# SEPARATION OF POLAR DRUG-LIKE COMPOUNDS USING SUPERCRITICAL FLUID CHROMATOGRAPHY COUPLED WITH ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY

**Lalida Wongbuth**



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# การแยกสารประกอบคล้ายยาที่มีขั้วด้วยโครมาโทกราฟีของไหลวิกฤตควบคู่กับอิเล็กโทรสเปรย์ไอออไนเซชันแทนเดมแมสสเปกโทรเมทรี

นางสาวลลิดา วงษ์บุตร



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# SEPARATION OF POLAR DRUG-LIKE COMPOUNDS USING SUPERCRITICAL FLUID CHROMATOGRAPHY COUPLED WITH ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

(Prof. Dr. Jatuporn Wittayakun)

Chairperson

(Asst. Prof. Dr. Patcharin Chaisuwan)

Member (Thesis Advisor)

(Assoc. Prof. Dr. Rapee Utke)

Member

(Asst. Prof. Dr. Sanchai Prayoonpokarach)

Member

(Prof. Dr. Santi Maensiri)

Acting Vice Rector for Academic Affairs and Internationalisation

(Prof. Dr. Santi Maensiri)

Dean of Institute of Science

ลลิดา วงษ์บุตร : การแยกสารประกอบคล้ายยาที่มีขั้วด้วยโครมาโทกราฟีของไหลวิกฤตควบคู่กับอิเล็กโทรสเปรย์ไอออไนเซชันแทนเดมแมสสเปกโทรเมทรี (SEPARATION OF POLAR DRUG-LIKE COMPOUNDS USING SUPERCRITICAL FLUID CHROMATOGRAPHY COUPLED WITH ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.พัชรินทร์ ชัยสุวรรณ, 82 หน้า.

โครมาโทกราฟีของไหลวิกฤต/แทนเดมแมสสเปกโทรเมทรี/การแยกสารมีขั้ว/SFC-ESI-MS/MS

โครมาโทกราฟีของไหลวิกฤตเป็นหนึ่งในเทคนิคการแยกที่มีประสิทธิภาพสูง เทคนิคดังกล่าวถือเป็นทางเลือกเพื่อใช้ในการวิเคราะห์สารประกอบที่มีขั้วซึ่งมักจะทำการวิเคราะห์แบบโครมาโทกราฟีของเหลวแบบดั้งเดิมด้วยนอร์มอลเฟส เนื่องจากในการวิเคราะห์ด้วย SFC ใช้สารที่มีความเป็นพิษน้อยทำให้เทคนิคนี้ถือเป็นเทคนิคที่เป็นมิตรต่อสิ่งแวดล้อม

งานวิจัยนี้ได้รายงานการพัฒนาวิธีวิเคราะห์ใหม่ที่ใช้เวลาสั้นและมีความน่าเชื่อถือด้วยโครมาโทกราฟีของไหลวิกฤตควบคู่กับอิเล็กโทรสเปรย์ไอออไนเซชันแทนเดมแมสสเปกโทรเมทรี (SFC-ESI-MS/MS) ในการแยกสารประกอบคล้ายยาที่มีขั้ว 7 ชนิด ได้แก่ 4-ethylanilene 4-amino-3-chloropyridine 2-amino-5-methyl-1,3,4-thiadiazole 4-amino-2-chloropyridine 5-aminoindole aristeromycin และ neplanocin โดยทำการหาสภาวะที่เหมาะสมสำหรับการตรวจวัดด้วยแมสสเปกโทรเมทรี และการแยกสารด้วยเทคนิค SFC ได้แก่ Cone voltage Collision energy สารปรุงแต่งในเฟสเคลื่อนที่ ชนิดของเฟสคงที่ อัตราการไหล และ การชะสารแบบเกรเดียนท์ พบว่าได้ผลเป็นที่น่าพอใจเมื่อทำการแยกบนคอลัมน์ Princeton Benzamide (3.0 x 50 mm, 3 μm) ด้วยเฟสเคลื่อนที่คาร์บอนไดออกไซด์กับสารละลายของ 2% (v/v) น้ำและ 30 mM แอมโมเนียมฟอร์เมตในเมธานอลเป็นองค์ประกอบ ที่อัตราการไหล 2 mL/นาที ในการวิเคราะห์ใช้เวลาเพียง 3.7 นาที ด้วยการชะแบบเกรเดียนท์ กราฟมาตรฐานที่ได้จากการวิเคราะห์สารทั้ง 7 ชนิดในตัวอย่างปัสสาวะมีค่าสัมประสิทธิ์สหสัมพันธ์มากกว่า 0.9988 ด้วยค่าขีดจำกัดต่ำสุดในการตรวจพบและค่าขีดจำกัดต่ำสุดในการหาปริมาณต่ำกว่า 0.6 mg/L และ 1.8 mg/L ตามลำดับ มีค่าร้อยละการคืนกลับที่วิเคราะห์ในตัวอย่างปัสสาวะระหว่างร้อยละ 94.6 ถึง 109.5 ความเที่ยงในวันเดียวกันและระหว่างวันจากค่าของความเบี่ยงเบนมาตรฐานสัมพัทธ์สำหรับพื้นที่พีคและเวลาการคงอยู่ของสารมีค่าน้อย

กว่าร้อยละ 5.54 และ 0.70 ตามลำดับ วิธีวิเคราะห์ดังกล่าวสามารถนำไปประยุกต์ใช้สำหรับการวิเคราะห์สารอื่นๆที่มีโครงสร้างที่คล้ายกับสารตัวอย่างได้



สาขาวิชาเคมี ลายมือชื่อนักศึกษา ลลิตา ออย์บุตร

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา พรสินทร์

LALIDA WONGBUTH : SEPARATION OF POLAR DRUG-LIKE COMPOUNDS USING SUPERCRITICAL FLUID CHROMATOGRAPHY COUPLED WITH ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY. THESIS ADVISOR : ASST. PROF. PATCHARIN CHAISUWAN, Ph.D. 82 PP.

SUPERCRITICAL FLUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY/POLAR COMPOUND SEPARATION/SFC-ESI-MS/MS

Supercritical fluid chromatography (SFC) is one of the most powerful separation techniques. The technique is an alternative choice for analysis of polar compounds which is usually performed by traditional normal phase liquid chromatography. Due to the relatively low toxicity of chemicals used in SFC, the method is considered as a green analytical technique.

This work reports a development of a new, rapid and reliable supercritical fluid chromatography - electrospray tandem mass spectrometry method (SFC-ESI-MS/MS) for the separation of seven polar drug-like compounds including 4-ethylanilene, 4-amino-3-chloropyridine, 2-amino-5-methyl-1,3,4-thiadiazole, 4-amino-2-chloropyridine, 5-aminoindole, aristeromycin and neplanocin. Parameters for the MS/MS detection and SFC separation including cone voltage, collision energy, mobile phase additive, type of stationary phase, flow rate and gradient elution were optimized. Satisfied results were obtained from separation on a Princeton Benzamide column (3.0 x 50 mm, 3 µm) with mobile phase of $CO_2$ (solvent A) and 2% (v/v)

water in methanol containing 30 mM ammonium formate (solvent B) at flow rate of 2.0 mL/min. The method required short analysis time of 3.7 min using gradient elution. Good linearity from matrix-matched standard calibration curves were achieved for all the compounds ($R^2$ > 0.9988) with limits of detection and quantification below 0.6 mg/L and 1.8 mg/L, respectively. Recoveries from analysis of spiked pooled urine sample were between 94.6% and 109.5%. Intra-day and inter-day method precision in terms of %RSD for peak area and retention time were less than 5.54% and 0.70%, respectively. The method can be applied and adopted for fast screening of other compounds with similar chemical structures.



School of Chemistry

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Student's Signature Lalida Wongbuth

Advisor's Signature P. Chaisuwan

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABPR | Automated backpressure regulator |
| ACN | Acetonitrile |
| BEN | Benzamide |
| CN | Cyano |
| CSPs | Chiral stationary phases |
| Da | Dalton |
| DEAP | Diethyl-Amino |
| El | Electron ionization |
| 4EP | 4-Ethyl-pyridine |
| FA | Formic Acid |
| GC | Gas chromatography |
| HAD | HA-Dipyridyl |
| HA-DHP | HA-Dihydroxypropyl |
| HPLC | High performance liquid chromatography |
| i.d | Inner diameter |
| LC | Liquid chromatography |
| L/hr | Liter per hour |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| $[M+H]^+$ | Protonated molecule |

# LIST OF ABBREVIATIONS (Continued)

| | |
|---|---|
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MS/MS | Mass spectrometry/Mass spectrometry |
| M.W. | Molecular weight |
| m/z | Mass-to-charge ratio |
| N/A | Not available |
| $NH_2$ | Amino |
| OD | Outer diameter |
| PA | Propyl-acetamide |
| PDA | Photodiode array |
| Psi | Pounds per square inch |
| $R^2$ | Coefficient of determination |
| SFC | Supercritical fluid chromatography |
| SFE | Supercritical fluid extraction |
| SFC-MS | Supercritical fluid chromatography - Mass spectrometry |
| Si | Silica |
| SIR | Selected ion recording |
| TIC | Total ion chromatogram |
| UHPSFC | Ultra-high performance supercritical fluid chromatography |
| $UPC^2$ | Ultra performance convergence chromatography |
| UPLC | Ultra performance liquid chromatography |

# CHAPTER I

# INTRODUCTION

## 1.1 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is a chromatographic technique based on gas chromatography (GC) and liquid chromatography (LC) principles which supercritical fluid is used as the mobile phase. The separation is based on distribution of the analyte between chromatographic stationary phase and mobile phase existing in supercritical fluid state. The supercritical fluid can pass through the stationary phase like a gas and dissolve compounds like a liquid.

Many compounds have been used as mobile phase in SFC including carbon dioxide, pentane, nitrous oxide, dichlorofluormethane, trifluoromethane and ammonia. Carbon dioxide ($CO_2$) is the most widely used since its supercritical fluid can be easily obtained. In addition to the low critical point of $CO_2$, $CO_2$ has several other favorable advantages including superior thermal stability, ability to dissolve most organic compounds, does not absorb ultraviolet or visible radiation, low cost, low toxicity and flammability. It is considered as a green reagent since it can be recycled and easily removed (Tarafder, 2016). $CO_2$ phase diagram (Figure 1.1) shows the characteristics of state transitions in a pressure-temperature diagram. Carbon dioxide exists in fluid state when the temperature and pressure reach or are higher than its critical point. At this state the supercritical fluid $CO_2$ is able to diffuse like a gas and has solvating power like a liquid. In SFC, the mobile phase strength can be increased

by changing the temperature and pressure to increase the density or using co-solvent modifier and additive.

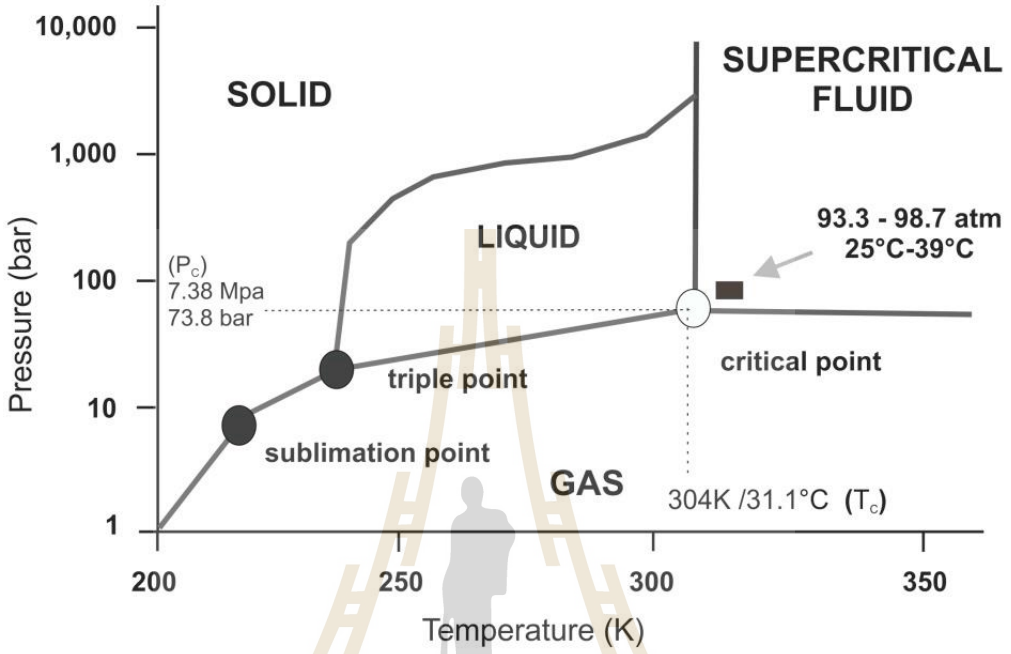



**Figure 1.1** $CO_2$ phase diagram (Budisa and Schulze-Makuch, 2014).

Supercritical fluid mobile phases have much greater solvating power than gaseous mobile phases allowing the separation of non-volatile and high molecular mass compounds unsuitable for GC. SFC allows the use of higher flow-rates than that used in high-performance liquid chromatography (HPLC) with lower pressure drop because of the lower viscosity and higher diffusivity of the supercritical fluid mobile phase. The SFC method generally provides greater efficiency in analysis time typically a three to five times faster than HPLC (Garzotti and Hamdan, 2002). Moreover, the SFC requires much lower solvent and reagent consumption compared to LC methods. The SFC is thus referred as a green separation technique. A comparison of chromatograms obtained from HPLC and SFC methods is presented in Figure 1.2.

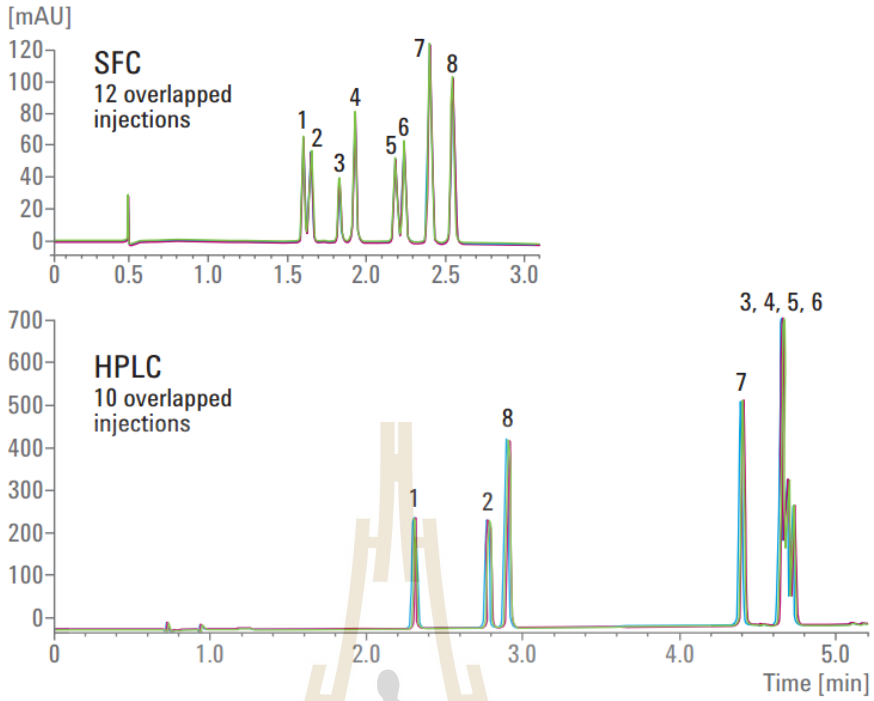



**Figure 1.2** Comparison of chromatograms obtained by HPLC and SFC for a mixture of: 1. caffeine, 2. theophyline, 3. cortisone, 4. prednisone, 5. hydrocortisone, 6. prednisilone, 7. sulfamerazine, 8. sulfaquinoxaline. SFC Conditions: 4 mL/min of 5 to 25% methanol in $CO_2$ in 3 min with a RX-SIL column (4.6 i.d. x 150 mm, 5 µm). HPLC conditions: 1.5 mL/min of 10 to 90% methanol in water in 4.5 min, 40 ºC on a Poroshell C18 column (4.6 i.d. x 150 mm, 2.7 µm) (Berger, 2015).

## 1.2 Mass spectrometry

Mass spectrometry (MS) is a highly specific and sensitive technique used to detect, identify and quantify molecular or atomic ions based on their mass-to-charge ratio (m/z). The molecules or atoms are ionized prior to induce to a mass analyzer. The ions are introduced into the ionization source to acquire ions in positive or negative charge. The ions are then induced through the mass analyzer for mass separation based on the m/z. Finally, the signal of ions detected is recorded by a data processing system

and displayed as a mass spectrum presenting the relative abundance of the signals (y axis) versus their m/z ratio (x axis).

Over a few years, electrospray ionization mass spectrometry (ESI-MS) has been increasingly used in clinical laboratories. It provides a sensitive and reliable tool for analysis with low sample volumes. The method can be applied for non-volatile and thermally labile bio-molecules.

ESI uses electrical energy to generate and induce ions of the interests into mass spectrometric analysis. Electrospray (charge droplets) is produced by applying a high electric field to a liquid sample passing through a capillary which is maintained at a high voltage (2.5 - 6.0 kV). The charge droplets then are continuously reduced in size by evaporation of desolvation gas flow, generally $N_2$ gas, and/or ESI-source temperature. Finally, the ions are then accelerated into the mass analyzer for measurement the ion intensity and molecular mass analysis. The most commonly used mass analyzer is quadrupole which determined by applying radio frequency and direct current.

MS can perform on a tandem system, commonly denoted as MS/MS for more specificity determination. When an ion of interest (usually called precursor ion) is selected by the first mass analyzer and fragmented to produce daughter ions in the second MS, the daughter ions can be monitored to provide structural information of the molecular ions.

## 1.3 Analytes of interest

Many pharmaceutical drugs contain amine functionality which can be protonated to provide a cationic center for binding to drug targets including receptors and

enzymes in the body (Muegge, 2003). In this study, seven aromatic amines were interested as they are drug-like compounds. The analytes of interest are as following.

### 1.3.1 4-Ethylaniline

4-Ethylaniline is an aromatic amine. A chemical structure of 4-ethylaniline is shown in Figure 1.3.

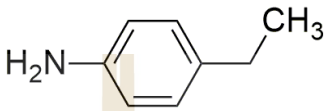



**Figure 1.3** Chemical structure of 4-ethylaniline.

| | |
|---|---|
| IUPAC name: | 4-Ethylaniline |
| Molecular formula: | $C_8H_{11}N$ |
| Average mass: | 121.180 Da |
| Boiling point: | 217.5 ºC at 760 mmHg |
| pKa: | 4.93 |
| LogP: | 2.10 |

### 1.3.2 4-Amino-3-chloropyridine

4-Amino-3-chloropyridine is a chlorinated heteroarylamine and is used in the preparation of inhibitor of phosphodiesterase type 4 (PDE4) as antiasthmatic agents as well as other pyridine based pharmaceutical agents. A chemical structure of 4-amino-3-chloropyridine is shown in Figure 1.4.

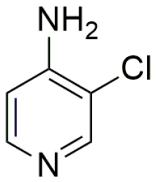



**Figure 1.4** Chemical structure of 4-amino-3-chloropyridine.

| | |
|---|---|
| IUPAC name: | 3-Chloro-4-pyridinamine |
| Molecular formula: | $C_5H_5ClN_2$ |
| Average mass: | 128.560 Da |
| Boiling point: | 250.9 ± 20.0 ºC at 760 mmHg |
| pKa: | 7.22 |
| LogP: | 0.53 |

### 1.3.3 2-Amino-5-methyl-1,3,4-thiadiazole

2-Amino-5-methyl-1,3,4-thiadiazole was used as a reagent in the synthesis of substituted 5H-benzo[i][1,3,4]thiadiazolo[3,2-a]quinazoline-6,7-diones which presented good cytotoxic activities. 2-Amino-5-methyl-1,3,4-thiadiazole was also used in the design of new phenothiazine-thiadiazole hybrids for development of antitubercular agents. A chemical structure of 2-amino-5-methyl-1,3,4-thiadiazole is shown in Figure 1.5.

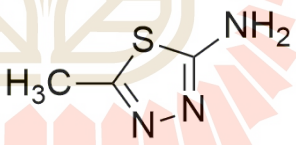



**Figure 1.5** Chemical structure of 2-amino-5-methyl-1,3,4-thiadiazole.



| | |
|---|---|
| IUPAC name: | 5-Methyl-1,3,4-thiadiazol-2-amine |
| Molecular formula: | $C_3H_5N_3$ |
| Average mass: | 115.157 Da |
| Boiling point: | 261.2 ± 23.0 ºC at 760 mmHg |
| pKa: | 1.34 |
| LogP: | -0.27 |

### 1.3.4 4-Amino-2-chloropyridine

4-Amino-2-chloropyridine is an aminopyridine derivative with potential herbicidal activity. A chemical structure of 4-amino-2-chloropyridine is shown in Figure 1.6.

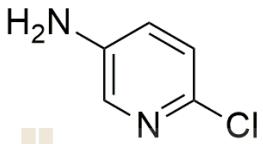



**Figure 1.6** Chemical structure of 4-amino-2-chloropyridine.

| | |
|---|---|
| IUPAC name: | 2-Chloropyridin-4-amine |
| Molecular formula: | $C_5H_5ClN_2$ |
| Average mass: | 128.560 Da |
| Boiling point: | 299.9 ± 20.0 ºC at 760 mmHg |
| pKa: | 4.99 |
| LogP: | 0.75 |

### 1.3.5 5-Aminoindole

5-Aminoindole is an aromatic amine. A chemical structure of 5-aminoindole is shown in Figure 1.7.

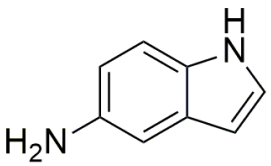



**Figure 1.7** Chemical structure of 5-aminoindole.

IUPAC name: 1H-indol-5-amine

Molecular formula: $C_8H_8N_2$

Average mass: 132.163 Da

Boiling point: 354.0 ± 15.0 ºC at 760 mmHg

pKa: 3.79

LogP: 1.24

### 1.3.6 Aristeromycin

Aristeromycin is an adenosine analog. Aristeromycin has the ability to inhibit enzymes needed for DNA synthesis and shows strong antiviral activity. A chemical structure of aristeromycin is shown in Figure 1.8.

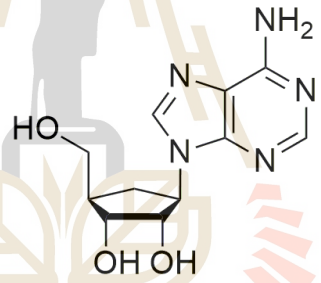



**Figure 1.8** Chemical structure of aristeromycin.

IUPAC name: (1R,2S,3R,5R)-3-(6-amino-9H-purin-9-yl)-5-(hydroxymethyl)cyclopentane-1,2-diol

Molecular formula: $C_{11}H_{15}N_5O_3$

Average mass: 265.268 Da

Boiling point: 595.2 ± 60.0 ºC at 760 mmHg

pKa: 3.67

LogP: -2.13

### 1.3.7 Neplanocin

Nepanocin, a cyclopentenyl analog of adenosine, is an antibiotic agent exhibiting significant antitumor activity. A chemical structure of neplanocin is shown in Figure 1.9.

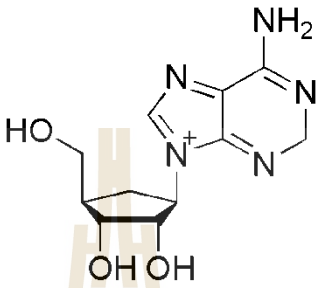



**Figure 1.9** Chemical structure of neplanocin.

| | |
|---|---|
| IUPAC name: | 6-Amino-9-[(1R,2S,3S)-2,3-dihydroxy-4-(hydroxymethyl)cyclopentyl]-2H-9λ$^5$-purin-9-ylium |
| Molecular formula: | $C_{11}H_{16}N_5O_3$ |
| Average mass: | 266.276 Da |
| Boiling point: | N/A |
| pKa: | 2.55 |
| LogP: | -5.86 |

Separation of polar compounds by the traditional chromatographic techniques is challenging since most of the polar compounds are not easily volatile and thus not amenable to be analyzed by GC unless derivatization process is performed. In order to perform the analysis for polar compounds by LC, normal-phased LC is needed. However, relatively toxic organic solvents such as hexane or ethyl acetate are required

for normal-phased LC. Unlike normal-phased LC method, SFC uses supercritical fluid with some additive as a mobile phase for separation of polar compounds on hydrophilic stationary phases. Due to the less chemical consumption and relatively nontoxic reagents used, the SFC is considered as a green analytical method and an alternative choice for separation of polar compounds.

In this study, a rapid and green SFC-ESI-MS/MS method was developed for the separation of seven drug-like compounds. Various experimental parameters including MS conditions (cone voltage and collision energy) and chromatographic conditions of SFC (mobile phase additive, type of stationary phase, flow rate and gradient elution) were optimized in order to obtain the best separation performance in the shortest analysis time. Furthermore, the SFC-ESI-MS/MS method was validated and applied for the analysis of spiked urine sample. The developed method can be adopted to use in pharmaceutical manufacturing process for fast screening of other new drug candidates, precursor and impurities with similar chemical structures.

## 1.4 Research objectives

1. To develop a rapid and green SFC-ESI-MS/MS screening method for separation of seven drugs-like compounds.

2. To evaluate analytical performance of the developed SFC-ESI-MS/MS method and apply to the analysis in urine sample.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is considered as a green separation technique with combination of liquid chromatography (LC) and gas chromatography (GC) principles. SFC has become a remarkable technique among analytical chromatographic techniques due to its advantages over traditional LC which are allowing the use of higher flow-rates with lower column back pressure, greater efficiency, shorter analysis time and lower solvent consumption (Lee *et al*., 2012; Zhou *et al*., 2014). The SFC is capable for analysis of thermally labile and nonvolatile compounds which are hardly analyzed by GC.

The first used of a supercritical fluid in chromatography was reported in 1962 by Klesper *et al*.. They successfully used high pressure GC (HPGC) for metal porphyrins separation using supercritical fluid dichlorodifluoromethane as a mobile phase by operating at the pressure above the critical point of the mobile phase. The first commercial supercritical fluid chromatography became available in 1988.

## 2.2 Method development in SFC

SFC has been reported for analysis of a wide range of analytes including chiral compounds (Toribio *et al.*, 2006; Hicks, 2016; Novakova and Dousa, 2017), polar and non-polar compounds (Turner *et al.*, 2001; Señoránsa and Ibañez, 2002).

SFC can also be described as an alternative to normal phase chromatography by performing on polar stationary phases and adding polar additive ('modifiers') or polar organic co-solvent into the common $CO_2$ mobile phase (Uchikata *et al.*, 2012; Sen *et al.*, 2016).

### 2.2.1 SFC methods for chiral compounds

More than half of commercial drugs currently are chiral and marketed as racemic mixtures of two drug's enantiomers (Hutt, 2002; Nguyen *et al.*, 2006). The two enantiomers are non-superimposable mirror images with similar chemical structure (Fasel *et al.*, 2005), and usually show different biological activities such as pharmacology, toxicology, pharmacokinetics and interactions with receptors (Hutt and Tan, 1996; Ribeiro *et al.*, 2012; Nguyen *et al.*, 2006). One enantiomer may have desired effects, while the other may have no effect or bad side effects (Tanret *et al.*, 2009). To avoid the undesirable side effects from inactive enantiomer and to control production quality in pharmaceutical manufacturing, various analytical methods have been developed for the enantiomeric separation and quantitative analysis of chiral drugs.

SFC is one of the most popular techniques for separation of chiral compounds in the pharmaceutical industry due to the fast separation and low solvent consumption. A wide variety of chiral stationary phases (CSPs) is used for the chiral separation using SFC and the most popular CSPs are the commercial polysaccharide-

based CSPs such as amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak® AD-H), cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel® OD-H), cellulose tris(4-methylbenzoate) (Chiralcel® OJ-H) and amylose tris((S)-α- methylbenzylcarbamate) (Chiralpak® AS-H).

In 2012, De Klerck *et al.* reported enatioseparation of promethazine on Chiralcel® OJ-H column. Hicks *et al.*, 2016 studied enantiopurity analysis of several pharmaceutical intermediates and the results achieved from SFC provided superior resolution compared to those from HPLC method. Cinacalset enantiomers were successfully separated using cellulose tris(3,5-dimethylphenylcarbamate) (Phenomenex Lux 3u Cellulose-1) as CSP (Novakova and Dousa, 2017), the separation is shown in Figure 2.1.

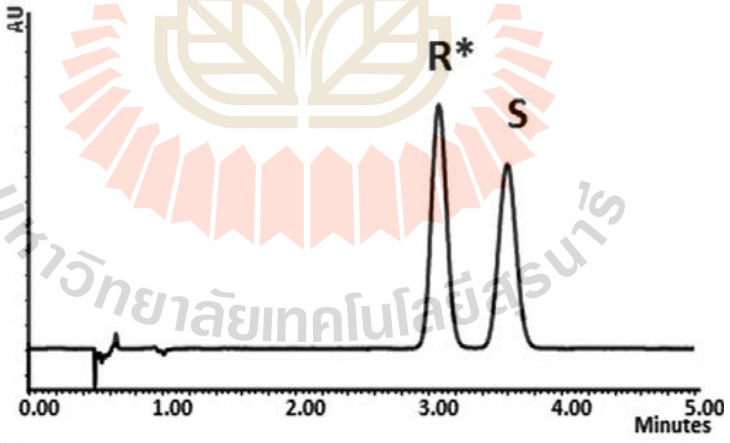



**Figure 2.1** Chromatogram obtained by SFC for cinacalset enantiomers using cellulose tris(3,5-dimethylphenylcarbamate) (Phenomenex Lux 3u Cellulose-1) column with a mobile phase of 10% of 0.1% trifluoroacetic (TFA) and 0.1% diethylamine (DEA) containing MeOH in $CO_2$ (Novakova and Dousa, 2017).

### 2.2.2 SFC methods for nonpolar compounds

SFC has been applied for separation of various nonpolar compounds. Most of the nonpolar solutes were separated by using $CO_2$ mobile phase with highly nonpolar modifier such as heptane.

Matsubara *et al.* (2012) reported a highly sensitive and selective analysis of epoxy carotenoids by SFC-ESI-MS/MS in a short analysis time using Merck Puroshere RP-18e column. In 2012, Uchikata *et al.* presented SFE–SFC/MS/MS method for 74 lipids analysis in dried blood spots. Other nonpolar compounds were also analyszed by SFC method such as lipids (Lee *et al.*, 2012; Yamada *et al.*, 2013), fat-soluble vitamins (Turner *et al.*, 2001; Señoránsa and Ibañez, 2002), organic pesticides (Ishibashi *et al*., 2012).

### 2.2.3 SFC methods for polar compounds

SFC can perform as a normal phase in LC technique, where polar stationary phase and the relatively nonpolar mobile phase are used. The SFC method provides advantages in terms of relatively nontoxic mobile phase and low column back pressure.

Achiral SFC separations for polar solutes typically require normal-phase type polar stationary phases, such as silica, diol, amino and cyano columns. In the last few years, a number of new stationary phases have been developed specifically for SFC such as 2-ethylpyridine, diethylamino and benzamide. Figure 2.2 shows a diol phase, an example of polar column for separation polar compounds.

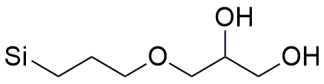



**Figure 2.2** Chemical structure of diol stationary phase.

For analysis of polar compounds, co-solvent (mobile-phase modifier) is usually added to the common $CO_2$ mobile phase to improve the elution power and peak shape (Zou *et al.*, 2000). The most often used co-solvents are alcohol such as methanol, ethanol or isopropanol.

The strong interaction of highly polar solutes with the stationary phase can cause incomplete elution or poor peak shapes (peak tailing) of the compounds. Therefore, highly polar additives such as a strong acid or base is required as an additive to reduce the interaction so that good peak shapes and resolution can be achieved (Berger *et al.*, 1989). The additives can adsorb onto the polar stationary phase surface forming mono-layer and can cover active sites on the stationary phase resulting in less interaction between the solutes and the stationary phase.

Sen *et al.* (2016) studied the influence of ammonium salts (formate, acetate and hydroxide) additives, the results revealed that the additives were beneficial for separation of polar compounds. However, each additive presented differently separation efficiency. Results of the effect of the additives are shown in Figure 2.3.

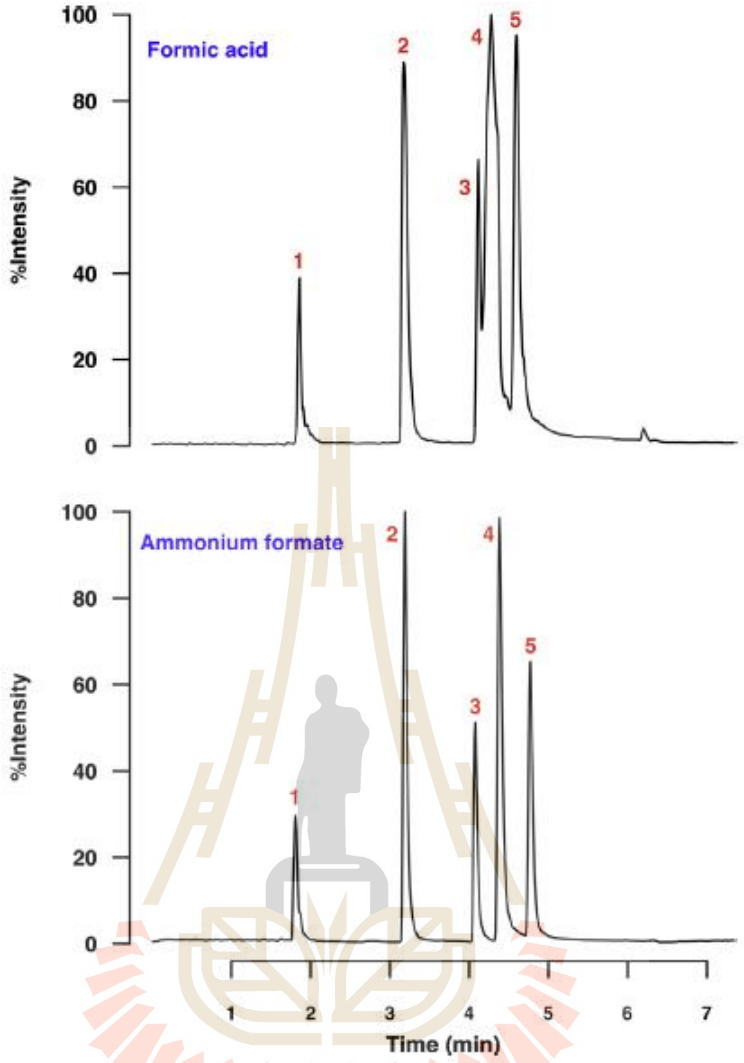



**Figure 2.3** Effect of additives (using 20 mM ammonium formate and 0.5% (v/v) formic acid) on chromatographic performance in SFC-MS. The chromatograms show separation of the nucleobase mixture (1:caffeine, 2:uracil, 3:adenosine, 4:cytosine and 5:cytidine) on a Torus Diol column (3.00 i.d. x 100 mm, 1.7 µm dP) at 40 ºC, using the 7.35 min gradient elution (Sen *et al.*, 2016).

### 2.2.4 SFC methods for pharmaceutical analysis

The availability of highly efficient analytical separation methods is essential for pharmaceutical industry since analysis of drug ingredients and precursors is needed at every stage of the drug development process.

Many groups studied the effect of chromatographic conditions in SFC i.e. stationary phase, mobile phase composition and injection solvent. Patel *et al.* (2012) demonstrated the interest of additive (e.g. water) to implement some alternative retention mechanism, such as HILIC-like, for the retention of peptides. Dispas *et al.* (2014) studied an importance of sample preparation as well as the influence of solvents used for the determination of amoxicillin hydrochloride in tablets by ultra-high performance SFC (UHPSFC). The obtained method could be used for quantitative analysis for the control of active substance in a pharmaceutical product. The influence of chromatographic conditions and injection solvent were examined for analysis of antimalarial molecules by Dispas *et al.* (2016). Unfortunately, only quinine sulfate can be analyzed under the investigation.

## 2.3 SFC coupled with MS

Supercritical fluid chromatography coupled with electrospray tandem mass spectrometry (SFC-ESI-MS/MS) is an analytical technique that combines the highly efficient separation of SFC with the mass selective detection capabilities of mass spectrometry. Due to the high separation efficiency and selective detection, fast separation is achieved.

SFC-ESI-MS/MS has been successfully used for analysis of small organic analytes in many applications. Garzotti and Hamdan (2002) developed the high throughput method of SFC-ESI-MS/MS for analysis of chiral mixtures with three different chiral stationary phases and different pressure/temperature working conditions. Uchikata *et al.* (2012) presented a reliable SFE–SFC/MS/MS method for analysis of lipids. The analysis of a mixture of polar metabolites in human urine using SFC-ESI-MS/MS was studied by Sen *et al.* (2016). Antiepileptic drugs were analyzed using SFC-ESI-MS/MS for pharmacokinetic study and finding the optimum dosages of the drugs (Wang *et al*., 2016). In 2017, Deng *et al.* developed a reliable and rapid method for the simultaneous determination of amine compounds in mainstream cigarette smoke using modified dispersive liquid liquid microextraction (DLLME) and SFC-MS/MS.

# CHAPTER III

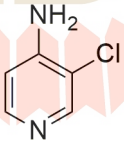
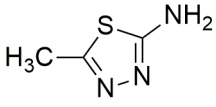
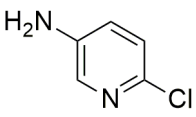
# MATERIALS AND METHODS

## 3.1 Chemicals and solvents

Information for the analytes of interest, solvent and chemical used are shown in Table 3.1 and Table 3.2.

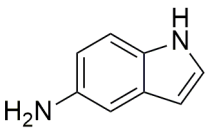
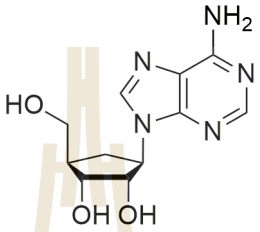
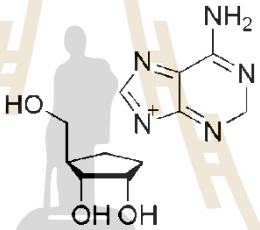
### 3.1.1 Analytes of interest

**Table 3.1** Chemical structure, Mw and logP of the analytes of interest.

| Name | Structure | Mw (Da) | LogP* |
|---|---|---|---|
| 4-Ethylaniline |  | 121.18 | 2.1 |
| 4-Amino-3-chloropyridine |  | 128.56 | 0.53 |
| 2-Amino-5-methyl-1,3,4-thiadiazole |  | 115.157 | -0.27 |
| 4-Amino-2-chloropyridine |  | 128.56 | 0.75 |

*P (Partition coefficient) = $[Analyte]_{1\text{-octanol}}$ / $[Analyte]_{aq}$

**Table 3.1** Chemical structure, Mw and logP of the analyte of interest (Continued).

| Name | Structure | MW (Da) | LogP* |
|---|---|---|---|
| 5-Aminoindole |  | 132.163 | 1.24 |
| Aristeromycin |  | 265.268 | -2.13 |
| Neplanocin |  | 266.276 | -5.86 |

*P (Partition coefficient) = $[Analyte]_{1\text{-octanol}} / [Analyte]_{aq}$

### 3.1.2 Solvents and chemicals used

**Table 3.2** Solvents and chemicals used.

| Chemical | Grade | Supplier |
|---|---|---|
| Methanol | LC-MS | Sigma-Aldrich (Poole, UK) |
| Methanol | HPLC | Sigma-Aldrich (Poole, UK) |
| Water | HPLC | Sigma-Aldrich (Poole, UK) |
| Liquid $CO_2$ | Food fresh (99.8% purity) | BOC (UK) |
| Formic acid | Analytical | Sigma-Aldrich (Poole, UK) |

**Table 3.2** Solvents and chemicals used (Continued).

| Chemical | Grade | Supplier |
|---|---|---|
| Acetic acid | Analytical | Sigma-Aldrich (Poole, UK) |
| Ammonium formate | Analytical | Sigma-Aldrich (Poole, UK) |
| Ammonium acetate | Analytical | Sigma-Aldrich (Poole, UK) |

## 3.2 Preparation of solutions

### 3.2.1 Preparation of co-solvent for mobile phase

#### 3.2.1.1 5% (v/v) water in MeOH

A 12.50 mL of water was added into a 250 mL volumetric flask and filled up with MeOH to obtain the final volume.

#### 3.2.1.2 20 mM ammonium formate in MeOH

A 0.3153 g of ammonium formate was dissolved and made up to 250.00 mL in a volumetric flask with MeOH.

#### 3.2.1.3 2% (v/v) water in MeOH containing 20 mM ammonium formate

A 0.3153 g of ammonium formate was dissolved in 5.00 mL of water and made up to final volume in a 250 mL volumetric flask with MeOH.

#### 3.2.1.4 2% (v/v) water in MeOH containing 30 mM ammonium formate

A 0.4730 g of ammonium formate was dissolved in 5.00 mL of water and diluted with MeOH in a volumetric flask to a final volume of 250.00 mL

#### 3.2.1.5 2% (v/v) water in MeOH containing 20 mM ammonium acetate

A 0.3854 g of ammonium acetate was dissolved in 5.00 mL of water in a 250 mL volumetric flask and made up to final volume with MeOH.

### 3.2.2 Preparation of make-up solvents

#### 3.2.2.1 0.1% (v/v) formic acid in MeOH

A 0.25 mL of formic acid was added into a 250 mL volumetric flask. MeOH was then filled up until the final volume was obtained.

#### 3.2.2.2 0.2% (v/v) formic acid in MeOH

A 0.50 mL of formic acid was added into a 250 mL volumetric flask and made up to final volume with MeOH.

### 3.2.3 Preparation of stock and working solutions

Stock and working solutions for each compound were individually prepared as shown details in Table 3.3.

**Table 3.3** Preparation of stock and working solutions.

| Compound | Stock solution | | Working solution | |
|---|---|---|---|---|
| | Solvent | Conc. (mg/mL) | Make up solvent | Conc. (mg/mL) |
| 4-Ethylaniline | MeOH | 2.93 | MeOH | 0.003 |
| 4-Amino-3-chloropyridine | MeOH | 3.11 | MeOH | 0.003 |
| 2-Amino-5-methyl-1,3,4-thiadiazole | MeOH | 2.47 | MeOH | 0.003 |
| 4-Amino-2-chloropyridine | MeOH | 1.88 | MeOH | 0.003 |
| 5-Aminoindole | MeOH | 2.88 | MeOH | 0.030 |

**Table 3.3** Preparation of stock and working solutions (Continued).

| Compound | Stock solution | | Working solution | |
|---|---|---|---|---|
| | Solvent | Conc. (mg/mL) | Make up solvent | Conc. (mg/mL) |
| Aristeromycin | 2:1 MeOH:water | 1.59 | MeOH | 0.030 |
| Neplanocin | 1:1 MeOH:water | 0.80 | MeOH | 0.300 |

### 3.2.4 Sample preparation

Pooled normal human urine sample was collected freshly from three healthy volunteers and stored at -20 ºC. Before analysis, the frozen pooled urine sample was thawed and filtered through a 0.2 µm Minisart® syringe filter. The sample was then diluted with MeOH in the ratio of 1:9 (v/v).

## 3.3 Instrumentations

### 3.3.1 SFC-MS/MS system

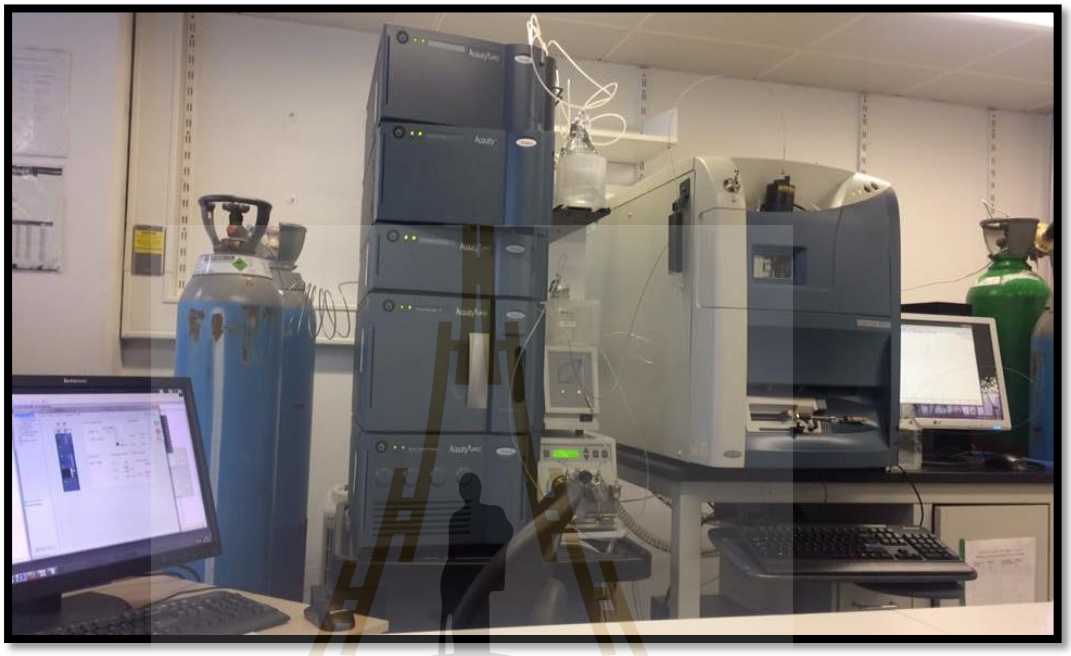


**Figure 3.1** Waters ACQUITY UPC$^2$-MS/MS system.

The supercritical fluid chromatographic (SFC) analysis was performed on a Waters ACQUITY UPC$^2$ (Ultra Performance Convergence Chromatography) system consisting of a binary solvent manager, a sample manager (control temperature at 4 ºC and fitted with a 10 µL injection loop), a convergence manager containing an automated backpressure regulator (ABPR) for controlling the system pressure to maintain the $CO_2$ supercritical properties, column manager (with an active column heater) and photodiode array detector (PDA).

The Waters ACQUITY UPC$^2$ was coupled to Waters Quattro Premier tandem mass for MS detection. Schematic figure of the UPC$^2$-MS/MS system is shown in Figure 3.2. A 515 HPLC-pump from Waters was used for propelling make-

up solvent into the liquid flow from the PDA detector to the MS/MS detector. An ABPR was used to maintain a flow at a pressure of 2,000 psi (138 bar) to maintain the $CO_2$ supercritical properties during a run. The flow was sprayed with an electrospray ion capillary via a length of 50 μm PEEKsil tubing. The software used for data acquisition was Waters MassLynx v.4.1.

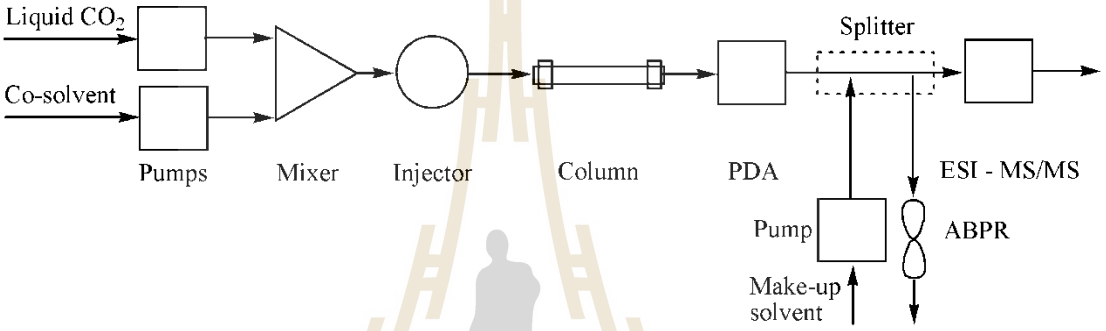



**Figure 3.2** Schematic diagram of basic component in the SFC-MS/MS system.

Schematic diagram in Figure 3.3 shows basic components of the electrospray ionization tandem mass spectrometer (ESI-MS). Electrospray (fine charged droplets) was produced by applying a high electric field to a liquid sample passing through a capillary. The charge droplets can be evaporated by desolvation gas flow, generally $N_2$. Then cone voltage, typically are in the region of 10 to 60 V was applied to extract and induce ions to vacuum region of the mass analyzer. Afterward, the ions are selected and analyzed according to their mass to charge ratio (m/z) by the quadrupole 1 which determined by applied radio frequency.

The extracted ions pass into collision cell where they collide with collision gas (Argon or Helium (Bordas-Nagy *et al.*, 1992)) which break ions from precursor ions (parent) to product ions (daughter). The fragment ions are selected by the

quadrupole 2 before detected at the detector by a conversion dynode, phosphor and photomultiplier detection system (Waters, 2005).

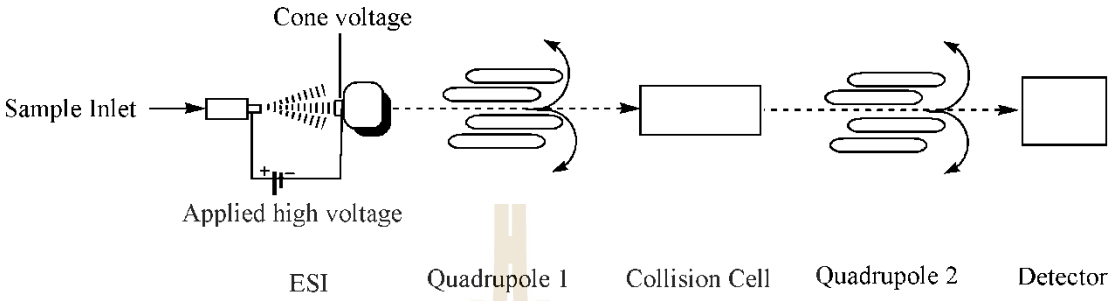



**Figure 3.3** Schematic representation of the flow of ions in Waters Quattro Premier tandem mass spectrometer.

## 3.4 Method optimization

### 3.4.1 Mass spectrometric conditions

Analytes of interest were directly injected into the Waters Quattro Premier tandem mass spectrometer and detected in total ion current (TIC) mode by using nitrogen as desolvation gas. Source conditions were capillary voltage, 3.5 kV; source temperature, 120 ºC; desolvation temperature, 250 ºC; cone gas flow, 300 L/hr and desolvation gas flow, 700 L/hr. Each compound was screened at four cone voltages in positive ESI MS mode (10, 20, 30 and 40 V) to gain the highest signal intensity.

Multiple reaction monitoring (MRM) was employed to perform fragmentation. Argon gas was used as the collision gas. The scan dwell time was set at 0.05 s for each channel. The collision energy was varied to find an optimum energy.

### 3.4.2 Mobile phase optimization

In order to screen for an optimum mobile phase, the analytes of interest were preliminary screened with the initial chromatographic conditions applied from the study of polar urinary metabolites (Sen *et al.*, 2016) which present desirable result for polar compound analysis using SFC-MS. The analytes were analyzed on an Acquity UPC$^2$ Torus DIOL column with dimension of 3.00 mm i.d. x 100 mm and particle size (dp) of 1.7 µm, set at 40 ºC with a mobile phase composed of $CO_2$ (solvent A) and co-solvent (solvent B). The flow-rate was set at 1 mL/min.

Gradient elution method of 7.35 min (Figure 3.4) was used for the investigation. The composition of mobile phase changed from 2% B at 0 min to 50% B in 4 min. The composition of 50% B was then held for 1.5 min and returned to the initial stage of 2% B in 0.8 min. The 2% B mobile phase was kept for 1.05 min for re-equilibrating the column before the next run.

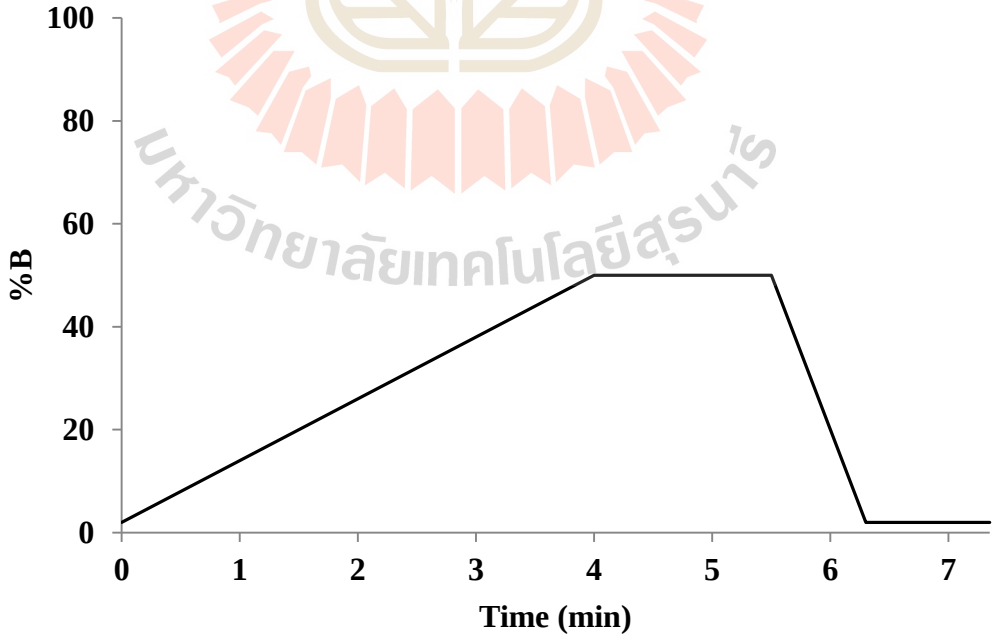



**Figure 3.4** The 7.35 min gradient elution method.

MeOH was used as co-solvent (solvent B) with three different co-solvent additives; water, ammonium formate and ammonium acetate.

Make-up solvent was added to the system prior to MS detection to provide greater ionization of the analytes. The addition of a protonation source such as formic acid to the make-up solvent can be used to enhance ionization and increase sensitivity. In this system MeOH containing formic acid was used as make-up solvent.

### 3.4.3 Types of column (stationary phase)

Eleven SFC columns with lengths of 50 mm (Table 3.4) were investigated to obtain the best separation for the seven drug-like compounds. The separation was performed by using gradient elution (Figure 3.4), $CO_2$ supercritical fluid (solvent A), 2% (v/v) water in MeOH containing 30 mM ammonium formate as co-solvent (solvent B) and make-up solvent was 0.2% (v/v) formic acid in MeOH as the make-up solvent.

**Table 3.4** Structure and feature of the studied columns.

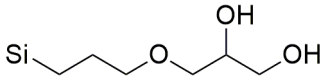
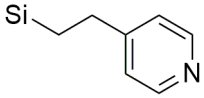
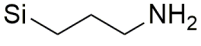
| Column | Functional group | Particle size (µm) | Pore size (Å) | ID (mm) |
|---|---|---|---|---|
| DIOL |  | 3 | 60 | 3.0 |
| 4-Ethyl-pyridine (4EP) |  | 3 | 60 | 3.0 |
| Amino |  | 3 | 60 | 3.0 |

**Table 3.4** Structure and feature of the studied columns (Continued).

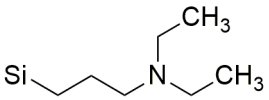
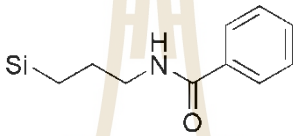
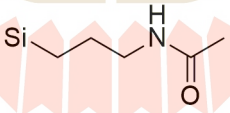
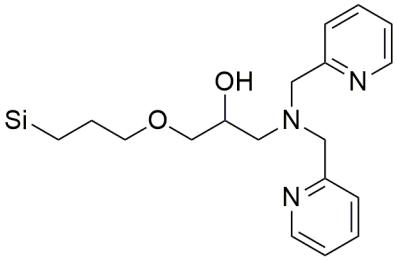
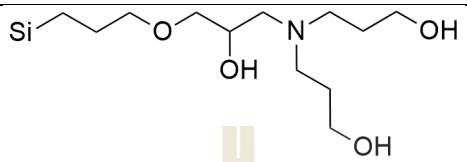
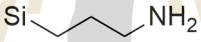
| Column | Functional group | Particle size (µm) | Pore size (Å) | ID (mm) |
|---|---|---|---|---|
| Diethyl-Amino (DEAP) |  | 5 | 60 | 4.6 |
| Benzamide (BEN) |  | 3 | 100 | 3.0 |
| Cyano (CN) |  | 3 | 60 | 3.0 |
| Propyl-acetamide (PA) |  | 3 | 60 | 3.0 |
| HA-Dipyridyl (HAD) |  | 3 | 100 | 3.0 |

**Table 3.4** Structure and feature of the studied columns (Continued).

| Column | Functional group | Particle size (µm) | Pore size (Å) | ID (mm) |
|---|---|---|---|---|
| HA-DHP |  | 3 | 100 | 3.0 |
| Luna-Silica |  | 3 | 100 | 3.0 |
| Luna-$NH_2$ |  | 3 | 100 | 3.0 |

### 3.4.4 Gradient system

Six linear gradient elutions were tested during method development to improve the separation obtained from the selected BEN column. The flow rate of 2 mL/min was set throughout the experiment. The linear gradient was operated from 2% B at 0 min to 40% B in 1.5 min (Grad 1), to 30% B in 1.8 min (Grad 2), to 30% B in 2 min (Grad 3), to 30% B in 2.2 min (Grad 4), to 25% B in 2.5 min (Grad 5), to 25% B in 3 min (Grad 6).

## 3.5 Method validation

### 3.5.1 Linearity

Linearity of the method was evaluated by constructing five concentration calibration curves of the analytes in diluted blank urine. The five concentrations for calibration curve construction were 2.5, 5, 10, 15 and 20 mg/L for 4-ethylaniline, 4-amino-3-chloropyridine, 2-amino-5-methyl-1,3,4-thiadiazole, 4-amino-2-chloropyridine and 5-aminoindole and 1.25, 2.5, 5, 7.5 and 10 mg/L for aristeromycin and neplanocin. Calibration curves were constructed by plotting peak area observed versus the concentration.

### 3.5.2 Precision and accuracy

Precision (repeatability) of the method was evaluated by intra-day and inter-day replicate injections of a diluted urine containing 10 mg/L of 4-ethylaniline, 4-amino-3-chloropyridine, 2-amino-5-methyl-1,3,4-thiadiazole, 4-amino-2-chloropyridine and 5-aminoindole and 5 mg/L of aristeromycin and neplanocin. The solution was injected for five replicates within one day for intra-day precision study and three consecutive days for inter-day precision study. Accuracy was evaluated by spiking known amounts of the analytes into the diluted blank urine. The found amounts of spike standard were compared with nominal values for recovery calculations.

### 3.5.3 Detection and quantification limits

Limit of detection (LOD) and limit of quantification (LOQ) were calculated using the following equations (ICH, 2005):

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

Where σ is standard deviation of response based on the calibration curve and S is the slope of the calibration curve.

# CHAPTER IV

# RESULTS AND DISCUSSIONS

## 4.1 Optimization of MS conditions

To develop a SFC-MS/MS method for the analysis of the seven analytes of interest (4-ethylaniline, 4-amino-3-chloropyridine, 2-amino-5-methyl-1,3,4-thiadiazole, 4-amino-2-chloropyridine, 5-aminoindole, aristeromycin and neplanocin), the optimization of cone voltage and collision energy for MS/MS detection were studied by direct injection of individual analytes into the MS/MS device to find an optimum MS parameters.

### 4.1.1 Optimization of cone voltage (CV)

The analytes of interest were directly injected and scanned by a mass spectrometer. The detection used electrospray ionization (ESI) as an ionization source to generate analyte ions before mass detection. As all the analytes are protonated producing positive ions, the ESI in positive mode (ESI+) in which positive ions are detected was chosen. A common acidic additive, formic acid was used as a make-up solvent to facilitate the protonation of the analytes before mass detection process.

One important parameter in the MS operation is cone voltage which is applied to extract and induce analyte ions into the mass analyzer after the liquid sample is ionized. To gain the highest signal intensity of the ions detected, cone voltage was optimized. Signal intensity obtained for protonated molecular ions for

each compound at cone voltage of 10-40 V are presented in Table 4.1.

**Table 4.1** Signal intensity of $[M+H]^+$ at studied cone voltages.

| Compound | Signal intensity ($x10^7$) | | | |
|---|---|---|---|---|
| | Cone voltage of ESI+ (V) | | | |
| | 10 | 20 | 30 | 40 |
| 4-Ethylaniline | 0.61 | 2.00 | 2.00 | 1.79 |
| 4-Amino-3-chloropyridine | 2.04 | 4.95 | 5.97 | 5.60 |
| 2-Amino-5-methyl-1,3,4-thiadiazole | 1.16 | 2.55 | 2.93 | 6.93 |
| 4-Amino-2-chloropyridine | 1.39 | 4.76 | 6.41 | 4.00 |
| 5-Aminoindole | 10.2 | 13.4 | 13.4 | 13.4 |
| Aristeromycin | 0.85 | 1.61 | 2.38 | 2.20 |
| Neplanocin | 0.51 | 1.17 | 1.57 | 1.18 |

It was found that the ion intensity tended to increase when cone voltage increased as the result of more ions accelerated into the mass analyzer. However, when the cone voltage was too high the reduction of intensity was observed due to more ion fragmentation causing many product ions and less signal intensity.

The optimum cone voltages and observed mass to charge ratio (m/z) for each compound are included in Table 4.2

**Table 4.2** Optimum cone voltages and observed m/z.

| Compound | Cone Voltage (V) | m/z |
|---|---|---|
| 4-Ethylaniline | 30 | 122, 94, 148 |
| 4-Amino-3-chloropyridine | 30 | 129, 131, 95 |
| 2-Amino-5-methyl-1,3,4-thiadiazole | 40 | 116, 75, 231 |
| 4-Amino-2-chloropyridine | 30 | 129,131, 151 |
| 5-Aminoindole | 30 | 133, 116, 263 |
| Aristeromycin | 30 | 266, 288, 105 |
| Neplanocin | 30 | 264, 136, 59 |

*The underline m/z (s) are base ions for each compound.

Full-scan product ion mass spectra acquired from applying the optimum cone voltages for each compound are shown in Figure 4.1.

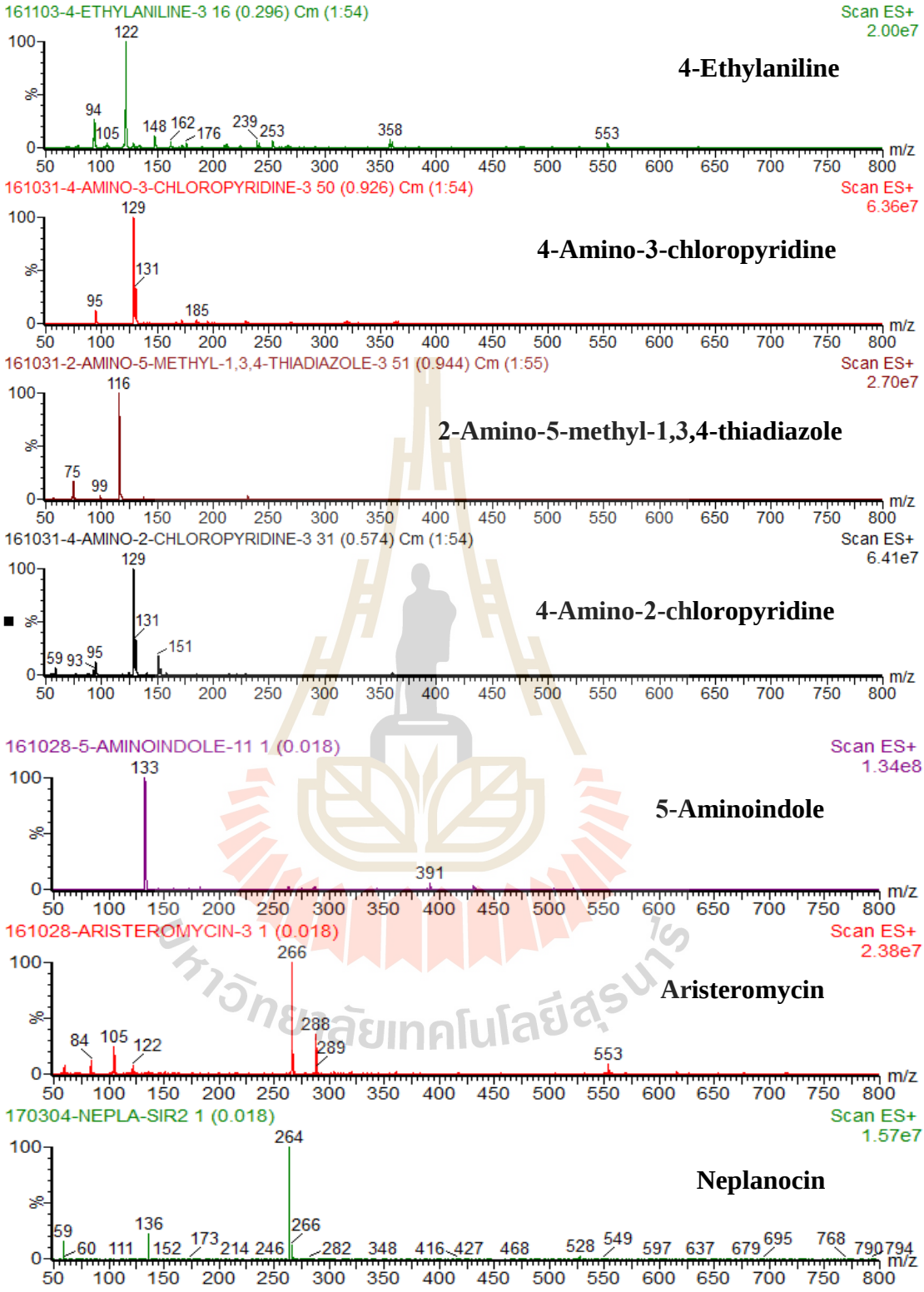



**Figure 4.1** Full-scan product ion mass spectra at optimum cone voltages.

The obtained mass spectra in Figure 4.1 shows peak characteristic for each compound. As expected, the $[M+H]^+$ ions were observed as the base ions (major ions) due to the soft ionization characteristic of the ESI-MS technique.

### 4.1.2 Optimization of collision energy (CE)

In order to increase selectivity of the detection, the target ions obtained from Section 4.1.1 were scanned by multiple reaction monitoring (MRM) to perform the second MS, called MS/MS. In the MS/MS detection, the precursor ion (parent ion) is collided to fragment and produce smaller ions (daughter ion). A product ion is selected and monitored for more selective detection. Although signal intensity is reduced when the MRM is applied, the fragmentation pattern provides structural information and quantitative data.

One important key for adjusting sensitivity of the method is the collision energy for the fragmentation in MRM. In this experiment, the collision energy was adjusted in the range of 10 to 40 eV. The optimum collision energy, parent and daughter ions for each compound are summarized in Table 4.3. Full-scan product mass spectra for each compound are shown in Figure 4.2.

**Table 4.3** Parent and daughter ions, optimum cone voltages and collision energy for the MRM method.

| Compound | Transition | | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|
| | Parent ion (m/z) | Daughter ion (m/z) | | |
| 4-Ethylaniline | 122 | 94 | 30 | 13 |
| 4-Amino-3-chloropyridine | 129 | 93 | 30 | 20 |
| 2-Amino-5-methyl-1,3,4-thiadiazole | 116 | 75 | 40 | 13 |
| 4-Amino-2-chloropyridine | 129 | 93 | 30 | 20 |
| 5-Aminoindole | 133 | 116 | 30 | 18 |
| Aristeromycin | 266 | 136 | 30 | 25 |
| Neplanocin | 264 | 136 | 30 | 18 |

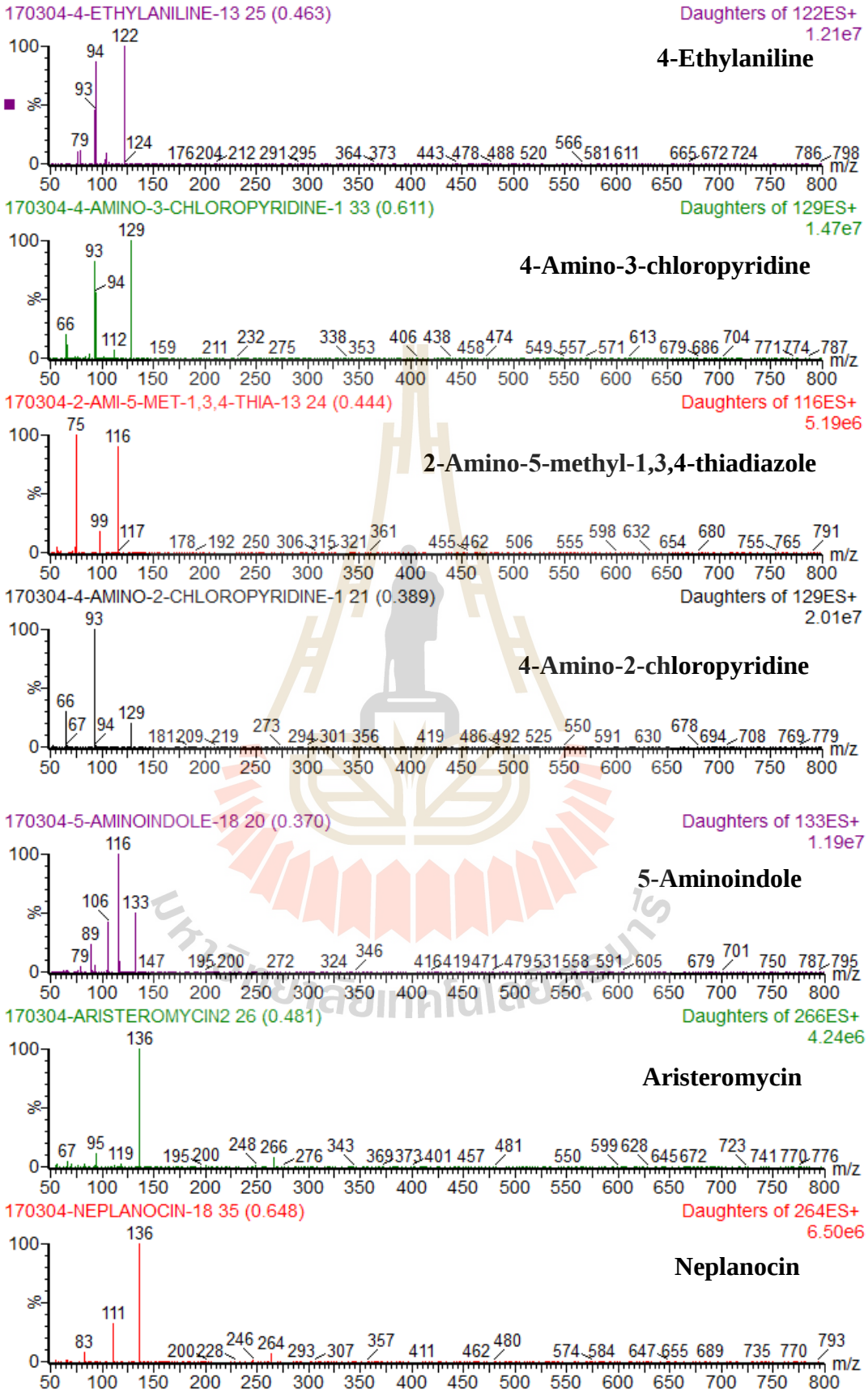



**Figure 4.2** The MS/MS spectra of the analytes of interest.

## 4.2 Optimization of SFC conditions

In this second part, the main objective was to study the influence of chromatographic conditions on the separation of the analytes using SFC-MS technique. Investigation of mobile phase composition, type of stationary phase, flow rate and gradient elution were performed to find an optimum condition for the analysis.

### 4.2.1 Effect of mobile phase additive

The common $CO_2$ was selected as the main component of the mobile phase for because of its low critical point, ready availability, remarkably low cost, low toxicity and flammability. It is considered as a green reagent since it can be recycled and also easily eliminated.

However, the non-polarity of the $CO_2$ makes it unsuitable for separation of polar compounds. In order to separate polar compounds by SFC with $CO_2$ mobile phase, some co-solvent and polar additive are required.

In this work, methanol was used as co-solvent since it is completely miscible with $CO_2$ and highly volatile, which can promote the analyte ionization for the MS detection. Highly polar additive was also added to the co-solvent to improve the SFC separation as by Philibert and Olesik (2011) reported. However, the polar additives can interfere with MS detection due to their poor volatility, the additive must be carefully selected.

In order to screen for an optimum mobile phase, the analytes were separated on a DIOL column, which has been previously shown good selectivity for polar compounds (Berger, 2015; Sen *et al.*, 2016). The mobile phase of $CO_2$ (solvent A) and

MeOH co-solvent (solvent B) without additive was preliminary used with gradient elution. The separations are shown in Figure 4.3.

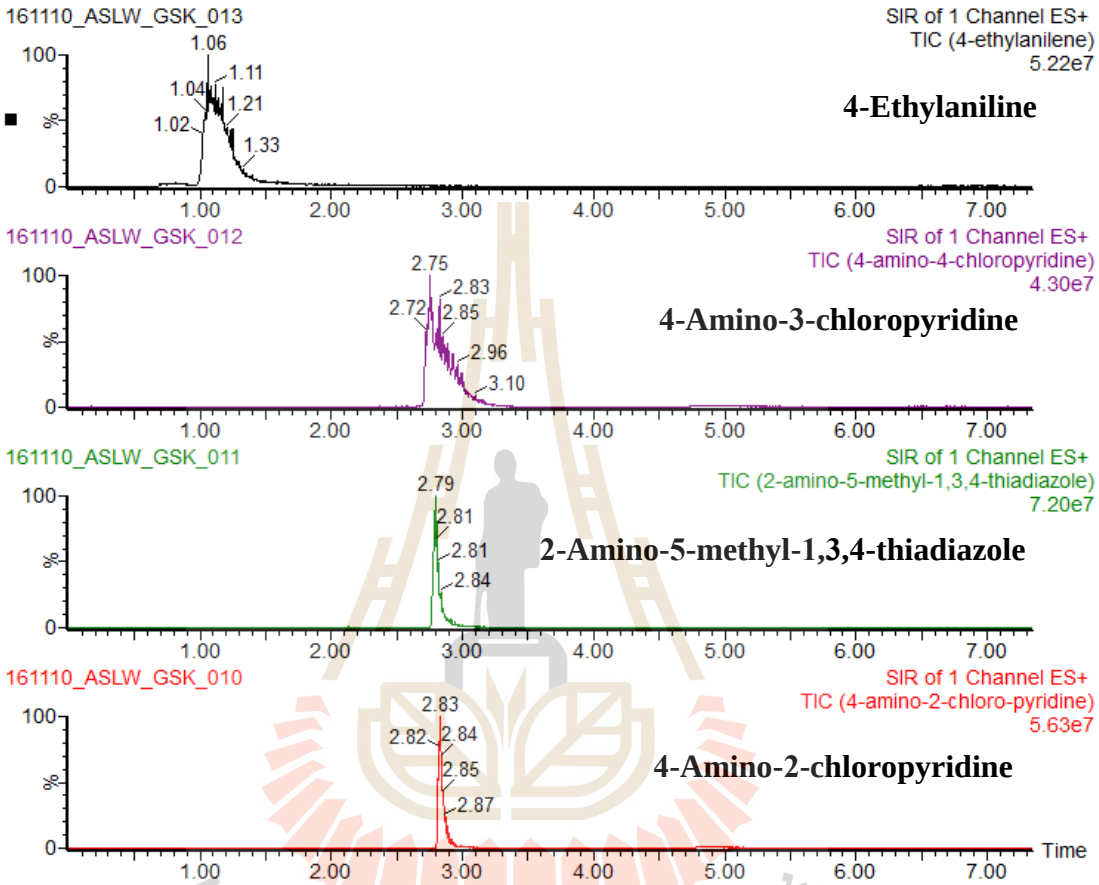



**Figure 4.3** Chromatograms obtained by SFC-MS method. Conditions: gradient elution (see Section 3.4.2) of $CO_2$ (solvent A) and MeOH (solvent B), DIOL column, 1 mL/min, 0.1% (v/v) formic acid in MeOH make-up solvent.

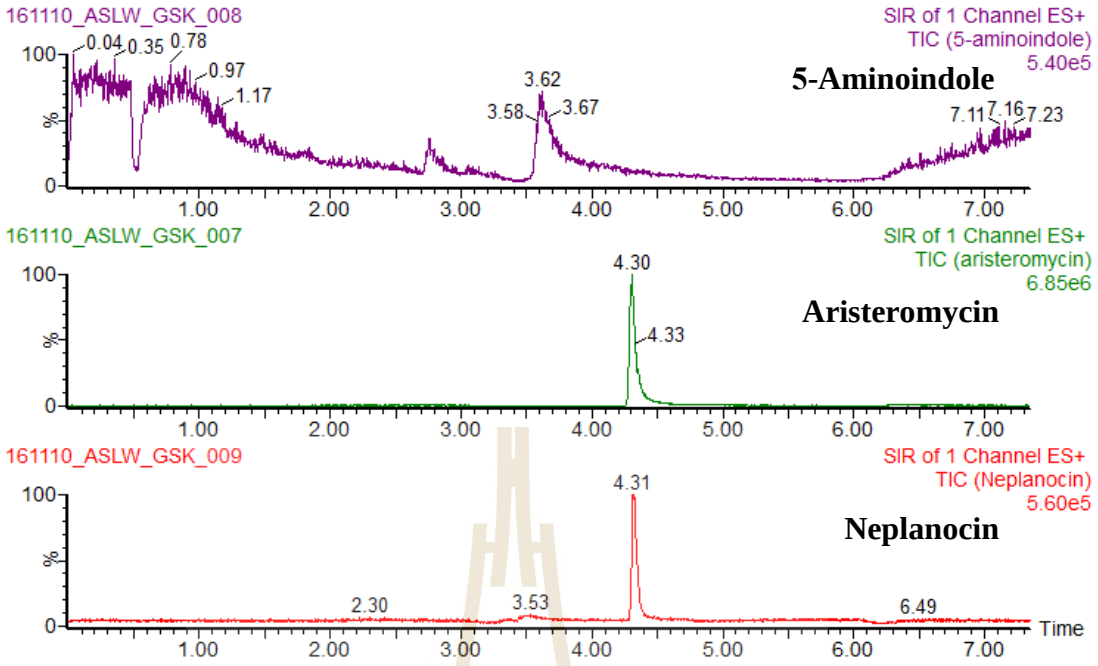



**Figure 4.3** Chromatograms obtained by SFC-MS method. Conditions: gradient elution (see Section 3.4.2) of $CO_2$ (solvent A) and MeOH (solvent B), DIOL column, 1 mL/min, 0.1% (v/v) formic acid in MeOH make-up solvent (Continued).

The results show that without additive, unsatisfied peaks were observed for 4-ethylaniline, 4-amino-3-chloropyridin and 5-aminoindole. Since addition of a small amount of a highly polar additive into the co-solvent has been reported for improving the separation of polar compounds in SFC (Berger *et al.*, 1989). Therefore, water and ammonium formate were examined as the additive in this experiment. The results in Figure 4.4 and Figure 4.5 showed that when water or ammonium formate were used, the separation was improved which could be due to (i) the strong adsorption of the additive onto the stationary phase surface causing less interaction between the analyte and the stationary phase and (ii) increasing of solvating power of the mobile phase by the polar additive. In addition, ion pairing interaction between the positively charged

amino cation of the analyte and the formate anion from the additive possibly occurred, peak tailing was thus eliminated.

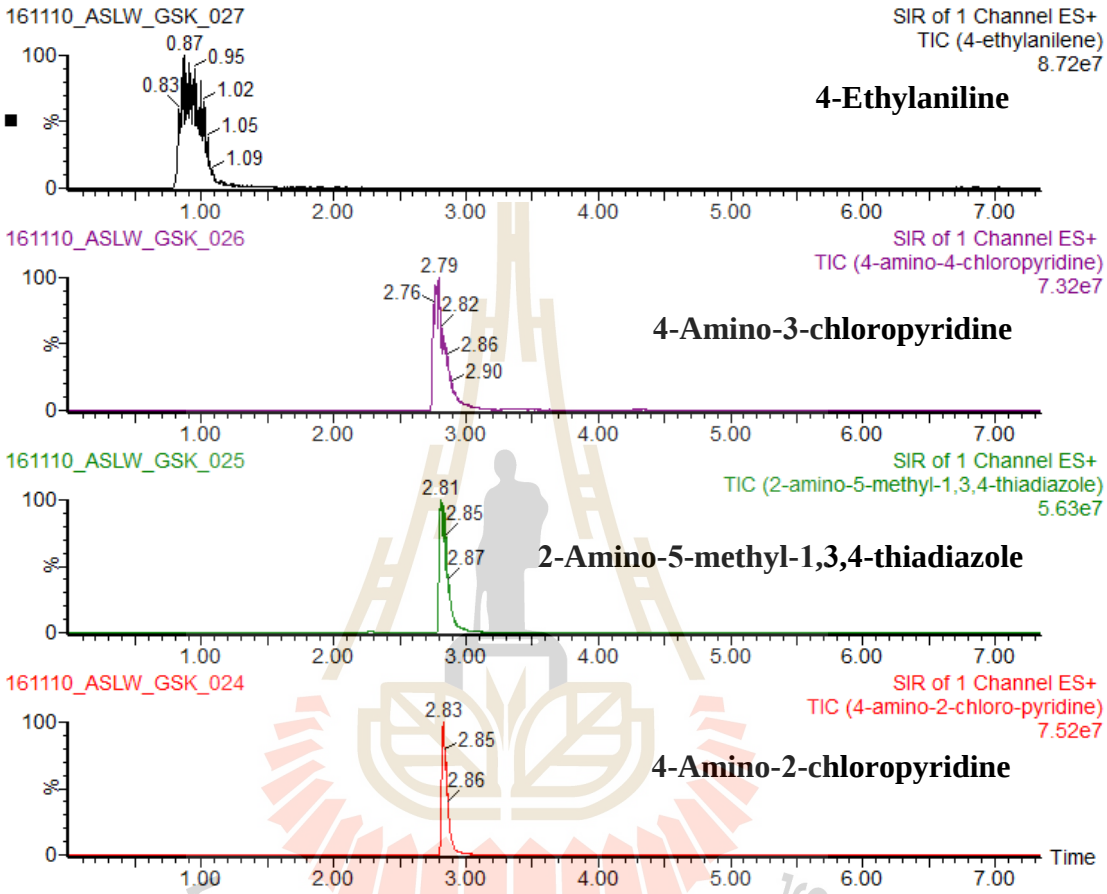



**Figure 4.4** Chromatograms obtained by SFC-MS method. Conditions: gradient elution (see Section 3.4.2) of $CO_2$ (solvent A) and 5% (v/v) water in MeOH (solvent B), DIOL column, 1 mL/min, 0.1% (v/v) formic acid in MeOH make-up solvent.

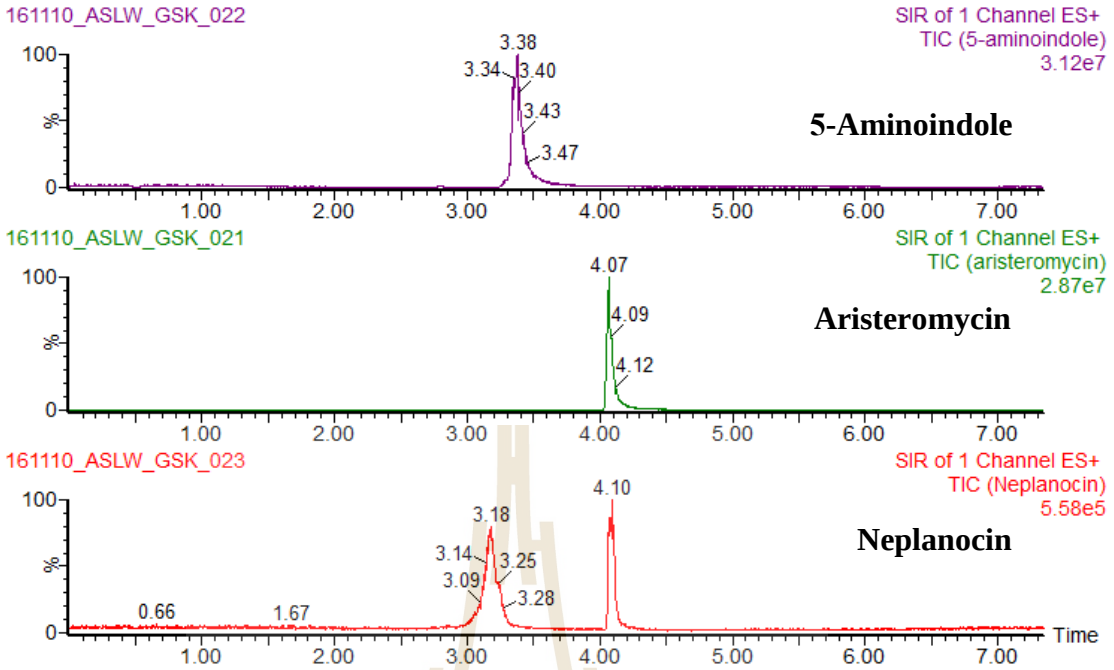



**Figure 4.4** Chromatograms obtained by SFC-MS method. Conditions: gradient elution (see Section 3.4.2) of $CO_2$ (solvent A) and 5% (v/v) water in MeOH (solvent B), DIOL column, 1 mL/min, 0.1% (v/v) formic acid in MeOH make-up solvent (Continued).

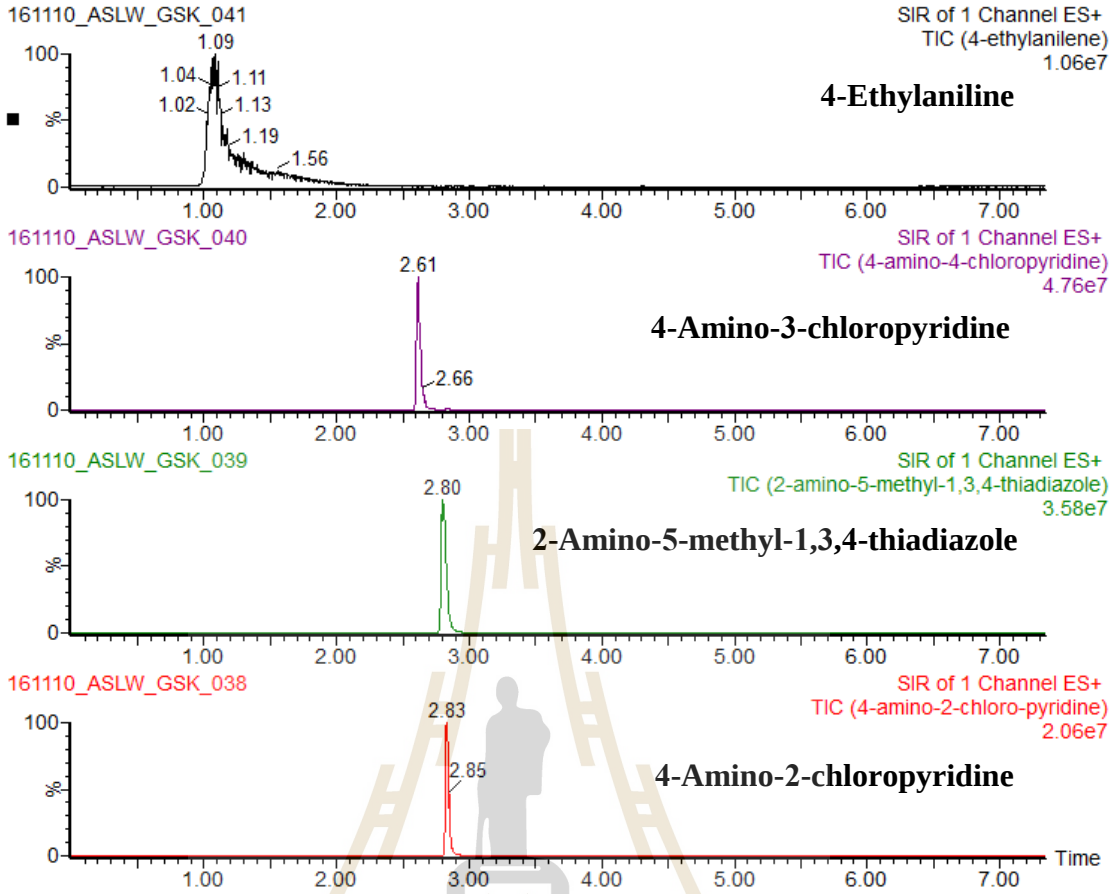



**Figure 4.5** Chromatograms obtained by SFC-MS method. Conditions: gradient elution (see Section 3.4.2) of $CO_2$ (solvent A) and 20 mM ammonium formate in MeOH (solvent B), DIOL column, 1 mL/min, 0.1% (v/v) formic acid in MeOH make-up solvent.

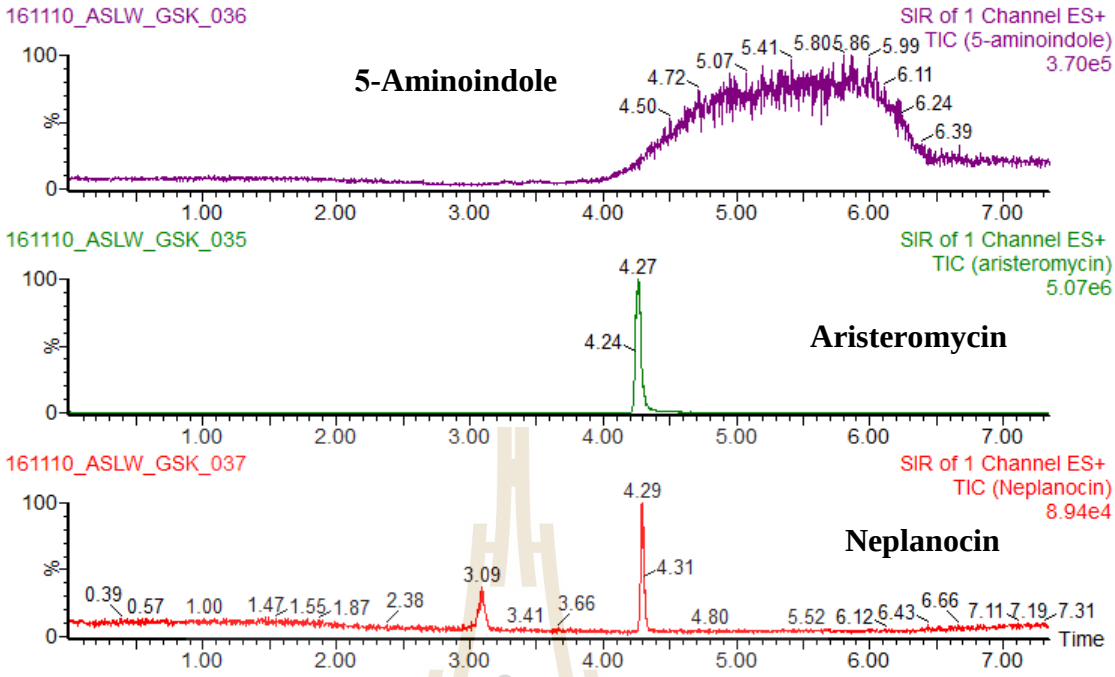



**Figure 4.5** Chromatograms obtained by SFC-MS method. Conditions: gradient elution (see Section 3.4.2) of $CO_2$ (solvent A) and 20 mM ammonium formate in MeOH (solvent B), DIOL column, 1 mL/min, 0.1% (v/v) formic acid in MeOH make-up solvent (Continued).

Results in Figure 4.4 and Figure 4.5 showed that when water was used as additive, the separation was improved for 5-aminoindole (Figure 4.4) while 20 mM ammonium formate showed peak improving for 4-amino-3-chloropyridine.

The binary additive of 2% (v/v) water with 20 mM ammonium formate was thus investigated to find the optimum mobile phase composition. Other binary additives were also studied including 2% (v/v) water with 30 mM ammonium formate and 2% (v/v) water with 20 mM ammonium acetate (results shown in Appendix A).

The separation obtained from the three binary additive systems revealed that both separation efficiency and peak intensity depended on type of additive.

From the results, the best additive system for the analysis was 2% (v/v) water with 30 mM ammonium formate. The separation from this condition is shown in Figure 4.6.

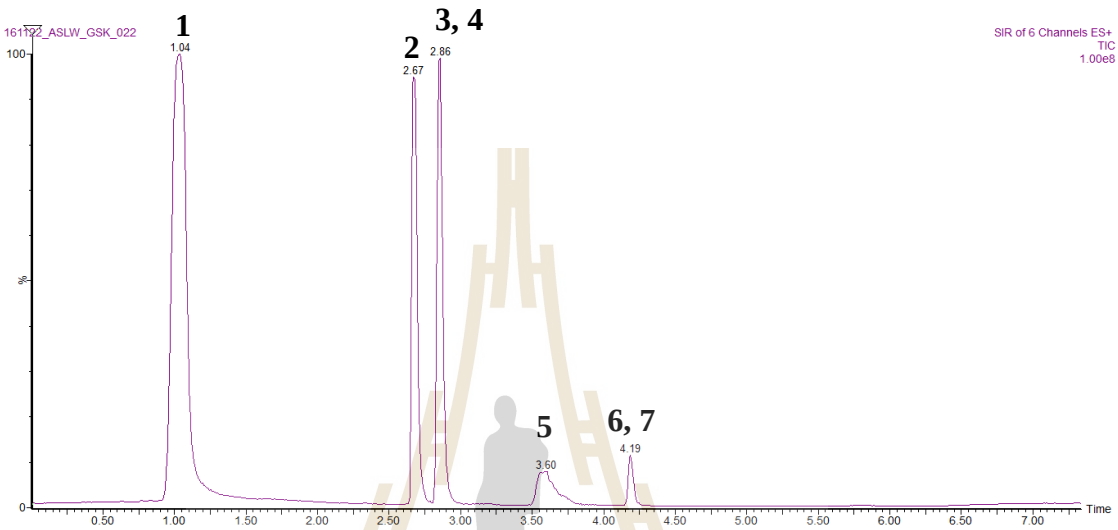



**Figure 4.6** Chromatogram obtained by SFC-MS method. (1) 4-Ethylaniline, (2) 4-amino-3-chloropyridine, (3) 2-amino-5-methyl-1,3,4-thiadiazole, (4) 4-amino-2-chloropyridine, (5) 5-aminoindol, (6) aristeromycin and (7) neplanocin. Conditions: gradient elution (see Section 3.4.2) of $CO_2$ (solvent A) and 2% (v/v) water in MeOH containing 30 mM ammonium formate (solvent B), DIOL column, 1 mL/min, 0.2% (v/v) formic acid in MeOH make-up solvent.

### 4.2.2 Effect of column

In order to achieve good selectivity for the separation of the polar analytes, polarity matching between the column and the analytes is needed. A total of eleven stationary phases with different functional group were investigated (see structures in Figure 3.4), nine columns were from PrincetonSFC column; DIOL, 4-Ethyl-pyridine (4EP), Amino, Diethyl-Amino (DEAP), Benzamide (BEN), Cyano (CN), Propyl-acetamide (PA), HA-Dipyridyl (HAD), HA-DHP and two columns were from Luna;

Luna-Silica and Luna-$NH_2$. The separations obtained from the eleven columns are shown in Figure 4.7

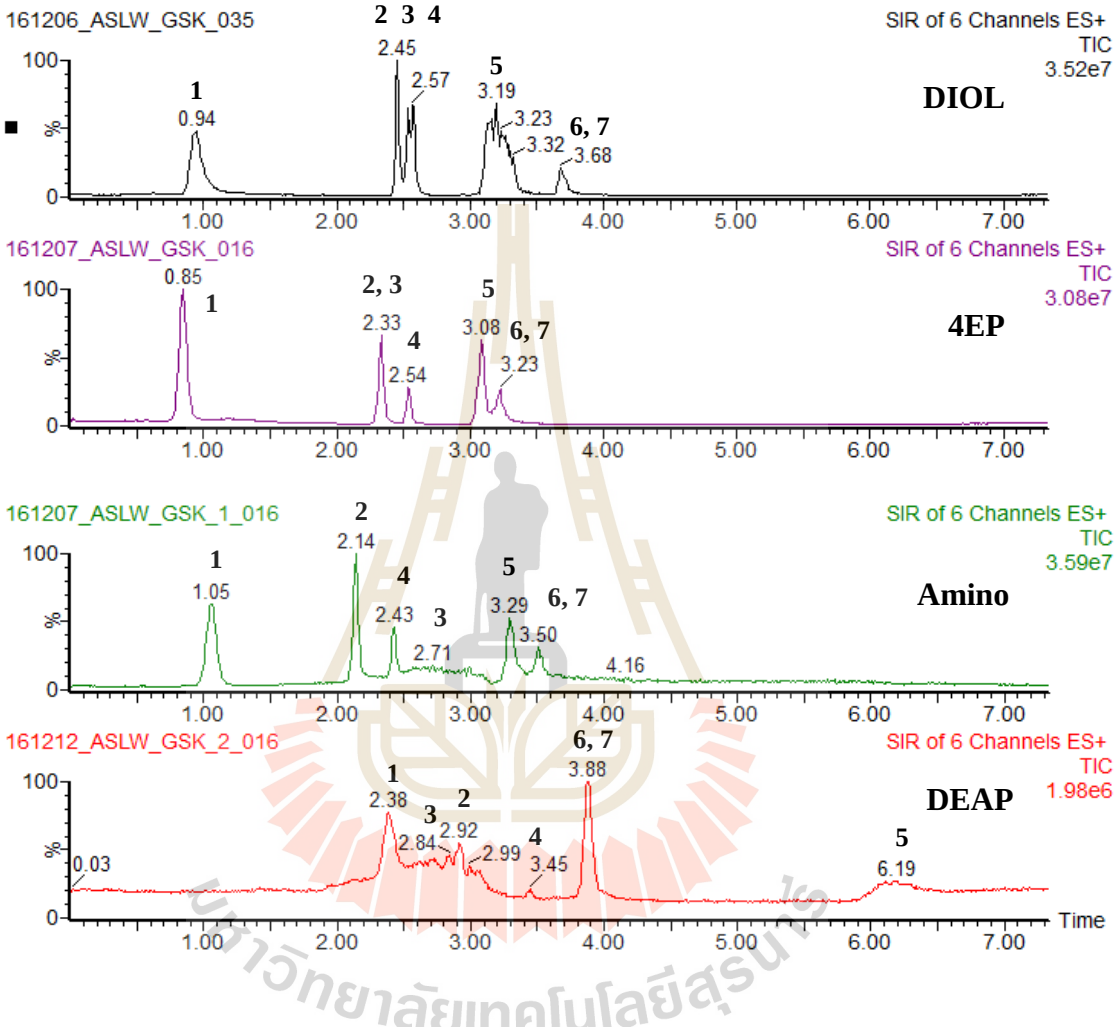



**Figure 4.7** Chromatogram obtained for the 11 columns by SFC-MS method. (1) 4-Ethylaniline, (2) 4-amino-3-chloropyridine, (3) 2-amino-5-methyl-1,3,4-thiadiazole, (4) 4-amino-2-chloropyridine, (5) 5-aminoindol, (6) aristeromycin and (7) neplanocin. Conditions: gradient elution (see Section 3.4.2) of $CO_2$ (solvent A) and 2% (v/v) water in MeOH containing 30 mM ammonium formate (solvent B), 1 mL/min, 0.2% (v/v) formic acid in MeOH make-up solvent.

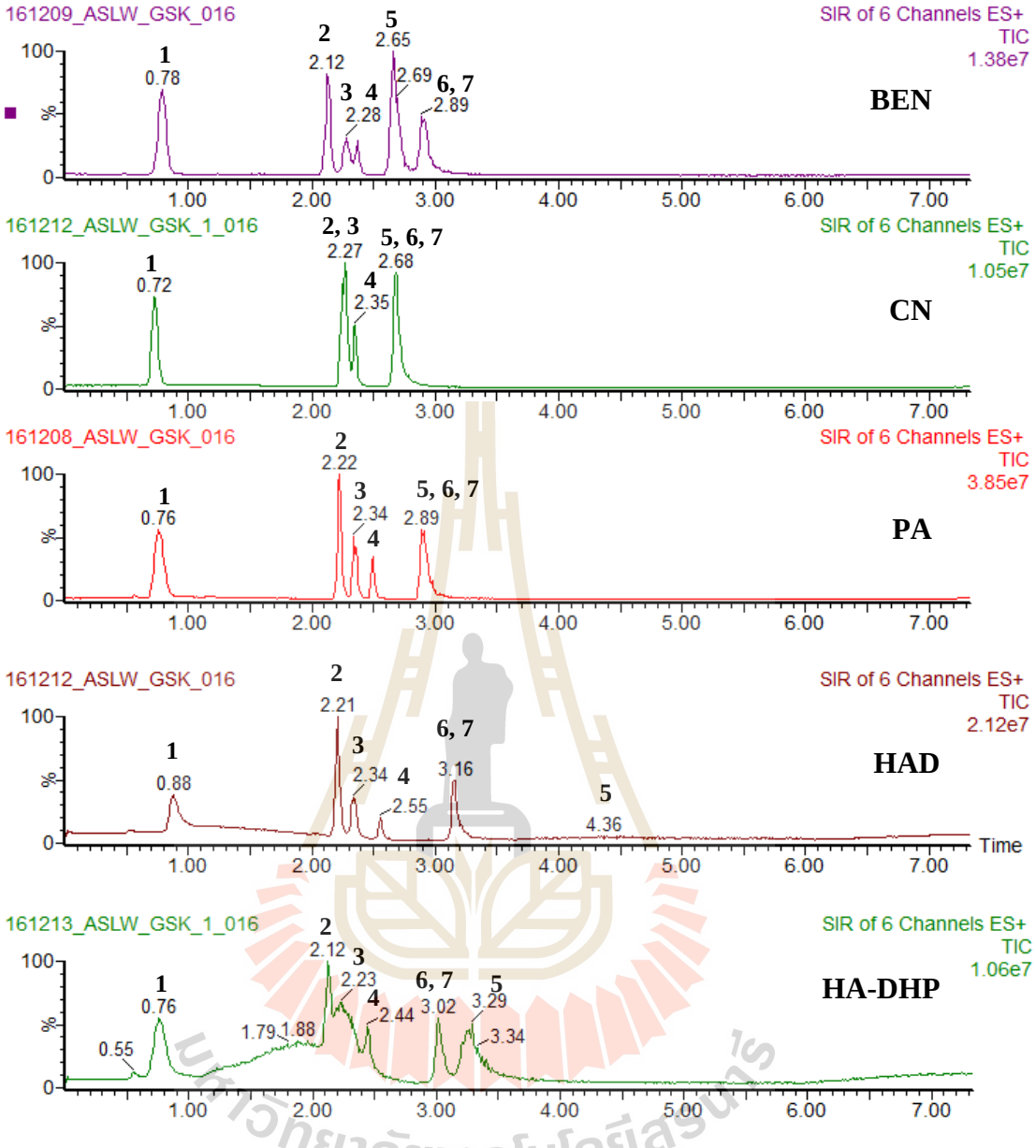



**Figure 4.7** Chromatogram obtained for the 11 columns by SFC-MS method. (1) 4-Ethylaniline, (2) 4-amino-3-chloropyridine, (3) 2-amino-5-methyl-1,3,4-thiadiazole, (4) 4-amino-2-chloropyridine, (5) 5-aminoindol, (6) aristeromycin and (7) neplanocin (Continued).

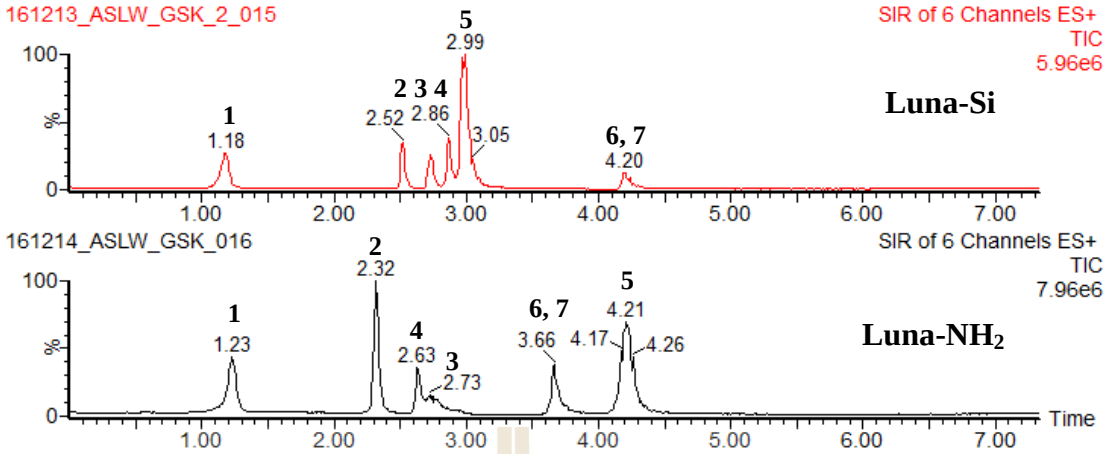



**Figure 4.7** Chromatogram obtained for the 11 columns by SFC-MS method. (1) 4-Ethylaniline, (2) 4-amino-3-chloropyridine, (3) 2-amino-5-methyl-1,3,4-thiadiazole, (4) 4-amino-2-chloropyridine, (5) 5-aminoindol, (6) aristeromycin and (7) neplanocin (Continued).

Since all the columns are relatively polar, the separation mechanisms are like in normal phase chromatography where the least polar compound is eluted first and the most polar compound come out last.

The elution orders obtained for the 11 columns were slightly different indicating different selectivities from the studied columns. Since the analytes contain both nonpolar aromatic and polar groups on their structures, the interactions to the stationary phases could be due to both hydrophobic and hydrophilic interactions.

The analytes were retained less on CN phase than the other phases whereas the analytes were more retained on the DIOL, Amino, DEAP, HA-DHP and Luna-$NH_2$ columns containing amino or hydroxyl groups on their surfaces which can caused more hydrophilic interaction. However the strong interaction by the amino or hydroxyl groups potentially caused poor peak shape for some compounds.

The separation were slightly better resolved on 4EP, BEN, PA and HAD which contain both nonpolar group like aromatic or alkyl group and also polar group like amino, hydroxyl or carbonyl group on the surfaces. The BEN column provided the best separation for the analytes since containing many functional groups including aromatic, alkyl, amino and carbonyl groups causing the highest selective in the separation. Otherwise, the analytes were also separated well on Luna-Si possibly due to there is more density of the functional group influenced in better separation obtained.

It was observed that the separation obtained from the BEN and Luna-Si were better than the others. The BEN column was selected due to its shorter separation time.

From the results, it is noticeably seen that no separation between aristeromycin and neplanocin was obtained due to their chemically structural similarities. However, both of them can be analyzed simultaneously according to the difference of their mass to charge ratio (m/z).

**4.2.3 Effect of flow rate**

In order to obtain fast separation, the analyte mixture was run at higher flow rates. Figure 4.8 shows chromatograms of the standard mixture run on BEN column at three different flow rates (1, 1.5 and 2 mL/min).

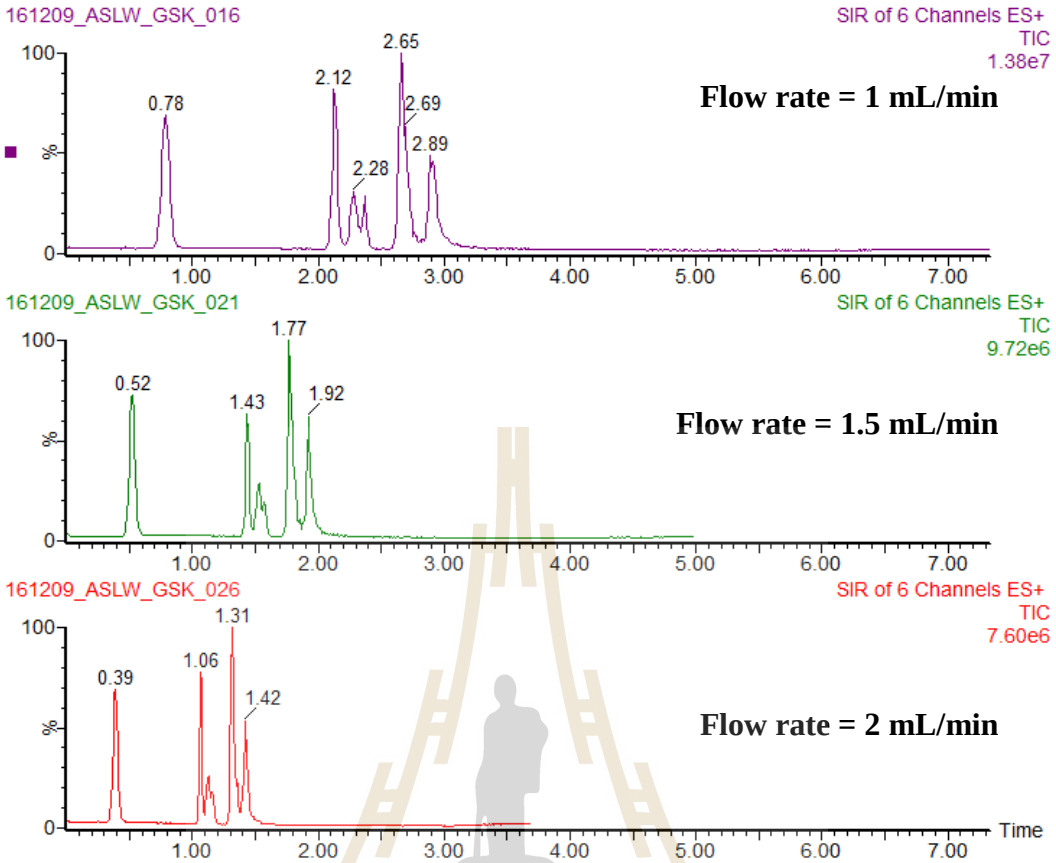



**Figure 4.8** Chromatograms obtained for the 3 flow rates by SFC-MS method. Conditions: gradient elution of $CO_2$ (solvent A) and 2% (v/v) water in MeOH containing 30 mM ammonium formate (solvent B), BEN column, 0.2% (v/v) formic acid in MeOH make-up solvent.

The above chromatograms demonstrated that at the higher flow rate (2 mL/min), the separation time was significantly decreased with slight loss of resolution. Thus, flow rate of 2 mL/min was selected for further optimization.

### 4.2.4 Gradient elution study

In this Section, linear gradient elution was optimized at flow rate of 2 mL/min to obtain good and fast separation. Six different linear gradients were examined as explained in Section 3.4.4. The six gradients started at 2% of co-solvent

(2% (v/v) water in MeOH containing 30 mM ammonium formate) and linearly increased to increase the mobile phase strength as shown in Figure 4.9. Chromatograms for each linear gradient are shown in Figure 4.10.

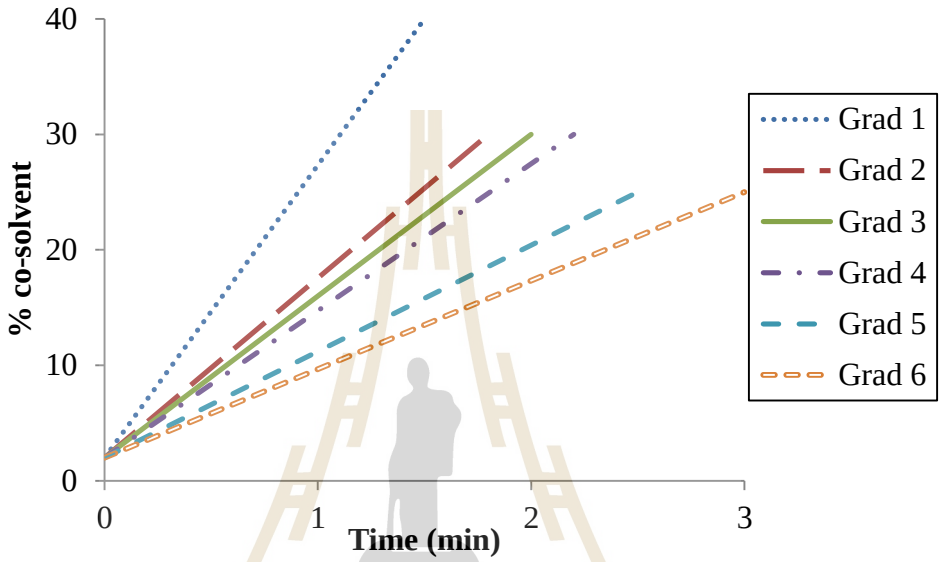



**Figure 4.9** Linear gradient elution for the analysis.

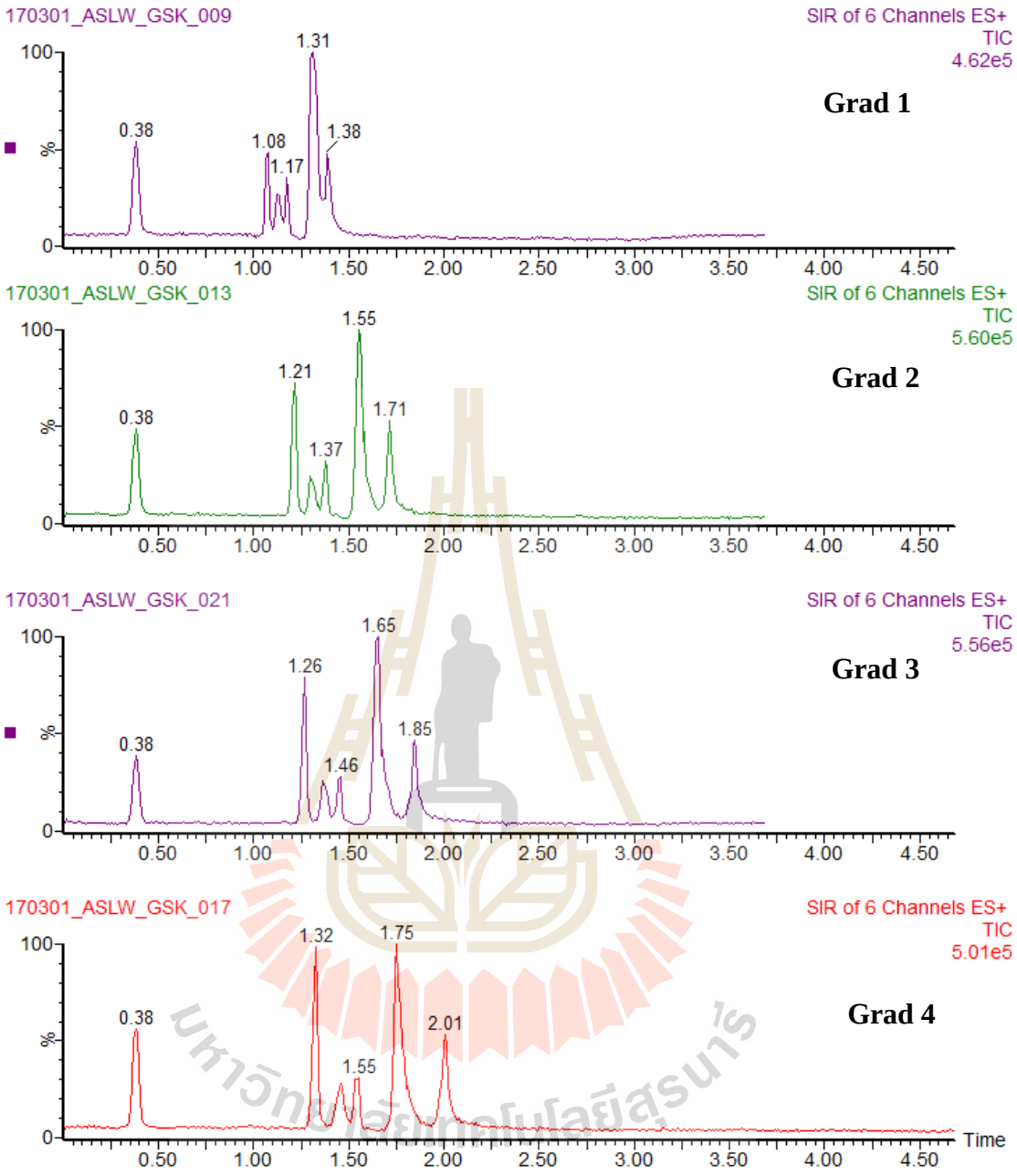



**Figure 4.10** Chromatograms obtained for the 6 gradient elutions by SFC-MS method. Conditions: gradient elution of $CO_2$ (solvent A) and 2% (v/v) water in MeOH containing 30 mM ammonium formate (solvent B), BEN column, 2 mL/min, 0.2% (v/v) formic acid in MeOH make-up solvent.

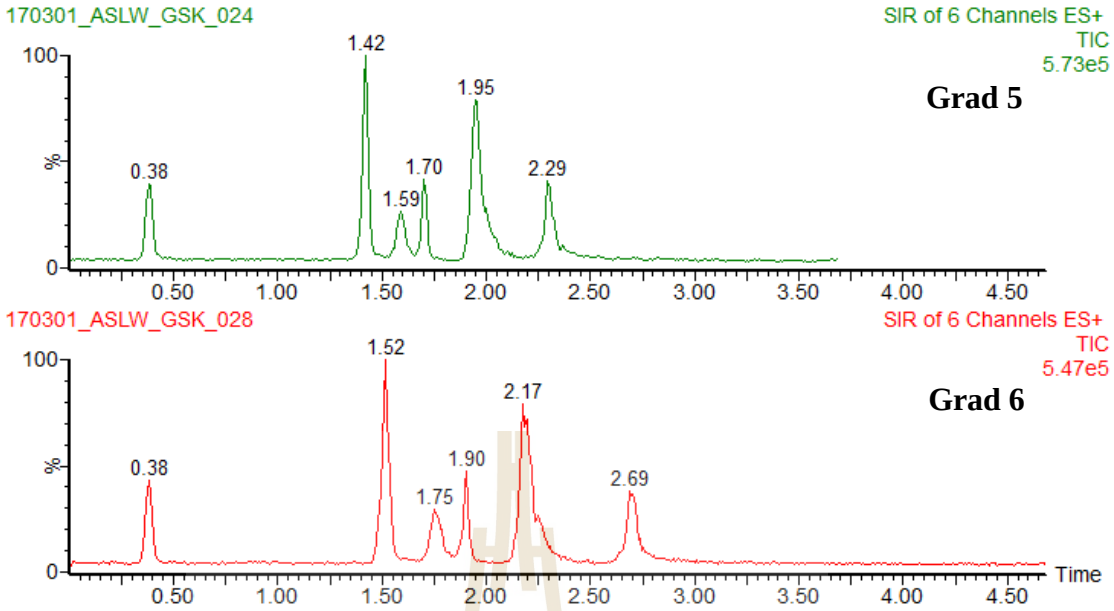



**Figure 4.10** Chromatograms obtained for the 6 gradient elutions by SFC-MS method. Conditions: gradient elution of $CO_2$ (solvent A) and 2% (v/v) water in MeOH containing 30 mM ammonium formate (solvent B), BEN column, 2 mL/min, 0.2% (v/v) formic acid in MeOH make-up solvent (Continued).

As expected, the results (Figure 4.10) showed that slower increasing rate of mobile phase strength provided longer retention or separation time with better resolution but also wider peaks. The Grad 1 gave the fastest separation with the worst resolution. To compromise between the resolution, peak width and separation time, gradient elution of Grad 5 was selected.

The optimum chromatographic conditions for analysis of the analytes are summarized in the table below.

**Table 4.4** Optimum chromatographic conditions for the separation.

| Parameter | Condition |
|---|---|
| Column | Benzamide |
| Primary mobile phase | $CO_2$ |
| Co-solvent | 2% (v/v) water in MeOH containing 30 mM ammonium formate |
| Make-up solvent | 0.2% (v/v) formic acid in MeOH |
| Flow rate | 2 mL/min |
| Linear gradient | Grad 5 (2% B at 0 min to 25% B in 2.5 min) |
| Run time | 3.7 min |

## 4.3 SFC-MS/MS for urine analysis

Another aim of this work is to apply the developed method for the analysis in urine sample. In order to increase the selectivity of the method for analysis of complex sample like urine, multiple reaction monitoring (MRM) was used as the second MS detection after the SF-MS, so called SFC-MS/MS.

A chromatogram for the spiked urine is shown in Figure 4.11.

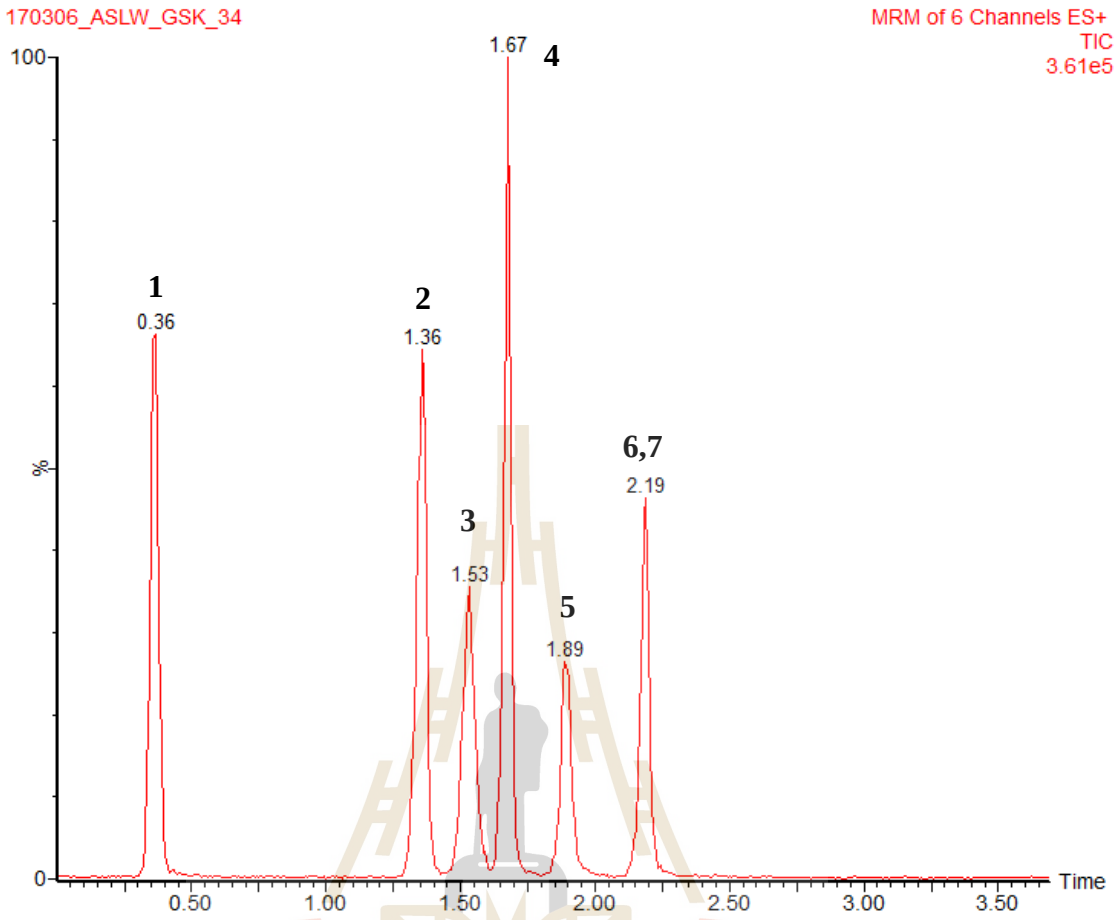



**Figure 4.11** Chromatogram obtained by SFC-MS/MS method. (1) 4-Ethylaniline, (2) 4-amino-3-chloropyridine, (3) 2-amino-5-methyl-1,3,4-thiadiazole, (4) 4-amino-2-chloropyridine, (5) 5-aminoindol, (6) aristeromycin and (7) neplanocin. Conditions: gradient elution (Grad 5) of $CO_2$ (solvent A) and 2% (v/v) water in MeOH containing 30 mM ammonium formate (solvent B), BEN column, 2 mL/min, 0.2% (v/v) formic acid in MeOH make-up solvent.

In Figure 4.11, only six chromatographic peaks for the seven compounds were obtained due to the peak overlapping of aristeromycin and neplanocin since the two compounds are structurally similar. The compounds are difficult to separate.

However, the selective monitoring by MS/MS allowed them to be analyzed in a single run. The results for analysis are shown in Table 4.5.

**Table 4.5** Quantification of spiked urine sample.

| Compound | Concentration (mg/L) | |
|---|---|---|
| | Added | Found |
| 4-Ethylaniline | 7.00 | 7.37 |
| 4-Amino-3-chloropyridine | 7.00 | 7.46 |
| 2-Amino-5-methyl-1,3,4-thiadiazole | 7.00 | 7.40 |
| 4-Amino-2-chloropyridine | 7.00 | 7.23 |
| 5-Aminoindole | 7.00 | 7.66 |
| Aristeromycin | 3.50 | 3.76 |
| Neplanocin | 3.50 | 3.31 |

To ensure that there is no interference from the urine matrix, blank urine was run under the optimum condition. Clean chromatogram of the blank urine in Figure 4.12 indicated that no signal from urine matrix was found.

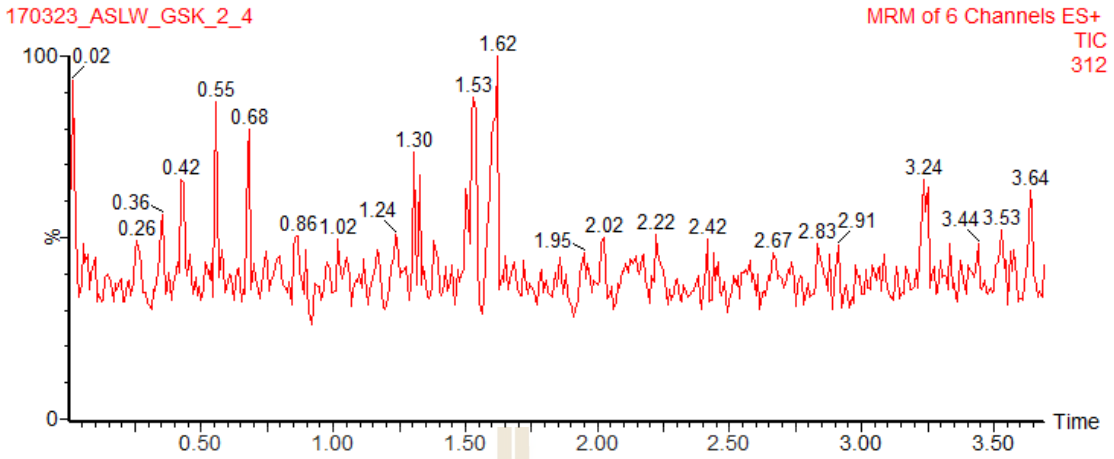



**Figure 4.12** Chromatogram of blank urine obtained by SFC-MS/MS method. Conditions: gradient elution (Grad 5) of $CO_2$ (solvent A) and 2% (v/v) water in MeOH containing 30 mM ammonium formate (solvent B), BEN column, 2 mL/min, 0.2% (v/v) formic acid in MeOH make-up solvent.

## 4.4 Method validation

In this part, the method was validated to evaluate the performance of the developed SFC-MS/MS method. Parameters and testes for method validation including linearity, precision, accuracy and sensitivity were studied.

### 4.4.1 Linearity study

Linearity of the method was studied by constructing calibration or standard curves from running series of five concentration standards in urine matrix (diluted with MeOH). The linear curves were constructed by plotting the peak area observed against the concentration. Linear calibration curves for all the compounds (Appendix B) were obtained in the range of ppm levels with good correlation coefficients ($R^2$>0.9988). The calibration equations and $R^2$ are summarized in Table 4.6.

**Table 4.6** Calibration equation and $R^2$ of each compound.

| Compound | Calibration equation | $R^2$ |
|---|---|---|
| 4-Ethylaniline | y = 106.78x - 37.227 | 0.9996 |
| 4-Amino-3-chloropyridine | y = 175.22x + 35.119 | 0.9995 |
| 2-Amino-5-methyl-1,3,4-thiadiazole | y = 157.64x - 73.159 | 0.9994 |
| 4-Amino-2-chloropyridine | y = 191.67x - 83.718 | 0.9994 |
| 5-Aminoindole | y = 71.255x - 11.006 | 0.9994 |
| Aristeromycin | y = 114.26x + 29.435 | 0.9988 |
| Neplanocin | y = 98.875x + 5.7924 | 0.9989 |

### 4.4.2 Precision and accuracy

Precision of the method was validated in terms of percentages of relative standard deviations (%RSD) for peak area and retention time. The analytes were injected for five replicates within one day for intra-day precision and for three consecutive days for inter-day precision.

The obtained %RSD for peak area were from 0.58 to 2.82% (intra-day precision) and 1.18 to 5.54% (inter-day precision) and for retention time were from 0.0 to 0.20% (intra-day precision) and 0.18 to 0.70% (inter-day precision).

Accuracy of the method was evaluated by calculating the percentage recoveries. %Recoveries for the seven compounds are shown in Table 4.7.

**Table 4.7** %RSD and %recovery obtained by the SFC-MS/MS.

| Compound | %RSD | | | | Relative recovery (%) |
|---|---|---|---|---|---|
| | Intra-day | | Inter-day | | |
| | Area | RT | Area | RT | |
| 4-Ethylaniline | 0.58 | 0.0 | 1.18 | 0.70 | 105.3 |
| 4-Amino-3-chloropyridine | 2.20 | 0.0 | 3.43 | 0.19 | 106.5 |
| 2-Amino-5-methyl-1,3,4-thiadiazole | 1.17 | 0.0 | 2.42 | 0.23 | 105.7 |
| 4-Amino-2-chloropyridine | 0.42 | 0.0 | 1.99 | 0.36 | 103.3 |
| 5-Aminoindole | 1.59 | 0.0 | 2.58 | 0.24 | 109.5 |
| Aristeromycin | 2.82 | 0.20 | 4.28 | 0.28 | 107.4 |
| Neplanocin | 1.50 | 0.0 | 5.54 | 0.18 | 94.6 |

The obtained results in Table 4.7 demonstrated good reliability and accuracy of the method for analysis of the analytes in urine sample.

**4.4.3 Sensitivity**

Sensitivity of the method was reported in terms of limit of quantification (LOQ) and limit of detection (LOD). The LOD is the lowest concentration of analyte in a sample that can be detected while the LOQ is the minimum level of the analyte which can be quantified with acceptable accuracy and precision.

The LOD and LOQ were found to be in the ranges of 0.4-0.6 ppm and 1.2-1.8 ppm, respectively. Table 4.8 shows LOD and LOQ for each analytes investigated under the optimum condition.

**Table 4.8** LOD(s) and LOQ(s) for the seven compounds analyzed by the developed SFC-MS/MS method.

| Compound | Sensitivity (mg/L) | |
|---|---|---|
| | LOQ | LOD |
| 4-Ethylaniline | 1.4 | 0.5 |
| 4-Amino-3-chloropyridine | 1.6 | 0.5 |
| 2-Amino-5-methyl-1,3,4-thiadiazole | 1.8 | 0.6 |
| 4-Amino-2-chloropyridine | 1.7 | 0.6 |
| 5-Aminoindole | 1.7 | 0.6 |
| Aristeromycin | 1.2 | 0.4 |
| Neplanocin | 1.2 | 0.4 |

# CHAPTER V

# CONCLUSIONS

In this work, a new, rapid and green analytical method was developed for determination of seven drug-like compounds including 4-ethylanilene, 4-amino-3-chloropyridine, 2-amino-5-methyl-1,3,4-thiadiazole, 4-amino-2-chloropyridine, 5-aminoindole, aristeromycin and neplanocin. The method is a hyphenated technique based on coupling of a SFC separation and a selective MS/MS detection, called SFC-MS/MS.

Various experimental parameters for MS/MS detection and SFC separation were optimized. The investigated parameters included cone voltage, collision energy, mobile phase additive, type of stationary phase, flow rate and gradient elution.

At the optimum condition, the compounds were separated on a Princeton Benzamide column (3.0 x 50 mm, 3 µm) with a flow rate of 2.0 mL/min using a $CO_2$ mobile phase with co-solvent of 2% (v/v) water in methanol containing 30 mM ammonium formate. The method required short analysis time of 3.7 min using gradient elution. Mass spectrometric detection was performed in positive ion electrospray mode (ESI+) with MRM.

Matrix-matched standards in urine were used for calibration construction. Good linearity for the analytes was observed ($R^2 > 0.9988$).

The limits of detection and quantification were between 0.4-0.6 mg/L and 1.2-1.8 mg/L, respectively. Precision in terms of %RSD for peak area and retention time were less than 5.54% and 0.70%, respectively. The method was applied for analysis of the compounds in spiked urine sample. Relative recoveries were in the range of 94.6% to 109.5%. The developed method has been shown acceptable reliability and accuracy. The method can be adopted for fast screening of other compounds with similar chemical structures.

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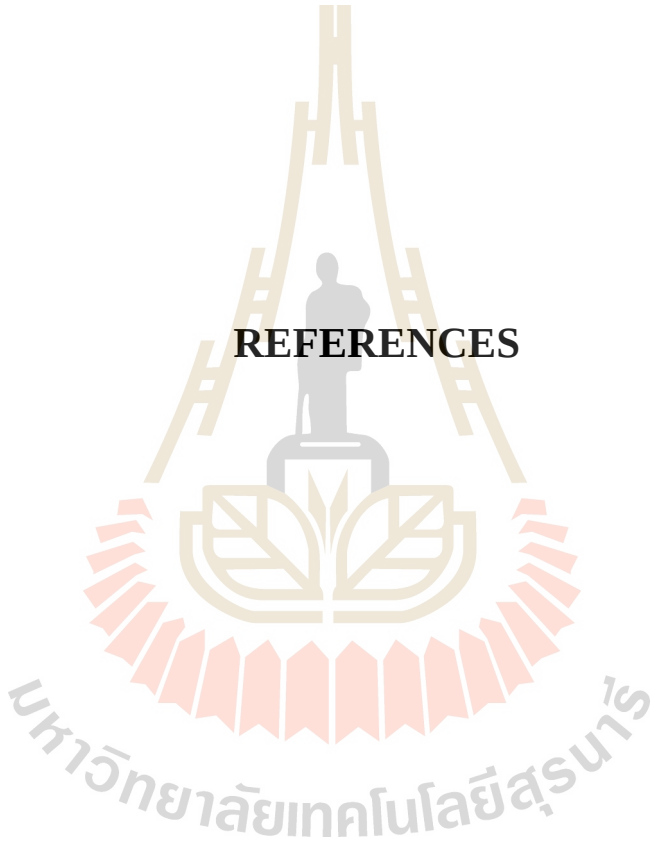

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# APPENDICES

# APPENDIX A

# CHROMATOGRAM: AN ADDITIVE VARYING

## A.1 Chromatogram of analyte mixture with 2% (v/v) water in MeOH containing 20 mM ammonium formate

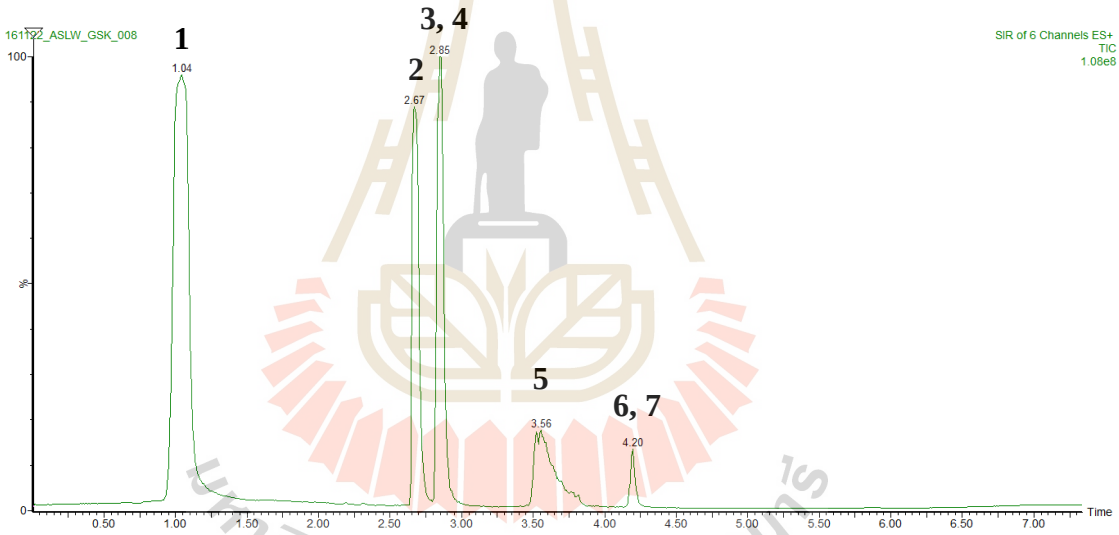


**Figure A.1** Chromatogram obtained by SFC-MS method. (1) 4-Ethylaniline, (2) 4-amino-3-chloropyridine, (3) 2-amino-5-methyl-1,3,4-thiadiazole, (4) 4-amino-2-chloropyridine, (5) 5-aminoindol, (6) aristeromycin and (7) neplanocin. Conditions: gradient elution (see Section 3.4.2) of $CO_2$ (solvent A) and 2% (v/v) water in MeOH containing 20 mM ammonium formate (solvent B), DIOL column, 1 mL/min, 0.2% (v/v) formic acid in MeOH make-up solvent.

## A.2 Chromatogram of analyte mixture with 2% (v/v) water in MeOH containing 20 mM ammonium acetate

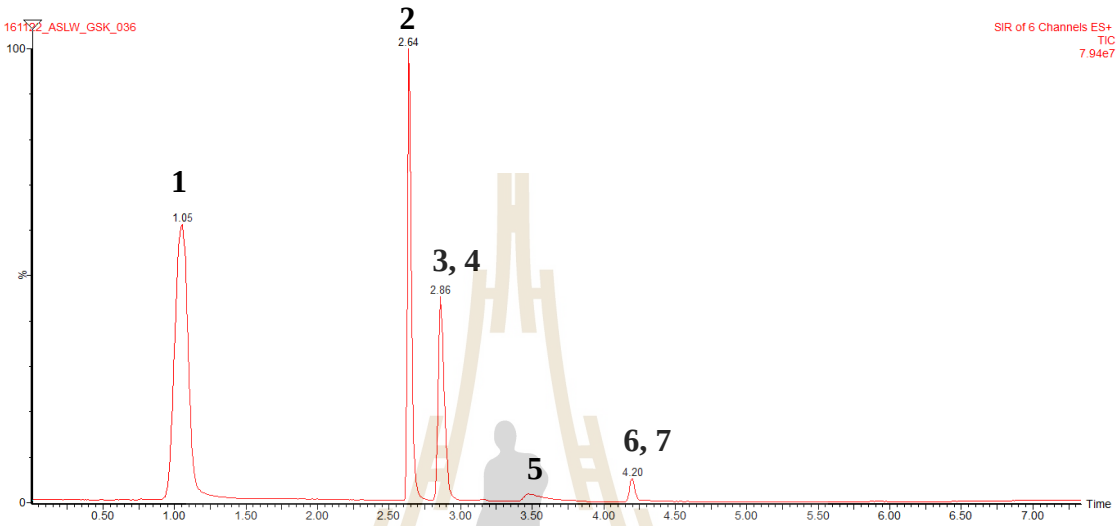


**Figure 4.8** Chromatogram obtained by SFC-MS method. (1) 4-Ethylaniline, (2) 4-amino-3-chloropyridine, (3) 2-amino-5-methyl-1,3,4-thiadiazole, (4) 4-amino-2-chloropyridine, (5) 5-aminoindol, (6) aristeromycin and (7) neplanocin. Conditions: gradient elution (see Section 3.4.2) of $CO_2$ (solvent A) and 2% (v/v) water in MeOH containing 20 mM ammonium acetate (solvent B), DIOL column, 1 mL/min, 0.2% (v/v) formic acid in MeOH make-up solvent.

# APPENDIX B

# LINEAR CALIBRATION

## B.1 Calibration curve of 4-ethylaniline

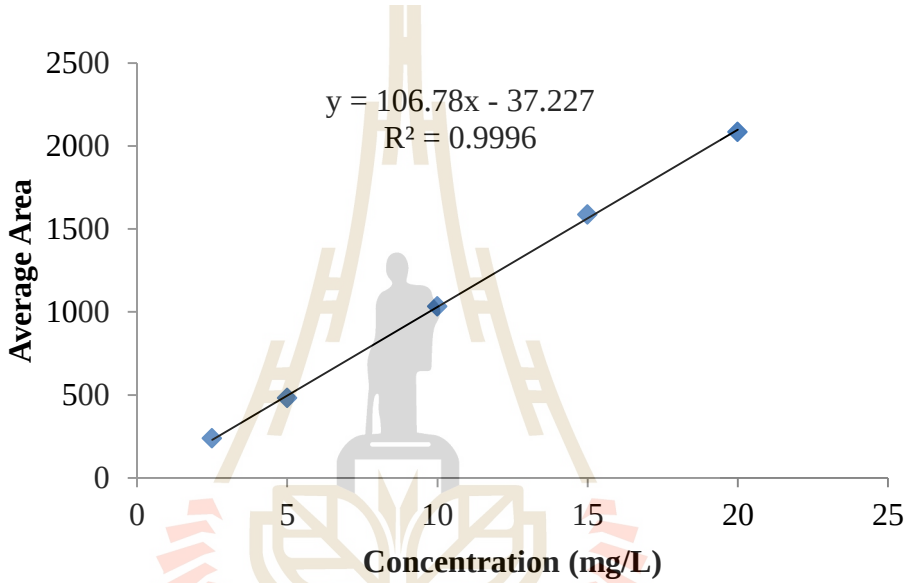


**Figure B.1** The calibration curve of 4-ethylaniline.

## B.2 Calibration curve of 4-amino-3-chloropyridine

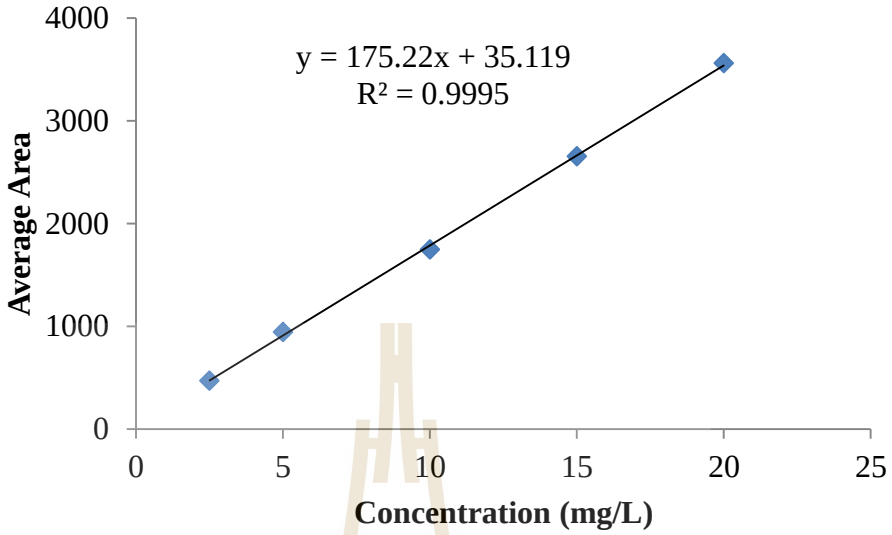


**Figure B.2** The calibration curve of 4-amino-3-chloropyridine.

## B.3 Calibration curve of 2-amino-5-methyl-1,3,4-thiadiazole

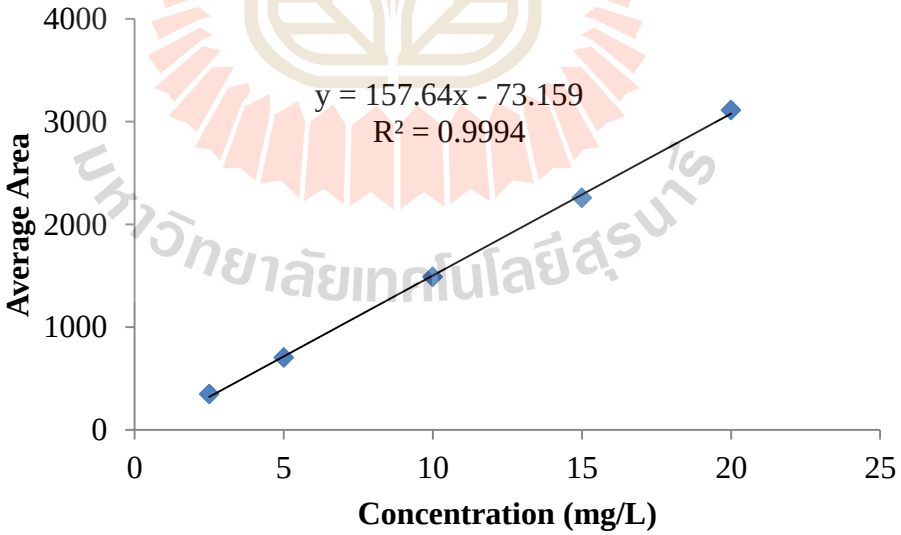


**Figure B.3** The calibration curve of 2-amino-5-methyl-1,3,4-thiadiazole.

## B.4 Calibration curve of 4-amino-2-chloropyridine

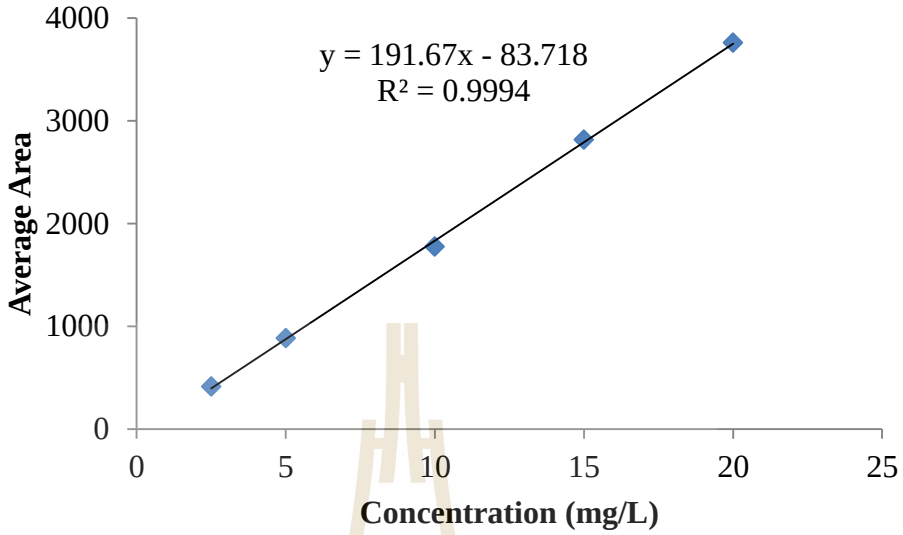


**Figure B.4** The calibration curve of 4-amino-2-chloropyridine.

## B.5 Calibration curve of 5-aminoindole

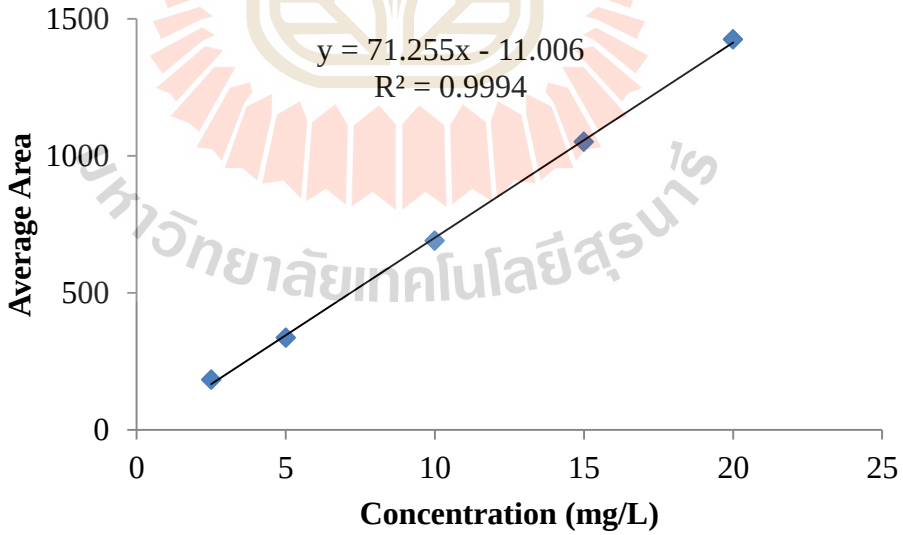


**Figure B.5** The calibration curve of 5-aminoindole.

## B.6 Calibration curve of aristeromycin

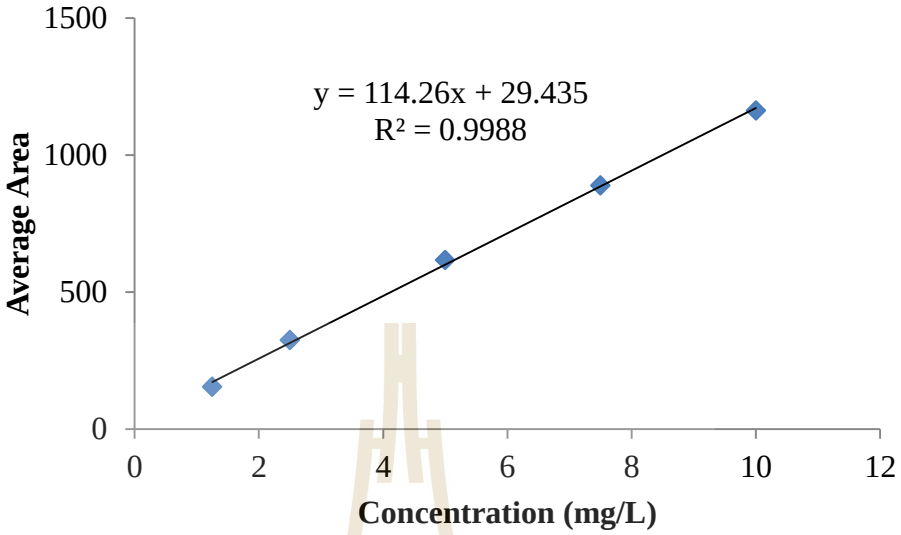


**Figure B.6** The calibration curve of aristeromycin.

## B.7 Calibration curve of neplanocin

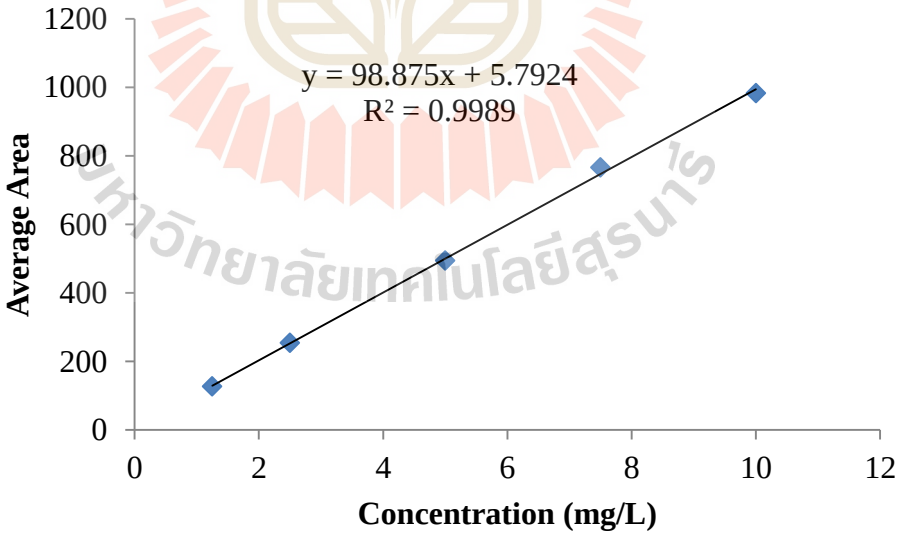


**Figure B.7** The calibration curve of neplanocin.

# CURRICULUM VITAE

**Name** Lalida Wongbuth

**Date of Birth** 29 April 1992

**Place of Birth** Maha Sarakham, Thailand

**Education**

2004 - 2009 Secondary school (Mathematics and Science Program) Mahasarakham University Demonstration School, Mahasarakham, Thailand

2010 - 2014 Bachelor of Science (Chemistry, 1st class honors), Khon Kaen University, Khon Kaen, Thailand

2014 - 2017 Student at School of Chemistry, Suranaree University of Technology, Nakhon Ratchasima, Thailand

**Grants and Fellowships**

Development and Promotion of Science and Technology Talents Project (Royal Government of Thailand scholarship)