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CHARACTERIZATION OF NON-VOLATILE CHEMICAL CONSTITUENTS IN THAI WINE USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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เปรมกมล การภักดี : การพิสูจน์เอกลักษณ์องค์ประกอบทางเคมีที่ระเหยยากในไวน์ไทยโดยใช้ลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมตรี (CHARACTERIZATION OF NON-VOLATILE CHEMICAL CONSTITUENTS IN THAI WINEUSING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.ธรรมนูญ หนูจักร, 65 หน้า.

ในงานวิจัยนี้ ได้พัฒนาและตรวจสอบความใช้ได้ของวิธีของเทคนิคไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟีแทนเดมแมสสเปกโทรเมตรี (HPLC/MS-MS) สำหรับการวิเคราะห์ปริมาณสารระเหยยากแบบจำเพาะคราวเดียวกัน ได้แก่ กรดอินทรีย์จำนวน 5 ชนิดและสารประกอบฟีนอลิกจำนวน 20 ชนิดในตัวอย่างไวน์ 19 ตัวอย่างซึ่งประกอบด้วย ไวน์แดงต่างประเทศ 2 ตัวอย่าง ไวน์แดงไทย 15 ตัวอย่าง และ ไวน์ผลไม้ 2 ตัวอย่าง โดยใช้ภาวะ HPLC-MS/MS ได้แก่ C18 คอลัมน์ และเฟสเคลื่อนที่แบบเกรเดียน A และ B ที่มีอัตราการไหล 0.4 มิลลิลิตร/นาที โดย A คือ 0.1% โดยปริมาตรของกรดฟอร์มิกในน้ำ และ B คือ 0.1% โดยปริมาตรของกรดฟอร์มิกในเมทานอล ขีดจำกัดการตรวจหาและขีดจำกัดการบอกปริมาณของการตรวจวิเคราะห์สารประกอบแบบจำเพาะนี้อยู่ในช่วง 0.0084-0.48 และ 0.026-1.45 มิลลิกรัมต่อลิตร ตามลำดับ จากการเติมสารมาตรฐานที่ทราบค่าความเข้มข้นลงในตัวอย่างไวน์เจือจาง ความถูกต้องของวิธีการวิเคราะห์ที่อยู่ในเกณฑ์การยอมรับคิดเป็น 84.9% ของข้อมูลทั้งหมดที่อยู่ในเกณฑ์การยอมรับได้ในช่วง 80-110% รวมไปถึงความเที่ยงที่มีค่าร้อยละส่วนเบี่ยงเบนมาตรฐานอยู่ระหว่าง 0.4-7% ซึ่งอยู่ในเกณฑ์การยอมรับได้ทั้งหมด สำหรับการวิเคราะห์ตัวอย่างจริง สารประกอบฟีนอลิกในตัวอย่างทั้งหมดพบในช่วง 0.00423-69.2 มิลลิกรัมต่อลิตร สารประกอบฟีนอลิกหลักที่มีความเข้มข้นสูงสุด ได้แก่ กรดแกลลิก กรดแคฟทาริก และแคชทิชิน ส่วนกรดอินทรีย์หลักที่มีความเข้มข้นสูงสุด ได้แก่ กรดแลคติก กรดทาทาริก และกรดซักซินิก จากการประมวลผลด้วยเทคนิค PCA พบว่าจำแนกได้เป็น 3 คลัสเตอร์ ได้แก่ คลัสเตอร์ A ประกอบด้วย 12 ไวน์แดงไทยจาก 3 แหล่งผลิต คลัสเตอร์ B ประกอบด้วย ไวน์แดงไทย 3 ชนิด และไวน์แดงต่างประเทศ 2 ชนิด และคลัสเตอร์ C ประกอบด้วยไวน์ผลไม้ 2 ชนิด นอกจากนี้ได้ตรวจพบสารที่มีความสำคัญสำหรับแต่ละคลัสเตอร์ ประกอบด้วย คลัสเตอร์ A ได้แก่ กรดไซรินจิก คลัสเตอร์ B ได้แก่ เคมเฟอรอล และคลัสเตอร์ C ได้แก่ กรดซิตริกและกรดมาร์ลิก ดังนั้นอาจใช้การวิเคราะห์ด้วยเทคนิคไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟีแทนเดม-แมสสเปกโทรเมตรีร่วมกับการประมวลผลด้วยเทคนิค PCA ในการควบคุมคุณภาพในการผลิตและการพิสูจน์ของแท้ของไวน์ได้

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PREMKAMOL KARAPAKDEE: CHARACTERIZATION OF NON-VOLATILE CHEMICAL CONSTITUENTS IN THAI WINEUSING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY. ADVISOR: ASSOC. PROF. THUMNOON NHUJAK, Ph.D., 65 pp.

In this work, high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was developed and validated for simultaneous determination of targeted non-volatile compounds, such as five organic acids and twenty phenolic compounds, in 19 wine samples including 2 imported red wines, 15 Thai red wines and 2 fruit wines, using the following HPLC-MS/MS conditions: a C18 analytical column and gradient elution of A: B mobile phase at flow rate 0.4 mL/min, where A and B are 0.1% v/v formic acid in water and 0.1% v/v formic acid in methanol. The limit of detection (LOD) and the limit of quantitation (LOQ) for these targeted compounds were obtained in a range of 0.0084-0.48 mg/L and 0.026-1.45 mg/L, respectively. By spiking known concentration standards in diluted pooled wine samples, the acceptable accuracy and precision were obtained with 84.9% of 225 data sets falling in acceptable criteria in the range of 80-110% and along with %RSD of 0.4-7%. For real sample analysis, the phenolic compounds in all samples were found in a range of 0.00423-69.2 mg/L. The predominant phenolic compounds with the highest concentrations found in all samples include gallic acid, caftaric acid and (+)-catechin. The predominant organic acid compounds with the highest concentrations found in all samples include lactic acid, tartaric acid and succinic acid. Using a PCA approach, three clusters were observed. Cluster A including 12 Thai red wines from 3 origins, cluster B including 3 Thai red wines and 2 imported red wines, and cluster C including 2 fruit wines. The following significant compounds were identified in each cluster, syringic acid for cluster A, kaempferol for cluster B and citric acid and malic acid for cluster C. Therefore, HPLC-MS/MS analysis combined with PCA analysis be used for quality control and authentication of wine.

Department: Chemistry Student's Signature .....

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LIST OF ABBREVIATION

A	slope of calibration curve
AOAC	association of official analytical chemists
$\bar{C}_{cal}$	the mean calibration concentration
C_{18}	octadecyl
C_8	octyl
CE	collision energy
C_i	each of calibration concentration values
CID	collision-induced dissociation
CL	Chateau De Loei
CPE	cloud-point extraction
DC	direct current
Dns	dilute and shoot
ESI	electrospray ionization
FT-ICR	Fourier-transform ion cyclotron resonance
GC	gas chromatography
GM	Granmonte
h_0	the leverage for the blank sample
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
	high performance liquid chromatography
HPLC-MS	mass spectrometry
	high performance liquid chromatography tandem
HPLC-MS/MS	mass spectrometry
ID	internal diameter
IUPAC	International Union of Pure and Applied Chemistry
l	the number of calibration samples
L/min	liter/min
LC	liquid chromatography

LC-MS/MS	liquid chromatography tandem mass spectrometry
LDA	linear discriminate analysis
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantitation
m/z	mass to charge ratio
mg	milligram
mg/L	milligram/liter
min	minute
μL	microliter
mL	milliliter
μm	micrometer
mm	millimeter
MRM	multiple reaction monitoring
MS	mass spectrometry/ mass spectrometer
ms	millisecond
MS/MS	tandem mass spectrometry
°C	degree celsius
PB	PB Valley
PCA	principle component analysis
PETE	polyethylene terephthalate
pH	potential of hydrogen ion
ppm	part per million
psi	pound per square inch
Q	quadrupole
Q1	first quadrupole
Q2	second Quadrupole
Q3	third Quadrupole
QqQ	triple quadrupole
Q-Trap	quadrupole- iontrap

RF	radio frequency
RSD	relative standard deviation
SFE	supercritical fluid extraction
SPE	solid-phase extraction
SV	Silverlake
Sy/x	residual standard deviation
THF	tetrahydrofuran
TOF	Time of flight
t_R	retention time
UHPLC	ultra high performance liquid chromatography
UV	UV-visible detector
UV-Vis	UV-Visible
v/v	volume by volume

CHAPTER I
INTRODUCTION

1.1 Problem Definition

Wine is an alcoholic beverage commonly made from grapes, widely consumed around the world due to its good taste and antioxidant property which provides health benefits [1]. Many studies suggest that moderate wine consumption may enhance heart function and blood pressure, prevent cancer and improve mental health. The moderate drinking means up to one drink per day for women and up to two drinks per day for men according to the United States (U.S.) Dietary Guidelines 2015 to 2020 [2]. Therefore, wine is not just a common alcoholic beverage but its benefits are huge. As a result, wine production areas are spread all over the world.

There are many wine productions and industries worldwide, particularly in Europe including France, Italy, and Spain which are top three wine productions in the world with 4,796,600, 4,607,850, and 4,293,466 tons produced in 2014 [3]. The other areas include United States, China, Argentina and etc. Therefore, wine production is a big industry which provides great benefits to many countries. In Thailand, there has been small wine production. However, some Thai wine products are among the high quality wine which has been documented with the winner award in the world wine competition. Moreover, the wine products are not only obtained from grape but also from other fruits such as lychee or mangosteen. This is an interesting issue in Thailand and the world wine market since Thai wine can be a new alternative for consumers as high-quality wine with the cheaper price. Quality and organoletptic properties of wine, such as color and taste, are mainly governed by its chemical components.

Generally, wine is manufactured from grape via a fermentation process by yeast. The process converses glucose and fructose to ethanol. Normally, wine can be classified into five fundamental groups namely red wine, white wine, Rosé Wine, Sparkling Wine and Fortified Wine [4]. Wine contains a large number of molecules including primary metabolites for example sugars, amino acids, organic acids, and lipids

and secondary metabolites such as phenolics, alkaloids, sterols, lignans, terpenes, and fatty acids. But the major compounds present in wine are organic acids and phenolic acids [5]. Moreover, varieties, geographical origin, production process and production year are important factors attributing to characteristics of wine. Although there are a plenty of researches in wine but Thai wine study is scanty. Thus, the study of Thai wine to a reliable scientific data may help to differentiate wine products in our country and comparative study to further improve Thai wine production. Important studies involves qualitative and quantitative analysis of major non-volatile compounds in Thai wine.

1.2 Literature review

There are several analytical techniques for qualitative and quantitative analysis of wine. In term of separation-based technique, the most widely utilized techniques are gas chromatography (GC) and high performance liquid chromatography (HPLC). With the focus on analysis of non-volatile, HPLC is an extensive technique. HPLC is operated with high pressure level by use of effective pumps to overcome the pressure drop along the packed column. Compared to conventional liquid chromatography (LC), HPLC results in better resolution and faster time of separation [6]. In addition, HPLC system requires lower amounts of sample and mobile phase. The technique is thus capable of analyzing a small sample volume and consuming less solvent.

HPLC hyphenated with mass spectrometry (HPLC-MS) or tandem mass spectrometry (HPLC-MS/MS) is a powerful tool for analysis of complex beverage samples such as wine with high accuracy and sensitivity. MS detection provides high selectivity and sensitivity, enabling identification of molecular masses and reliable quantification for a huge number of compounds in wine samples.

For targeted compound analysis in wine such as phenolic acid, organic acid and sugar, the LC/MS-MS detection usually employed quadrupole (Q), triple quadrupole (QqQ) and quadrupole- iontrap (Q-Trap) mass analyzers due to their selectivity and sensitivity towards targeted compounds.

There are many applications in the area of food and beverage analysis. The examples include analysis of total resveratrol in grape juice, cranberry juice, and wine

[7]. There are also many applications of LC-QqQMS (allowing MS/MS analysis) with various purposes such as classification of red wine with different geographic origin, grape variety and vintage according to the phenol contents [1], simultaneous determination of 20 organic acids and polyphenols in red wine [8], analysis of polyphenolic compounds in liquid samples of grape juice, green tea and coffee [9], rapid analysis of phenolic acids in beverages [10]. Quantitative analysis of sensometabolomic phenolic compounds in red wine [11] and orosensory identification of astringent mouthfeel and bitter-tasting compounds in red wine [12] were also reported by using HPLC-Q-trap operated with MS/MS analysis. Moreover, sample preparation is necessary prior to LC-MS/MS analysis in order to remove matrices which can interfere the detection, improve separation efficiency and prevent the contaminants to the column and MS instrument.

Sample preparation methods for wine analysis include solid-phase extraction (SPE) [13], liquid-liquid extraction (LLE) [14] supercritical fluid extraction (SFE) [15] and cloud-point extraction (CPE) [16]. However, most of the researchers often performed 'dilution and filtration' before LC-MS analysis since this sample preparation method is simple, fast, and cost effective.

Since the data of non-volatile profile can be complicated, the processing method is required to clarify the data. Chemometrics is a powerful mathematical and statistical method to analyze multivariate data with pattern recognition methods to simplify chromatographic results [17]. It provides ability to discrimination and grouping of the data due to its similarity or differentiation. The unsupervised pattern recognition, one of the chemometrics method, is widely used for data processing in analytical chemistry. It helps to discriminate and combine samples with the similar properties into the same group which can be separated from the others with different properties. Principle component analysis (PCA) is one of the most widely used techniques for reducing the amount of data when there is correlation between the variables present. The advantages of this method are to produce a pattern and relationship for all data, consistent answer and more reliable conclusion to researchers and to reduce number of experiments [17]. The examples of previous wine research applying chemomatrics

include discrimination of red wine samples with different geographical origin according to their polyphenol contents using HPLC-UV and fluorescence detection combined with PCA and linear discriminate analysis (LDA) [14] and discrimination of wine and grape samples according to their organic acid contents by direct infusion electrospray ionization mass spectrometry combined with PCA [18]

Although, there are a lot of research studying non-volatile compounds in wine, analysis in the aspect of simultaneous determination of non-volatile compounds (including organic acids and phenolic compounds) for characterization and differentiation of Thai wine samples according to their geographical origin and production year has not been reported. Moreover, the comparative study of Thai wine and the import wines is of interest.

1.3 Purpose of the study/Objective and scope of the study

In this study, the method to quantify non-volatile compounds was developed, validated and further applied for simultaneous detection of 25 targeted compounds in different Thai wine samples. The sample preparation approach was 'dilution and filtration' by diluting the samples in appropriate solvent and then passing the solutions through a nylon filter. Identification and quantification of the targeted compounds were accomplished by using LC-QqQMS operated with MS/MS mode. Evaluation of pattern recognition was performed for differentiation of the samples according to their production years and geographical origins. Comparative study of Thai wine and imported wine was also included.

CHAPTER II

THEORY

2.1 Non-volatile Compounds in Wine

Wine contains a large number of compounds which can be separated into volatile and non-volatile compounds. Non-volatile compounds are of interest due to their effects on color, taste, quality, and character of wine [5]. The important compounds are sugars, organic acids, phenolics [5]. The organic acids and phenolic compounds are dominant groups due to their effects on organoleptic and antioxidant properties. The detail of these groups will be provided in the next section.

Organic acids are important components along winemaking process which are present in both fresh grape and wine. Organic acids directly influence color and taste of wine products, as well as affecting growth of yeast during fermentation process and protecting wine from bacteria [19]. Generally, wine has pH between 2.3 and 3.9 which is acid. Organic acids play an important role to sour taste and the acidity balancing out the sweetness and bitter components in wine such as tannins [19]. The three primary organic acids in grape wine are tartaric, malic and citric acids. Other important acids present in wine are lactic, succinic, and acetic acid. The structure of major organic acids compounds are shown in Fig. 2.1

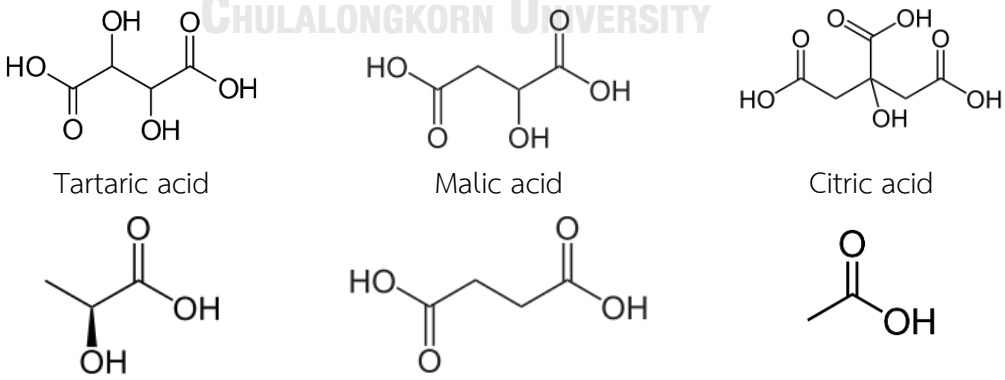
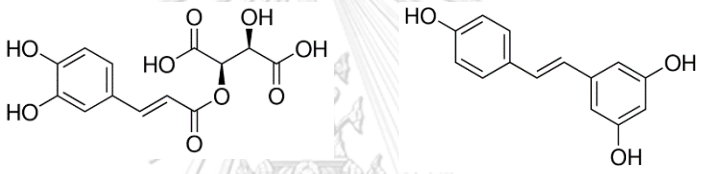


Figure 2.1 Major organic acid compounds in wine

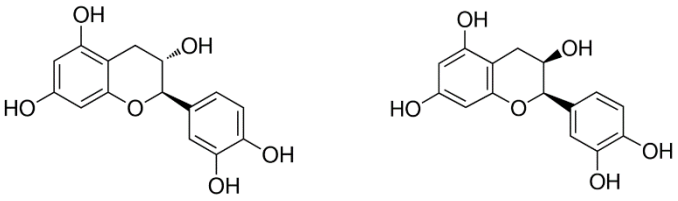
Major non-volatile compounds in wine are phenolic compounds which contains aromatic ring directly bonded at least a hydroxyl substituent. These compounds are found in wine contributing to the tastes of bitterness and astringency (especially in red wine), color and mouthfeel of wine. There are a large number of compounds present in wine including both of flavonoids and non-flavonoids. For non-flavonoid, there are many sub groups including hydroxycinnamic acid, benzoic acids, hydrolysable tannins and stilbenes. In general, various non- flavonoids found in wine include caftaric acid which is the most abundant hydroxycinnamic acid and resveratrol which is in stilbene group. The structure of non-flavonoid phenolic compounds are shown in Fig. 2.2



Caftaric acid Resveratrol

Figure 2.2 Non-flavonoid phenolic compounds in wine

The flavonoids, a majority of the phenols in red wine, are derived from extraction of the skins and seeds of grapes during the fermentation process which can be classified into three groups; flavan-3-ols, flavonols and anthocyanins. Catechin is one of the most important compounds present in wine as shown in Fig. 2.3. Other major flavonoids are shown in Figure 2.4



(+)-Catechin (-)- Epicatechin

Figure 2.3 Major flavonoid phenolic compounds in wine

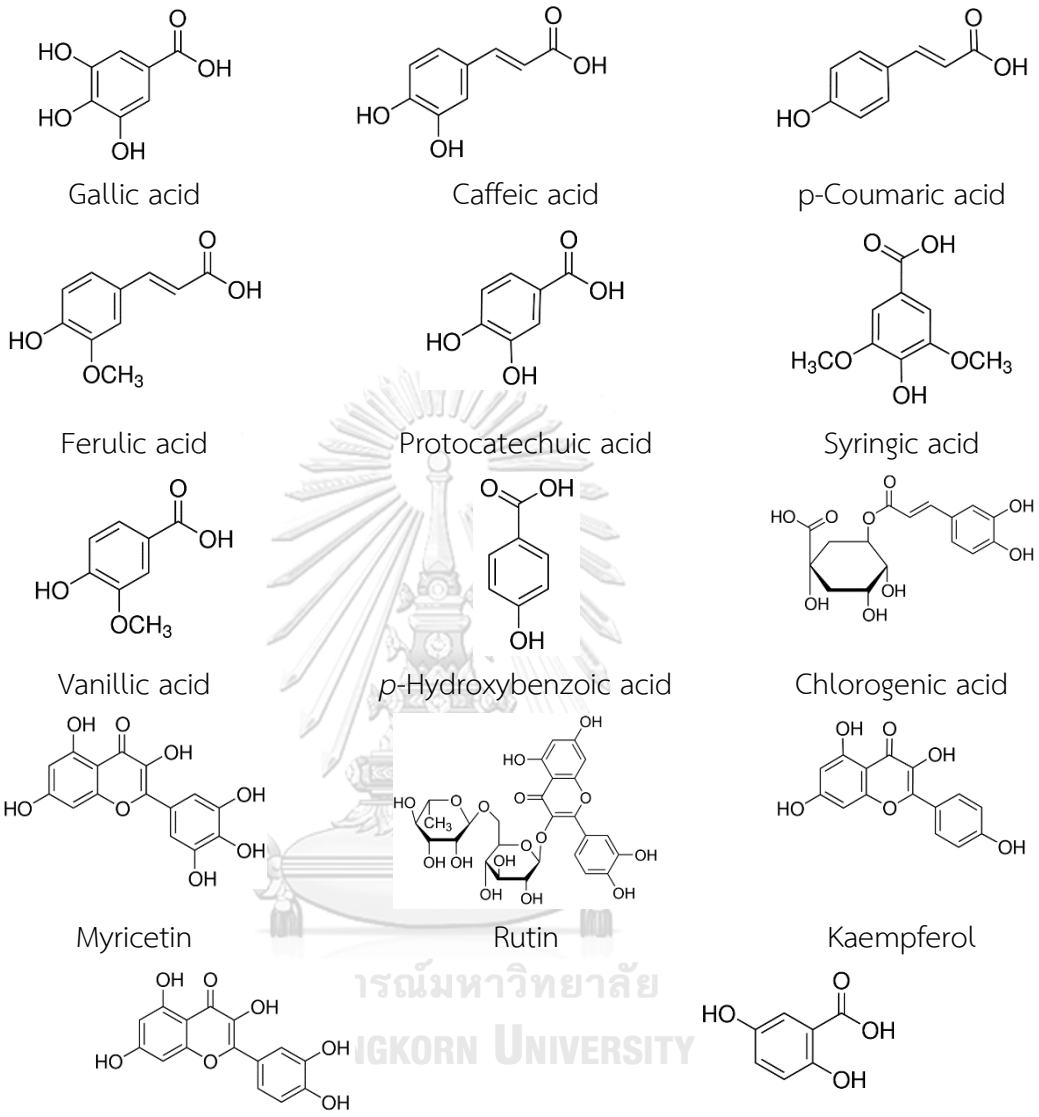
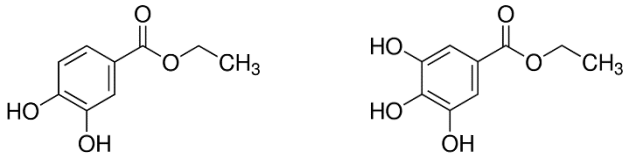


Figure 2.4 Another phenolic compounds in wine

Moreover, several phenolic acid ethyl esters which are phenolic acid bonded with ethyl alcohol are remarkable due to some research found that they also contribute to sensory taste [11, 12].



Protocatechuic acid ethyl ester Gallic acid ethyl ester

Figure 2.5 Phenolic acid ethyl esters compounds in wine

2.2 Sample Preparation

2.2.1 Dilution and filtration

Dilution and filtration or dilute and shoot (Dns) increasingly become an extensive sample preparation method in various samples such as clinical, forensic toxicology or food. These methods only require dilution with organic solvents such as acetonitrile, methanol, ethanol or mixtures such as mixture of water and methanol or acetonitrile. This is followed by filtration through membrane. The advantages of this method are simplicity, inexpensive preparation tools, compatibility with reverse-phase chromatographic conditions and minimizing matrix effect with good sensitivity and precision [20-22]. In wine analysis, this method is extensively used for sample preparation [1, 5, 23, 24]

2.3 Instrumental Determination

2.3.1 High Performance Liquid Chromatography (HPLC)

2.3.1.1 Basic Principle

Liquid chromatography (LC) is a chromatographic technique based on different interaction of chemical compounds between stationary phase and liquid mobile phase. LC can be classified into several types based on separation mechanism between solute and stationary phase including adsorption chromatography, partition chromatography, ion-exchange chromatography and size-exclusion chromatography.

High performance liquid chromatography (HPLC) is an advanced form of LC where mobile phase delivery system is operated at high pressure.

The chromatographic process initially occurs by solute is injected into injector. Then, mobile phase, which is forced by pumping at a constant flow rate, introduces the solute into an analytical column. The compounds that have stronger interaction with stationary phase will more slowly elute from the column. The different elution rate causes separation of various chemical compounds. After that, each component is detected by a detector due to its physicochemical properties such as UV absorption, fluorescence and molecular mass. After data processing, the output will be obtained as a chromatogram which is relation between signal and retention time (t_R). The separated components are displayed as peaks with different retention time in chromatogram. The difference in height and areas of each peak depends on amount of injected sample, amount of compounds, and sensitivity of detection method. From this fact, quantification of each compound can be determined with a calibration plot.

A schematic diagram of HPLC instrument is shown in Figure 2.6 the instrument consists of mobile phase and mobile phase reservoirs, pumping system, injector, column and detector.

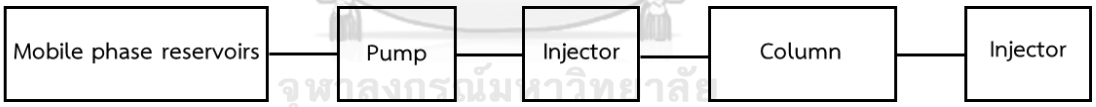


Figure 2.6 Schematic of HPLC instrument

2.3.2.1 Components of Instrument

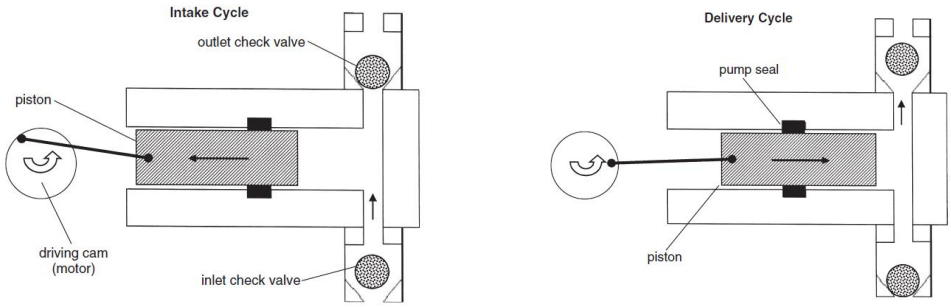
2.3.2.1.1 Mobile phase and mobile phase reservoirs

The mobile phase reservoirs are simply used for storing the mobile phase before being pumped into system. Reservoirs are often made of glass. Mobile phases in HPLC are usually mixed between solvents with or without additives. In general, there are two mobile phase elution modes including isocratic and gradient elutions. Isocratic elution is performed with constant composition of mobile phase, but gradient elution is performed with programming composition of mixed mobile phase. The advantage of gradient elution is better resolution and reduce analysis time. The proper

characteristics of mobile phase are high purity, analyte-dissolving ability, and detector compatibility.

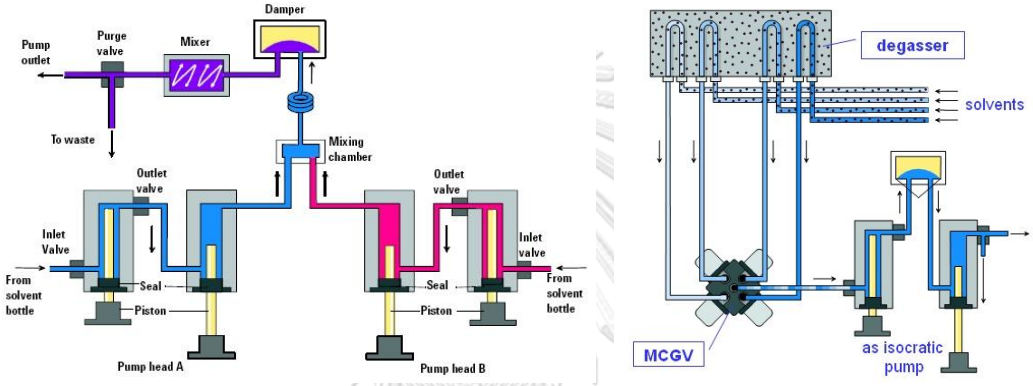
2.3.2.1.2 Pumping system

The role of pumping system is utilized for controlling and delivering mobile phase from reservoirs to a column with precise and reproducible flow rate which gives a certain mobile phase composition and thus reproducible chromatographic condition [25]. Most HPLC pumping systems are designed for analysis up to 6,000 psi (400 bar) but for high pressure work, using a sub-2 μm particle analytical column, is allowed to operate in a range from 8000 to 15,000 psi (550–1000 bar) [26]. The most common type of HPLC pumps using in HPLC pumping system is single-piston reciprocating pump. During the analysis, a piston is pulled out of the pump head which create low-pressure region inside allowing a mobile phase to enter the pump. After filling in the mobile phase, the piston is pushed back into the pump head, creating high-pressure region inside. Mobile phase then flows into the column. However, the type of pumps can be divided into 2 groups according to a number of solvent types used as mobile phase which includes a quaternary pump and a binary pump. The quaternary pump can be called as Low Pressure Mixing Gradient which is utilized to deliver the mobile phase to the system with only a single pump [27]. It can generate a gradient up to 4 different type of mobile phase with a proportioning valve at low pressure. In contrast, the binary pump or High Pressure Mixing Gradient [27] has 2 independent pumps providing specific flow for each solvent. The gradient can be generated through a mixing chamber after pumping. This creates a high pressure system due to the solvents are under the pressure before the mixing occurs. The gradient of mobile phase can be set from 0% to 100% v/v of two mobile phases to achieve an accurate analysis.



(a) (b)

Figure 2.7 Schematic of the single-piston reciprocating pump.
(a) Intake (fill) cycle, (b) delivery cycle. [26]



(a) (b)

Figure 2.8 Schematic of (a) binary pump and (b) quaternary pump [28].

2.3.2.1.3 Injector

The injector is a sample introduction system which introduces the extracted sample solution into a column in a narrow band. The characteristics of injector are precise and accurate injections of sample solution volume and can be operated under high back pressure. The core component of autosampler is a sample injection valve, is most commonly a six-port rotary valve type. It consists of sample loop for loading of the sample which generally contains sample solution between 1-100 μL but typically sample injection volume is around 2-20 μL. When loading process begins, the sample and waste ports are connected to fill in with two-to-five fold excess of sample flushing out the previous sample. Then, the rotor is moved to inject position by connecting the sample loop with high-pressure mobile phase resulting in the sample flow simultaneously swept into the column. There are both manual and

automatic injections but at present automatic injection is preferred. Conventional HPLC system also provides autosampler which can automatically analyze more than a hundred samples per day.

2.3.2.1.4 Column

The chromatographic column is employed in HPLC separation. The column packed with stationary phase separates the individual components in a presence of mobile phase flow. There are typically two kinds of columns; guard and analytical columns. The function of guard column is to prevent the contaminants approaching the analytical column which causes clogging and degrading analytical column's efficiency. It often has shorter length, similarly packing material as analytical column and it sometimes has larger particle size. An analytical column has longer length, larger internal diameter and particle size. Modern HPLC analytical column length is typically from 50 to 300 mm and 2.1-4.6 mm of internal diameter (ID) filled with 1.7-10 μm stationary phase particles. All of these feathers significantly affect separation profile and detection sensitivity especially when using gradient elution mode. A short, small diameter particle and small ID column generally results in high sensitivity analysis [29]. When sample solution flow is driven from injector by pumping system into the column, the separation of each component occurs. It is frequently retained inside the column depending on a selectivity of analyte on each stationary phase. The molecular characteristics affecting HPLC selectivity are hydrophobicity, size, intermolecular and intramolecular force [29]. In brief, the structure and chemistry of the column stationary phase determine the elution profile.

In addition, various types of chromatographic columns are available classified by separation mechanism; normal phase, reverse phase, ion exchange and size exclusion columns. For a normal phase column, the separation mechanism is based on hydrophilic interaction. The stationary phase is polar and mobile phase is non-polar solvent usually organic solvent such as hexane or methylene chloride. Example of this column type includes a packed column containing diol, cyano or amino functional group. Furthermore, there is another type of hydrophilic compounds analysis which names as hydrophilic interaction liquid chromatography (HILIC). Its separation

mechanism is considerably the same as normal phase separation but can employ semi-aqueous mobile phase with the addition of ionic compounds (e.g. ammonium acetate) in order to increase polarity of mobile phase. This results in improve solubility of hydrophilic analytes.

Reverse phase mode is the most versatile and commonly used for a wide range of different types of analytes. It has been estimated that around three-fouth of all HPLC analyses are currently employing reverse phase columns. The stationary phase is non-polar including bonded silica with octadecyl (C_{18}) and octyl (C_8) groups. The longer chain hydrocarbon group on the surface of particle is, the non-polar surface is. The mobile phase is aqueous and water-miscible organic solvent mobile phases, which are applied under both isocratic and gradient elution. Common solvents are acetonitrile, methanol, and tetrahydrofuran (THF). In reverse phase separation, the most hydrophilic components elute first, and the more hydrophobic compounds are retained longer and eluted later which is opposite of the normal phase.

In ion exchange mode, the stationary phase is a cross-linked polymer resin modified with acidic or basic with either cationic or anionic charged ions. The mobile phase is an aqueous buffer. The separation mechanism occurs on the basis of the attractive ionic force between charged analyte and oppositely charged stationary phase. This mode of separation is commonly used for separation of ionic or polar analytes including proteins, carbohydrates and amino acids.

Size exclusion mode utilizes a sieving effect based on molecular weight. The packing material contains semi-rigid organic gels, porous silica and controlled pore glasses with various pore sizes. A low molecular weight analytes can easily diffuse into the pores and they are also retained longer than higher molecular weight analytes. Size exclusion chromatography is mostly used for separation of polymers, carbohydrates and proteins.

2.3.2.1.5 Detector

The chromatographic detector is a transducer which changes a physical or chemical property of eluted analyte into an electrical signal related to analyte

concentration [26]. The ideal characteristics of detector should give a high sensitivity and selectivity response, be unaffected by temperature or flow rate variation and be responding to all solutes [26]. The most widely used chromatographic detectors are UV-Visible (UV-Vis) spectrophotometer, fluorescence detector and mass spectrometer (MS). MS is a powerful detector due to its high sensitivity and high selectivity. Moreover, it provides reliable confirmation of analytes according to their molecular and fragmented ions.

2.3.2 Mass Spectrometry (MS)

2.3.2.1 Basic Principle

MS is a powerful analytical technique which is used to tentatively identify unknown compounds and quantify known compounds by measurement of mass to charge ratio (m/z) of ionized atoms or molecules. This technique is commonly hyphenated with HPLC or UHPLC. A major instrumentation of mass spectrometry consists of ion source, mass analyzer and detector. The process is performed by transferring sample through the sample introduction into ion source under vacuum system to generate gaseous ions and accelerate into mass analyzer. Then, gaseous ions are separated according to their specific m/z by a mass analyzer. The ion detector determines their relative abundance and processes data. Finally, the MS spectrum of molecule is obtained by plotting between %relative abundance and m/z ratios. The general ion source for ionization is electrospray ionization (ESI) as detailed in the following part.

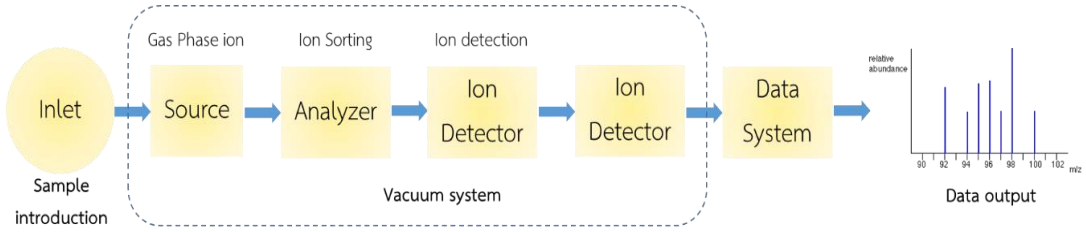


Figure 2.9 Schematic diagram of MS system [30, 31]

2.3.2.2 Components of MS

2.3.2.2.1 Ion source: Electrospray ionization (ESI)

ESI is a soft ionization technique which forms gaseous charged ions without fragmentation at atmospheric pressure. This technique is able to result in a single or multiple charged ions which can be either positive or negative. The analytes that can be ions in solution or preformed ions such as organic compounds with acid or basic groups are suitably ionized by this technique. The ionization process includes 3 steps; nebulization and charging, desolvation, and ion evaporation.

Firstly, the analyte solution is introduced by syringe pump or the eluent flow from HPLC. Then, it is sprayed from an electrospray capillary under high electric field and temperature leading to ion evaporation. The analyte solution is changed into small charged droplets. Finally, the droplets are repelled towards ionization chamber where the rapid solvent evaporation occurs resulting in gaseous ions formation.

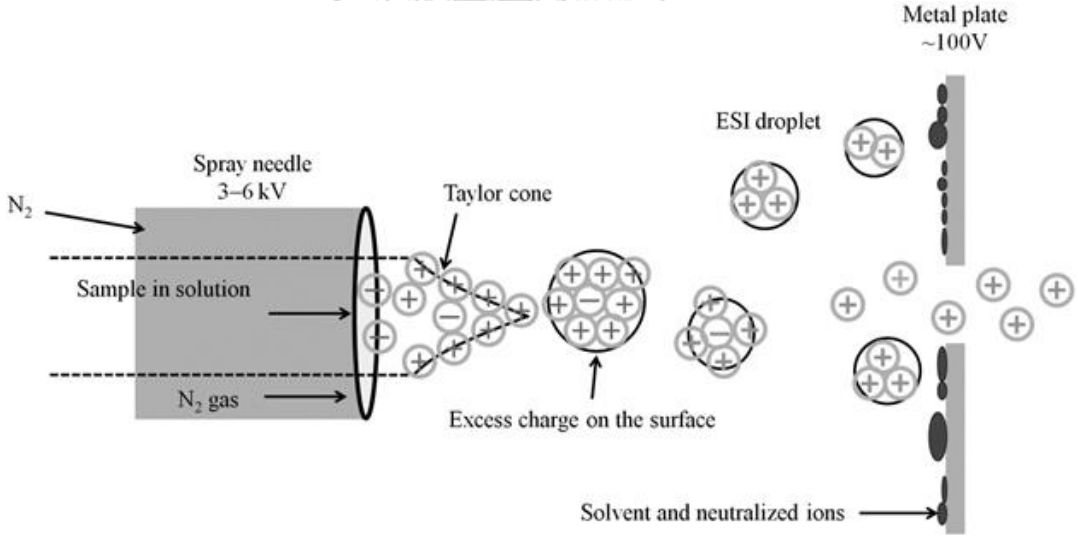


Figure 2.10 Schematic diagram of ESI source [32].

2.3.2.2.2 Mass analyzer

Mass analyzer, the most important part of MS, is used to differentiate ions according to their *m/z*. After ions are formed in ion source, they are accelerated into mass analyzer and separated by adjusting the electric and magnetic fields. Mass analyzer selection involves mass range limit, analysis speed, transmission, mass accuracy and resolution [33]. There are several types of mass analyzer for example

Quadrupole (Q), Time of flight (TOF), magnetic sector, ion trap and Fourier-transform ion cyclotron resonance (FT-ICR) mass analyzers. This work is performed with Quadrupole mass analyzer with the details given in the following part.

2.3.2.2.3 Quadrupole mass analyzer

Quadrupole is the most commonly used mass analyzer due to its fast scan rate and high transmission efficiency. This device operation relies on the stability of the trajectories in oscillating electric fields to separate ions according to their *m/z* ratios [m]. The Quadrupole mass analyzer consists of four metal rods which are connected in parallel. The two-opposite rods are connected to direct current (DC) voltage, while the others are connected to radio frequency (RF) voltage. When the ions pass through the Quadrupole, they are accelerated along the z axis and enter the space between the rods. The ion separation occurs by varying the applied voltage. Only ions of certain *m/z* ratios can pass through the analyzer and subsequently reach the detector. Other ions have unstable trajectories and will strike with the rods. The quadrupole is a real mass-to-charge ratio analyzer. It does not depend on the kinetic energy of the ions when they leave the source.

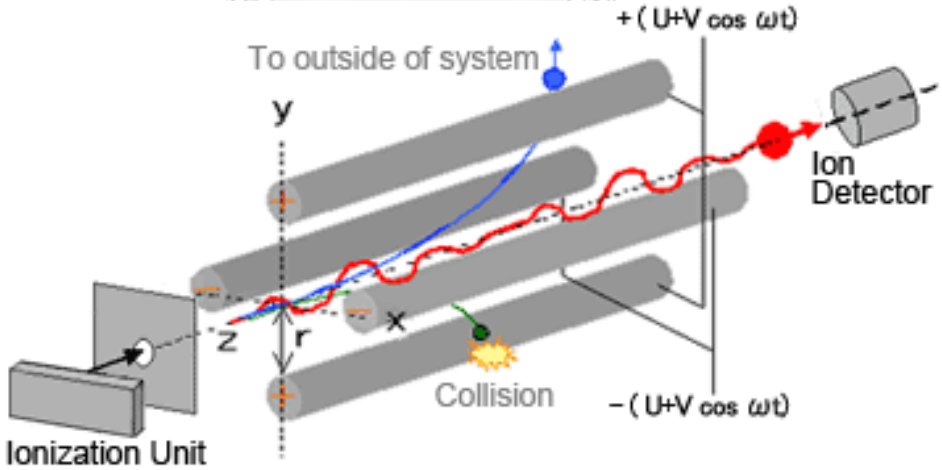


Figure 2.11 Quadrupole mass analyzer [34]

2.3.2.3 Tandem mass spectrometry (MS/MS): Triple Quadrupole (QqQ)

Tandem mass spectrometry (MS/MS) is a design of two or more mass analyzers coupled together in order to enhance detection performance. For hyphenation with HPLC, the triple Quadrupole configuration which consists of three quadrupole sets is

one of the most sensitive and reproducible quantitation. The particular precursor ions are selected by the first (Q1) and enter to the second Quadrupole (Q2). Q2 is a collision cell which involves high energy and inert collision gas such as N_2 to induce fragmentation of specific precursor ions into product ions. This process is called collision-induced dissociation (CID). Finally, the product ions are transmitted into the third quadrupole (Q3). The product ions are analyzed and subsequently detected. Components with the same molecular weight in a mixture can be specifically differentiated due to their dissimilar product ions.

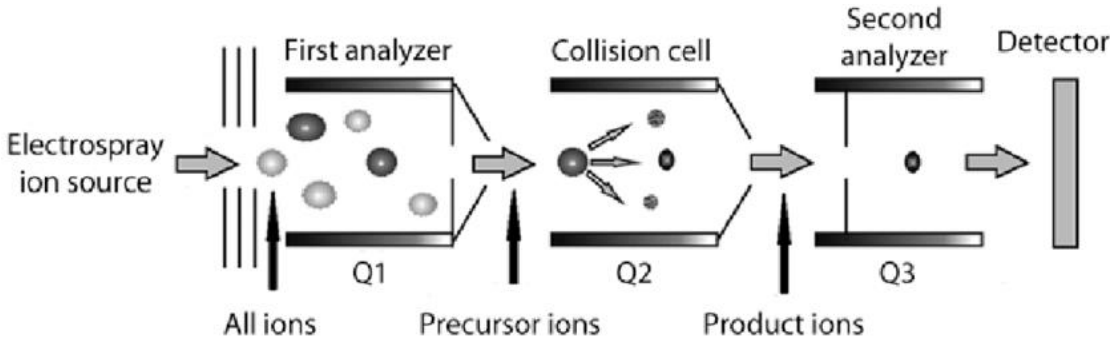


Figure 2.12 Schematic diagram of triple quadrupole mass analyzer

2.3.2.3.1 Multiple reaction monitoring (MRM)

Multiple reaction monitoring (MRM) is an operation mode of QqQMS for quantitative analysis of multi-targeted compounds. In the first step of operation, Q1 selects and transmits specific precursor ion. Then, the ions are induced and fragmented in Q2. Finally, specific fragmented ions with defined m/z are selected and transmitted in Q3 to analyze. The result is shown in a form of MS/MS fragmentation spectrum. When these two stages of operation are utilized, there is very little interference from background matrix which results in excellent sensitivity and high specificity due to specific MRM transitions. MRM can be employed for quantification a compound in complex mixtures and matrices [35].

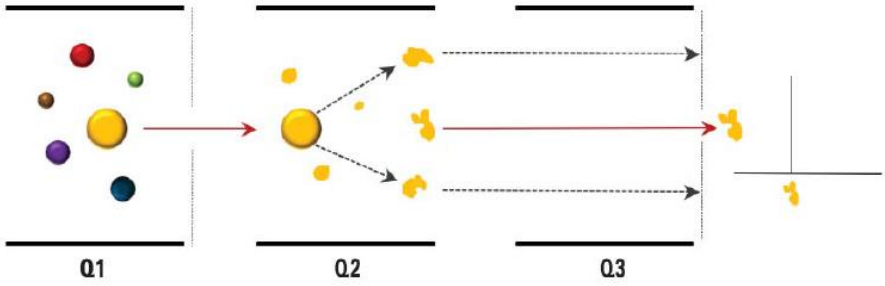


Figure 2.13 A schematic diagram of MRM mode on a triple quadrupole instrument [35].

2.3.2.3.2 Ion Detector

The ion detector is a device for measurement of electrical signal of ions passing through mass analyzer and the detector slit. The most common detector is an electron multiplier mass detector. It is a commonly horned-shaped vacuum-tube, which multiplies incident charges. The inner surface of this tube contains coated dynodes and emissive material where secondary emission occurs. When ions bombard on dynode, the electrons are emitted from material. These electrons are accelerated by electric potential resulting in more electron emission. A large amount of electrons results in electronic signal amplification and represents the signal of a particular *m/z* in a mass spectrum. The intensity of signal depends on the amount of ions, and each ion is found to correspond to a different mass to charge ratio. Figure 2.14 represents the horned-shaped electron multiplier mass detector.

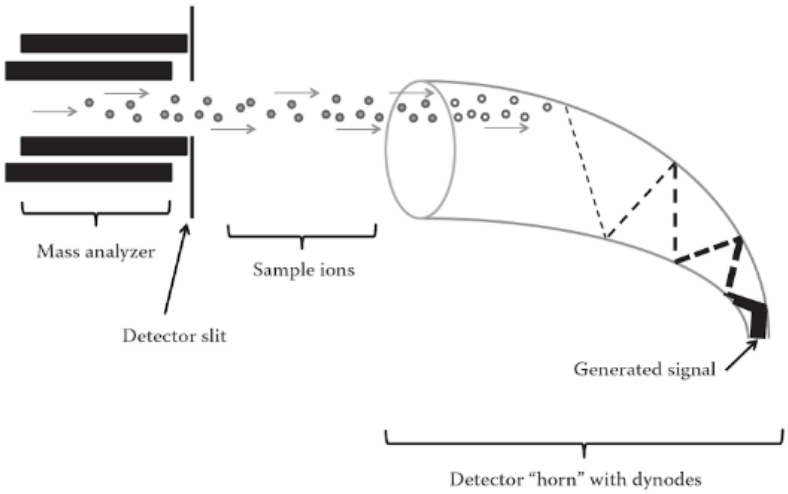


Figure 2.14 Schematic of the horned-shaped electron multiplier mass detector [36].

2.4 Data analysis

2.4.1 Chemometrics

Chemometrics involves the use of statistical and mathematical methods, to design or select the optimum procedures and experiments, and to provide maximum chemical information by analyzing chemical data [37]. The data from hyphenated analytical method are massive and complex. So, chemometrics is a powerful technique for analyzing multivariate data with pattern recognition methods to simplify chromatographic results.

2.4.2 Principal Component Analysis (PCA)

Principal component analysis (PCA) is a widely used technique for data visualization of a large number of samples and variables [17]. The aim of this method are dimension reduction of data and observing the patterns and relationships of multivariate data with simultaneously noise eliminating. The idea of PCA is to find principal components (PC) which are linear combinations of the original variables as shown in Equation 3.1

$$PC1 = a_{11}X_1 + a_{12}X_2 + a_{13}X_3 + \ldots a_{1n}X_n$$
$$PC2 = a_{21}X_1 + a_{22}X_2 + a_{23}X_3 + \ldots a_{2n}X_n$$
etc.

where PC_1 and PC_2 are a linear combinations of the original variables and a_{11} a_{12} and etc. are the coefficients of X_1, X_2, and etc.

The original data matrix is orthogonal projected by using PCA into PC space. The significance of each PC can be represented with the percentage variance. The first principal component (PC1) accounts for most of the variation with the largest percent variance in the data set and the second (PC2) accounts for the second largest variation respectively.

In PCA, data matrix (X) of I samples and J variables is decomposed into PCs consisting of a scores matrix (T), a loadings matrix (P) and a residual matrix (E) as shown in Equation 2.1.

$$x = TP' + E \qquad \textbf{(2.1)}$$

Fig. 2.15 presents the decomposition of data matrix (X) into a scores matrix (T), a loadings matrix (P') with PCA

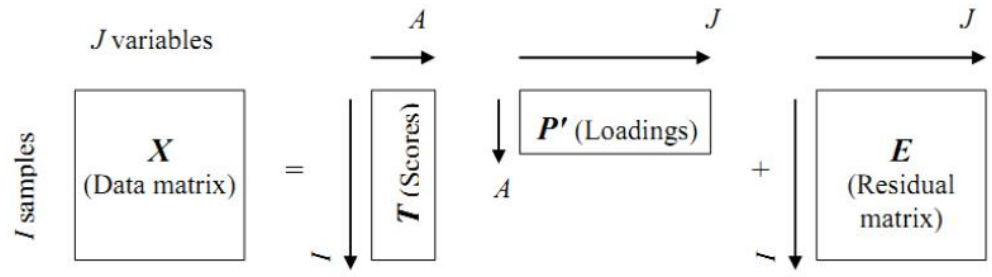


Figure 2.15 The decomposition of data matrix (X) into a scores matrix (T), a loadings matrix (P') with PCA

After PCA processing, the data matrix of each sample can be visualized the differentiation of samples. Sample with similar properties will be aligned in the same region of the plot and far from samples with different properties. Figure 2.16 presents the example of PCA data

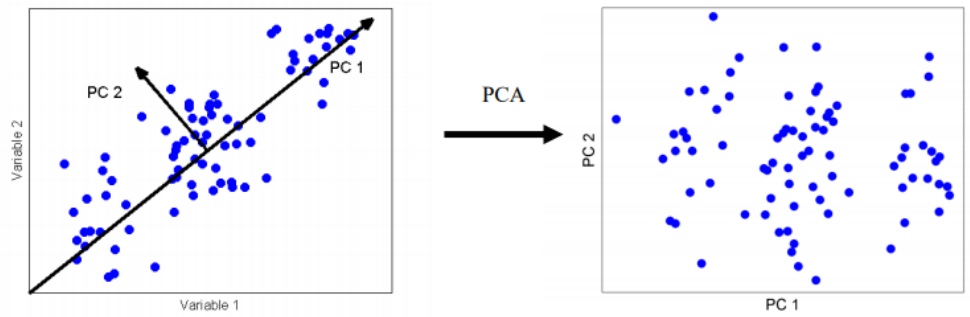


Figure 2.16 Data in original space (left) and the position of samples in PC spaces (right)

CHAPTER III

EXPERIMENTAL

3.1 Instrumental and apparatus

3.1.1 HPLC system: Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA) equipped with the Agilent 1290 Infinity binary pump and the Agilent 1290 Infinity thermostatted column compartment

3.1.2 MS system: Agilent 6490 (Agilent Technologies, Santa Clara, CA, USA) Triple Quadrupole mass spectrometer using an electrospray (ESI) interface and MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA)

3.1.3 HPLC column: Agilent InfinityLab Poroshell 120 SB-C18 (4.6 ×100 mm, 2.7 µm, Agilent, (CA, USA)

3.1.4 Milli-Q ultra pure water system, Merk (Germany)

3.1.5 HPLC vial: 2 mL amber vial with PETE cap (Agilent Technologies, Santa Clara, CA, USA)

3.1.6 Syringe filter: 0.22 µm nylon membrane (CNW technology, Thailand)

3.1.7 Micropipettes: 2-20, 20-200, 100-1000 and 500-5000 µL micropipettes, Eppendorf (Germany)

3.1.8 Syringe: 2 mL plastic syringe (Nipro, New Jersey, USA)

3.1.9 Glassware: volumetric flasks, beakers, solvent bottles, cylinders with various sizes (Duran, Germany)

3.2 Chemicals

3.2.1 Standard compounds

Flavan-3-ols: (+)-catechin (≥98%), (-)-epicatechin (≥97%)

Flavonols: Kaempferol (≥97%), Myricetin, Quercetin (≥95%), and Rutin (≥95%)

Organic acids: L-(+)-Lactic acid (90%), Malic acid (≥99%), Succinic acid (≥99%), Citric acid (≥99.5%) and Tartaric acid (≥99%)

Phenolic acid: Caffeic acid (≥98%), Caftaric acid (≥98%), Chlorogenic acid (≥95%), Ferulic acid (99%), Gallic acid (≥97.5%), Gentistic acid (≥99%), p-Coumaric acid (≥98%), *p*-Hydroxybenzoic acid (99%), Protocatechuic acid (≥99%), Syringic acid (≥95%), and Vanillic acid (≥97%)

Ethyl ester: Gallic acid ethyl ester (≥96%), Protocatechuic acid ethyl ester

Stillbens: Resveratrol (≥99%)

Internal standard: 3-amino-4-methylbenzoic acid (≥99%), and 3,5-difluorobenzoic acid (≥99%)

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) except for Myricetin purchased from Toronto Research Chemicals (North York, Canada).

3.2.2 Organic solvents

LC-MS/MS grade methanol was used as solvent for standard solution and mobile phase in HPLC analysis, and for dilution of sample in preparation step which was purchased from J.T. Baker (USA). Analytical grade formic acid used as mobile phase additive was obtained from Fisher scientific (Leicestershire, UK).

3.2.3 Samples

15 Thai grape (red), 1 Lychee and 1 mangosteen red wine samples and 2 imported wine samples were purchased from reliable market in Thailand. For Thai red wine, the authentically wine samples were made from *Syrah* varieties. Thai red wine samples were obtained from 3 different geographic origins including 3 vintages from 2012 to 2014. There are produced from various provinces as shown in Table 3.1.

Table 3.1 Commercial names and production provinces of Thai red wine samples

Commercial name	Production province
Chateau De Loei (CL)	Loei
Granmonte (GM)	Nakhon Ratchasima
PB Valley (PB)	Nakhon Ratchasima
Silverlake (SV)	Chonburi

Pooled red wines were used for method validation for determination of organic acids and phenolic compounds in wine. This validated method was also applied for all wine analyses. All samples were kept in refrigerator at 15 ºC until analysis.

3.3 Standard solution preparation

Stock standard solutions of organic acid and phenolic compounds (1000 ppm) were individually prepared by dissolving 10 mg of each standard with 50% v/v methanol in water and methanol in 10 mL volumetric flask. The stock solutions were stored at 0 ºC before analysis.

Stock internal standard solutions (2000 ppm) of 3-amino-4-methylbenzoic acid and 3,5-difluorobenzoic acid were individually prepared by dissolving 20 mg of each standard with methanol in 10 mL volumetric flask. Mixed internal standard were prepared by dissolving 500 μL of each 2000 ppm internal standard solution with 50% v/v methanol in water in 10 mL volumetric flask and stored at 0 ºC until analysis.

3.4 Working mixed standard solutions preparation

Mixed organic and phenolic standard was prepared by diluting individual stock solution with 50% v/v methanol in water adjusted to the desired final concentration of each compound and 5 mg/L for each internal standard. The mixed standard solution was daily prepared and transferred into 2 mL amber vials with PETE screw caps. The working mixed standard solutions were used in HPLC optimization and LOD & LOQ calculation.

3.5 Sample preparation

All Thai wine samples (500 μL) were diluted with 4500 μL of 50% v/v methanol in water. The diluted samples were filtered with 0.22 μL nylon syringe filter. Before analysis, the filtered solutions (950 μL) were transferred into 2 mL amber vials containing 50 μL of mixed internal standard solutions (100 ppm).

3.6 Optimization of mass spectrometric detection

The organic acid and phenolic standards (10 ppm) were individually prepared in 50% v/v methanol in water. MS/MS method for organic acid and phenolic compounds was optimized by operating MS/MS system in either positive or negative mode. Each targeted analyte (10 ppm) was directly injected into the MS source without HPLC column with 50% v/v of mobile phase B where mobile phases A and B were 0.1% v/v formic acid in water and methanol, respectively using flow rate of 0.4 mL/min. Mass spectra were obtained in both positive and negative ESI modes in the range of m/z 40-1000. The major parameters of MS/MS optimization are shown in the next section.

3.6.1 Precursor ion

The precursor ion of each targeted analyte and internal standard were monitored with ion scan mode of detection in both positive and negative modes. The m/z range detection covered all targeted analyte m/z. The mode resulting in the highest abundance was chosen for each targeted analyte.

3.6.2 Product ion and collision energy optimization

After the precursor ions were selected, the product ions were monitored using appropriate collision energy (CE) of each targeted analysts. The first two highest abundance product ions were selected as quantitative and qualitative ions, respectively. After that, MRM mode was performed to optimize the CE for each precursor ion. The selected MS conditions are shown in Table 4.1.

3.7 Optimization of HPLC separation

HPLC-MS/MS was carried out using an Agilent 1290 Infinity LC system coupled to Agilent 6490 Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), equipped with an electrospray (ESI) interface and operated in both negative and positive modes. MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA) was used to control the instruments, data acquisition, and data processing.

Chromatographic separation was performed using a C18 reverse phase column Agilent InfinityLab Poroshell 120 SB-C18 with 4.6 × 100 mm, 2.7 µm (Agilent, CA, USA). The separation temperature which was maintained at 30 ºC. The injected sample volume was 2 µL.

The mobile phase for HPLC-MS/MS analysis consisted of 0.1% v/v formic acid in water (mobile phase A) and 0.1% v/v formic acid in methanol (mobile phase B) at flow rate of 0.4 mL/min under gradient elution. The gradient profile was10%B at 0.00 min, 35%B at 4 min, 100%B at 18 min held at this condition until 19.5 min and back to initial condition of 10%B at 20 min. A multiple reaction monitoring (MRM) mode was used with 2 transitions for analysis of each analyte.

3.8 Method validation

3.8.1 Analytical limit

According to IUPAC recommendation [38], the limit of detection (LOD) is defined as a concentration level for which the risk of false is not detected. In order to obtain LOD and limit of quantitation (LOQ) values from an internal standard calibration plot, the mixture of organic acid and phenolic compounds with different concentration in the final solutions were prepared as described in section 3.4. The HPLC-MS/MS method was performed as described in section 3.7 with four triplicate concentration levels to obtain the internal calibration plots. The results are shown in section 4.3.

3.8.2 Standard calibration curve

Standard internal calibration curves were established using seven, ten and eleven concentrations levels of mixed standard solutions in 50% v/v methanol in water with three replicates for each level as shown in appendix A.

Table 3.2 Concentration range of standard calibration curve with 7 concentration levels.

Analyte	Concentration level (mg/L)						
	L1	L2	L3	L4	L5	L6	L7
Caffeic acid	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Caftaric acid	0.050	0.13	0.25	0.50	1.3	2.5	5.0
(+)-catechin	0.050	0.13	0.25	0.50	1.3	2.5	5.0
Chlorogenic acid	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Citric acid	0.10	0.25	0.50	1.0	2.5	5.0	10
Coumaric acid	0.050	0.13	0.25	0.50	1.3	2.5	5.0
(-)-epicatechin	0.050	0.13	0.25	0.50	1.3	2.5	5.0
Ferulic acid	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Gallic acid	0.050	0.13	0.25	0.50	1.3	2.5	5.0
Gallic acid ethyl ester	0.050	0.13	0.25	0.50	1.3	2.5	5.0
Gentistic acid	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Kaempferol	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Malic acid	0.10	0.25	0.50	1.0	2.5	5.0	10
Myricetin	0.010	0.025	0.050	0.10	0.25	0.50	1.0
p-Hydroxybenzoic acid	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Protocatechuic acid	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Protocatechuic acid ethyl ester	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Quercetin	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Resveratrol	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Rutin	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Syringic acid	0.010	0.025	0.050	0.10	0.25	0.50	1.0
Vanillic acid	0.010	0.025	0.050	0.10	0.25	0.50	1.0

Table 3.3 Concentration range of standard calibration curve with 10 and 11 concentration levels

Analyte	Concentration level (mg/L)										
	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11
Tartaric acid	0.10	0.25	0.50	1.0	2.5	5.0	10	25	50	100	-
Succinic acid	0.10	0.25	0.50	1.0	2.5	5.0	10	25	50	100	200
Lactic acid	0.10	0.25	0.50	1.0	2.5	5.0	10	25	50	100	200

3.8.3 Accuracy and precision

Accuracy and precision of the optimized method were evaluated by spiking mixed standard into the pooled Thai wine samples with three concentration levels. The six replicates of HPLC-MS/MS analysis were performed within three consecutive days to evaluate the values. The three concentration levels were shown in Table 3.4. The accuracy is presented by the percentage recovery and the precision is presented by the percentage relative standard deviation of the recovery. All the results are shown in Section 4.3.3.

Table 3.4 Spiked concentration of organic acids and phenolic in pooled sample for accuracy evaluation.

Analyte	Spiked concentration in pooled sample (mg /L)		
	Low	Medium	High
Organic acid			
Malic acid	10	15	20
Tartaric acid	5.0	10	15
Succinic acid	10	15	20
Lactic acid	5.0	10	20
Citric acid	10	15	20
Phenolic compounds			
Caffeic acid	0.060	0.30	0.60
Caftaric acid	1.0	1.5	2.8
(+)-Catechin	1.0	1.5	2.8
Chlorogenic acid	0.060	0.30	0.60

Table 3.4 (Continued)

Analyte	Spiked concentration in pooled sample (mg /L)		
	Low	Medium	High
Coumaric acid	1.0	1.5	2.8
(-)-Epicatechin	1.0	1.5	2.8
Ferulic acid	0.060	0.30	0.60
Gallic acid	1.0	1.5	2.8
p-Hydroxybenzoic acid	0.060	0.30	0.60
Rutin	0.060	0.30	0.60
Syringic acid	0.060	0.30	0.60
Vanillic acid	0.060	0.30	0.60
Kaempferol	0.060	0.30	0.60
Myricetin	0.060	0.30	0.60
Quercetin	0.060	0.30	0.60
Protocatechuic acid	0.060	0.30	0.60
Gentistic acid	0.060	0.30	0.60
Gallic acid ethyl ester	1.0	1.5	2.8
Protocatechuic acid ethyl ester	0.060	0.30	0.60
Resveratrol	0.060	0.30	0.60

3.8.4 Application in real sample

The developed and validated method was used for determination of targeted organic acids and phenolic compounds in Thai and imported wine samples. The recovery of organic acid and phenolic compounds were calculated according to internal standard calibration curves by analyzing of the spiked pooled samples of Thai red wine at known concentration levels (as shown in 3.8.3). The results of the determined targeted compound amounts are shown in Section 4.4.

CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Optimization of mass spectrometric detection

According to Section 3.6, the following parameters were investigated for MS/MS analysis of each organic acid, phenolic compound and also internal standard using both negative and positive polarity modes for the precursor ion: collision energy (CE) and Q1/Q2 product ions. Note that Q1 and Q2 are the product ions with the most and second most abundance, and are used for quantitative and qualitative analysis, respectively It can be seen from Table 4.1 that the better sensitivity for MS/MS detection of all 5 organic acids and 13 phenolic compounds are obtained with the negative polarity mode, while the rest 7 phenolic compounds are better detected with the positive polarity mode: (+)-catechin, chlorogenic acid, (-)-epicatechin, rutin, kaempferol, myricetin, resveratrol. This is possible because the carboxylic group of 5 organic acids is preferably deprotonated, while the phenolic compounds can be deprotonated or protonated depending on the structure of particular compounds.

Table 4.1 Precusor ion, product ion, collision energy (CE) of each organic acids, phenolic and internal standard

No.	Analyte	Polarity	Precursor ion		Q_1/Q_2	Product ion	CE (volt)
			Form	m/z			
OA01	Malic acid	Negative	$[M-H]^-$	134.02	Q_1	115.1	5
					Q_2	71.1	9
OA02	Tartaric acid	Negative	$[M-H]^-$	150.02	Q_1	87.1	9
					Q_2	58.4	37
OA03	Succinic acid	Negative	$[M-H]^-$	118.03	Q_1	73.1	5
OA04	Lactic acid	Negative	$[M-H]^-$	90.03	Q_1	43	9
OA05	Citric acid	Negative	$[M-H]^-$	192.03	Q_1	86.8	13
					Q_2	110.8	9
PC01	Caffeic acid	Negative	$[M-H]^-$	180.04	Q_1	135.2	9
					Q_2	134.2	25

Table 4.1 (Continued)

No.	Analyte	Polarity	Precursor ion		Q_1/Q_2	Product ion	CE
			Form	m/z			
PC02	Caftaric acid	Negative	$[M-H]^-$	312.05	Q_1	148.8	5
					Q_2	178.9	9
PC03	(+)-catechin	Positive	$[M+H]^+$	290.08	Q_1	138.9	9
					Q_2	122.9	29
PC04	Chlorogenic acid	Positive	$[M+H]^+$	354.10	Q_1	162.8	13
					Q_2	88.8	57
PC05	Coumaric acid	Negative	$[M-H]^-$	164.05	Q_1	118.9	9
					Q_2	92.9	33
PC06	(-)-epicatechin	Positive	$[M+H]^+$	290.08	Q_1	138.9	9
					Q_2	122.9	25
PC07	Ferulic acid	Negative	$[M-H]^-$	194.06	Q_1	133.7	13
PC08	Gallic acid	Negative	$[M-H]^-$	170.02	Q_1	124.9	9
					Q_2	78.9	21
PC10	*p*-Hydroxybenzoic acid	Negative	$[M-H]^-$	138.03	Q_1	92.9	13
					Q_2	65	33
PC11	Rutin	Positive	$[M+H]^+$	610.15	Q_1	302.7	25
					Q_2	464.7	5
PC13	Syringic acid	Negative	$[M-H]^-$	198.05	Q_1	181.9	9
					Q_2	123	21
PC14	Vanillic acid	Negative	$[M-H]^-$	168.04	Q_1	151.8	9
					Q_2	107.9	17
PC15	Kaempferol	Positive	$[M+H]^+$	286.05	Q_1	120.8	33
					Q_2	68.9	49
PC16	Myricetin	Positive	$[M+H]^+$	318.04	Q_1	152.8	33
					Q_2	68.9	60
PC17	Quercetin	Negative	$[M-H]^-$	302.04	Q_1	150.8	17
					Q_2	178.7	13
PC18	Protocatechuic acid	Negative	$[M-H]^-$	154.03	Q_1	108.9	13
					Q_2	90.8	25
PC20	Gentistic acid	Negative	$[M-H]^-$	154.03	Q_1	107.8	21
PEE01	Gallic acid ethyl ester	Negative	$[M-H]^-$	198.05	Q_1	123.9	21
					Q_2	168.7	13

Table 4.1 (Continued)

No.	Analyte	Polarity	Precursor ion		Q_1/Q_2	Product ion	CE
			Form	m/z			
PEE02	Protocatechuic acid ethyl ester	Negative	$[M-H]^-$	182.06	Q_1	107.8	25
					Q_2	153	13
ST01	Resveratrol	Positive	$[M+H]^+$	228.08	Q_1	107.1	21
					Q_2	77.1	45
IS01	3-amino-4-methylbenzoic acid	Positive	$[M+H]^+$	158.02	Q_1	92.9	17
					Q_2	106.8	21
IS02	3,5-difluorobenzoic acid	Negative	$[M-H]^-$	158.02	Q_1	112.8	9
					Q_2	92.8	25

Q_1 = quantifier ion, Q_2 = Qualifier ion

Table 4.2 Parameter of MS/MS system

MS parameter	Optimal condition
Capillary voltage (Positive and Negative)	3,000 V
Nozzle voltage	1,000 V
Gas flow	16 L/min
Nebulizer	20 psi
Sheath gas flow	11 L/min
Gas temperature	200 °C
Sheath gas temperature	400 °C
Dwell time	20 ms/cycle

4.2 Optimization of HPLC separation

From Section 3.7, the chromatographic separation of organic acids and phenolic compounds was performed using a Poroshell C18 HPLC column (4.6 x 100 mm, 2.7 µm) and a gradient mobile phase elution with the mobile phase A of a 0.1% v/v formic acid in water and mobile phase B of a 0.1% v/v formic acid in methanol. The following optimum gradient profile elution was obtained: 0.00 min: 10%B, 4 min: 35%B, 18 min: 100%B, 19.5 min: 100%B and at 20 min back to an initial condition of 10%B. Typically, the achieved baseline resolution of at least 2.0 for two adjacent peaks is accepted for quantitative analysis using chromatographic separation. However, HPLC-MS/MS provides high selectivity due to an MRM detection mode which can detect and display

different transition of particular precursor ions and its product ions with individual transition window. Therefore, partial or whole overlapping of targeted chromatogram peaks was not affected. The overall separation is shown in Fig. 4.1

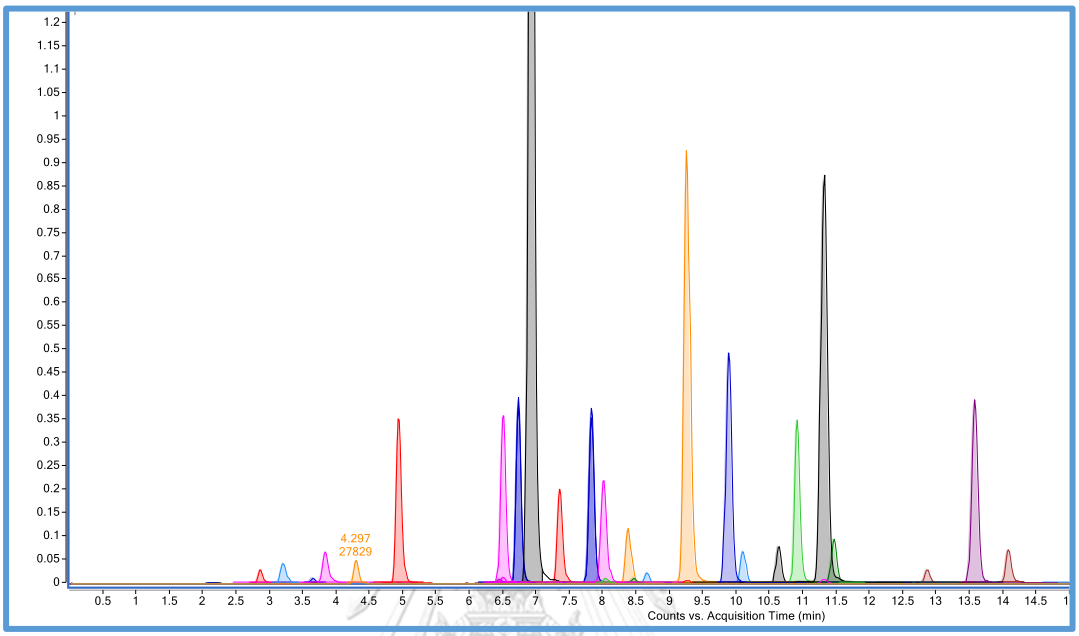


Figure 4.1 HPLC-MS/MS chromatogram of 25 targeted non-volatile compounds and 2 internal standards.

4.3 Method validation

4.3.1 Analytical limits

According to IUPAC recommendation [38], the limit of detection (LOD) is defined as a concentration level for which can be discriminated from the blank, the risk of false is non-detects. Therefore, the equation can be expressed in Equation 4.1.

$$LOD = \frac{3.3S_{x/y}}{A}\sqrt{1+h_0+\frac{1}{l}} \qquad (4.1)$$

A is the slope of a linear calibration plot between the signal against the analyte concentration

$S_{x/y}$ is the residual standard deviation.

l is the number of calibration samples

h_0 is the leverage for the blank sample

$$h_0 = \frac{\bar{c}_{cal}^{2}}{\sum_{i=1}^{l} (c_i - \bar{c}_{cal})^2} \quad (4.2)$$

$\bar{c}_{cal}$ is the mean calibration concentration

c_i is each of calibration concentration values.

LOQ calculation is similar to Equation 4.1 but replace the factor of 3.3 with 10. The equation of LOQ is then shown in Equation 4.3

$$LOQ = \frac{10S_{x/y}}{A} \sqrt{1+h_0+\frac{1}{l}} \quad (4.3)$$

Table 4.3 shows the results of calibration plots obtained from four triplicate concentrations levels of standard in 50% v/v methanol in water (l of 12 in Equation 4.1). From the results in Table 4.3, LOD and LOQ values are found in the range of 0.0084-0.48 mg/L and 0.026- 1.45 mg/L, respectively.

Table 4.3 Analytical limits obtained from internal standard calibration results

Analyte	Conc. range	Calibration plot				
		Slope	Intercept	R^2	LOD (mg/L)	LOQ (mg/L)
Organic acid						
Malic acid	0.10-10	3.04×10^{-6}	0.0013	0.9986	0.071	0.22
Tartaric acid	0.10-100	5.37×10^{-6}	0.016	0.9776	0.13	0.38
Succinic acid	0.10-100	6.64×10^{-6}	0.092	0.9711	0.18	0.53
Lactic acid	0.10-200	1.61×10^{-6}	0.013	0.9702	0.48	1.4
Citric acid	0.10-10	1.45×10^{-6}	0.054	0.9898	0.15	0.45
Phenolic compound						
Caffeic acid	0.010-1.0	3.31×10^{-4}	0.052	0.9574	0.0094	0.028
Caftaric acid	0.050-5.0	9.13×10^{-5}	-0.0053	0.9986	0.03	0.032
(+)-catechin	0.050-5.0	3.17×10^{-5}	0.0011	0.9989	0.044	0.13
Chlorogenic acid	0.010-1.0	2.20×10^{-5}	0.000057	0.9996	0.01	0.031
Coumaric acid	0.050-5.0	2.83×10^{-4}	0.023	0.9980	0.033	0.1
(-)-epicatechin	0.050-5.0	3.51×10^{-5}	0.00099	0.9995	0.031	0.094
Ferulic acid	0.010-1.0	3.94×10^{-5}	-0.0004	0.9991	0.012	0.036
Gallic acid	0.050-5.0	1.33×10^{-4}	-5.8	1.000	0.034	0.1
Gentistic acid	0.010-1.0	1.51×10^{-4}	-0.0015	0.9993	0.012	0.036
p-Hydroxybenzoic acid	0.010-1.0	1.89×10^{-4}	0.0009	0.9997	0.066	0.2
Rutin	0.010-1.0	1.53×10^{-6}	-0.00001	0.9995	0.0084	0.026
Syringic acid	0.010-1.0	1.42×10^{-5}	0.011	0.9158	0.027	0.081
Vanillic acid	0.010-1.0	1.31×10^{-5}	-0.00012	0.9995	0.17	0.52
Kaempferol	0.010-1.0	7.91×10^{-6}	-0.00014	0.9971	0.013	0.039
Myricetin	0.010-1.0	8.61×10^{-6}	-0.00017	0.9967	0.023	0.069
Quercetin	0.010-1.0	4.77×10^{-4}	-0.0023	0.9979	0.019	0.058
Protocatechuic acid	0.010-1.0	1.67×10^{-4}	-0.0022	0.9994	0.01	0.032
Resveratrol	0.010-1.0	4.02×10^{-5}	-0.00033	0.9996	0.021	0.063
Gallic acid ethyl ester	0.050-5.0	3.21×10^{-4}	0.058	0.9925	0.019	0.057
Protocatechuic acid ethyl ester	0.010-1.0	5.66×10^{-4}	0.013	0.9946	0.054	0.16

4.3.2 Standard calibration curve

A standard calibration curve for quantitative analysis is generally obtained from the relationship between the response and multi- replicate analyte concentrations. In the work, the internal calibration plots between relative responses of analyte against the known standard concentration at 7 to 10 levels were used as shown in Appendix. Good linearity was obtained with correlation coefficient (R^2) higher than 0.91.

4.3.3 Accuracy and Precision

As detailed in Section 3.8.3, pooled red Thai wine samples from 15 bottles were divided into 3 batches, and each batch was spiked with all targeted standards at different known concentration levels. The amounts of organic acids and phenolic compounds in pooled wine samples before and after spiking standards were determined by HPLC-MS/MS using six replicate runs and internal standard calibration curves. Results of the accuracy and precision evaluated by recovery and %RSD, are shown in Table 4.4. According to acceptable criteria of % recovery by AOAC method [39], it can be seen that 84.9% of 225 data sets for %recovery falls within the acceptable criteria values. Out of acceptable ranges of %recovery is possible due to matrix effects on these followings analytes: chlorogenic acid, (-)-epicatechin, gallic acid kaempferol, myricetin, resveratrol, rutin and vanillic acid which higher than acceptable range. For intraday precision, all of the %RSD values in a range of 0.4-7% were found to fall in a range acceptable criteria which is calculated using the Horwitz equation where acceptable criteria = $0.67 \times 2^{(1-0.5\log C)}$ %

Table 4.4 Accuracy and precision in recovery of organic acids and phenolic compounds spiked in pooled sample at three level (n = 6 replicate runs)

Analyte	Conc. (mg/L)	% Recovery (%RSD)			
		Acceptable criteria	Day1	Day2	Day3
Organic acid					
Malic acid	10	80-110(7.6)	102(2)	110(3)	88(1)
	15	80-110(7.1)	94(0.4)	106(1)	94(2)
	20	80-110(6.8)	95(1)	105(3)	102(1)
Tartaric acid	5	80-110(8.4)	108(1)	108(1)	103(2)
	10	80-110(7.6)	104(2)	104(2)	92(2)
	15	80-110(7.1)	97(1)	97(1)	100(1)
Succinic acid	10	80-110(7.6)	95(0.5)	95(0.5)	98(0.4)
	15	80-110(7.1)	91(0.8)	91(0.8)	96(1)
	20	80-110(6.8)	99(0.4)	99(0.4)	98(1)
Lactic acid	5	80-110(8.4)	80(1)	99(3)	80(4)
	10	80-110(7.6)	85(1)	97(3)	80(1)
	20	80-110(6.8)	88(1)	93(3)	91(2)
Citric acid	10	80-110(7.6)	104(2)	104(4)	93(3)
	15	80-110(7.1)	102(1)	103(3)	99(2)
	20	80-110(6.8)	102(2)	102(4)	101(3)
Phenolic compound					
Caffeic acid	0.06	80-110(16.4)	82(1)	108(1)	97(3)
	0.3	80-110(12.8)	94(1)	103(3)	97(2)
	0.6	80-110(11.6)	97(1)	95(1)	99(3)
Caftaric acid	1	80-110(10.7)	93(1)	104(1)	100(2)
	1.5	80-110(10.1)	87(1)	99(1)	105(1)
	2.75	80-110(9.2)	98(1)	86(2)	103(2)
(+)-Catechin	1	80-110(10.7)	101(1)	103(2)	106(3)
	1.5	80-110(10.1)	103(3)	102(1)	102(1)
	2.75	80-110(9.2)	108(1)	108(1)	112(2)
Chlorogenic acid	0.06	80-110(16.4)	106(1)	113(1)	115(3)
	0.3	80-110(12.8)	114(1)	108(3)	122(2)
	0.6	80-110(11.6)	118(1)	108(1)	129(3)

Table 4.4 (Continued)

Analyte	Conc. (mg/L)	% Recovery (%RSD)			
		Acceptable criteria	Day1	Day2	Day3
Coumaric acid	1	80-110(10.7)	89(1)	91(1)	86(2)
	1.5	80-110(10.1)	83(1)	84(3)	81(1)
	2.75	80-110(9.2)	86(1)	82(1)	84(1)
(-)-Epicatechin	1	80-110(10.7)	118(1)	118(1)	118(2)
	1.5	80-110(10.1)	120(1)	121(1)	117(2)
	2.75	80-110(9.2)	119(2)	118(2)	122(3)
Ferulic acid	0.06	80-110(16.4)	81(2)	78(2)	82(6)
	0.3	80-110(12.8)	85(2)	89(2)	89(3)
	0.6	80-110(11.6)	87(1)	93(2)	95(1)
Gallic acid	1	80-110(10.7)	84(1)	89(1)	115(2)
	1.5	80-110(10.1)	85(1)	85(2)	112(1)
	2.75	80-110(9.2)	88(1)	84(1)	103(1)
p-Hydroxybenzoic acid	0.06	80-110(16.4)	85(2)	79(1)	90(5)
	0.3	80-110(12.8)	86(1)	87(2)	94(2)
	0.6	80-110(11.6)	87(2)	84(2)	91(2)
Rutin	0.06	80-110(16.4)	88(2)	86(4)	82(5)
	0.3	80-110(12.8)	108(5)	106(5)	102(3)
	0.6	80-110(11.6)	108(5)	109(4)	113(1)
Syringic acid	0.06	80-110(16.4)	119(2)	92(2)	80(3)
	0.3	80-110(12.8)	87(1)	89(2)	88(1)
	0.6	80-110(11.6)	78(1)	78(2)	81(2)
Vanillic acid	0.06	80-110(16.4)	111(5)	89(1)	95(3)
	0.3	80-110(12.8)	100(2)	93(2)	110(1)
	0.6	80-110(11.6)	98(3)	94(3)	109(1)
Kaempferol	0.06	80-110(16.4)	74(7)	67(6)	62(6)
	0.3	80-110(12.8)	109(6)	108(4)	104(6)
	0.6	80-110(11.6)	115(2)	116(3)	114(3)
Myricetin	0.06	80-110(16.4)	108(4)	100(3)	103(6)
	0.3	80-110(12.8)	104(2)	96(2)	131(2)
	0.6	80-110(11.6)	120(2)	117(5)	150(1)

Table 4.4 (Continued)

Analyte	Conc. (mg/L)	% Recovery (%RSD)			
		Acceptable criteria	Day1	Day2	Day3
Quercetin	0.06	80-110(16.4)	25(3)	54(2)	107(5)
	0.3	80-110(12.8)	83(2)	90(3)	99(3)
	0.6	80-110(11.6)	94(1)	94(2)	107(1)
Protocatechuic acid	0.06	80-110(16.4)	91(1)	92(1)	107(4)
	0.3	80-110(12.8)	85(1)	84(2)	102(1)
	0.6	80-110(11.6)	90(1)	85(1)	106(2)
Gentisic acid	0.06	80-110(16.4)	80(1)	80(1)	98(1)
	0.3	80-110(12.8)	89(1)	86(2)	97(2)
	0.6	80-110(11.6)	90(1)	88(2)	96(1)
Gallic acid ethyl ester	1	80-110(10.7)	87(1)	86(1)	90(2)
	1.5	80-110(10.1)	84(1)	85(2)	88(1)
	2.75	80-110(9.2)	82(1)	81(1)	82(1)
Protocatechuic acid ethyl ester	0.06	80-110(16.4)	94(1)	101(2)	104(3)
	0.3	80-110(12.8)	96(1)	97(1)	98(1)
	0.6	80-110(11.6)	92(1)	95(1)	94(2)
Resveratrol	0.06	80-110(16.4)	97(1)	91(1)	108(3)
	0.3	80-110(12.8)	102(3)	108(1)	119(1)
	0.6	80-110(11.6)	116(2)	107(1)	120(1)

4.4 Application in real sample

As detailed in Section 3.8.4, the optimum method was used for determination of organic acids and phenolic compounds in 19 samples including 15 Thai red wines, 1 Mangosteen wines, 1 Lychee wine and 2 imported red wines obtained from supermarket and distributors in Thailand. The results are shown in Table 4.5 and 4.6.

Table 4.5 Amounts of organic acids and phenolic compounds in 11 Thai red wine samples.

Analyte	Concentration range (mg/L)										
	GM 11	GM 14-1	GM 14-2	PB 12-1	PB 12-2	CL 12-1	CL 12-2	CL 13-1	CL 13-2	CL 14-1	CL 14-2
Organic acid											
OA01	24.9	19.5	20.3	42.7	44.2	69.6	69.1	70.0	69.6	52.8	50.7
OA02	7.20	590	592	703	707	714	736	733	716	635	617
OA03	4.78	330	339	397	410	369	389	388	380	409	388
OA04	1233	1165	1194	873	867	1179	1183	1187	1125	1041	1028
OA05	11.3	61.7	62.6	174	178	6.61	14.8	98.4	104	10.4	8.80
Phenolic compound											
PC01	1.62	2.20	2.21	3.57	3.86	2.98	3.69	2.75	2.73	6.00	5.92
PC02	26.5	27.4	28.5	19.0	19.0	21.7	23.7	25.8	26.1	37.5	36.2
PC03	12.0	9.08	9.73	11.1	16.3	6.15	7.51	6.15	6.78	8.04	9.01
PC04	N/A	0.781	1.25	1.75	1.49	3.16	27.0	18.7	13.1	7.47	5.00
PC05	2.79	8.58	8.78	6.81	6.92	3.08	3.22	5.13	4.73	4.09	3.94
PC06	9.79	7.22	7.69	8.72	14.3	2.20	2.88	3.57	3.94	4.75	5.57
PC07	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PC08	18.6	10.5	11.4	41.3	42.0	23.2	24.6	32.7	32.4	27.9	27.0
PC10	ND	ND	ND	N/A	N/A	N/A	N/A	N/A	N/A	N/A	ND
PC11	0.00423	ND	ND	ND	ND	1.03	1.00	0.322	0.340	1.24	1.37
PC13	0.0191	N/A	N/A	7.42	6.24	1.99	2.25	4.54	4.21	2.34	2.35
PC14	N/A	N/A	N/A	8.46	8.30	5.48	5.73	6.25	6.06	N/A	N/A
PC15	0.751	0.784	0.819	N/A	N/A	N/A	N/A	N/A	ND	ND	ND
PC16	1.40	3.60	4.13	0.977	1.97	N/A	0.746	N/A	N/A	1.37	1.40
PC17	0.0163	5.30	6.03	0.824	N/A	N/A	N/A	N/A	N/A	ND	ND
PC18	0.663	1.25	1.26	4.96	4.85	5.06	5.42	5.30	5.21	3.22	2.93
PC20	0.778	0.793	0.804	0.467	0.811	ND	N/A	ND	N/A	0.475	0.447
PEE01	7.45	2.72	2.76	8.64	8.81	4.69	5.03	5.59	5.32	6.90	6.57
PEE02	ND	ND	ND	N/A	N/A	N/A	N/A	N/A	N/A	N/A	ND
ST01	0.0854	12.7	13.2	3.23	3.66	3.72	3.99	1.83	1.92	5.85	6.01
OAs	1281	2166	2208	2190	2206	2338	2392	2716	2732	2476	2395
PAs	82.5	92.9	98.6	127	139	84.4	117	129	150	119	113

Table 4.6 Amounts of organic acids and phenolic compounds in 4 Thai red wine, 2 fruit wines, and imported red wine samples.

Analyte	Concentration (mg/L)							
	Thai wine				Fruit wine		Imported wine	
	SV12-1	SV12-2	SV14-1	SV14-2	MW-1	LW-1	CAPE15	FAT16
Organic acid								
OA01	72.4	72.7	111	109	816	93.9	101	45.1
OA02	541	494	574	519	132	663	884	990
OA03	518	516	478	453	461	376	559	383
OA04	1424	1492	1202	1195	293	656	921	775
OA05	161	157	134	125	170	813	144	55.2
Phenolic compound								
PC01	5.63	6.09	2.80	2.55	0.759	0.948	3.45	2.23
PC02	9.10	8.97	32.6	30.5	1.24	5.50	31.3	48.7
PC03	1.68	19.5	24.0	26.1	ND	25.8	16.4	43.9
PC04	25.0	17.5	15.0	7.24	16.6	2.98	3.06	3.07
PC05	9.62	10.6	9.99	9.58	ND	ND	2.25	1.45
PC06	ND	9.59	16.4	18.0	ND	25.3	16.8	43.2
PC07	N/A	N/A	N/A	N/A	ND	N/A	N/A	N/A
PC08	53.8	55.0	69.2	67.0	1.37	9.68	25.4	50.8
PC10	N/A	ND	N/A	N/A	ND	ND	ND	ND
PC11	N/A	N/A	ND	ND	N/A	ND	ND	ND
PC13	1.47	N/A	6.45	5.64	ND	ND	0.814	2.39
PC14	5.75	N/A	12.0	11.4	ND	N/A	4.86	3.77
PC15	N/A	N/A	ND	ND	ND	ND	1.05	1.57
PC16	N/A	2.69	2.93	4.60	N/A	N/A	17.1	10.3
PC17	N/A	2.11	ND	ND	ND	0.618	12.2	11.9
PC18	5.90	4.77	14.5	13.4	1.67	0.480	2.85	1.85
PC20	N/A	0.389	1.03	1.06	ND	0.380	N/A	N/A
PEE01	10.2	10.4	19.7	18.9	N/A	2.34	7.93	14.3
PEEE02	N/A	N/A	2.67	2.57	ND	ND	ND	ND
ST01	0.920	2.54	3.81	4.02	ND	N/A	2.81	6.09
OAs	2499	2401	2148	2093	1872	2602	2609	2248
PAs	233	223	117	114	21.6	74.0	148	246

From Tables 4.5 and 4.6, the validated method was used for analysis of 5 targeted organic acid and 20 targeted phenolic compounds in real wine samples. The result show that most of all targeted compounds of interest were found in all wine samples, except for ferulic acid and *p*-hydroxybenzoic acid which were not detected in these real wine samples.

All targeted organic acids were determined in all wine samples ranging from 4.0 to 1,500 mg/L. In comparison with to all 25 targeted compounds, lactic acid was found in the highest concentration in the range of 650-1,500 mg/L. This is the fact that naturally malic acid is converted into lactic acid during the malolactic fermentation in a winemaking process. Another predominant organic acid compounds are tartaric acid and succinic acid were found in range of 500-1000 and 330-560 mg/L, respectively, except for 290 mg/L of lactic acid and 130 mg/L of tartaric acid in mangosteen wine, and comparably small amount of 7.2 mg/L of tartaric acid and 4.8 mg/L of succinic acid in Granmonte Thai red wine (GM11).

Among 19 samples and 20 targeted phenolic compounds, the following targeted phenolic compound were found in all 17 red wines (Thai red wines and imported red wines) including caffeic acid, caftaric acid, (+)-catechin, coumaric acid, gallic acid, protocatechuic acid, gallic acid ethyl ester, resveratrol, chlorogenic acid and (-)-epicatechin in except for non-detected amounts of the latter two phenolic compounds in Granmonte Thai red wine (GM11) and Silverlake Thai red wine (SV12-1), respectively. Moreover, two phenolic compounds including ferulic acid and *p*-hydroxybenzoic acid were not detected in all samples. The phenolic compounds in all samples were found in a range of 0.00423-69.2 mg/L. Note that the predominant phenolic compounds in all samples are gallic acid, caftaric acid and (+)-catechin.

Among 15 Thai red wines samples, the major phenolic compounds include caftaric acid, (+)-catechin and chlorogenic acid, 28.5, 26.1 and 25.0 mg/L, respectively, whereas the major phenolic compounds found in 2 imported wines include caftaric acid, (+)-catechin and (-)-epicatechin, 48.7, 43.9 and 43.2 mg/L, respectively. For 2 fruit wine analysis, the major phenolic compounds found in these 2 fruit wines include (+)-catechin, (-)-epicatechin and chlorogenic acid, 25.8, 25.3 and 16.6 mg/L, respectively.

In comparison with the total phenolic compound content of all wine samples as in Figure 4.2, imported wine (FAT16) was found to have the highest total phenolic content. However, among Thai red wine, SV-14-1 was found to have the highest total phenolic compound content. In comparison with the total organic acids content in all samples as shown in Figure 4.3, insignificance difference in the total organic acid content was obtained with the average amount of 2,350 ± 220 mg/L, except for 1,281 mg/L found in Granmonte Thai red wine (GM11). Moreover, a total phenolic content for each sample is higher than a total organic acid content as shown in Figure 4.4, implying that organic acids were the major component of all wine samples. In comparison with all targeted compounds, organic acids were detected as the major component. Therefore, the predominant compounds in wine samples were lactic acid, tartaric acid and succinic acid, respectively.

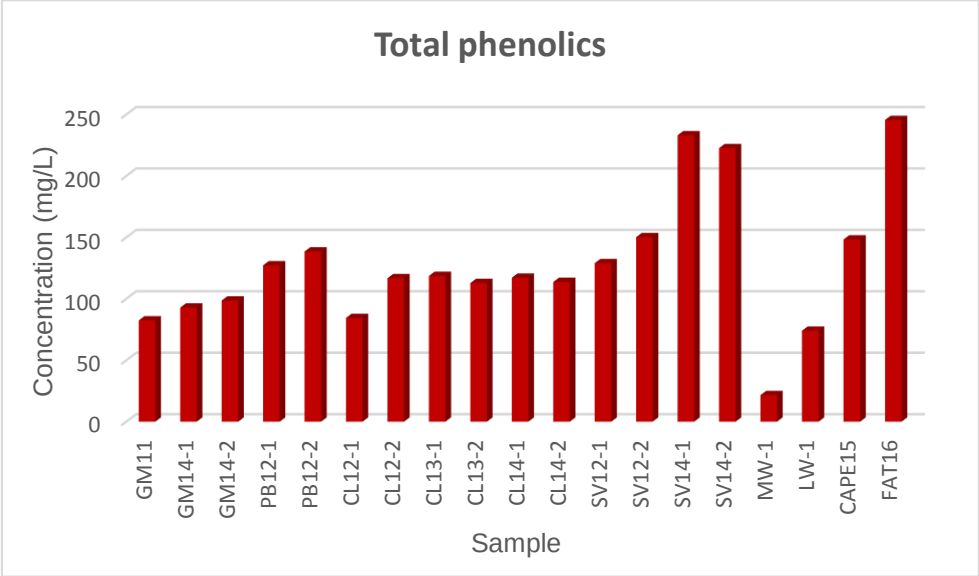


Figure 4.2 The amount of total phenolic compound content in all wine samples

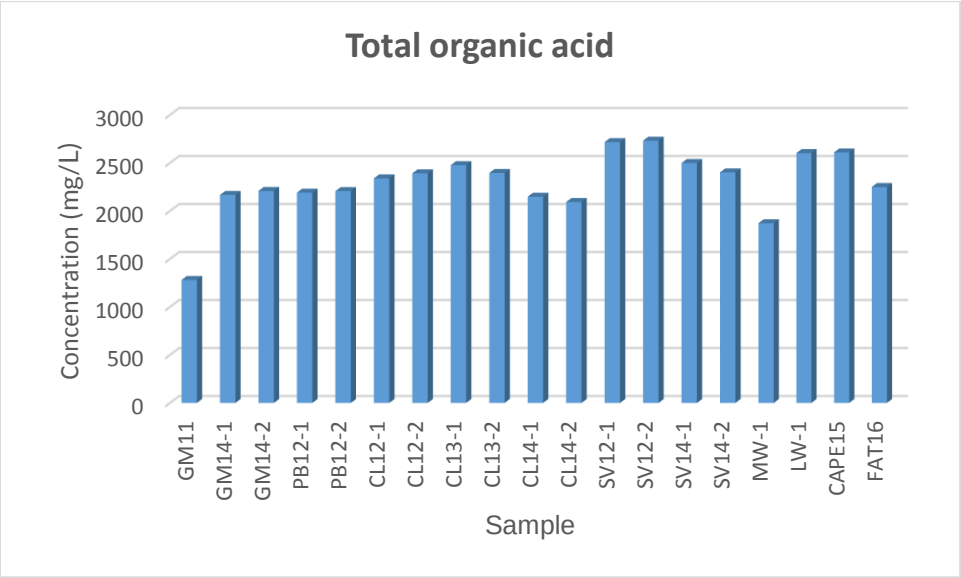


Figure 4.3 The amount of total organic acid content in all wine samples

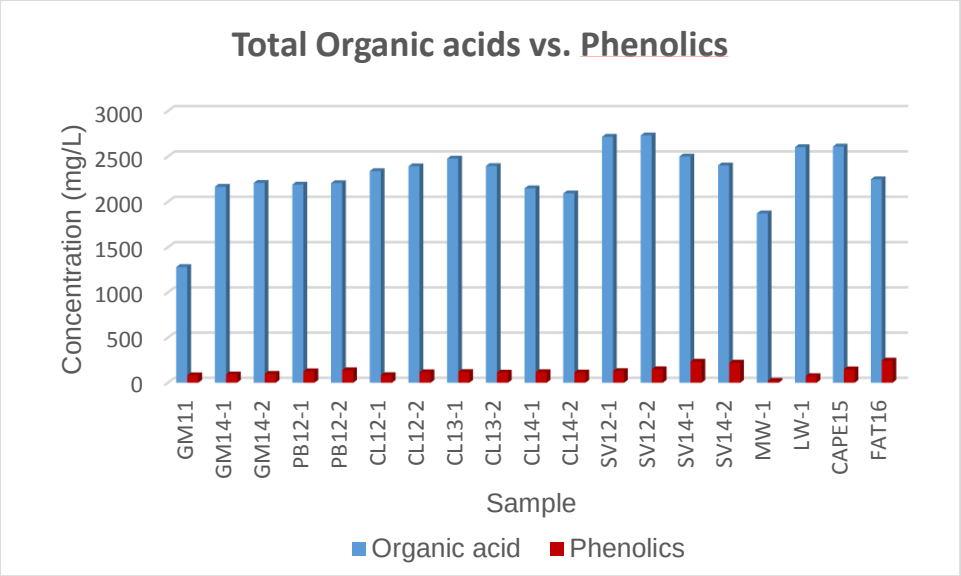


Figure 4.4 The amount of total phenolic compound content compared with total organic acid content

4.5 Data analysis

4.5.1 Principal component analysis (PCA)

Due to the huge and complicated data of non-volatile compounds, PCA was applied to visualize the data of Thai wines with different origins and production years, and imported wines. The data matrix consisted of concentrations of 25 non-volatile compounds and 19 samples. Before PCA processing, each analyte concentration was normalized by the total concentration of all compounds in the same sample. The PCA score plot was shown in Figure 4.5. The criteria in grouping samples is based on the originated samples including Thai wine, imported wines and fruit wines. Three clusters were observed including cluster A, cluster B and cluster C. Cluster A includes 12 Thai red wines (PB12-1, PB12-2, CL12-1, CL12-2, CL13-1, CL13-2, CL14-1, CL14-2, SV12-1, SV12-2, SV14-1 and SV-14-2) from 3 origins, cluster B includes 3 Thai red wines (GM11, GM14-1 and GM14-2) and 2 imported red wines (CAPE15 and FAT 16), and cluster C includes 2 fruit wines (MW-1 and LW-2). The samples in the same cluster have a similar chemical profile.

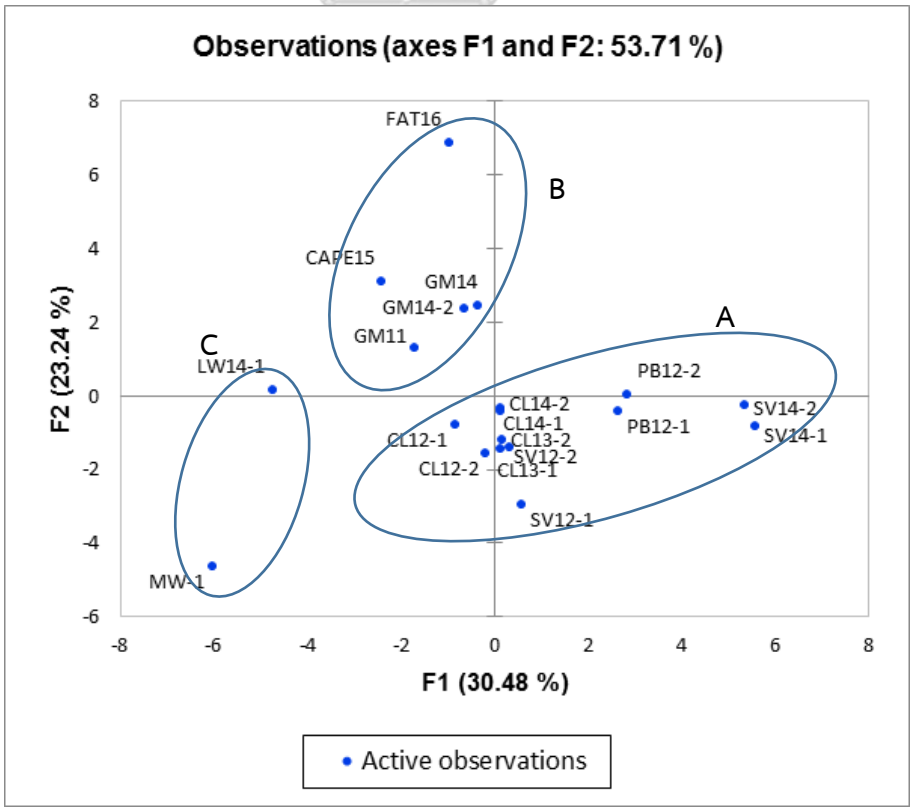


Figure 4.5 PCA score plot of 19 samples

Figure 4.6 shows the biplot of 19 samples and 25 non-volatile compounds which represent a relationship between the correlated compounds and their sample group. The significant compounds (markers) of each cluster can be selected from the compounds projected onto the region of that cluster. The results show that important compounds for cluster A is synringic acid, for cluster B is kaempferol, and for cluster C are citric acid and malic acid. Moreover, the distribution plots of significant compounds are shown in Figures 4.7 – 4.10. This implies that these compounds may be used as discriminant markers of each cluster

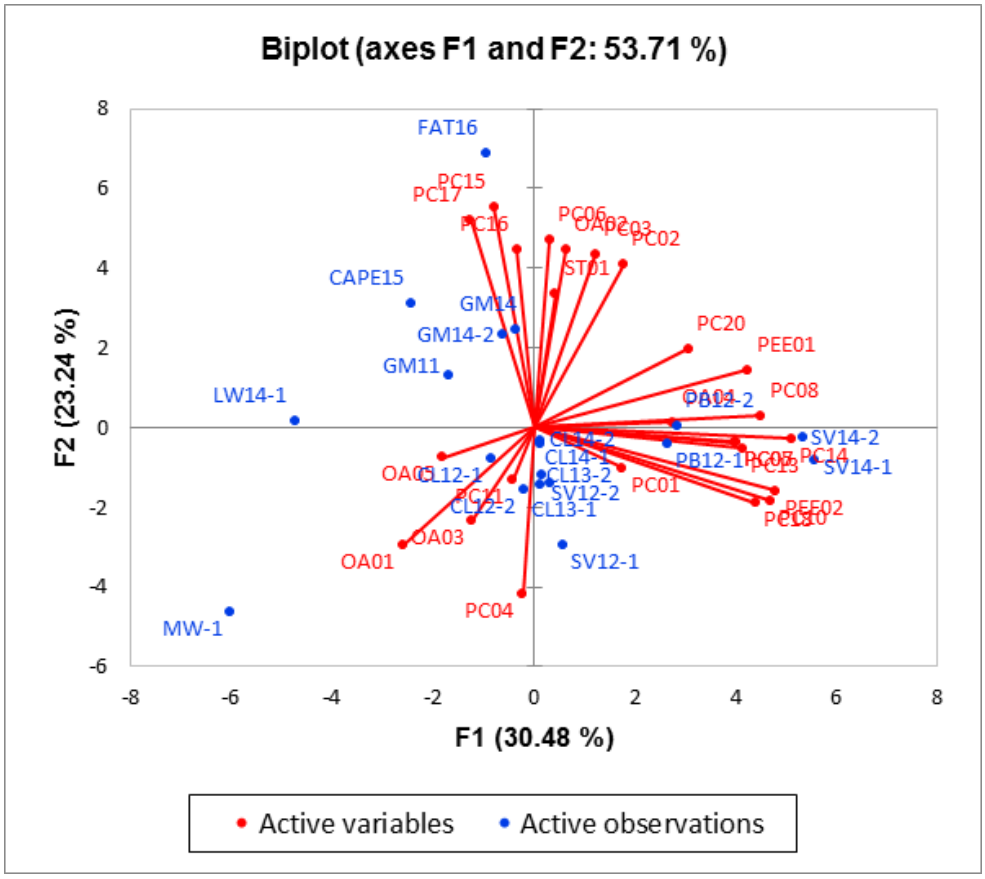


Figure 4.6 PCA biplot of 19 samples and 25 non-volatile compounds

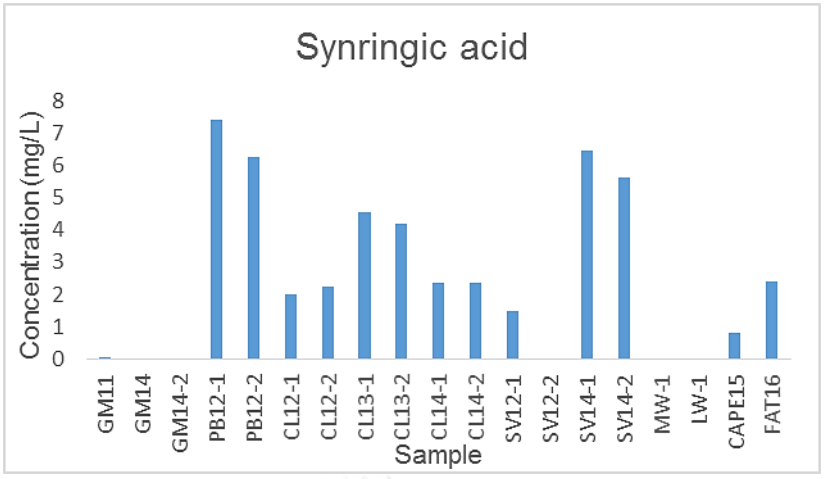


Figure 4.7 The distribution plots of significant compounds of cluster A

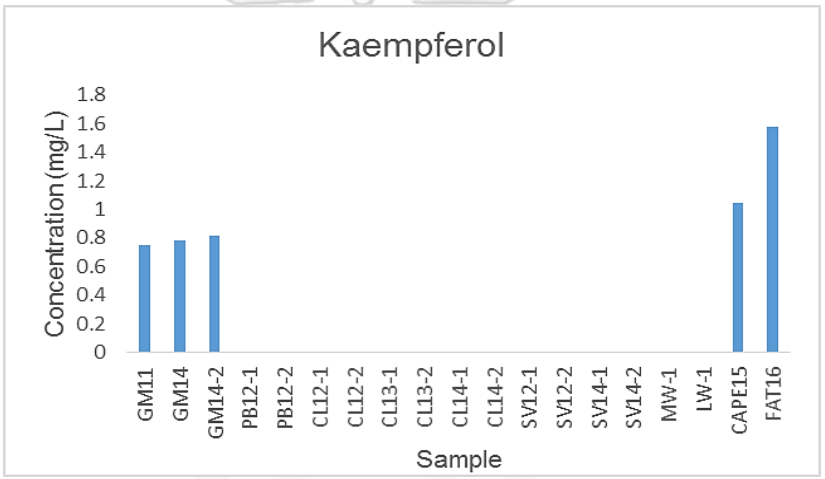


Figure 4.8 The distribution plots of significant compounds of cluster B

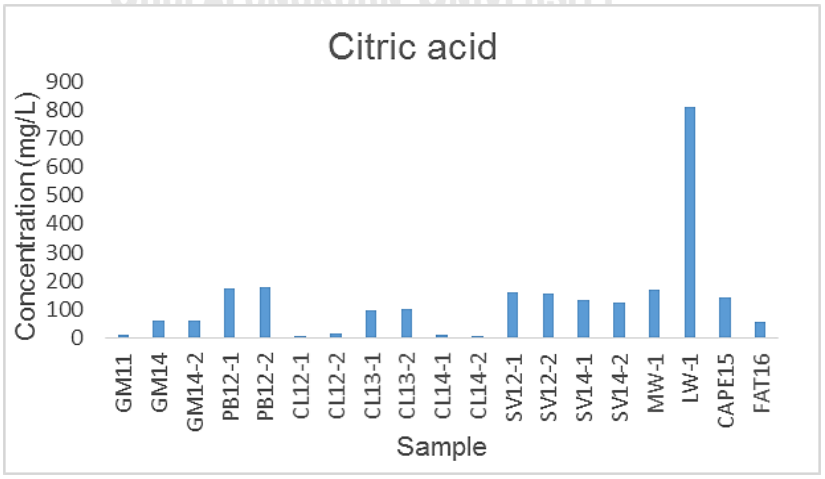


Figure 4.9 The distribution plots of significant compounds of cluster C (1)

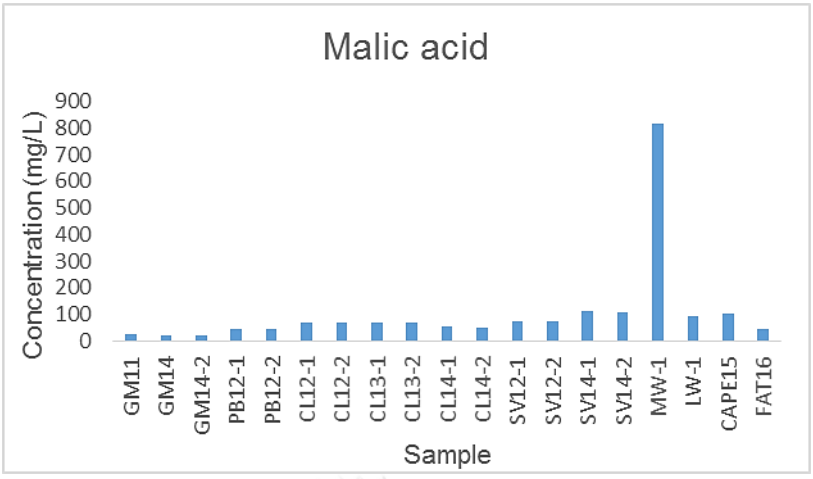


Figure 4.10 The distribution plots of significant compounds of cluster C (2)

CHAPTER IV
CONCLUSIONS

The HPLC-MS/MS method was developed for a simultaneous determination of 25 targeted non-volatile compounds, including 5 organic acids and 20 phenolic compounds, in 19 Thai wines with different geographical origins and production years. The optimal HPLC conditions were performed with a C18 reverse phase column separation. The mobile phase consisting of 0.1% v/v formic acid in water and 0.1% v/v formic acid in methanol was used at a flow rate of 0.4 mL/min in a gradient elution mode. The HPLC-MS/MS triple quadrupoles mass analyzer with electrospray ionization (ESI) interface was performed. A multiple-reaction monitoring (MRM) was operated in both positive and negative modes. MassHunter Workstation software was used to control the instruments, data acquisition, and processing. The rapid sample preparation was carried out by diluting the wine sample with 50% methanol in water and filtrating the diluted sample with 0.22 μm nylon syringe filters.

For method validation, analytical limits, internal standard curve, accuracy and precision were validated. The analytical limits including LOD and LOQ were obtained in the range of 0.0084-0.48 mg/L and 0.026-1.45 mg/L, respectively. Good linearity for internal standard calibration curves was found with correlation coefficient (R^2) higher than 0.91. By spiking known amount of standards in diluted pooled wine samples, the satisfactory accuracy was evaluated with the recovery showing that 84.9% of 225 data sets for %recovery falls within the acceptable criteria values, along with acceptable intraday precision, with the %RSD values in a range of 0.4-7% which all data set fall in a range acceptable criteria.

Using this validated HPLC-MS/MS analysis of targeted organic acids and phenolic compounds in real samples including 15 Thai red wines, 2 imported red wines and 2 fruit wines, most of all targeted compounds of interest were found in these real samples except for non-detected amount of ferulic acid and *p*-hydroxybenzoic acid. The organic acids found in all samples were the range of 4.0-1500 mg/L, especially the major amount of lactic acid of 650-1500 mg/L, tartaric acid of 500-1000 mg/L and

succinic acid of 330-560 mg/L except for 290 mg/L of lactic acid and 130 mg/L of tartaric acid in mangosteen wine, and comparably small amount of 7.2 mg/L of tartaric acid and 4.8 mg/L of succinic acid in Granmonte Thai red wine (GM11).

Among all 19 samples and 20 targeted phenolic compounds, two phenolic compounds including ferulic acid and *p*-hydroxybenzoic acid were not detected in all samples. The following targeted phenolic compound were found in all 17 red wines: caffeic acid, caftaric acid, (+)-catechin, coumaric acid, gallic acid, protocatechuic acid, gallic acid ethyl ester, resveratrol, ferulic acid, *p*-hydroxybenzoic acid, chloragenic acid and (-)-epicatechin in except for non-detected amounts of the latter two phenolic compounds in Granmonte Thai red wine (GM11) and Silverlake Thai red wine (SV12-1), respectively. In addition, phenolic compounds in all samples were ranged in 0.00423-69.2 mg/L. Note that the predominant phenolic compounds in all samples is gallic acid, caftaric acid and (+)-catechin.

Among Thai red wines, the major phenolic compounds include caftaric acid, (+)-catechin and chlorogenic acid, 28.5, 26.1 and 25.0 mg/L, respectively, while the major phenolic compounds found in 2 imported wines include caftaric acid, (+)-catechin and (-)-epicatechin, 48.7, 43.9 and 43.2 mg/L, respectively. For 2 fruit wine analysis, the major phenolic compounds found in 2 fruit wines include (+)-catechin, (-)-epicatechin and chlorogenic acid, 25.8, 25.3 and 16.6 mg/L, respectively.

In comparison of the total phenolic compound content of all samples, imported wine (FAT16), was found to have the highest total phenolic content and Thai red wine (SV-14-1) was found to have the highest total phenolic compound content. In comparison of the total organic acids content in all samples except for Granmonte Thai red wine (GM11), insignificance difference in the total organic acid content of are obtained with average amount of 2,350 $\pm$ 220 mg/L, but the much lower amount of total organic acids content was found in GM11 at 1,281 mg/L . Moreover, a total phenolic content for each sample is higher than a total organic acid content, implying that organic acids were the major component of all samples.

By using a PCA approach for 19 samples and 25 variables, three clusters were observed. Cluster A includes 12 Thai red wines (PB12-1, PB12-2, CL12-1, CL12-2, CL13-1, CL13-2, CL14-1, CL14-2, SV12-1, SV12-2, SV14-1 and SV-14-2) from 3 origins, cluster B 1includes 3 Thai red wines (GM11, GM14-1 and GM14-2) and 2 imported red wines (CAPE15 and FAT 16), and cluster C includes 2 fruit wines (MW-1 and LW-2). It can be implied that most of Thai red wines with different geographical origins and production years have the similar targeted non-volatile compositions, except Granmonte Thai red wines (GM11, GM14-1 and GM14-2) From the PCA biplot, the obtained significant compounds of cluster A is syringic acid, cluster B is kaempferol and cluster C are citric acid and malic acid.

In future work, this developed HPLC-MS/MS method should be investigated for interlaboratory precision in order to complete the validation method for a wide range of another type of wine sample and beverage. Moreover, this developed HPLC-MS/MS method can be applied in determination of targeted non-volatile compounds in other samples or only determination of significant compounds for quality control and authentication with faster and cheaper analysis.

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APPENDIX

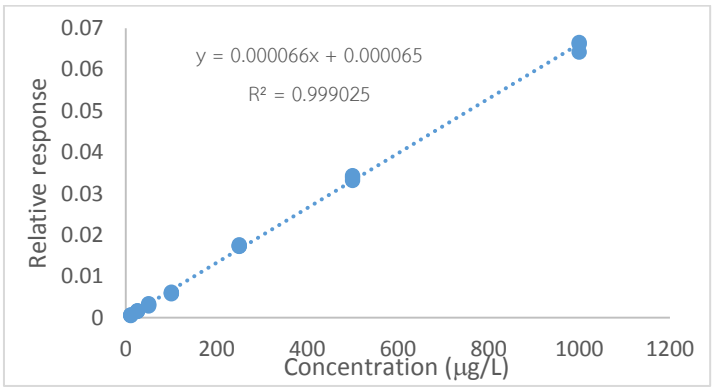

Figure A1 Internal standard calibration curve of Caffeic acid

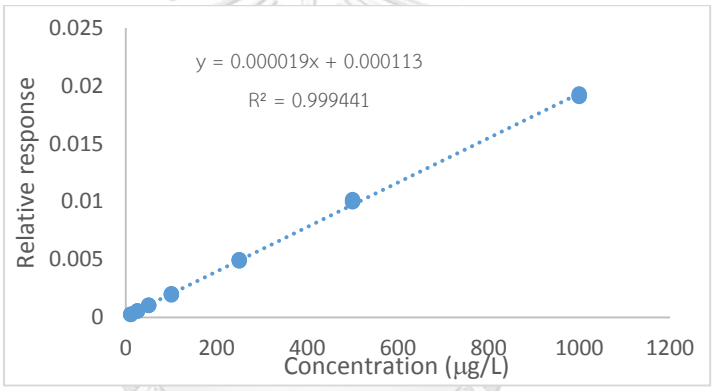

Figure A2 Internal standard calibration curve of Chlorogenic acid

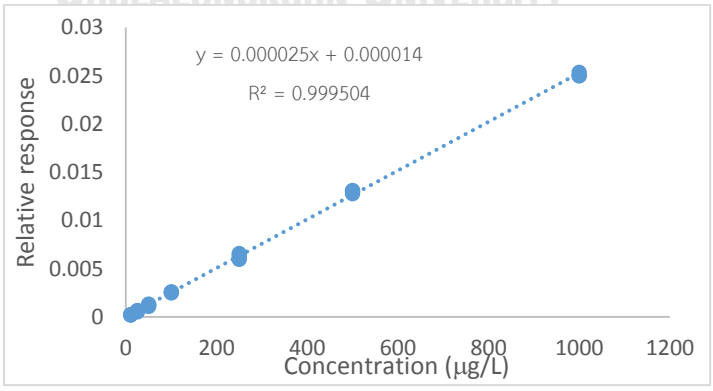

Figure A3 Internal standard calibration curve of Ferulic acid

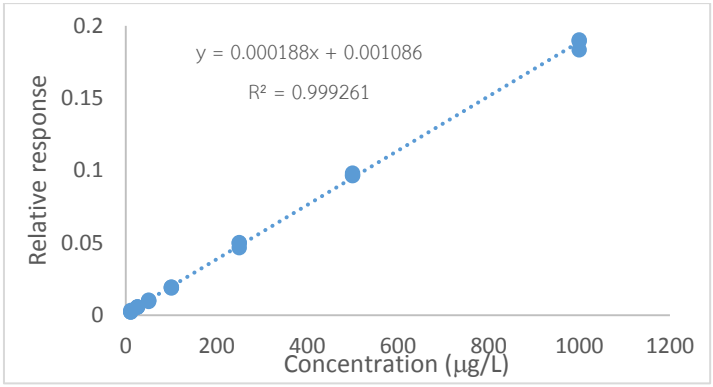

Figure A4 Internal standard calibration curve of *p*-Hydroxybenzoic acid

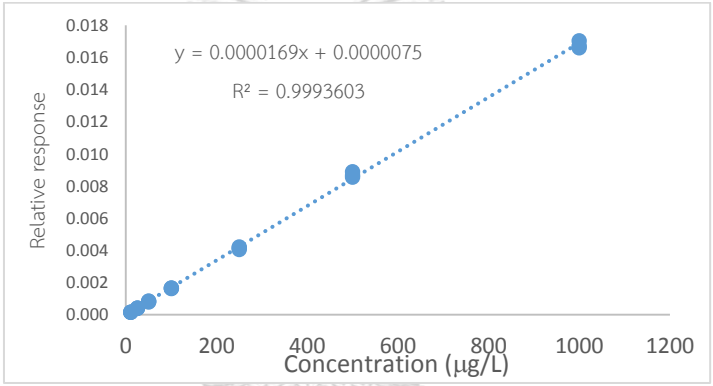

Figure A5 Internal standard calibration curve of rutin

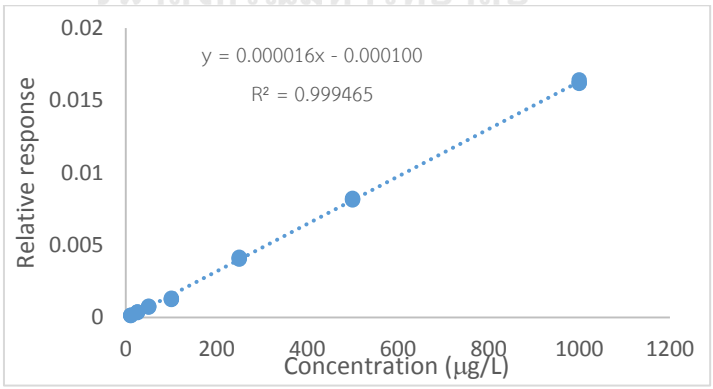

Figure A6 Internal standard calibration curve of Synringic acid

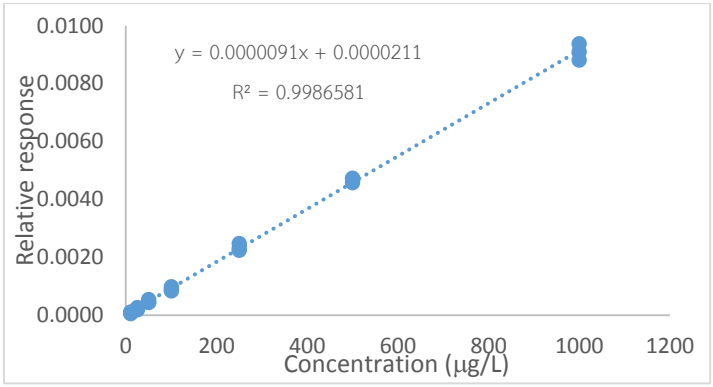

Figure A7 Internal standard calibration curve of Vanillic acid

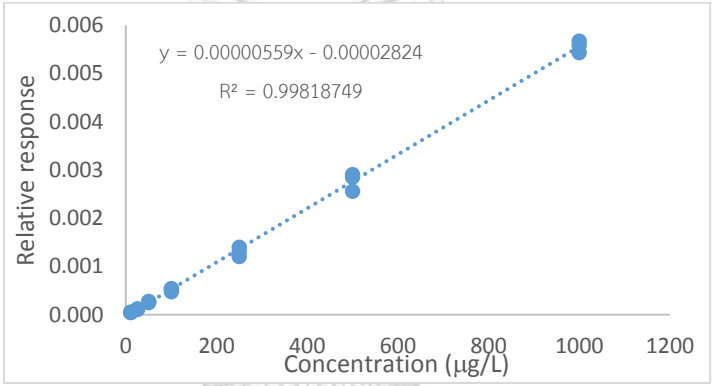

Figure A8 Internal standard calibration curve of Kaempferol

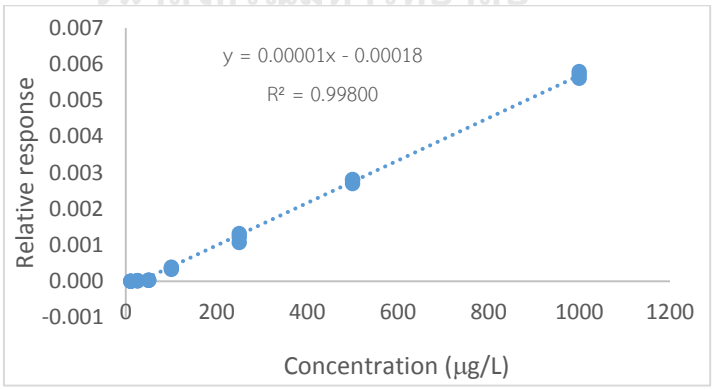

Figure A9 Internal standard calibration curve of Myricetin

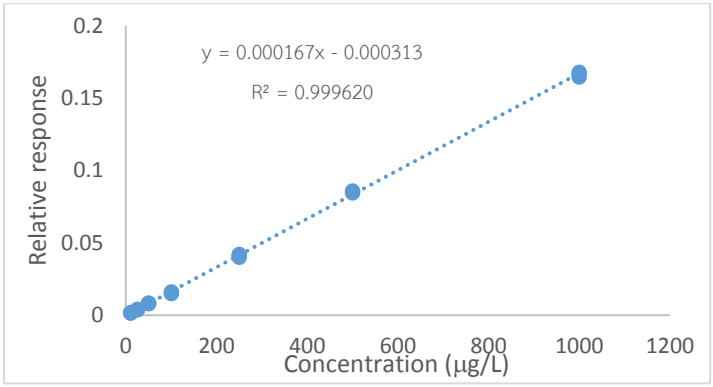

Figure A10 Internal standard calibration curve of Gentistic acid

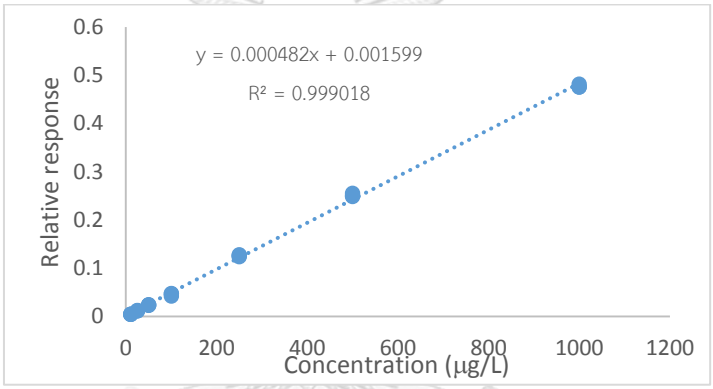

Figure A11 Internal standard calibration curve of Protocatechuic acid ethyl ester

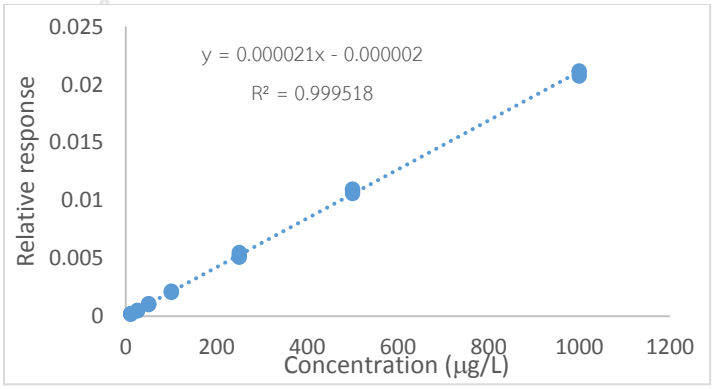

Figure A12 Internal standard calibration curve of Resveratrol

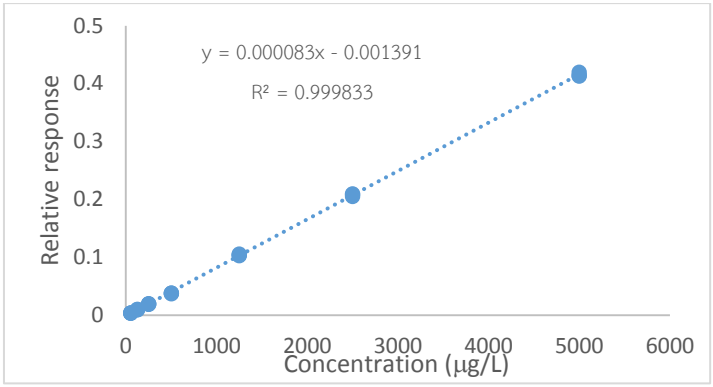

Figure A13 Internal standard calibration curve of Caftaric acid

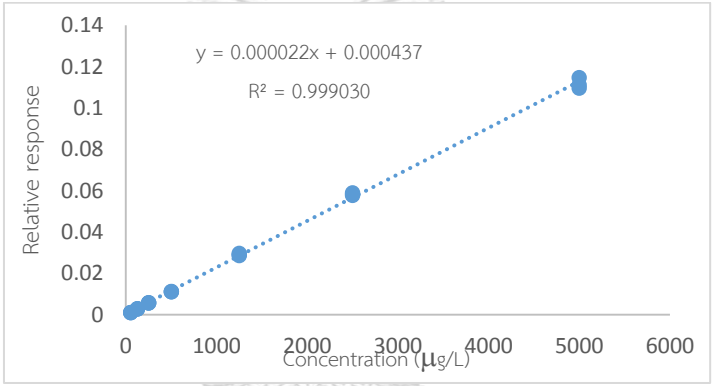

Figure A14 Internal standard calibration curve of (+)-catechin

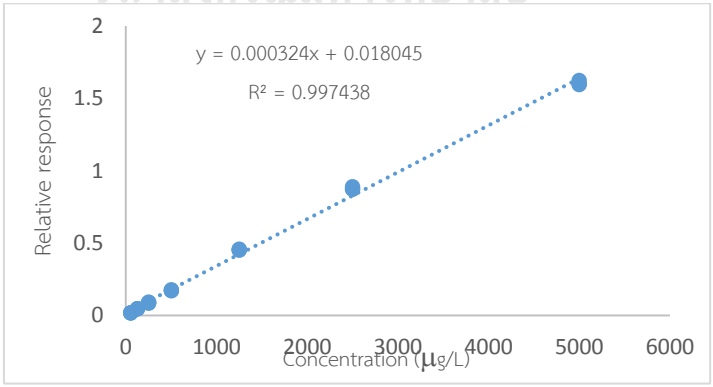

Figure A15 Internal standard calibration curve of Coumaric acid

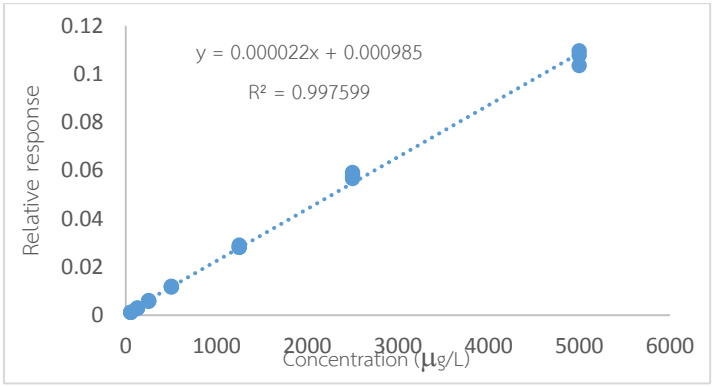

Figure A16 Internal standard calibration curve of (-)-epicatechin

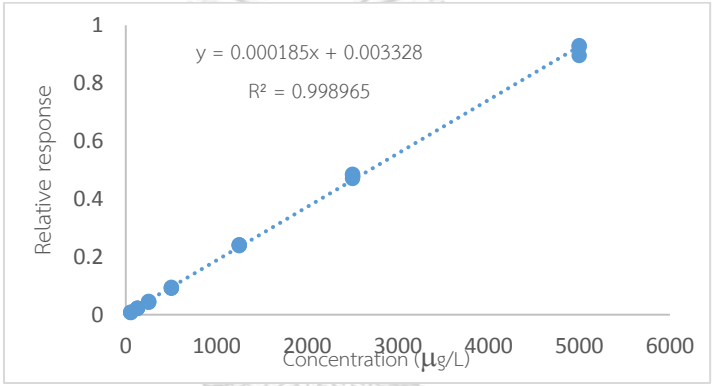

Figure A17 Internal standard calibration curve of gallic acid

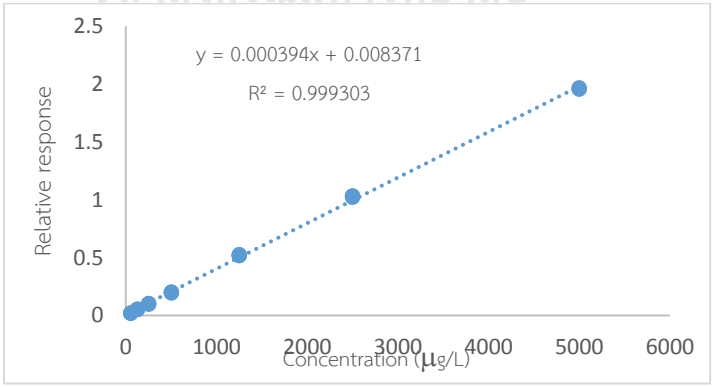

Figure A18 Internal standard calibration curve of gallic acid ethyl ester

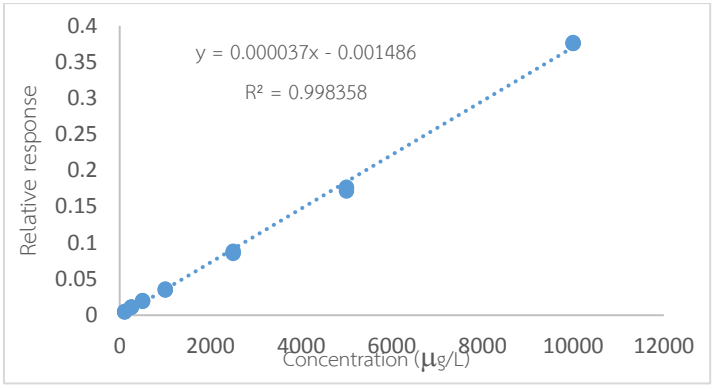

Figure A19 Internal standard calibration curve of malic acid

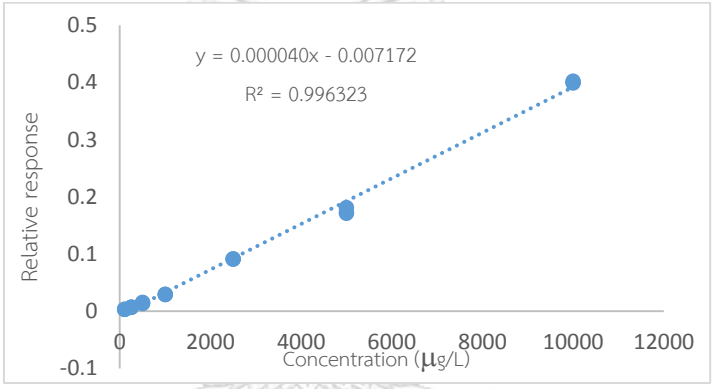

Figure A20 Internal standard calibration curve of citric acid

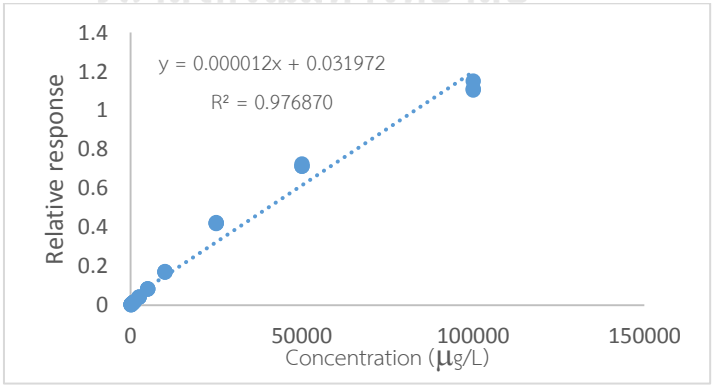

Figure A21 Internal standard calibration curve of tartaric acid

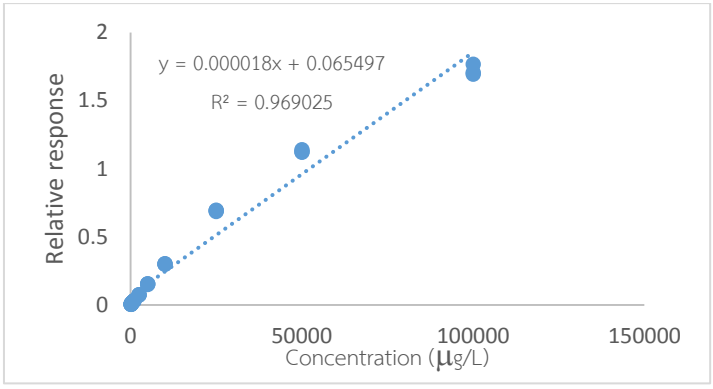

Figure A22 Internal standard calibration curve of succinic acid

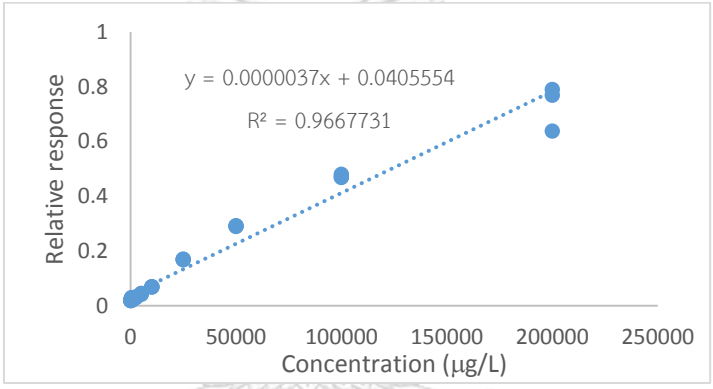

Figure A23 Internal standard calibration curve of lactic acid

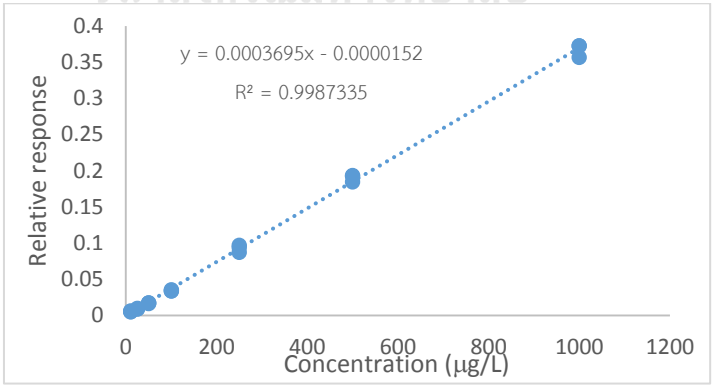

Figure A24 Internal standard calibration curve of quercetin

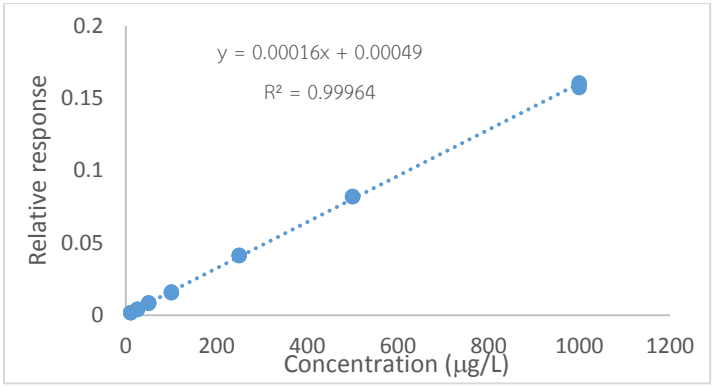

Figure A25 Internal standard calibration curve of Protocatechuic acid

VITA

Miss Premkamol Karapakdee was born on September 17, 1992, in Saraburi, Thailand. She graduated with Bachelor's of science in Chemistry from Mahidol University 2015. Afterwards, she continued her study for Master degree in Analytical Chemistry at Department of Chemistry, Faculty of Science, Chulalongkorn University and graduated with a Master's degree in 2018.