCI) | 44 |
| Ion trap mass spectrometer | 46 |
| Sector mass spectrometer | 47 |
| Quadruple mass spectrometer | 48 |
| Time of flight mass spectrometer (TOF) | 50 |
| Mass spectrometry-mass spectrometer (MS/MS) | 51 |
| 1.4 Fragmentation pathways of anthocyanin glycoside | 53 |
| 1.5 Aims of this research | 61 |
| CHAPTER II EXPERIMENTAL | |
| 2.1 Apparatus and Chemicals Chiang Mai University | 62 |
| 2.1.1 Apparatus | 62 |
| 2.1.2 Chemicals | 63 |
| 2.2 Black rice samples | 63 |
| 2.3 Extraction of anthocynins from Thai black rice sample | 64 |
| 2.3.1 Selection of solvents | 64 |

| | Page |
|--|-----------------|
| 2.3.2 Clean up of black rice sample extracts | 65 |
| 2.4 Separation of anthocyanins from black rice bran extracts by LC-DAD and | |
| LC-ESI-MS | |
| 2.4.1 Optimization of separation condition | 66 |
| 2.4.1.1Optimization of mobile phase composition | 66 |
| 2.4.1.2 Optimization of mobile phase flow rate | 67 |
| 2.4.1.3 Optimization of column dimension | 68 |
| 2.4.1.4 Optimization of gradient profile of mobile phase | 69 |
| 2.5 Identification of anthocyanins in Thai black rice extracts by LC-ESI-MS | 70 |
| and LC-ESI-MS/MS | |
| 2.5.1 Optimization of electrospray ionization condition | 71 |
| 2.5.1.1 Fragmentor voltage | 71 |
| 2.5.1.2 Capillary voltage | 72 |
| 2.5.1.3 Drying gas temperature | 72 |
| 2.5.1.4 Drying gas flow rate | 72 |
| 2.5.1.5 Nebulizer pressure | 72 |
| 2.5.2 Optimization of MS/MS | S ⁷³ |
| 2.6 Determination of the relative contents of anthocyanins in extracts of leaves | 75 |
| and seed of the two black rice cultivars at different growth stages | |

| | Page |
|---|------|
| CHAPTER III RESULTS AND DISCUSSION | |
| 3.1 Extraction of anthocynins from bran of Thai black rice | 77 |
| 3.2 Separation of anthocyanin components from Thai black rice extracts by | 79 |
| LC-DAD and LC-ESI-MS | |
| 3.2.1 Effect of type of mobile phase on component separation | 79 |
| 3.2.2 Effect of mobile phase flow rate on component separation | 81 |
| 3.2.3 Effect of column dimension on component separation | 82 |
| 3.2.4 Effect of mobile phase composition on component separation | 83 |
| 3.3 Identification of anthocyanins in the black rice sample extracts by LC-DAD, | 86 |
| ESI-MS and MS/MS | |
| 3.3.1 Optimization of electrospray ionization mass parameters for | 86 |
| analysis of anthocyanin standards | |
| 3.3.1.1 Fragmentor voltage in positive ionization mode | 86 |
| 3.3.1.2 Capillary voltage in positive ionization mode | 88 |
| 3.3.1.3 Drying gas temperature in positive ionization mode | 90 |
| 3.3.1.4 Drying gas flow rate in positive ionization mode | 92 |
| 3.3.1.5 Nebulizer pressure in positive ionization mode | 94 |
| 3.3.2 Optimization of collision energy inCID process for the identification | 97 |
| of anthocyanins components | |
| 3.3.3 Identification of anthocyanins in the black rice leaf, seed, and bran | 103 |
| extracts by LC-DAD, LC-ESI-MS and LC-ESI-MS/MS | |

xiii

| | Page |
|---|------|
| 3.3.3.1 Anthocyanins identified by LC-DAD | 103 |
| 3.3.3.2 Anthocyanins identified by LC-ESI-MS | 105 |
| 3.3.3.3 Anthocyanins identified by LC-ESI-MS/MS | 106 |
| 3.3.3.4 Anthocyanins in bran of the Thai black rice | 110 |
| Cyanidin-3-O-glucoside | 111 |
| Peonidin-3-O-glucoside | 115 |
| 3.3.3.5 Anthocyanins in leaves of the Thai black rice | 119 |
| Cyanidin-3-O-glucoside-5-O-rhamnoside | 120 |
| Cyanidin-3-O-diglucoside-5-O-glucoside | 123 |
| Cyanidin-3-O-xyloside-glucoside | 126 |
| Cyanidin-3-O-xyloside-glucoside (isomer) | 128 |
| Cyanidin-3-O-diglucoside | 131 |
| Cyanidin-3-O-(p-coumaroyl)glucoside-5-O-glucoside | 134 |
| Cyanidin-3-O-(feruloyl)glucoside-5-O-glucoside | 137 |
| Peonidin-3-O-diglucoside | 140 |
| Peonidin-3- <i>O</i> -(<i>p</i> -coumaroyl)glucoside-5- <i>O</i> -xyloside | 143 |
| Malvidin-3- <i>O</i> -(<i>p</i> -coumaroyl)glucoside-5- <i>O</i> -glucoside | 146 |
| 3.4 Relative contents of the identified anthocyanons in extracts of the two black rice cultivars at different growth stages | 154 |
| CHAPTER IV CONCLUSION | 166 |
| REFERENCES | 168 |
| CERRICULUM VITAE | 173 |

xiv

| LIST OF TABLES | |
|--|------|
| Table | Page |
| 1.1 Naturally occurring anthocyanins | 15 |
| 3.1 The optimum LC-DAD and LC-ESI-MS condition | 85 |
| 3.2 Peak areas and ion counts of cyanidin-3-O-glucoside at varied fragmentor | 87 |
| voltages in positive ionization mode 3.3 Peak areas and ion counts of cyanidin-3- <i>O</i> -glucoside at varied capillary | 89 |
| voltage in positive ionization mode | |
| 3.4 Peak areas and ion counts of cyanidin-3- <i>O</i> -glucoside at varied drying gas | 91 |
| temperatures in positive ionization mode | |
| 3.5 Peak areas and ion counts of cyanidin-3-O-glucoside at varied drying gas | 93 |
| flow rates in positive ionization mode | |
| 3.6 Peak areas and ion counts of cyanidin-3-O-glucoside at varied nebulizer | 95 |
| pressures in positive ionization mode. | |
| 3.7 The optimum ESI-MS conditions for analysis of anthocyanins | 96 |
| 3.8 The optimum collision energy conditions | 102 |
| 3.9 Anthocyanins identified in leaves of the Thai black rice cultivars Khumdoisakhet and BGMSN 11 | 149 |
| 3.10 Anthocyanins identified in seed of the Thai black rice cultivars | 150 |
| Khumdoisakhet and BGMSN 11 | |

| Table | Page |
|--|------|
| 3.11 Anthocyanins identified in bran of the Thai black rice cultivars | 150 |
| Khumdoisakhet and BGMSN 11 | |
| 3.12 % Relative contents of the identified anthocyanins in leaves of | 155 |
| BGMSN11extracts at different growth stages | |
| 3.13 % Relative contents of the identified anthocyanins in leaves of | 156 |
| Khumdoisakhet extracts at different growth stages | |
| 3.14 % Relative contents of the identified anthocyanins in seed of BGMSN11 | 157 |
| extracts at different growth stages | |
| 3.15 % Relative contents of the identified anthocyanins in seed of | 158 |
| Khumdoisakhet extracts at different growth stages | |

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่ Copyright[©] by Chiang Mai University All rights reserved

| LIST OF FIGURES | |
|---|------|
| Figure | Page |
| 1.1 Schematic of rice | 2 |
| 1.2 Germination to emergence stages of rice growth | 3 |
| 1.3 Seeding stage of rice growth | 4 |
| 1.4 Tillering stage of rice growth | 4 |
| 1.5 Stem elongations stage of rice growth | 5 5 |
| 1.6 Panicle initiations to booting stages of rice growth | 5 |
| 1.7 Heading or panicle exsertion stages of rice growth | 6 |
| 1.8 Flowering stage of rice growth | 6 |
| 1.9 Milk grain stage of rice growth | 7 |
| 1.10 Dough grain of rice growth | 8 |
| 1.11 Mature grain stages of rice growth | 8 |
| 1.12 Schematic of the structure of pigmentd rice kernel | 2 10 |
| 1.13 Basic structures of many classes of flavonoids | 14 |
| 1.14 Basic structure of anthocyanidins | 15 |
| 1.15 Structure of the anthocyanidins most commonly found in foods | 16 |
| 1.16 Chemical structures of many classes of sugar | 19 |
| 1.17 Chemical structures of many classes of acylate | 20 |
| 1.18 Chemical transformations of anthocyanins | 20 |
| 1.19 Diagram of high performance liquid chromatography (HPLC) | 28 |

xvii

| Figure | Page |
|---|-------|
| 1.20 A schematic diagram of UV-Vis absorption detector | 31 |
| 1.21 A schematic of photo diode array detector diagram | 33 |
| 1.22 Schematic diagram of a mass spectrometer | 35 |
| 1.23 Schematic of the mechanism of fast atom bombardment ionization | 38 |
| mass spectrometry (FAB) | |
| 1.24 A schematic diagram of the mechanism of MALDI | 39 |
| 1.25 A schematic of an ESI interface | 42 |
| 1.26 A schematic of the mechanism of ion formation in ESI interface | 42 |
| 1.27 A schematic of the components of an APCI source | 45 |
| 1.28 A schematic of more detailed view of the mechanism of APCI | 45 |
| 1.29 A schematic of a quadrupole ion trap mass analyzer | 47 |
| 1.30 A schematic of a sector mass spectrometer | 48 |
| 1.31 Schematic of a quadrupole mass analyzer | 49 |
| 1.32 Schematic of a TOF mass analyzer | 50 |
| 1.33 Schematic diagram of relative applicability of LC-MS techniques compar | ed 52 |
| With of GC-MS by Chiang Mai Univer | |
| 1.34 Ion nomenclature adopted for anthocyanin glycosides fragmentation | 53 |
| (A) anthocyanin aglycone (B) anthocyanidin glycoside | |

| Figure | Page |
|--|---------------|
| 1.35 Formation of the radical aglycone product ion (\mathbf{Y}_0^+) by a hemolytic | 55 |
| cleavage of the glycosidic bond between the aglycone and the glycan residu | e |
| 1.36 Characteristic product ions formed by cross-ring cleavage in a hexose and | 56 |
| pentose residue | |
| 1.37 Characteristic product ions formed by di-O-glucoside and O-diglucoside | 57 |
| anthocyanins | |
| 2.1 Two Thai black rice cultivars used in the experiment: (A) BGMSN 11 (B) | 64 |
| Khumdoisakhet | |
| 2.2 Schematic diagram of the Agilent LC-MS electrospray spray chamber setting | g 70 |
| 3.1 Four extracts of Khumdoisakhet rice bran; (A) methanol containing | 77 |
| 0.5% formic acid (B) methanol (C) dichrolomethane: methanol (1:4) and | |
| (D) isopropanol | |
| 3.2 Contents of anthocyanins in the black rice bran extracts expressed by UV-Vi | i s 78 |
| absorbance at wavelength 520 nm | |
| 3.3 LC-DAD chromatograms (at wavelength 520 nm) of the extract from bran of | f 80 |
| Khumdoisakhet rice using (A) methanol : water (90:10), (B) methanol : 0.5 | % |
| acetic acid in water (90:10) and (C) acetronitril : water (90:10) as mobile ph | ase |
| 3.4 LC-DAD Chromatograms (at wavelength 520 nm) of the extract from bran of | of 81 |
| Khumdoisakhet rice using (A) 0.3 ml/min and (B) 0.4 ml/min as flow rates | |

of mobile phase

- 3.5 LC-DAD Chromatograms (at wavelength 520 nm) of the extract from bran of 82
 Khumdoasakhet rice using (A) Zorbaxe eclipse plus C₁₈ (B) Hypersil BDS
 C₁₈ as column on LC system
- 3.6 LC-ESI-MS Chromatograms of the extracts from the black rice leaves,
 Khumdoisakhet, using (A) 90% 50% of 0.5 % acetic acid in water,
 (B) 90% 55% of 0.5 % acetic acid in water and (C) 90% 0% of 0.5 %
 acetic acid in water mixed with methanol as composition of mobile phase
 3.7 TICs of cyanidin-3-*O*-glucoside at fragmentor voltage 110, 120, 130,140,
 and 150 V in positive ionization mode
 3.8 TICs of cyanidin-3-*O*-glucoside at capillary voltage 3000, 3500, 4000, 4500,
 and 5000 V in positive ionization mode
- 3.9 TICs of cyanidin-3-O-glucoside at drying gas temperature 300, 310, 320, 330, 91340 and 350 °C in positive ionization mode
- 3.10 TICs of cyanidin-3-*O*-glucoside at drying gas flow rate 8, 9, 10, 11 and 93 12 l/min in positive ionization mode
- 3.11 TICs of cyanidin-3-O-glucoside at nebulizer pressure 26, 28, 30, 32, and9534 psi in positive ionization mode
 - 3.12 Product ion mass spectra of malvidin-3-*O*-glucoside obtained by
 LC-ESI-MS/MS (Q-TOF) of *m/z* 493 using collision energy of (A) 22 V,
 (B) 20 V, (C) 17 V, and (D) 15 V

| Figure | Page |
|--|------|
| 3.13 Product ion mass spectra of cyanidin-3-O-xyloside glucoside obtained by | 99 |
| LC-ESI-MS/MS (Q-TOF) of ion at m/z 581 using collision energy of | |
| (A) 25 V, (B) 22 V, (C) 20 V, (D) 17 V, and (D) 15 V | |
| 3.14 Product ion mass spectra of cyanidin-3-O-(p-coumaroyl)glucoside-5- | 101 |
| O-glucosidexx obtained by LC-ESI-MS/MS (Q-TOF) of m/z 757 using | |
| collision energy of (A) 25 V, (B) 22 V, (C) 20 V, (D) 17, and (E) 15 V | |
| 3.15 Chromatograms obtained by LC-DAD at wavelength 520 nm of an extract | 104 |
| from bran of the black rice | |
| 3.16 UV-Vis spectra obtained by LC-DAD of an extracts from bran of the | 104 |
| Khumdoisakhet rice (A) Peak 1 and (B) Peak 2, of which their chromatogram | n |
| is shown in Figure 3.15 | |
| 3.17 Full scan ESI-MS spectrum of cyanidin-3-O-glucoside having molecular | 105 |
| ion at <i>m/z</i> 449 | |
| 3.18 Chromatograms obtained from LC-ESI-MS of bran extract Khumdoisakhet | 106 |
| extract; (A) Mass chromatogram at m/z 449 (B) Total ion chromatogram | |
| 3.19 Chromatograms obtained from LC-ESI-MS of extracts from leaves of two | 108 |
| black rice cultivars; (A) BGMSN 11 (B) Khumdoisakhet | |
| 3.20 Chromatograms obtained from LC-ESI-MS of extracts from seed of two | 108 |
| black rice cultivars; (A) BGMSN 11 (B) Khumdoisakhet | |
| 3.21 Chromatograms obtained from LC-ESI-MS of extracts from bran of two | 109 |
| black rice cultivars: (A) BGMSN 11 (B) Khumdoisakhet | |

| Figure | Page |
|--|------|
| 3.22 ESI-MS and ESI-MS/MS spectrum of cyanidin-3-O-glucoside | 109 |
| -5-O-rhamnoside having molecular ion at m/z 595 obtained from of the Thai | |
| black rice extract; (A) ESI-MS (B) ESI-MS/MS | |
| 3.23 Chromatograms obtained from LC-ESI-MS of an extracts from bran of the | 112 |
| black rice cultivar Khumdoisakhet; (A) Mass chromatograms at m/z 449 | |
| (B) Total ion chromatogram | |
| 3.24 Full scan mass spectra of an extract from bran of the black rice cultivar | 113 |
| Khumdoisakhet obtained by; (A) ESI-MS (B) ESI-MS/MS | |
| 3.25 MS/MS spectra of the parent ion at m/z 287 obtained from LC-ESI-MS/MS | 114 |
| of the extract from leaves of the black rice cultivar, Khumdoisakhet showing | 5 |
| fragmentation pathway of some ion as well as the neutral losses | |
| 3.26 Chromatograms obtained from LC-ESI-MS of the extract from bran of the | 117 |
| black rice cultivar BGMSN 11; (A) Mass chromatograms of m/z 463 | |
| (B) Total ion chromatogram | |
| 3.27 Full scan mass spectra of an extract from bran of the black rice cultivar | 118 |
| Khumdoisakhet obtained by; (A) ESI-MS (B) ESI-MS/MS | |
| 3.28 MS/MS spectra of the parent ion at m/z 301 obtained from LC-ESI-MS/MS | 119 |
| of the extract from leaves of the black rice cultivar, Khumdoisakhet showing | S of |
| fragmentation pathway of some ion as well as the neutral losses | |
| 3.29 Chromatograms obtained from LC-ESI-MS of an extract from leaves of | 121 |
| the black rice cultivar Khumdoisakhet; (A) Mass chromatograms at m/z 595 | |
| (B) Total ion chromatogram | |

xxii

xxiii

| Figure | Page |
|--|-------|
| 3.30 Full scan mass spectra of an extract from leaves of the black rice cultivar | 122 |
| Khumdoisakhet obtained by; (A) ESI-MS (B) ESI-MS/MS | |
| 3.31 Chromatograms obtained from LC-ESI-MS of an extract from leaves of the | 124 |
| black rice cultivar Khumdoisakhet; (A) Mass chromatograms at m/z 773 | |
| (B) Total ion chromatogram | |
| 3.32 Full scan mass spectra of an extract from leaves of the black rice cultivar | 125 |
| Khumdoisakhet obtained by; (A) ESI-MS (B) ESI-MS/MS | |
| 3.33 Chromatograms obtained from LC-ESI-MS of the extracts from leaves of th | e 126 |
| black rice cultivar, Khumdoisakhet; (A) Mass chromatograms at m/z 581 | |
| (B) Total ion chromatogram | |
| 3.34 Full scan mass spectra of an extract from leaves of the black rice cultivar | 127 |
| Khumdoisakhet obtained by; (A) ESI-MS (B) ESI-MS/MS | |
| 3.35 Chromatograms obtained from LC-ESI-MS of an extract from leaves of the | 128 |
| black rice cultivar Khumdoisakhet; (A) Mass chromatograms at m/z 581 | |
| (B) Total ion chromatograms | |
| 3.36 Full scan mass spectra of an extract from leaves of the black rice cultivar | 130 |
| Khumdoisakhet obtained by; (A) ESI-MS (B) ESI-MS/MS | |
| 3.37 Chromatograms obtained from LC-ESI-MS of an extract from leaves of | 132 |
| the black rice cultivar Khumdoisakhet; (A) Mass chromatograms at m/z 611 | |
| | |

(B) Total ion chromatogram

| Figure | | |
|--|-----|--|
| 3.38 Full scan mass spectra of an extract from leaves of the black rice cultivar | | |
| Khumdoisakhet obtained by; (A) ESI-MS (B) ESI-MS/MS | | |
| 3.39 Chromatograms obtained from LC-ESI-MS of an extract from leaves of | 135 | |
| the black rice cultivar, Khumdoisakhet; (A) Mass chromatograms at m/z 75 | 7 | |
| (B) Total ion chromatogram | | |
| 3.40 Full scan mass spectra of an extract from leaves of the black rice cultivar | 136 | |
| Khumdoisakhet obtained by; (A) ESI-MS (B) ESI-MS/MS | | |
| 3.41 Chromatograms obtained from LC-ESI-MS of an extract from leaves of | 138 | |
| the black rice cultivar Khumdoisakhet; (A) Mass chromatogram at m/z 787 | | |
| (B) Total ion chromatogram | | |
| 3.42 Full scan mass spectra of an extract from leaves of the black rice cultivar | 139 | |
| Khumdoisakhet obtained by; (A) ESI-MS (B) ESI-MS/MS | | |
| 3.43 Chromatograms obtained from LC-ESI-MS of an extract from leaves of | 141 | |
| the black rice cultivar Khumdoisakhet extract; (A) Mass chromatograms at | | |
| m/z 625 (B) Total ion chromatogram | | |
| 3.44 Full scan mass spectra of an extract from leaves of the black rice cultivar | 142 | |
| Khumdoisakhet obtained by; (A) ESI-MS (B) ESI-MS/MS | | |
| 3.45 Chromatograms obtained from LC-ESI-MS of an extract from leaves of | 144 | |
| the black rice cultivar Khumdoisakhet and BGMSN 11; (A) Mass | | |
| chromatograms at m/z 741 (B) Total ion chromatograms | | |

xxiv

| Figure | Page |
|--|------|
| 3.46 Full scan mass spectra of an extract from leaves of the black rice cultivar | 145 |
| Khumdoisakhet obtained by; (A) ESI-MS (B) ESI-MS/MS | |
| 3.47 Chromatograms obtained from LC-ESI-MS of an extract from leaves of | 147 |
| the black rice cultivar, Khumdoisakhet ; (A) Mass chromatograms at m/z 80 | 1 |
| (B) Total ion chromatogram | |
| 3.48 Full scan mass spectra of the extracts from the black rice cultivar | 147 |
| Khumdoisakhet | |
| 3.49 MS/MS spectra of the parent ion at m/z 331 obtained from LC-ESI-MS/MS | 148 |
| of the extracts from leaves of the black rice cultivar Khumdoisakhet showin | g |
| fragmentation pathway of some ions as well as the neutral losses | |
| 3.50 Total ion chromatograms of the extracts from leaves of the black rice | 151 |
| cultivar; (A) Khumdoisakhet and (B) BGMSN 11, obtained by LC-ESI-MS | |
| showing the presence of the identified anthocyanins and their positions | |
| 3.51 Total ion chromatograms of the extracts from seed of the black rice | 152 |
| cultivar; (A) Khumdoisakhet and (B) BGMSN 11, obtained by LC-ESI-MS | |
| showing the presence of the identified anthocyanins and their positions | |
| 3.52 Total ion chromatograms of the extracts from bran of the black rice | 153 |
| cultivar; (A) Khumdoisakhet and (B) BGMSN 11, obtained by LC-ESI-MS | |
| showing the presence of the identified anthocyanins and their positions | |
| 3.53 Relative contents of anthocyanins obtained from LC-ESI-MS of the | 159 |
| extract from leaves and seed at different growth stages of the black rice | |
| cultivars, Khumdoisakhet and BGMSN 11 | |

XXV

ABBREVIATIONS AND SYMBOLS

| | MeCN | acetonitrile |
|--------|-----------------|---|
| | C ₁₈ | octadecyl |
| | °C | degree Celsius |
| | CID | collision-induced dissociation |
| | DCM | dichloromethane |
| | ESI | electrospray ionization |
| | eV | electron volt |
| | FI | flow injection |
| | G | gram |
| | HPLC | high performance liquid chromatography |
| | Kg | kilogram |
| | kV | kilovolt |
| | 1 | liter |
| | LC | liquid chromatography |
| Copyri | LC-ESI-MS | liquid chromatography- electrospray ionization mass |
| | | spectrometry |
| | LC-MS | liquid chromatography-mass spectrometry |
| | MS | mass spectrometry |
| | MS/MS | mass spectrometry-mass spectrometry |
| | min | minute |
| | | |

xxvi

milliliter ml meter 2/02/03/19 m molecular weight MW mass-to-charge ratio m/z, MeOH methanol mg milligram MCP micro channel plate nanometer nm PDA photodiode array part per million ppm pound per square inch psi Q-TOF hybrid quadrupole time-of-flight total ion chromatograms TICs TOF time-of-flight μl micro liter micrometer μm ultraviolet **NGAGISCON** ^{vol}by Chiang Mai University ghts reserved Copyrigh ig

xxvii