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โดยไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมตรี

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CHARACTERIZATION OF NON-VOLATILE COMPOUNDS IN TOM YUM SOUP USING
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Thesis Title	CHARACTERIZATION OF NON-VOLATILE COMPOUNDS IN TOM YUM SOUP USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY
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วนบุษป์ วงศ์บุปผา : การพิสูจน์เอกลักษณ์ของสารระเหยยากในต้มยำโดยไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมตรี (CHARACTERIZATION OF NON-VOLATILE COMPOUNDS IN TOM YUM SOUP USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.ธรรมนูญ หนูจักร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร.ชฎิล กุลสิงห์, 79 หน้า.

ในงานวิจัยนี้ได้พัฒนาและตรวจสอบความใช้ได้ของวิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมตรี (HPLC-MS/MS) สำหรับวิเคราะห์ปริมาณสารระเหยยากแบบจำเพาะคราวเดียวกัน ได้แก่ กรดอินทรีย์จำนวน 2 ชนิด, กรดอะมิโนจำนวน 20 ชนิด และสารประกอบฟีนอลิกจำนวน 9 ชนิดในตัวอย่างวัตถุดิบหลักของต้มยำ, ต้มยำที่ปรุงขึ้นจากวัตถุดิบสด และเครื่องปรุงต้มยำสำเร็จรูปที่เป็นองค์ประกอบหลักของต้มยำ ได้แก่ ข่า, ตะไคร้, ใบมะกรูด, พริกขี้หนู, มะนาว และน้ำปลา โดยกลุ่มสารระเหยง่ายดังกล่าวถูกแยกโดยใช้ภาวะ HPLC-MS/MS ได้แก่ PoroShell C18 คอลัมน์ (4.6 x 100 มิลลิเมตร, 2.7 ไมโครเมตร) ในระบบการชะแบบเกรเดียน A:B ที่อัตราการไหล 0.3 มิลลิลิตรต่อนาที เฟสเคลื่อนที่ A ประกอบด้วย 0.1% โดยปริมาตรของกรดฟอร์มิกในน้ำ และ B ประกอบด้วย 0.1% โดยปริมาตรของกรดฟอร์มิกในเมทานอล ขีดจำกัดในการวิเคราะห์ประกอบด้วยขีดจำกัดในการตรวจหาและขีดจำกัดการบอกปริมาณของการตรวจวิเคราะห์ สารประกอบแบบจำเพาะนี้อยู่ในช่วง 0.010 – 0.62 และ 0.032 – 1.9 มิลลิกรัมต่อลิตร ตามลำดับ จากการเติมสารมาตรฐานที่ทราบค่าความเข้มข้น 3 ความเข้มข้นลงในตัวอย่างต้มยำเจือจางพบว่ามีความถูกต้องของวิธีการวิเคราะห์ที่อยู่ในเกณฑ์ยอมรับคิดเป็น 98.6% ของข้อมูลทั้งหมดที่อยู่ในเกณฑ์ยอมรับในช่วง 80-110% รวมไปถึงความเที่ยงที่มีค่าร้อยละส่วนเบี่ยงเบนมาตรฐานอยู่ระหว่าง 9-16 ซึ่งอยู่ในเกณฑ์การยอมรับได้ทั้งหมด

สำหรับการวิเคราะห์ด้วยเทคนิค HPLC-MS/MS ในวัตถุดิบหลักของต้มยำและต้มยำที่ปรุงขึ้นจากวัตถุดิบสดพบสารระเหยยากหลักจากแหล่งวัตถุดิบต่างๆ ได้แก่ กรดอินทรีย์จำนวน 2 ชนิด ประกอบด้วยกรดซิตริก 2714 มิลลิกรัมต่อกิโลกรัม และกรดมาลิก 212 มิลลิกรัมต่อกิโลกรัมจากน้ำมะนาว ส่วนกรดอะมิโน 14 ชนิดจากน้ำปลา และกรดอะมิโนจำนวน 6 ชนิดจากน้ำมะนาว และสารประกอบฟีนอลิกจากแหล่งสมุนไพรหลายชนิดด้วยกัน สำหรับปริมาณกรดอะมิโนหลักสูงสุด 5 อันดับแรกในต้มยำ ได้แก่ กรดแอสปาร์ติก, ฟีนิลอะลานีน, ไอโซลิวซีน, วาลีน และลิวซีน ในช่วง 370-623 มิลลิกรัมต่อกิโลกรัม และสารประกอบฟีนอลิกหลักในต้มยำ ได้แก่ อะซิทอกซีคาวิคอลอะซิเตตซึ่งเป็นสารสำคัญในข่า ดังนั้นการวิเคราะห์โดยเทคนิค HPLC-MS/MS สามารถตรวจวัดสารระเหยยากทั้งในเชิงปริมาณและสามารถระบุแหล่งที่มาของสารสำคัญในต้มยำได้

ภาควิชา	เคมี	ลายมือชื่อนิสิต ....
สาขาวิชา	เคมี	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
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WANABUD WONGBUBPHA: CHARACTERIZATION OF NON-VOLATILE COMPOUNDS IN TOM YUM SOUP USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY. ADVISOR: ASSOC. PROF. THUMNOON NHUJAK, Ph.D., CO-ADVISOR: CHADIN KULSING, Ph.D., 79 pp.

In this work, high performance liquid chromatography hyphenated with tandem mass spectrometry (HPLC-MS/MS) was developed and validated for simultaneous determination of 31 targeted non-volatile compounds, such as 2 organic acids, 20 amino acids and 9 phenolic acids, in Tom Yum ingredient, Tom Yum soup made from the fresh ingredients and commercial Tom Yum pastes, where the main Tom Yum ingredients include galangal, lemongrass, kaffir lime leaves, Bird's eye chili, lime juice and fish sauce. The following HPLC-MS/MS conditions were used: a PoroShell C18 column (4.6 x 100 mm, 2.7 μm) using a gradient elution of A:B mobile phase at a flow-rate of 0.3 mL/min, where A consisted of 0.1%v/v formic acid in water and B consisted of 0.1%v/v formic acid in methanol. This developed HPLC-MS/MS method can determine these targeted non-volatile compounds with the limit of detection and the limit of quantitation in the range of 0.010 – 0.62 and 0.032 – 1.9 mg/L, respectively. Using pooled diluted Tom Yum soup spiked with standard at known concentrations, acceptable accuracy for quantitative analysis was obtained with the recoveries in a range of 62-115%, which is 98.6% of the recovery data being within 80-110% for the analytes concentration in the range of 0.06-3 ppm. An accepted level of precision intraday and interday were also obtained in the range of 9-16 with RSD.

From the HPLC-MS/MS analysis of ingredient and Tom Yum soup, the main sources of targeted non-volatile compounds were obtained: two organic acids including citric acids of 2714 mg/kg and malic acid of 212 mg/kg from lime juice, fourteen amino acids from fish sauce, another six amino acids from lime juice, phenolic compounds from various sources. The top five amino compounds found in Tom Yum soup were aspartic acid, phenylalanine, isoleucine, valine and leucine in the range 370-623 mg/kg. In addition, acetoxy chavicol acetate of 98 mg/kg, mainly from galangal, was found to be the predominant phenolic compounds found in Tom Yum soup. Note that the unit of mg/kg here refers to the mass of a hot water-dissolving compounds in prepared Tom Yum paste. This developed HPLC-MS/MS analysis can be used for quantitative determination and identify the sources of non-volatile compounds in Tom Yum soup.

Department:	Chemistry	Student's Signature ....
Field of Study:	Chemistry	Advisor's Signature ....
Academic Year:	2017	Co-Advisor's Signature ....

ACKNOWLEDGEMENTS

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A	slope of calibration curve
ACN	acetonitrile
ACA	acetoxy chavicol acetate
℃	degree Celsius
CE	collision energy
CID	collision-induced dissociation
cm	centimeter
DC	direct current voltage
ESI	electrospray ionization
FA	formic acid
g	gram
h_0	Leverage for the blank sample
HPLC	high performance liquid chromatography
HPLC-MS/MS	high performance liquid chromatography-tandem mass spectrometer
I	Number of calibration samples
I.D.	Internal diameter
kg	kilogram
L	liter
L	levels of calibration
LC	liquid chromatography
LOD	limits of detection
LOQ	limits of quantitation
M	molar
MeOH	methanol
mg	milligram
min	minute
mL	milliliter

MW	molecular weight
MS	mass spectrometer
m/z	mass to charge ratio
Quad	quadrupole
r^2	correlation coefficient
RSD	relative standard deviation
SD	standard deviation
$S_{y/x}$	Residual standard deviation
v/v	volume by volume
y_i	experimental response values for sample
$\hat{y}$	estimated response values for sample
µg	microgram
µL	microliter

CHAPTER I

INTRODUCTION

1.1   Problem Definition

Thai food is renowned worldwide for its variation of flavors and rich nutrients. The utilization of Thai ingredients is essential for preparing the recipe, in order to create the aromatic taste and aroma. Tom Yum is one of the most famous sour and spicy soups, which is prepared by boiling several ingredients including bird's eye chili, galangal root, lemongrass, kaffir lime leaves and acid fruits such as lime juice or tamarind pulp [1]. This menu is one of the healthiest Thai foods, provides several health benefits such as cancer prevention and antioxidant properties [2]. However, these fresh vegetables and spices cannot be kept for a long time before cooking. Therefore, the frozen and dried ingredient are used to preserve the unique flavor of the ingredients. Herbs and Spices are commonly used in traditional Thai's dishes and contain combination of tastes such as sour, sweet, salty, bitter and even the tastes of human feeling; spiciness, astringent etc. These were contributed by the presence of non-volatile compounds such as phenol compounds, organic acids, sugar, fatty acids, alkaloids, vitamin etc.

Nowadays, Ready-to-cook convenient foods are becoming popular. Thai's food industrial is developing Tom Yum seasoning to export the characteristic Thai's food into International market. Generally, there are several types of commercial Tom Yum such as instant product, powder and dried seasoning [3] that may not be exactly like the traditional soup mostly prepared with fresh ingredients such as citric acid; one of the most common additives in majority of food and drink products. These products are labeled under either citric acid or its E number, E330 [4] and used in soup instead of fresh ingredients.

Tastes of authentic Tom Yum soup were studied in Thai food industry in order to guarantee the original taste of Tom Yum and to serve a consumer demand in different countries around the world. Tom Yum products available in markets and

department stores in Thailand contain many chemical components of Tom Yum that is nutritious, flavorful and colorful to improve taste, texture and appearance such as spices, natural and artificial flavors. However, the products' tastes may be different. This study thus develops approaches to characterize non-volatile compounds in Tom Yum prepared according to the same basic ingredients including lemongrass, galangal, fish sauce, kaffir lime leaves, bird's eye chili, lime juice and fish sauce.

1.2   Literature review

Chemical analysis of food was interested to guarantee food quality. The analysis involves several types of samples from raw ingredients and materials to the processed products. Targeted compound analysis in food includes volatiles, semi-volatiles and non-volatiles such as flavor, antioxidants, polyphenols, food additives as well as the contaminants [5]. The amounts of major compounds in different food depend on their physical properties such as their polarity or solubility in plants [6]. The chemical components of several ingredients used in Tom Yum have been analyzed.

Limes (*Citrus aurantifolia* (Christm.) Swingle.) are citrus fruits that have a green round shape, which contain major organic acids such as citric and malic acids with a trace amount of tartaric acid, benzoic acid, oxalic acid and succinic acid reported [7]. So limes are mostly used as fresh or as juice in order to preserve their nutritional values and special flavors which are mainly sour or tart. Furthermore, lime juice can increase in bitterness when held at room temperature for an extended period of time due to the delayed conversion of the non-bitter precursor molecule to limonin. Limonin is commonly analyzes in commercial juice [8].

Bird's eye chilies (*Capsicum frutescens* L.) are an important ingredient in Thai cuisine due to it's hot and strong pungency spices. Thai's name called Prik kee Noo. These fruits contain capsaicinoids, a family of compounds that give them the characteristic pungent taste. The two major capsaicinoids are capsaicin and

dihydrocapsaicin followed by nordihydrocapsaicin, homodihydrocapsaicin and homocapsaicin [9, 10]

Galangal (*Alpinia galanga* L.) is a pungent and aromatic rhizome, which is a member of the ginger family. It called Thai galangal, greater galangal, or Kha in Thailand. The rhizomes are widely used as a spice or ginger substitute for flavoring food throughout southeastern Asian countries. The major compounds include acetoxy chavicol acetate (ACA) and hydroxyl chavicol acetate (HCA) that the pungent principle compounds are exhibit anti-inflammatory, antitumor, and analgesic actions [11].

Kaffir lime leaf (*Citrus hystrix* DC.) is a member of the genus *Citrus*. These leaves are dark green and have a glossy sheen at different ages. The main use of kaffir lime leaves is as a flavouring, especially in Asian cuisines such as Tom Yum and curries dishes. Previous study showed that the leaf contains alkaloid, flavonoid, tannin and saponin compounds. Moreover, Its exhibit antioxidant activity, antimicrobial activity and anti-inflammatory [2, 12].

Lemongrass (*Cymbopogon citratus* DC. Stapf) is a native herb from tropical and subtropical countries. It can be used in Asian cuisine. Commonly used in soups, curries and teas for its lemon flavor, lemongrass is used in fresh, dried and powdered form and also used in medicine to treat fever conditions and as a relaxant and sleeping aid. The leaves constitute a source of essential oil for the flavor and fragrance industries [13]. Moreover, the investigation of the chemical constituents was founded alkaloids, non-volatile terpenoids, flavonoids, carotenoid and tannins from every part of *Cymbopogon* species [14].

Fish sauce is a rich source of variety essential amino acids that made from tropical fish species, there have been numerous investigation of fish sauce fermentation using different types of raw material. The fermentation process was extended the shelf- life and also enhances the flavor and nutritional quality of fish sauce[15]. Normally, fish sauce were reported high amino acids including methionine, histidine and lysine [16].

Currently, several sample preparation methods for analysis of food ingredients have been reported such as organic solvent extraction using ethanol, hexane, methanol, acetone or ethyl acetate [17-20], the extraction performance depends on compounds solubility in different solvents. Other conventional extraction techniques include heating-reflux extraction, maceration and hydrodistillation. However, these sample preparation processes are often time consuming and use toxic solvents [21, 22].

Alternatively, water is the green extraction solvent, nontoxic, nonflammable and pollution prevention [23]. Besides, water has unique properties to dissolve water soluble non-volatile species such as organic acids, phenolic acids, alcohol, sugar, as well as for inorganic substances [24].

High performance liquid chromatography (HPLC) is one of the most versatile techniques to separate and determine a variety chemical compounds. The technique has been applied in the area of food analysis for quality control, safety of food, detection of authentic product, control of contaminant, etc. [25]. In the context of food authenticity, various types of detectors can be used such as single or multiple wavelengths UV-Vis detector or fluorescence for determination of low analyte levels. The different detection techniques enable not only highly sensitive but also highly selective analysis of compounds. Among different approaches, mass spectrometer (MS) is the most widely used detector for identification, qualitative and quantitative analysis. HPLC hyphenated with MS (HPLC-MS) is thus a very useful technique for analysis of many compounds which are non-volatile, extremely polar, or thermally labile. The compounds can be separated successfully with LC before detection with MS. Qualitative and quantitative analysis of targeted compounds can be further improved by using MS/MS mode allowing detection of the compounds according to the specific fragmentation pathways of each compound. This eliminates interferences which enables targeted analyte confirmation and quantification with high selectivity and sensitivity.

1.3   Aim and expected benefits of this work

The qualitative and quantitative analysis of bioactive compounds from food ingredients have been reported. Water is widely used as polar solvent for extraction of natural water-soluble products such as proteins, sugars and organic acids, etc. Spices and herbs are food ingredients, which have been used as flavoring, seasoning, coloring agents and sometimes as preservatives that useful around the world especially in southeastern Asian countries [26]. Tom Yum soup is a combination of various spices depending on characteristic taste in this menu. Furthermore, spices are usually added as flavoring agents to food preparations in raw, crushed paste and cooked types.

In the previous work, non-volatile compounds found to contribute to taste include phenolic compounds, alkaloids, amino acids, organic acids, ions and others [27-29]. In this work, qualitative and quantitative analyses of targeted non-volatile taste substances and major compounds in the extracted samples of individual boiled ingredient and cooked Tom Yum were performed. The work focuses on three groups of compounds, including free amino acids, phenols compounds and organic acids. Water will be used as solvent to extract the samples prior to the analysis with HPLC-MS/MS.

CHAPTER II

THEORY

2.1 Non-volatile compounds and major component in Tom Yum spices

2.1.1 Phenolic compounds

Presence of phenolic compounds in fruits and vegetables can lead to two characteristics. First, they contribute to the sensory qualities: color, taste and aroma depending on the chemical composition. Second, some phenolic compounds possess pharmacological properties and can be used for therapeutic purposes [30]. Phenolic compounds comprise an aromatic ring, one or more hydroxyl substituents [31], which can be categorized into several classes as shown in Table 2.1

Table 2.1 Classes of phenolic compounds in plants [32]

Class	Carbon number
Simple phenolics	C_6
Hydroxybenzoic acids	C_6-C_1
Acethophenones, phenylacetic acid	C_6-C_2
Hydrocinnamic acids, phenylpropanoids	C_6-C_3
Napthoquinones	C_6-C_4
Xanthones	$C_6-C_1-C_6$
Stibenes, anthraquinones	$C_6-C_2-C_6$
Flavonoids, isoflanoids	$C_6-C_3-C_6$
Lignans, neolignans	$(C_6-C_3)_2$
Biflavonoids	$(C_6-C_3-C_6)_2$
Lignins	$(C_6-C_3)_n$
Condensed tannins (proanthocyanidins or flavolans)	$(C_6-C_3-C_6)_n$

There are a lot of major and minor phenolic compounds in Tom Yum spices. In this study 9 targeted phenolic compounds in spices that are major components as well as compounds expressing a unique taste of the spices.

Table 2.2   Targeted phenolic compounds in Tom Yum spices

Compound Name	Source	Taste attribute
Limonin	Citrus fruits [33]	Bitter [34]
Naringin	Citrus fruits [35]	Bitter [34]
Cathechin	Galangal [36]	Bitter [37]
p-Coumaric acid	Lemongrass [13]	-
Chlorogenic acid	Lemongrass [13]	-
Caffeic acid	Lemongrass [13]	-
Acetocxy chavical acetate (ACA)	Galangal [38]	Pungent [38]
Capsaicin	Chili [39]	Pungent [40]
Dihydrocapsaicin	Chili [39]	Pungent [40]

2.1.2 Organic acids

Organic acids contribute to the sourness or acidity, particularly as flavor, color and aroma. The qualitative and quantitative analysis of major organic acids is important in food and beverage industries. Sourness is determined by the concentrations of predominant organic acids which are found in citrus juices. Furthermore, citric and malic acids and some amino acids such as aspartic and glutamic acids also contribute to sourness [41].

2.1.3 Free amino acids

Free amino acids (AAs) are omnipresent compounds in foodstuffs, plants and living organisms. AAs are known to contribute to sensory perceptions in foods some of which express taste attributes. For example, sour taste is contributed from asparagine and glutamic acid, and bitter taste is contributed from leucine and valine [42-44]. In fish sauce, non-volatile compounds such as free amino acids, peptides, neocleotides and organic acids are responsible for the flavor and taste [45]. In the previous studies, many fish sauce samples have been analyzed which showed chemical compounds related to flavor. They have reported that amino acids were associated with favorable properties of fish sauce [46] Amino acid constituents of peptides in fish sauces such as glutamic acid, aspartic acid, glycine, etc were found [47].

2.2 High performance liquid chromatography

High performance liquid chromatography (HPLC) is an advantageous and widely used type of elution chromatography. The technique is used for separating and determining compounds in a variety of samples such as organic, inorganic and biological materials. HPLC separations involve use of both the mobile phase (a liquid phase) and the stationary phase (usually materials of varying chemical bonded to a solid support which may be hydrophobic or hydrophilic based on the chemical properties of analytes). In HPLC, several instrument and column chemistry parameters need to be optimized in order to generate a satisfying separation result which is suitable for qualitative or quantitative proposes. Typically, HPLC instrument consists of mobile phase reservoirs, pump, injector, column and detector.
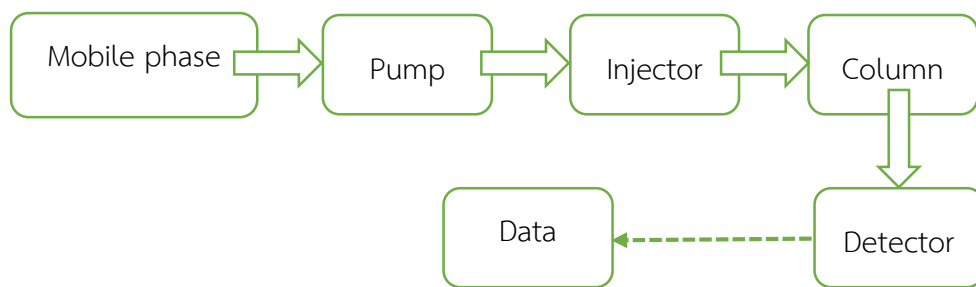
Figure 2.1   HPLC or UHPLC system diagram. Adapted from [48]

2.2.1 Mobile phase reservoirs

Currently, the modern HPLC instrument is used with glass reservoirs to remove dissolved gases and some impurity from the liquids. In order to perform reliable analysis, types of mobile phase should be high purity, analyte-dissolving solvent, non-reactive with the stationary phase and compatible with the detector. An elution with a single or constantly mixed solvent is termed an isocratic elution. In gradient elution, there are two or more solvent mixtures the compositions of which are varied in a series of steps during the separation. Use of gradient elution frequently improves separation efficiency and fastens analysis time. The instrumental parts are equipped with proportioning valves that continuously introduce solvents from reservoirs [48].

2.2.2 Pump

The high pressures are generated by liquid chromatographic pumps including pulse-free output, flow reproducibility and resistance to corrosion by various solvents. There are neat pump designs that are capably and exactingly proportionate. Two major types of pumps are used in HPLC instruments including a binary pump and a quaternary pump. Binary pumps consist of two pumps working together with each delivering a different volume fraction of the total flow. These systems are recognized to give the most reproducible gradient profile and deliver more quickly the lowest mixing volumes than other system. A quaternary pump is a device simultaneously mixing up to four different solvents at low pressure which is located prior to the pump

that delivers the mixed solvent. Quaternary systems use only one pump head so the price is lower than binary pump. The ability of quaternary pump to mix solvents gives great flexibility when developing gradient separations particularly for separations with complex gradients.
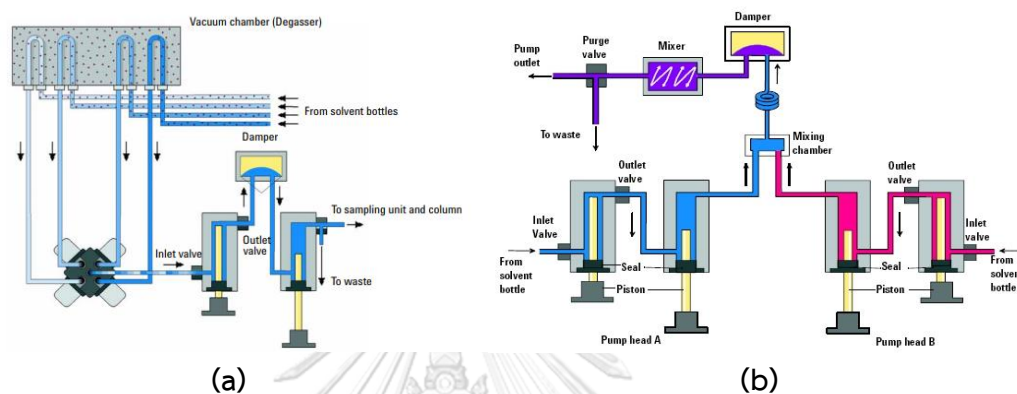
Figure 2. 2   Schematic of (a) the binary pump (b) the quaternary pump

2.2.3   Injector

Sample injection valve is used to introduce liquid sample into the HPLC eluent without significant change of pressure or flow which includes manually and automatically types. Valve is an efficient system which can control eluent through the loop filling and sweep the eluent in the loop onto the HPLC under high backpressure with precise and accurate volume. Valve injection allows the rapid, reproducible and delivery of a wide range of sample volumes (10 to 50 μL).

2.2.4   Chromatographic column

Liquid chromatographic columns are usually made from stainless steel tubing packed with the stationary phase material. Column length mostly ranges from 5 to 25 cm and the particle size of the material is typically from 3 to 5 μm. The mobile phase transports through the column and the analytes are separated by selective distribution between mobile phase and stationary phase. LC column can be grouped according to particle platforms such as fully porous, core shell or nonporous particles.

In addition, chemical composition can be separated into silica-based and polymer-based [49].

Nowadays, the most widely used type of HPLC is partition chromatography, which can be divided into two types: normal phase and reversed phase. The character of normal phase is polar stationary phase and organic mobile phase(s). The slightest polar constituent in liquid samples will be eluted first. In the other hand, reversed-phase chromatography, stationary phase is non-polar and aqueous-organic mobile phases conventionally employing the gradient with increasing polarity, where the most polar constituent can elute first. Nowadays, high-resolution and fast analysis will require for improve detection with system stability and reproducibility [50].

2.2.5 Detectors

A detector is the part of instrument that converts sample concentration into the electrical signal. The selection depends on concentration range of samples, detector sensitivity and eluent compatibility. Generally, the most widely used detector is ultraviolet or visible radiation because of their low cost and easy to use for detection of versatile compounds. Nowadays, mass spectrometric detectors have become the new choice enabling qualitative and quantitative analysis with low analyte concentrations [50].

2.3 Mass spectrometry

Mass spectrometry is popular analytical technology that allows detection of ions according mass to charge ratio (m/z), to provide structural for identification of chemical structures. MS consists of ion source, mass analyzer and ion detector. The combination of liquid chromatographic and mass spectrometric parts consists of two techniques, first step, LC eluent vaporization to remove solvent and LC column interfacing [51].
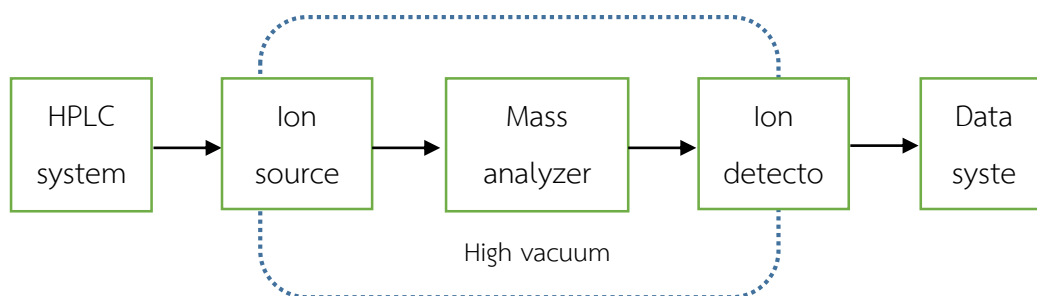
Figure 2. 3   Block diagram of an LC/MS system. Adapted from [50]

2.3.1   Ion source: electrospray ionization

Electrospray ionization (ESI) is the most commonly applied ionization source providing superior performance, improved sensitivity for polar molecules. ESI works by evaporating ions from a solution at atmospheric pressure. Sample solution is sprayed through a metal capillary where high voltage is applied (typically 3-4 kV). Sample solution becomes highly charged droplets by application of high voltage potential into the ESI ion source. The evaporation of the solvent is assisted by a stream of nitrogen gas to assist desolvation. The droplets become smaller until they reach a critical point (Rayleigh limit) in which the sample ions are released from the droplets according to their electrostatic repulsion.
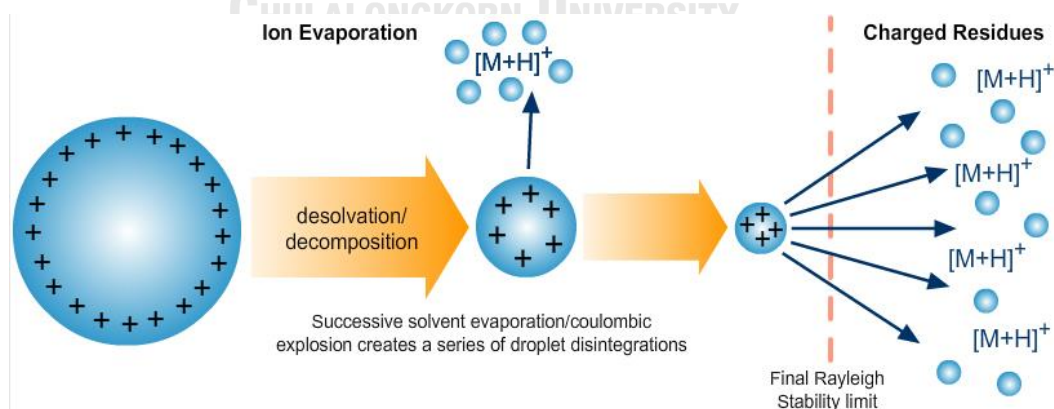
Figure 2.4   Schematic representation of electrospray ionization [52].

2.3.2 Mass analyzer

The mass analyzer is an essential part of mass spectrometer. Different types of ions are separated based on mass to charge ratio (m/z) and pass to the detector. Nowadays, several types of mass analyzers have been developed [53]. All mass analyzers use static or dynamic electric and magnetic fields that can be alone or combined. The commonly used mass analyzers include quadrupole (Quad), ion trap and time-of-flight (TOF), etc.

2.3.2.1 Quadrupole (Q)

Quadrupole (Quad), electric fields are used to separate ions according to their mass-to-charge ratio (m/z). The analyzer consists of four metal rods, which are connected to DC and radio-frequency (RF) voltages. Quadrupole analyzers act like mass filter, voltages is created for ions of a certain m/z ratio to pass through the analyzer to the transducer. These analyzers are used for selection of targeted ions, quantitative applications or scan of m/z range [54].
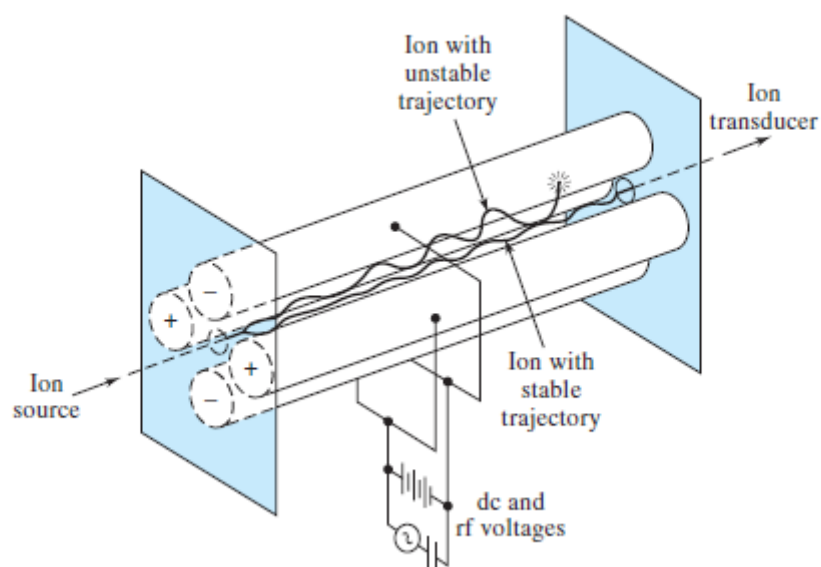
Figure 2.5   A quadrupole mass analyzer [50]

2.3.2.2 Tandem mass spectrometry

MS/MS is the combination of two or more MS operations (of the same or different kind). Aim is either to get structural information of ions by specific fragmentation pathways of the ions or achieve selectivity and sensitivity for quantitative analysis by selection of ion transitions. However, the conventional instrument with the MS/MS capability is the triple quadrupole (QQQ), which is widely used in an analytical laboratories in the pharmaceutical, food/beverage and chemical industries. The fragmentation patterns and the mass product ions are determined. First quadrupole (Q1) selects specific mass entering high ionization potential and high pressure in the second quadrupole (Q2), called the collision cell where collision-induced dissociation (CID) occurs to produce fragmentation(s) of molecular ions into smaller fragments. These product ions can be analyzed by the third quadrupole (Q3) as shown in Figure 6. Multiple reaction monitoring (MRM) has previously been shown to be specific, accurate and reproducibility method, which has been used for determination of targeted compounds.
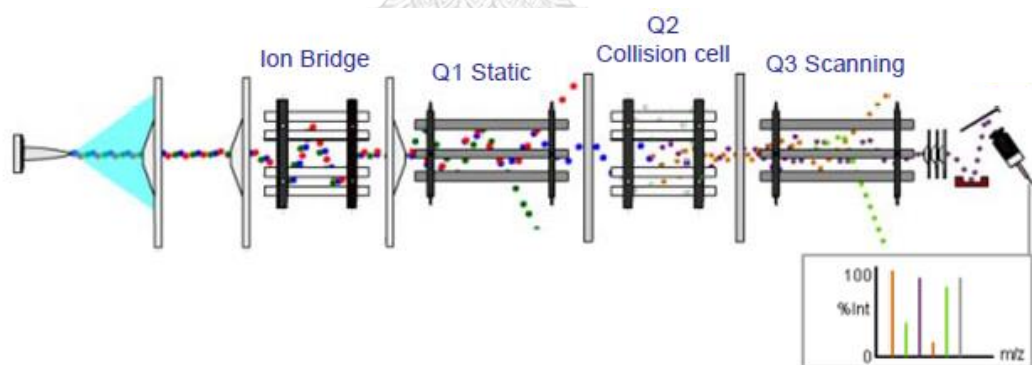
Figure 2.6   Schematic diagram of a triple quadrupole mass analyzaer. Reproduced from [55]

2.3.3   Ion detector

The detection of ion emerging from mass analyzer involves the counting, converting and multiplying the number of electrons to amplify the signal. Mostly, the general design is an electron multiplier consisting of dynodes. A dynode is

coated with copper-beryllium or lead doped glass, to be holding at higher voltages. Continuous dynode electron multipliers employ a single horn-shaped dynode to a power supply, which are the channel electron multipliers collecting the signal. The dynode emits electrons and accelerated them to the next dynodes causing the total number of electrons to increase.
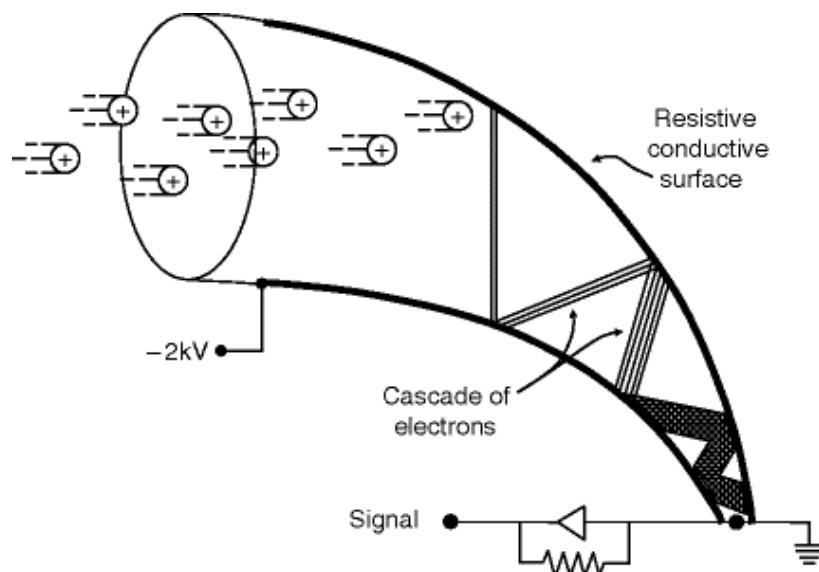
Figure 2.7   Schematic of the horned-shaped electron multiplier mass.
Reproduced from [56]

CHAPTER III

EXPERIMENTAL

3.1 Instrumental and apparatus

3.1.1 High performance liquid chromatograph (HPLC), Agilent Technologies Model 1290 (CA, USA), consisting of vacuum degasser, binary pump, Agilent jet weaver, autosampler and column compartment

3.1.2 Mass spectrometer (MS), Agilent Technologies Model 6490 (CA, USA), consisting of triple quadrupoles mass analyzer, electrospray ionization (ESI) interface and MassHanter software processing

3.1.3 LC-MS/MS column: PoroShell C18 column (4.6 x 100 mm, 2.7 μm)

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

3.1.5 Balance: Sartorius Model AC211S-00MS (Germany)

3.1.6 Micropipettes: 2-20 μL, 20-200 μL, 100-1000 μL, 500-2500 μL and 1000-5000 μL, Eppendorf (Germany)

3.1.7 Volumetric flasks: 10, 25, 50, 100, 250, 500, 1000 mL

3.1.8 Filter: Nylon membrane filter 47 mm. 0.2 μm, Alltech Associates Inc (IL, USA)

3.1.9 LC vial: 2 mL amber vials with PTFE cap (Agilent Technologies, Santa Clara, CA, USA)

3.1.10 Glass wares: solvent bottles, beakers, cylinders in various sizes (Schott, Elmsford, NY, USA)

3.2 Chemicals

3.2.1 Standard compounds and internal standard

All standard chemicals were amino acids, organic acids and phenollic compounds) : Twenty solid amino acid standards were purchased from Sigma-Aldrich (MO, USA): alanine (≥98%), arginine (≥98%), asparagine (≥98%), aspartic acid (≥98%),

glutamine (≥98%), glutamic acid (≥98%), histidine (≥98%), hydroxyproline (≥98%), isoleucine (≥98%), leucine (≥98%), lysine (≥98%), metionine (≥98%), phenylalanine (≥98%), proline (≥98%), serine (≥98%), threonine (≥98%), tyrosine (≥98%), trptophan (≥98%), valine (≥98%), glutathione (≥98%). Two organic acids: citric acid (≥99.5%) and malic acid (≥99.5%). Nine polyphenols: limonin (≥99.5%), naringin (95%), p-coumaric acid (≥98%), chlorogenic acid (≥95%), caffeic acid (≥98%), acetoxychavicol acetate (≥98%), capsaicin (≥98%), dihydrocapsaicin (≥97%) and cathechin (≥95%). Internal standards used in positive and negative modes were 3-Amino-4-methylbenzoic acid (AMBZA) and 3,5 – difluorobenzoic acid (DFBZA), respectively, which were also purchased from Sigma-Aldrich.

3.2.2   Organic solvents

LC-MS grade methanol used for HPLC-MS/MS techniques was supplied by J.T. Baker Chemical (Center Valley, PA, USA), Ethanol (analytical grade) used for dissolving standards was purchased from J.T. Baker Chemical (Center Valley, PA, Acetonitrile (analytical grade) used for dissolving standards was purchased from J.T. Baker Chemical (Center Valley, PA and analytical grade of formic acid was purchased from Merck (Darmstadt, Germany).

3.2.3   Samples

The samples in this experiment can be divided in two parts. First, the sample was fresh spices of Tom Yum recipe consisting of lime, chili, lemongrass, galangal, kaffir lime leaves and Thai's fish sauce which was purchased from the local market in Bangkok. The spices were washed and kept in the refrigerator at 5 °C until the analysis. The second group is difference band of Tom Yum pastes which were purchased from Gourmet market Thailand at Siam Paragon.

3.2.4 **Preparation of stock standard solutions and internal standard solutions**

3.2.4.1 Stock solutions of standard phenolic compounds and organic acids were prepared with the concentration of at 1000 mgL^{-1}, by dissolving 10 mg of each compound in 10 mL of the suitable solvents in a 10 mL volumetric flask. The solutions were stored at -20 °C in a refrigerator until use.

3.2.4.2 Standard solutions of amino acids were prepared with the concentration of 2000 mgL^{-1}, by dissolving 20 mg each compound in 10 mL of the suitable solvent in a 10 mL volumetric flask. The solutions were stored at 5 °C in a refrigerator until use

3.2.4.3 Internal standard solutions of 3-Amino-4-methylbenzoic acid (AMBZA) and 3,5 – difluorobenzoic acid (DFBZA) were prepared at 2,000 mgL^{-1} by dissolving 20 mg of each compound in MeOH in a 10 mL volumetric flask.

3.2.5 **Working mixed targeted non-volatile standard preparation**

A mixture of targeted non-volatile standard was prepared by diluting an individual stock solution in 50, v/v methanol in water to result in the final solution containing 10 mgL^{-}1 of each compound.

3.3 **Optimization of triple quadrupole mass spectrometer for quantitation**

Triple quadrupole mass spectrometer is considered as an instrument to characterize structural and to calculate the *m/z* values of the known compounds. MS optimization can be divided into three steps: ionization, mass analysis and detection. The sample was ionized by ESI, soft ionization technique by applying a high electric charge to the sample needle and introduced into the mass spectrometer. This technique is particularly suitable for polar compounds, and can be operated in positive and negative mode. In positive ionization mode, the spraying nozzle is kept at positive potential which the charging occurs protonation. During the negative ionization mode, charging occurs deprotonation of the analytes when the spraying is kept at negative

potential. The positive and negative charges would be repelled by the high voltage capillary and towards the liquid surface at the capillary outlet [57].

The molecular mass of the targeted non-volatile compounds is calculated based on *m/z* ratio [19]. Several parameters have to be considered for quantitative analysis of known compounds. The parameters were optimized separately for each compound by direction of the compounds into HPLC-MS/MS system (without use of a column), and 50, v/v methanol in water containing 0.1% v/v formic acid were applied as a mobile phase with a flow rate of 0.3 mLmin^{-1}.

3.3.1   Molecular ion

Molecular ions of each targeted compound and the internal standards were determined by using an MS2Scan mode, where *m/z* values of the molecular weights of the individual compounds were set as the precursor ions in both positive and negative modes of the detection. However, the mode showing higher sensitivity was only applied for each analyte.

3.3.2   Product ion and collision energy optimization

Agilent MassHanter Optimizer software was used to select a proper product ions and collision energy for each compound. In brief for each precursor ion, the software selected four product ions with the highest abundance. The maximum abundance was selected as a quantitative *m/z* ion, while another ion with the lower abundance was a qualitative *m/z* ion. Moreover, the collision energies yielding the highest abundance of the selected product ions were applied in an MRM mode. The MS condition were shown in Section 4.1.

3.4   Sample preparation

3.4.1   Fresh spices

The samples; lime juice, fish sauce, lemongrass, galangal, chili and kaffir lime leaves were prepared individually according to the cooking recipe from Suan Dusit International Culinary School. Each spice (10 g) was boiled in 50 mL of hot water for 5 min. Then, the solution was filtered with a colander for separation of the crude ingredient. The solution was further diluted 10X with 50% v/v methanol in water and the final solution was filtered through a 0.22 µm-Nylon syringe filter. This solution (1 mL) was used as the samples for UHPLC-MS/MS analysis.

3.4.2   Mixed spices for Tom Yum soup

The samples; lime juice, fish sauce, lemongrass, galangal, chili and kaffir lime leaves were mixed and prepared according to the cooking recipe above. The mixed ingredients were boiled. Then, the sample solution was filtered with a colander to separate the crude ingredient. The solution was further diluted 10X with 50% v/v methanol in water. The final solution was filtered through a 0.22 µm-Nylon syringe filter, and then 1 mL of this solution was transferred into a 2 mL vial for UHPLC-MS/MS analysis.

3.4.3   Commercial bands of Tom Yum Paste

Each paste was weighted (50 g) and boiled in hot water at 250 mL for 5 min. Then, the solution was filtered with a colander to separate the crude ingredient. The sample solution was diluted 10X with 50% v/v methanol in water and the final solution was filtered through a 0.22 µm-Nylon syringe filter. 1 mL of this solution was transferred into a 2 mL vial for UHPLC-MS/MS analysis. Moreover, the control Tom Yum paste was prepared with weight Tom Yum ingredients following the standard recipe and spin overall of ingredient around 10 minutes. The control TYS paste was weighted (50 g) and prepared the same process of any Tom Yum paste.

3.5   Optimization of HPLC separation

The mixture of targeted non-volatile compounds was prepared in 50% v/v methanol in water at 10 mg L^{-1} for each compound. Chromatographic separation was performed using a PoroShell C$_{18}$ column (4.6 x 100 mm, 2.7 µm) with binary system of mobile phases under a gradient elution mode. Mobile phase A was an aqueous solution of 0.1% v/v formic acid, while mobile phase B was 0.1% v/v formic acid in methanol. The selected gradient profile was applied as follow: 5% B from 0.0-5.0 min, increased to 100% B from 5.0 to 24.50 min and decreased again to 5% B from 24.50 to 25.00 min. The flow rate was set at 0.3 mL min^{-1} and column temperature was 30 °C. The injection volume was 2 µL.

3.6   Method validation

3.6.1   Limits of detection and quantification

Limit of detection (LOD) is defined as the minimum of concentration of a compound that results in a peak which is significantly higher than the noise level. Limit of quantification (LOQ) is defined as the minimum concentration of a compound that is usable for generation of the internal calibration curve (a plot of concentration vs ratio of analyte peak area to that of the internal standard). The calibration curve was constructed according to three replicate analysis of each concentration level by injection of solutions with different concentrations of each standard spiked with the same concentration of the internal standard. The calibration plots were performed with three triplicate concentration level of each analyte spiked in the final solvent as shown in section 4.5.1.

3.6.2   Standard calibration curve

Standard calibration curves were constructed using AMBZA and DFBZA as the internal standards in positive and negative mode, respectively. The mixtures of targeted standard non-volatile compounds with seven concentration levels were

prepared in 50% v/v methanol in water. Triplicate analysis was performed for each level as shown in Table 3.1 and Table 3.2. The linear regression plots were performed using the relative response, the ratios of peak areas of the analyte to the internal standard which are shown in Section 4.5.2.

Table 3.1   Concentration range of standard calibration curve in ingredients samples

Analyte	Concentration range (mgL^{-1})						
	L1	L2	L3	L4	L5	L6	L7
Group I; the concentration range of 0.01 – 1.0 mgL^{-1}							
Histidine	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Hydroxyproline	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Limonin	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Naringin	0.01	0.025	0.05	0.1	0.25	0.5	1.0
p-Coumaric acid	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Caffeic acid	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Dihydrocapsaicin	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Cathechin	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Group II; the concentration range of 0.05 – 5.0 mgL^{-1}							
Arginine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Methionine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Tryptophan	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Tyrosine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Glutathione	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Capsaicin	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Group III; the concentration range of 0.1 – 10 mgL^{-1}							
Lysine	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Phenylalanine	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Proline	0.1	0.25	0.5	1.0	2.5	5.0	10.0

Serine	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Threonine	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Alanine	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Asparagine	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Aspartic acid	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Glutamic acid	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Glutamine	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Isoleucine	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Leucine	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Valine	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Citric acid	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Malic acid	0.1	0.25	0.5	1.0	2.5	5.0	10.0
Chlorogenic acid	0.1	0.25	0.5	1.0	2.5	5.0	10.0
ACA	0.1	0.25	0.5	1.0	2.5	5.0	10.0

Table 3.2 Concentration range of standard calibration curve in Tom Yum soup based on Suan Dusit International Culinary School recipe.

Analyte	Concentration range (mgL^{-1})						
	L1	L2	L3	L4	L5	L6	L7
Group I; the concentration range of 0.01 – 1.0 mgL^{-1}							
Histidine	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Hydroxyproline	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Methionine	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Tryptophan	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Limonin	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Naringin	0.01	0.025	0.05	0.1	0.25	0.5	1.0
p-Coumaric acid	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Chlorogenic acid	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Caffeic acid	0.01	0.025	0.05	0.1	0.25	0.5	1.0
ACA	0.01	0.025	0.05	0.1	0.25	0.5	1.0

Capsaicin	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Dihydrocapsaicin	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Cathechin	0.01	0.025	0.05	0.1	0.25	0.5	1.0
Group II; the concentration range of 0.05 – 5.0 mgL^{-1}							
Alanine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Arginine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Asparagine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Aspartic acid	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Glutamic acid	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Glutamine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Isoleucine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Leucine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Lysine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Phenylalanine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Proline	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Serine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Threonine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Tyrosine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Valine	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Glutathione	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Malic acid	0.05	0.125	0.25	0.5	1.25	2.5	5.0
Group III; the concentration range of 0.1 – 10 mgL^{-1}							
Citric acid	0.1	0.25	0.5	1.0	2.5	5.0	10.0

3.6.3 Accuracy and precision

Accuracy and precision in the quantitative method were evaluated by analyzing spiked standards in the sample at three concentration levels on three different days, as shown in Table 3.3. The accuracy is expressed by percentage recovery as shown in Equation 3.1. The precision values expressed by the percentage relative standard deviation of the recovery are shown in Section 4.5.3.

Table 3.3   Spiking concentration range of thirty-one standards in Tom Yum soup for accuracy study

Analyte	Spiking concentration (mgL^{-1})		
	Low	Medium	High
Group I; the concentration range of 0.01 – 1.0 mgL^{-1}			
Histidine	0.060	0.40	0.60
Hydroxyproline	0.060	0.40	0.60
Limonin	0.060	0.40	0.60
Naringin	0.060	0.40	0.60
p-Coumaric acid	0.060	0.40	0.60
Caffeic acid	0.060	0.40	0.60
Dihydrocapsaicin	0.060	0.40	0.60
Cathechin	0.060	0.40	0.60
Group II; the concentration range of 0.05 – 5.0 mgL^{-1}			
Arginine	0.08	0.48	0.98
Methionine	0.08	0.48	0.98
Tryptophan	0.08	0.48	0.98
Tyrosine	0.08	0.48	0.98
Glutathione	0.08	0.48	0.98
Capsaicin	0.08	0.48	0.98
Group III; the concentration range of 0.1 – 10 mgL^{-1}			
Lysine	0.75	2.0	3.0
Phenylalanine	0.75	2.0	3.0
Proline	0.75	2.0	3.0
Serine	0.75	2.0	3.0
Threonine	0.75	2.0	3.0
Alanine	0.75	2.0	3.0
Asparagine	0.75	2.0	3.0
Aspartic acid	0.75	2.0	3.0

Glutamic acid	0.75	2.0	3.0
Glutamine	0.75	2.0	3.0
Isoleucine	0.75	2.0	3.0
Leucine	0.75	2.0	3.0
Valine	0.75	2.0	3.0
Citric acid	0.75	2.0	3.0
Malic acid	0.75	2.0	3.0
Chlorogenic acid	0.75	2.0	3.0
ACA	0.75	2.0	3.0

3.7   Application to real samples

Targeted non-volatile compounds were determined by HPLC-MS/MS in each ingredient (lemongrass, galangal, chili, kaffir lime leaves, lime juice, fish sauce), Tom Yum soup (mixed ingredients according to Suan Dusit International Culinary School recipe) and four commercial Tom Yum paste samples obtained from department store in Bangkok were determined by HPLC-MS/MS. The recovery of analytes before and after spiking with standards in Tom Yum soup were determined at known levels in range of 3.0-150 mg/kg), the results are shown in Table 4.7. And the quantitative analysis was also applied in commercial Tom Yum paste, the results are shown in Table 4.8. The resulting non-volatile compound were prepared and discussed in Section 4.

CHAPTER IV

RESULTS AND DISCUSSION

4.1   Optimization of Mass spectrometry

The following parameter for MS/MS analysis of targeted compounds were optimized according to Section 3.3: polarity modes, Q1/Q2 product ions and collision energy, where the Q1 product ion with the most abundance is used for quantitative determination and the second most abundance Q2 product ion. Results are shown in Table 4.1. It can be seen that the better MS/MS analysis of all 20 amino acids is performed using a positive polarity mode, but that of 2 carboxylic acids, citric acid and malic acid is performed by a negative polarity mode. Using an acidic mobile phase condition, it is possibly because the amino acid are preferably protonated during an ESI process, while these 2 carboxylic acids are preferably deprotonated. For the MS/MS detection of 9 phenolic compounds, the positive mode provides better sensitivity for 5 compounds but the negative mode provides better sensitivity for another 4 compounds. This implies that the phenolic compounds can be deprotonated or protonated depening on the structure of the particular compound.

Table 4.1   Molecular ion, product ion, collision energy (CE) and abundance

Analytes	Polarity	Molecular ion		Q1/Q2	Product ion	CE	Abundance
		form	m/z				
Group I: Free amino acids							
Alanine	Positive	[M+H]$^+$	90.06	Q1	44	5	656
Arginine	Positive	[M+H]$^+$	175.12	Q1	70	25	298653
				Q2	59.9	13	73548
Asparagine	Positive	[M+H]$^+$	133.06	Q1	74	13	2612
				Q2	28	33	980
Aspartic acid	Positive	[M+H]$^+$	134.05	Q1	87.9	5	2073
				Q2	73.9	9	2340
Glutamic acid	Positive	[M+H]$^+$	146.07	Q1	56.1	29	4373
				Q2	41	29	3344
Glutamine	Positive	[M+H]$^+$	147.08	Q1	130	5	7422
				Q2	83.9	17	11144
Histidine	Positive	[M+H]$^+$	156.08	Q1	109.9	9	147413
				Q2	82.6	29	11711

Q_1 = Quantitative m/z, Q_2 = Qualitative m/z

Table 4.1   Molecular ion, product ion, collision energy (CE) and abundance (continued)

| Analyte | Polarity | Molecular ion | | Q1/Q2 | Product ion | CE | Abundance |
		form	m/z				
Hydroxyproline	Positive	[M+H]⁺	132.07	Q1	85.9	13	74729
				Q2	68	21	39994
Isoleucine	Positive	[M+H]⁺	132.10	Q1	86	5	31160
				Q2	44	25	4691
Leucine	Positive	[M+H]⁺	132.10	Q1	85.9	5	31255
				Q2	29.9	13	8516
Lysine	Positive	[M+H]⁺	147.12	Q1	130	5	18820
				Q2	84	13	47547
Methionine	Positive	[M+H]⁺	150.06	Q1	60.9	21	10700
				Q2	56	13	16401
Phenylalanine	Positive	[M+H]⁺	166.09	Q1	120	9	125982
				Q2	102.9	25	47169
Proline	Positive	[M+H]⁺	116.07	Q1	70	13	88749
				Q2	28	45	8572

Q_1 = Quantitative m/z, Q_2 = Qualitative m/z

Table 4.1   Molecular ion, product ion, collision energy (CE) and abundance (continued)

Analyte	Polarity	Molecular ion		Q1/Q2	Product ion	CE	Abundance
		form	m/z				
Serine	Positive	$[M+H]^+$	106.05	Q1	60	5	2193
				Q2	42.1	25	1004
Thronine	Positive	$[M+H]^+$	120.07	Q1	74	5	3826
				Q2	56	13	2713
Tryptophan	Positive	$[M+H]^+$	205.10	Q1	188.1	1	63427
				Q2	146	13	35037
Tyrosine	Positive	$[M+H]^+$	182.08	Q1	135.9	9	8921
				Q2	90.8	29	9555
Valine	Positive	$[M+H]^+$	118.09	Q1	72	5	8288
				Q2	55	21	2708
GSH	Positive	$[M+H]^+$	308.09	Q1	84	29	21697
				Q2	76	17	28109

Q_1 = Quantitative m/z, Q_2 = Qualitative m/z

Table 4.1 Molecular ion, product ion, collision energy (CE) and abundance (continued)

Analyte	Polarity	Molecular ion		Q1/Q2	Product ion	CE	Abundance
		form	m/z				
Group II: Phenols compounds							
Limonin	Positive	[M+H]+	471.20	Q1	160.9	21	2397
				Q2	94.8	57	2450
Naringin	Positive	[M+H]+	279.17	Q1	271	29	4784
				Q2	150.8	41	5767
p-Coumaric acid	Negative	[M-H]-	163.04	Q1	119	9	172835
				Q2	93.1	37	13791
Chlorogenic acid	Negative	[M-H]-	353.09	Q1	190.8	53	10205
				Q2	85	57	732
Caffeic acid	Negative	[M-H]-	179.03	Q1	134.7	9	155752
				Q2	88.8	33	4654
ACA	Positive	[M+H]+	133.1	Q1	104.8	17	99068
				Q2	76.7	33	82430
Capsaicin	Positive	[M+H]+	306.21	Q1	137	13	2641
				Q2	94	57	1106

Q_1 = Quantitative m/z, Q_2 = Qualitative m/z

Table 4.1 Molecular ion, product ion, collision energy (CE) and abundance (continued)

Analyte	Polarity	Molecular ion		Q1/Q2	Product ion	CE	Abundance
		form	m/z				
Dihydrocapsaicin	Positive	$[M+H]^+$	308.22	Q1	136.9	25	2684
				Q2	93.9	53	1262
Cathechin	Negative	$[M-H]^-$	289.07	Q1	244.8	9	10076
				Q2	203	17	4560
Group III: Organic acids							
Citric acid	Negative	$[M-H]^-$	191.02	Q1	110.8	9	11421
				Q2	86.8	13	2905
Malic acid	Negative	$[M-H]^-$	133.01	Q1	71	9	5404
				Q2	42.9	17	1613
Internal standards							
AMBZA	Positive	$[M+H]^+$	152.07	Q1	92.8	17	1390
				Q2	76.9	33	1958
DFBZA	Negative	$[M-H]^-$	157	Q1	112.9	9	65933
				Q2	92.8	21	32527

Q_1 = Quantitative m/z, Q_2 = Qualitative m/z

4.2   HPLC separation of targeted non-volatile standards

From Section 3.5, targeted non-volatiles compounds were optimized for chromatographic separation using a PoroShell C_{18} column (4.6 x 100 mm, 2.7 µm) with binary mobile phase in a gradient elution mode. Mobile phase A was an aqueous solution of 0.1% (v/v) formic acid, while mobile phase B was 0.1% (v/v) formic acid in methanol. A binary gradient elution system was applied as follow: 0.0-5.0 min, 5% B; 5.0-24.50 min, 100% B; 24.50-25.00 min, 5% B, along with the mobile phase flow rate of 0.3 mL/min, column temperature of 30 °C and the sample injection volume of 2 µL.

Results of the data of quantitative m/z and qualitative m/z for MS/MS detection are shown in Figure 4.1. Note that the HPLC chromatogram for each analyte are obtained from MS/MS analysis using an MRM mode that can display an individual transition window.
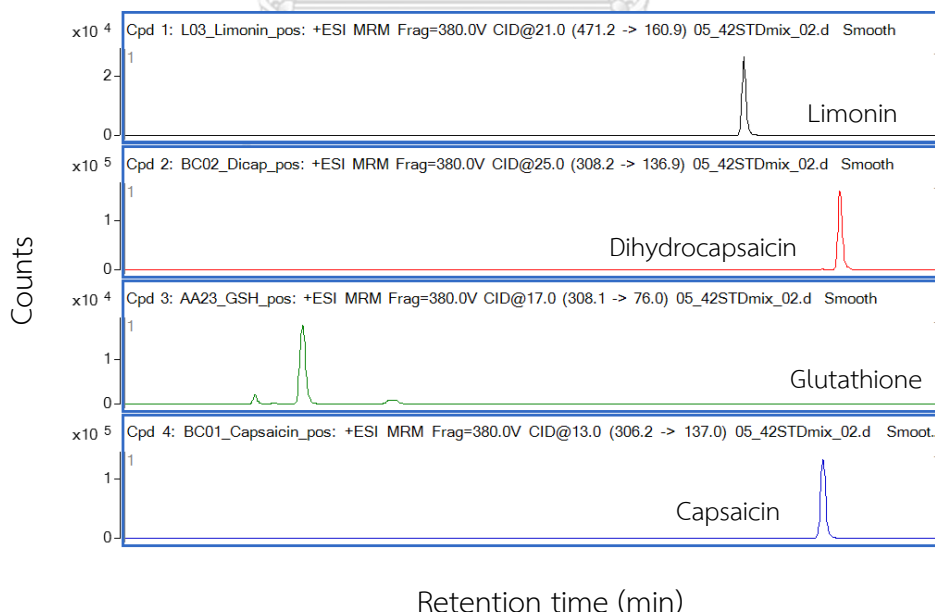
Figure 4.1   HPLC-MS/MS chromatogram of the standards
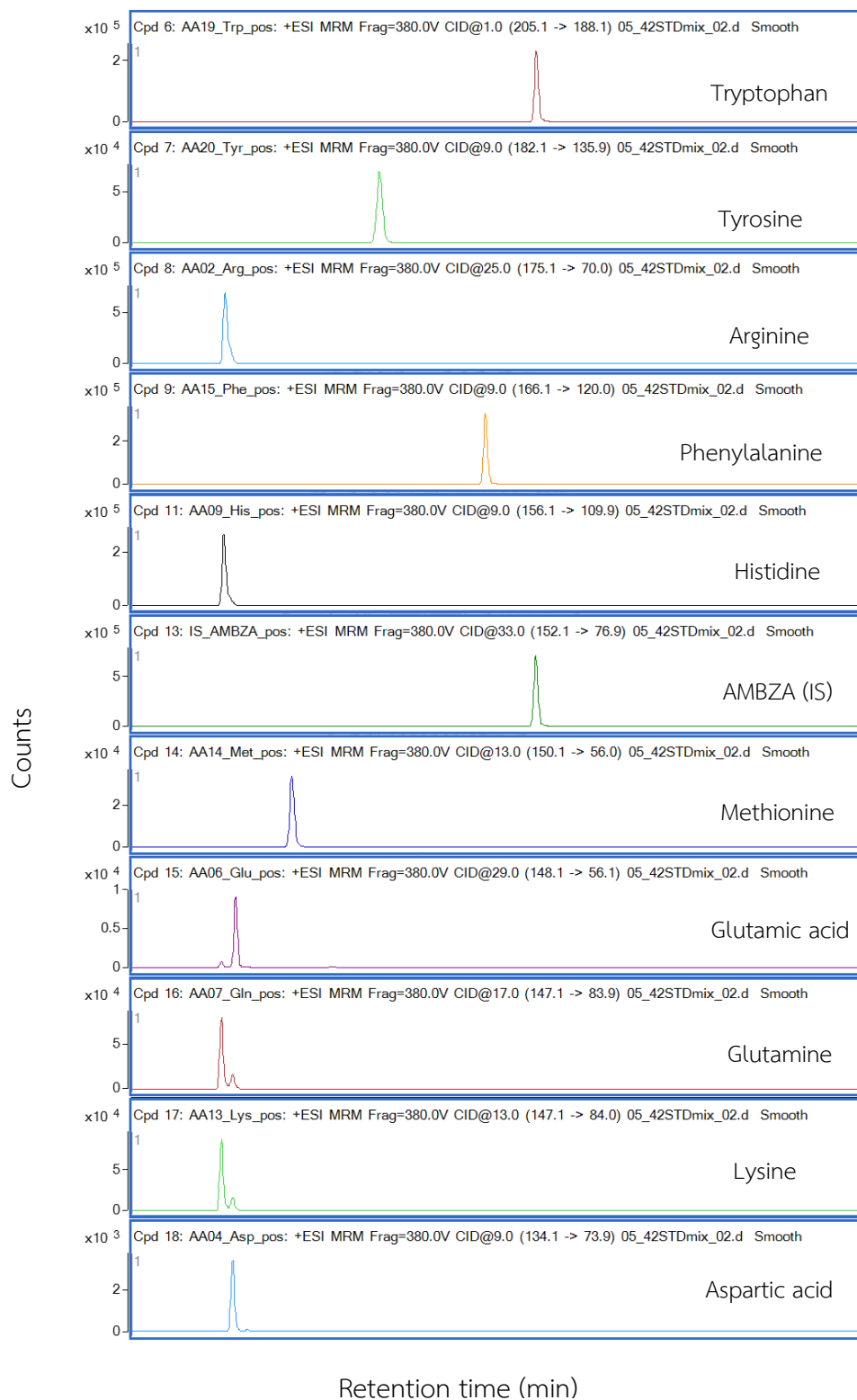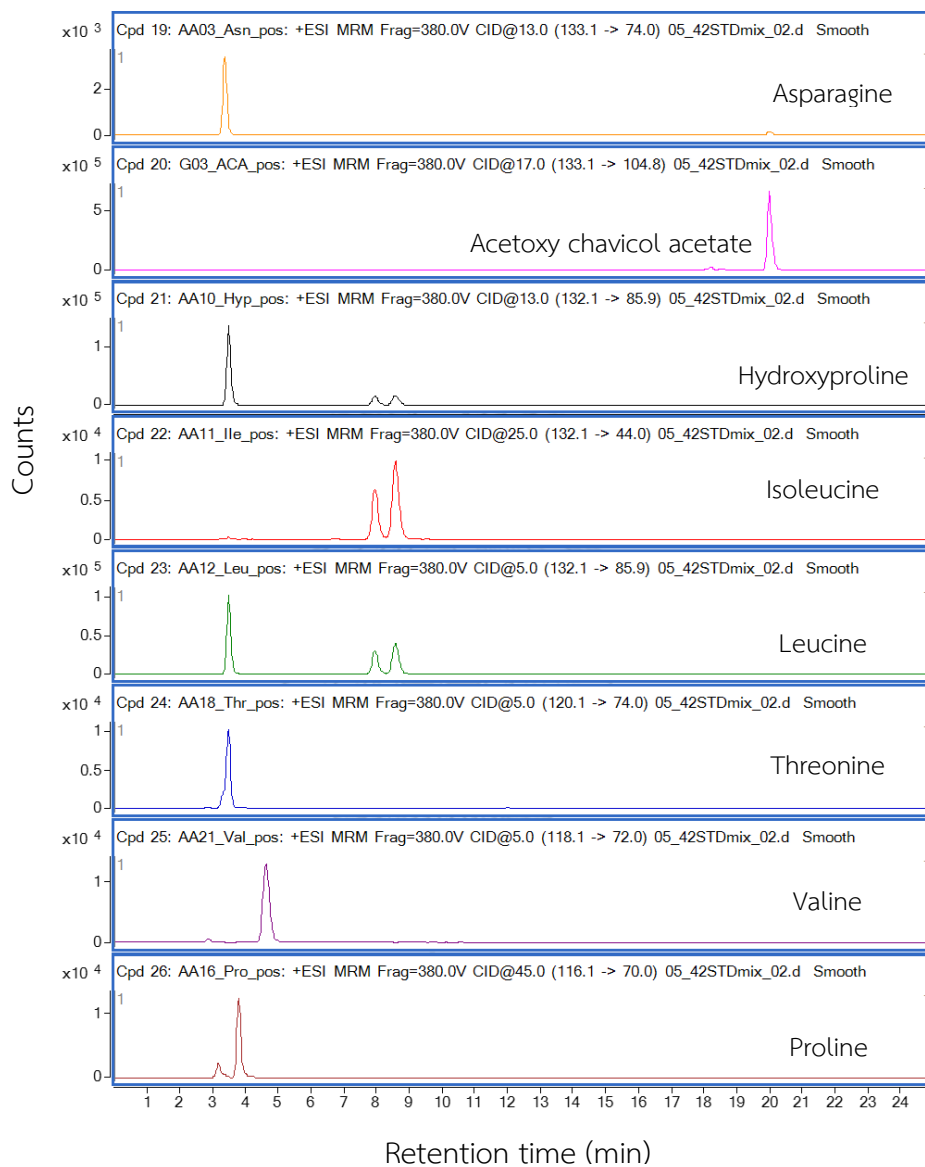
Figure 4.1   HPLC-MS/MS chromatogram of the standards (continued)

Figure 4.1   HPLC-MS/MS chromatogram of the standards (continued)

Cpd 27: AA17_Ser_pos: +ESI MRM Frag=380.0V CID@5.0 (106.1 -> 60.0) 05_42STDmix_02.d Smooth — Serine

Cpd 28: AA01_Ala_pos: +ESI MRM Frag=380.0V CID@5.0 (90.1 -> 44.0) 05_42STDmix_02.d Smooth — Alanine

Cpd 29: L04_Naringin_neg: -ESI MRM Frag=380.0V CID@41.0 (579.2 -> 150.8) 05_42STDmix_02.d Smooth — Naringin

Cpd 31: LG02_Chlorogenic acid_neg: -ESI MRM Frag=380.0V CID@57.0 (353.1 -> 85.0) 05_42STDmix_02.d... — Chlorogenic acid

Cpd 33: Cathechin_neg: -ESI MRM Frag=380.0V CID@9.0 (289.1 -> 244.8) 05_42STDmix_02.d Smooth — Cathechin

Cpd 34: L01_Citric acid_neg: -ESI MRM Frag=380.0V CID@9.0 (191.0 -> 110.8) 05_42STDmix_02.d Smooth — Citric acid

Cpd 35: LG03_Caffeic acid_neg: -ESI MRM Frag=380.0V CID@9.0 (179.0 -> 134.7) 05_42STDmix_02.d Smo... — Caffeic acid

Retention time (min)

Figure 4.1   HPLC-MS/MS chromatogram of the standards (continued)

4.3   HPLC fingerprint study

In this work, the chemical profiles of targeted compounds were analyzed based on an MRM mode according the overall parameters as shown in Section 4.1. The total ions chromatogram is shown in Figure 4.2 and the retention times are listed in Table 4.2. It should be noted that the retention time of each standard and the Q1/Q2 product ion from MRM analysis were used to identify each analyte in real sample for qualitative and also for quantitative analysis using Q1,
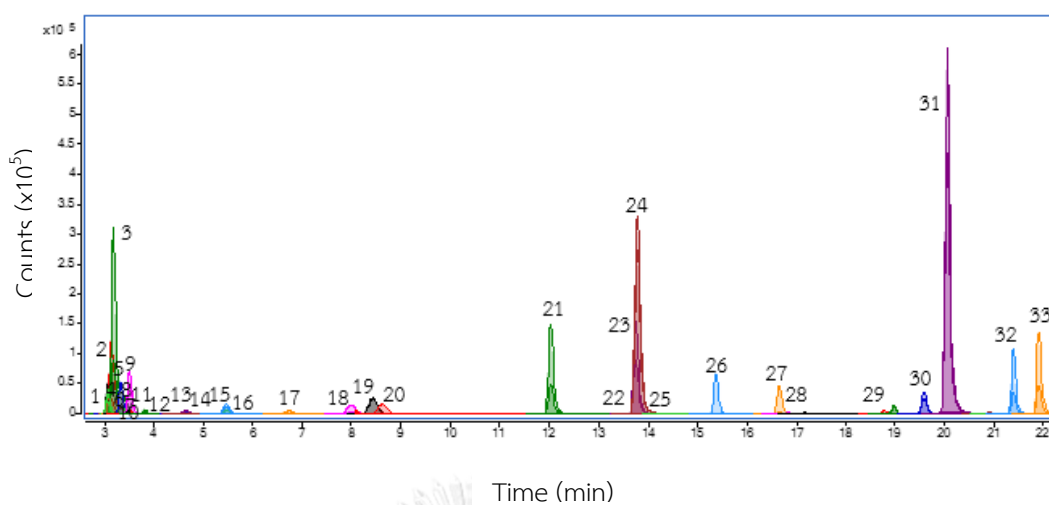
Figure 4.2   Total ions chromatogram of targeted non-volatile compounds by an MRM mode.

Table 4.2   The retention time of targeted non-volatile standards in this work

Peak No.	Analytes	t_R (min)	Peak No.	Analytes	t_R (min)
1	Lysine (Lys)	3.05	2	Histidine (His)	3.12
3	Arginine (Arg)	3.17	4	Serine (Ser)	3.35
5	Asparagine (Asn)	3.36	6	Alanine (Ala)	3.42
7	Aspartic acid (Asp)	3.42	8	Glutamine (Gln)	3.42
9	Threonine (Thr)	3.47	10	Hydroxyproline (Hyp)	3.49
11	Glutamic acid (Glu)	3.53	12	Proline (Pro)	3.79
13	Malic acid (MA)	4.61	14	Valine (Val)	4.66
15	Metionene (Met)	5.46	16	Glutathione (GSH)	5.47
17	Citric acid (CA)	6.77	18	Isoleucine (Ile)	8.01
19	Tyrosine (Tyr)	8.51	20	Leucine (Leu)	8.64
21	Phenylalanine (Phe)	12.02	22	Cathechin (Cat)	13.73
23	Tryptophan (Trp)	13.74	24	AMBZA (ISP)	13.67
25	Chlorogenic acid (Chloro)	14.36	26	Caffeic acid (Caf)	15.33
27	p-Coumaric acid (Cou)	16.61	28	Naringin (Nar)	16.78
29	Limonin (Lim)	18.91	30	DFBZA (ISN)	19.52
31	Acetoxy chavicol acetate (ACA)	19.99	32	Capsaicin (Cap)	21.33
33	Dihydrocapsaicin (Dicap)	21.85			

4.4   The amounts of targeted non-volatile compounds in real samples

4.4.1   The amounts of targeted non-volatile compounds in each ingredient of Tom Yum

The targeted non-volatile compounds in each ingredient were quantified by an MRM mode of HPLC-MS/MS, where each ingredient was prepared according to Section 3.4.1. Table 4.3 shows the amounts of all compounds in each ingredient. Figures 4.3-4.8 also compare the amounts of non-volatile compounds in individual ingredient, classified by three groups organic acids, amino acids, phenolic acid.

Table 4.3   The amounts of non-volatile compounds in each ingredient

Comp.	The concentration of targeted non-volatile compounds (mg/kg)					
	KL*	G*	LG*	C*	FS*	LJ*
Organic acids						
CA	704	333	403	1.55×10^3	89.7	2.73×10^4
MA	1.34×10^3	1.39×10^3	261	3.16×10^3	165	2.04×10^3
Amino acids						
Ala	205	28.9	400	98.5	1.06×10^3	223
Arg	9.25	2.04	105	24.7	2.68	55.7
Asn	101	330	1.07×10^3	1.13×10^3	1.16	770
Asp	112	79.6	97.3	299	102	1.45×10^3
Gln	98.0	42.7	306	447	971	15.5
Glu	130	184	131	290	351	729
GSH	3.52	3.05	114	18.4	0.00	177
His	5.39	2.77	7.23	10.9	35.0	3.35
Hyp	4.09	0.260	0.58	2.25	3.30	2.03
Ile	17.8	3.97	33.2	45.1	2.97×10^3	37.7
Leu	20.7	3.36	31.0	27.7	3.87×10^3	46.6
Lys	20.1	8.22	34.7	27.6	280	30.9
Met	5.79	1.17	8.74	15.0	2.23×10^3	14.6
Phe	86.0	5.20	27.2	56.1	2.94×10^3	52.1
Pro	660	5.89	62.5	28.3	591	190

Ser	699	18.9	94.5	150	36.5	162
Thr	158	23.3	78.8	98.1	162	55.1
Trp	27.7	2.24	10.3	27.7	495	5.19
Tyr	37.4	2.73	55.9	49.7	610	6.57
Val	31.3	7.52	56.4	70.0	2.29×10^{3}	75.7
Phenol compounds						
ACA	1.12	470	0.480	0.250	0.160	0.130
Caf	1.49	0.460	28.7	2.56	1.46	2.48
Cap	0.700	0.430	0.440	219	0.710	0.550
Cat	1.18	30.1	0.570	0.76	0.540	0.880
Chloro	1.17	0.00	13.0	262	1.14	0.510
Cou	5.59	4.16	44.7	0.27	0.00	0.340
Dicap.	0.75	0.650	0.650	50.7	0.760	0.690
Lim	61.2	0.520	0.480	0.490	0.460	36.5
Nar	11.2	0.140	0.180	0.15	0.130	1.72

KL*: kaffir lime leaves, G*: galangal, LG*: lemongrass, C*: chili, FS*: fish sauce, LJ*: lime juice

4.4.1.1 The amounts of targeted non-volatile compounds in kaffir lime leaves

As shown in Figure 4.3, the total amounts of targeted non-volatile compounds in kaffir lime leaves were found in order organic acids > amino acids > phenolic compounds, malic acid of 1340 mg/kg and citric acid of 704 mg/kg. The top three major amino acids includes serine of 699 mg/kg, proline of 660 mg/kg and alanine of 205 mg/kg. Among the targeted 9 phenolic compounds, limonin was found as a major amount of the 61.2 mg/kg, the rest were each less than 12 mg/kg.
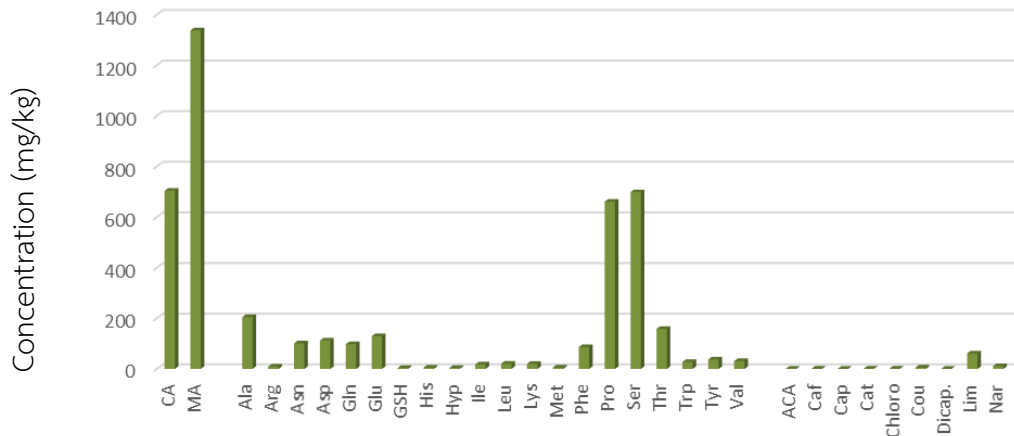
Figure 4.3   The amounts of non-volatile compounds in Kaffir lime leaves

4.4.1.2 The amounts of targeted non-volatile compounds in galangal

As seen in Figure 4.4, Galangal rhizome is found to contain higher amount of organic acids, including malic acid of 1390 mg/kg and citric acid of 333 mg/kg, than amino acid and phenolic acid. Among the targeted 9 phenolic compounds, acetoxychavicol acetate (ACA) and cathechin are two key component, 470 and 30.1 mg/kg, respectively, while the rest are each less than 5 mg/kg. Two major amino acids are asparagine and glutamic acid, 330 mg/kg and 184 mg/kg, respectively.
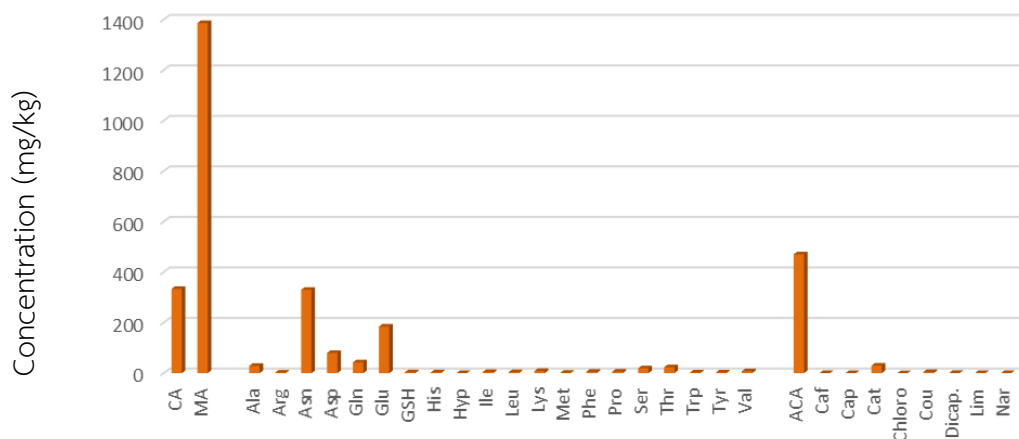

Figure 4.4   The amounts of non-volatile compounds in Galangal

4.4.1.3 The amounts of targeted non-volatile compounds in lemongrass

As shown in Figure 4.5, the key non-volatile compounds found in lemongrass are amino acid, including asparagine, alanine and glutamine, 1070, 400 and 306, respectively. The high amount of organic acids are also seen, citric acid and malic acid of 403 and 201 mg/kg., respectively. Two major phenolic compounds found are *p*-coumaric acid of 44.7 mg/kg and caffeic acid of 28.7 mg/kg, while the rest are less than 13 mg/kg, but mostly less than 1 mg/kg.

Figure 4.5   The amounts of non-volatile compounds in Lemongrass

4.4.1.4 The amounts of targeted non-volatile compounds in chili

As shown in Figure 4.6, the key non-volatile compounds found in chili are organic acids, including malic acid and citric acid of 3160 mg/kg and 1550 mg/kg, respectively. The high amount of amino acids include asparagine, glutamine and aspartic acid, 1130, 447 and 299 mg/kg, respectively. Among the targeted 9 phenolic compounds, chlorogenic acid, capsaicin and dihydrocapsaicin are the major three components, 262, 219 and 50.7 mg/kg, respectively, while the rest are each less than 3 mg/kg.
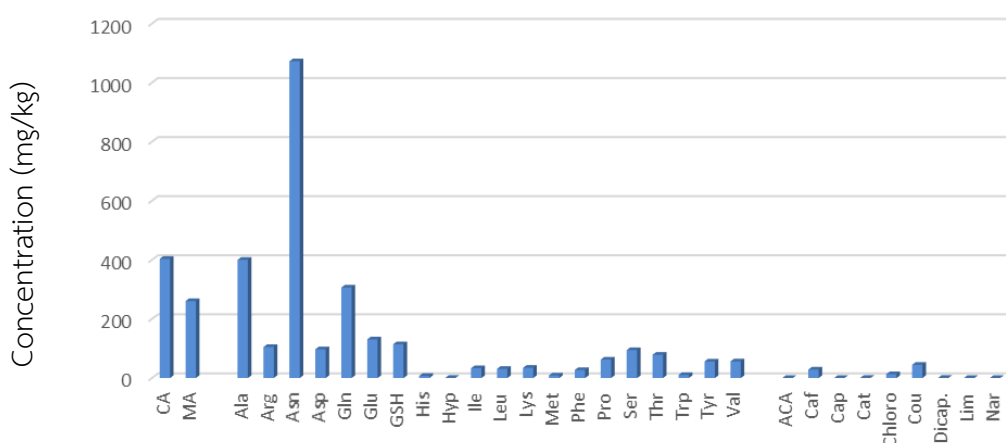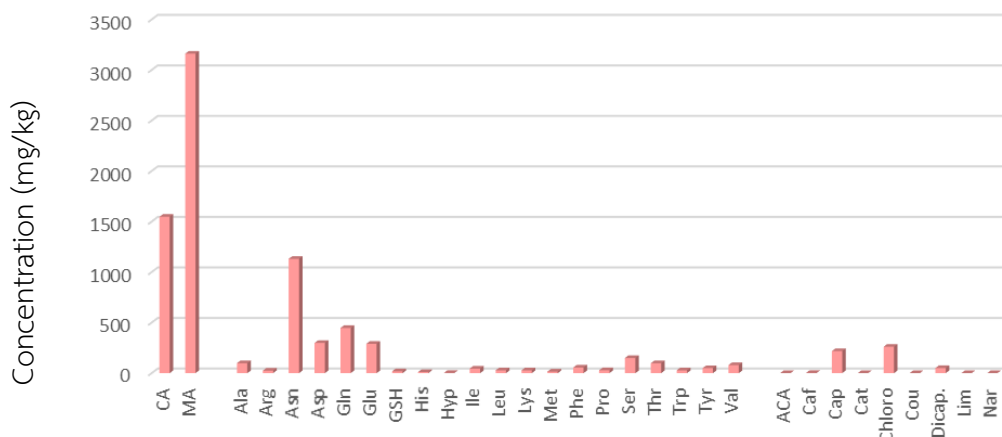
Figure 4.6   The amounts of non-volatile compounds in Chili

4.4.1.5 The amounts of targeted non-volatile compounds in fish sauce

As shown in Figure 4.7, the key non-volatile compounds in fish sauce were obtained in order amino acids > organic acid > phenolic acid. These amino include alanine, leucine, isoleucine, phenylalanine, valine and methionine, 10600, 3870, 2970, 2940, 2290 and 2230 mg/kg, respectively. It can be seen that the amount of organic acids found in fish sauce, citric acid of 90 and malic acid of 165 mg/kg, were much less than those in other ingredients. In addition, the targeted 9 phenolic compounds found in fish sauce are each less than 2 mg/kg.

Figure 4.7   The amounts of non-volatile compounds in Fish sauce

4.4.1.6 The amounts of targeted non-volatile compounds in lime juice

According to the results as shown in Figure 4.8, It is the fact that, among targeted non-volatile compounds in lime juice, the much higher amount of organic acids including citric acid of 27300 mg/kg and malic acid of 2040 mg/kg. The major amounts of amino acids include, aspartic acids, asparagine and glutamic acid of 1450, 770 and 729 mg/kg, respectively. Among the targeted 9 phenolic compounds, limonin of 36.5 mg/kg is the key component, while the rest are each less than 3 mg/kg.

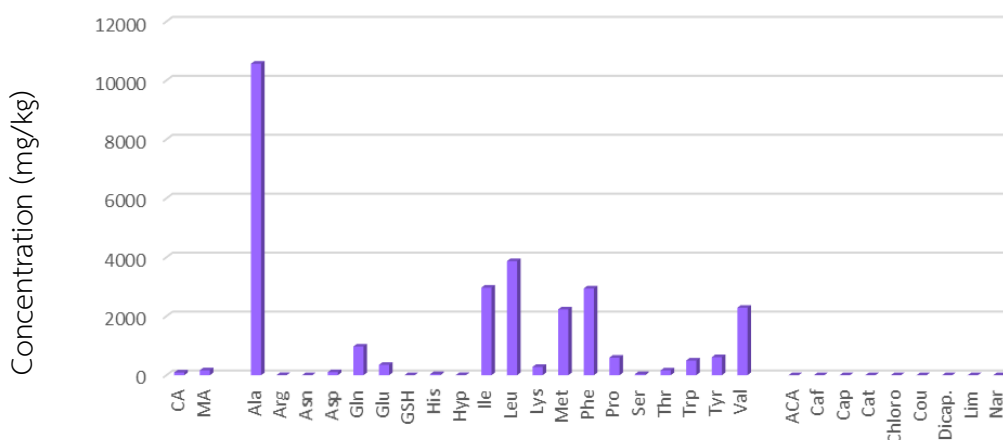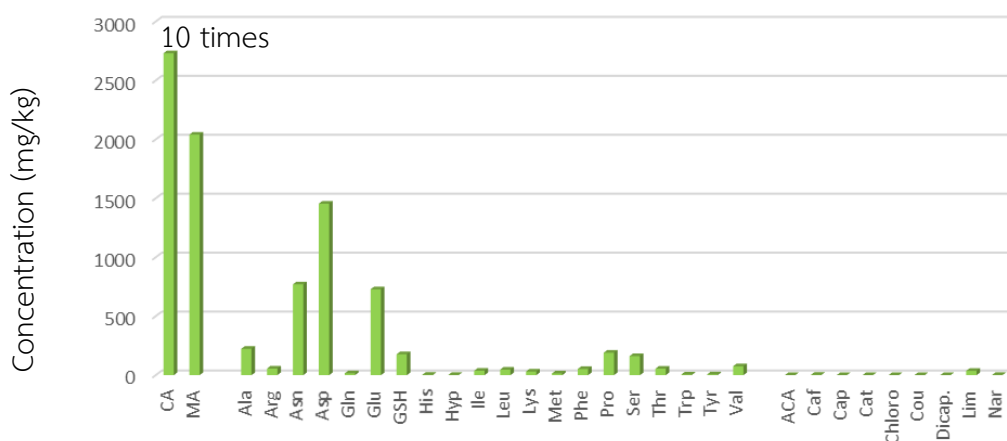**Figure 4.8**   The amounts of non-volatile compounds in Lime juice

4.4.2   The amounts of targeted non-volatile compounds in observed Tom Yum soup compared with expected Tom Yum Soup

Tom Yum soup was prepared with mixed ingredients according to Tom Yum recipe suggested by the Suan Dusit International Culinary School. The Tom Yum soup contains the total weights of mixed ingredient of about 71 g in water of 250 mL, where the weight of each ingredient is shown in Table 4.4. Using an HPLC-MS/MS method developed in this work, the amounts of non-volatile compounds in Tom Yum soup were determined, and compare with the expected amounts that are calculated from the original amount of non-volatile compounds of individual ingredient before mixing. In addition, the main ingredient sources of targeted non-volatile compounds

found in Tom Yum soup were identified using information in Table 4.3 and Figures 4.3-4.9. Results are showed in Table 4.4 and Figure 4.9-4.11.

It can be seen that the main source of organic acids found in Tom Yum soup are from lime juice. The comparably observed an expected amounts of citric acid were obtained, 2714 and 2694 mg/kg, respectively, while the observed less than expected amounts of malic acid is obtained, 212 and 339 mg/kg. In most case, the lower amounts of individual phenolic compound and amino acid in observed than expected TYS were obtained, except for the following two phenolic compounds and five amino acids with the higher amounts for observed TYS: acetoxy chavicol acetate (98/67), cathechin (6.2/4.9), lysine (184/84), threonine (176/85), hydroxyproline (3.8/1.8), histidine (23/12), serine (125/114), where the first/second value in the bracket refer to the amounts of observed and expected TYS in unit of mg/kg. It should be noted that the expected amounts are evaluated under particular pH of hot water for individual ingredient dissolved in the hot water, while the observed amounts are obtained under a low pH acidic condition due to the amounts of organic acids originated from lime juice. In the latter case, a chemical reaction, especially maillard reaction between organic acids and amino acid or hydrolysis reaction. These reactions may result in an increase or decrease in the amounts of targeted non-volatile compounds. In addition, the new compounds may occur under the above mentioned condition that should be investigated using HPLC-MS/MS with Q-TOF mass analyzer.

Table 4.4 The amounts of targeted non-volatile compounds in observed TYS compared with expected TYS and main source of analytes in Tom Yum soup

Comp.	Amount of individual analyte in each ingredient /total crude of mixed ingredient (%)						Conc. (mg/kg)		Main source of analytes in TYS
	KL* (2g)	G* (10g)	LG* (15g)	C* (4g)	FS* (16g)	LJ* (24g)	Expected TYS*	Observed TYS*	
Organic acid									
CA	0.209	0.495	0.898	0.919	0.213	97.3	2694	2714	Lime juice
MA	3.16	16.4	4.61	14.9	3.12	57.8	339	212	Lime juice, Galangal, Chili
Amino acid									
Ala	0.226	0.159	3.31	0.217	93.1	2.95	2.56×10^{4}	523	Fish sauce
Arg	0.598	0.661	50.9	3.19	1.39	43.2	43.5	8.91	Lime juice, Lemongrass
Asn	0.474	7.74	37.8	10.6	0.0430	43.4	600	123.34	Lime juice, Lemon grass
Asp	0.559	1.98	3.63	2.97	4.06	86.8	566	371.39	Lime juice
Gln	0.855	1.86	20.1	7.80	67.8	1.62	323	39.94	Fish sauce, Lemongrass
Glu	0.915	6.50	6.93	4.10	19.8	61.7	399	154.54	Lime juice, Fish sauce
GSH	0.116	0.502	28.2	1.21	0.000	70.0	24.3	12.3	Lime juice, Lemongrass
His	1.30	3.34	13.1	5.23	67.8	9.69	11.7	23.1	Fish sauce, Lemongrass
Hyp	6.29	2.04	6.68	6.94	40.6	37.4	1.83	3.80	Fish sauce, Lime juice

KL*: kaffir lime leaves, G*: galangal, LG*: lemongrass, C*: chili, FS*: fish sauce, LJ*: lime juice, Expected TYS*: expected Tom Yum soup, Observed TYS*: observed Tom Yum soup

Table 4.4 The amounts of targeted non-volatile compounds in observed TYS compared with expected TYS and main source of analytes in Tom Yum soup (continued)

Comp.	Amount of individual analyte in each ingredient /total crude of mixed ingredient (%)						Conc. (mg/kg)		Main source of analytes in TYS
	KL* (2g)	G* (10g)	LG* (15g)	C* (4g)	FS* (16g)	LJ* (24g)	Expected TYS*	Observed TYS*	
Ile	0.0720	0.0810	1.01	0.367	96.6	1.84	692	452.15	Fish sauce
Leu	0.0650	0.0530	0.730	0.174	97.2	1.75	897	622.68	Fish sauce
Lys	0.674	1.38	8.70	1.85	75.0	12.4	84.2	174.12	Fish sauce
Met	0.0320	0.0320	0.361	0.165	98.5	0.963	511	279	Fish sauce
Phe	0.350	0.106	0.829	0.456	95.7	2.54	693	412.67	Fish sauce
Pro	8.03	0.358	5.70	0.689	57.5	27.8	232	158.60	Fish sauce
Ser	17.3	2.33	17.6	7.43	7.24	48.1	114	124.68	Lime juice, Lemongrass, Kaffir lime leaves
Thr	5.22	3.86	19.6	6.50	42.9	21.9	85.1	176.41	Fish sauce, Lime juice, Lemongrass
Trp	0.660	0.267	1.84	1.32	94.4	1.48	118	45.9	Fish sauce
Tyr	0.676	0.246	7.58	1.80	88.3	1.42	156	77.40	Fish sauce
Val	0.158	0.190	2.13	0.806	92.1	4.58	559	499.07	Fish sauce

KL*: kaffir lime leaves, G*: galangal, LG*: lemongrass, C*: chili, FS*: fish sauce, LJ*: lime juice, Expected TYS*: expected Tom Yum soup, Observed TYS*: observed Tom Yum soup

Table 4.4 The amounts of targeted non-volatile compounds in observed TYS compared with expected TYS and main source of analytes in Tom Yum soup (continued)

Comp.	Amount of individual analyte in each ingredient /total crude of mixed ingredient (%)						Conc. (mg/kg)		Main source of analytes in TYS
	KL* (2g)	G* (10g)	LG* (15g)	C* (4g)	FS* (16g)	LJ* (24g)	Expected TYS*	Observed TYS*	
Phenol compounds									
ACA	0.0480	99.7	0.152	0.0210	0.0550	0.0640	66.5	98.0	Galangal
Caf	0.562	0.861	81.1	1.92	4.38	11.2	7.49	2.79	Lemongrass
Cap	0.155	0.474	0.732	96.0	1.25	1.44	12.8	7.40	Chili
Cat	0.684	87.3	2.50	0.882	2.49	6.15	4.85	6.20	Galangal
Chloro	0.183	0.00	15.3	82.1	1.43	0.970	17.9	4.14	Chili
Cou	1.53	5.69	91.5	0.147	0.000	1.12	10.3	3.50	Lemongrass
Dicap.	0.604	2.60	3.90	81.4	4.86	6.61	3.51	2.00	Chili
Lim	12.0	0.513	0.709	0.193	0.729	85.8	14.4	8.05	Lime juice
Nar	31.7	2.04	3.73	0.834	3.02	58.7	0.993	0.295	Lime juice, Kaffir lime leaves

KL*: kaffir lime leaves, G*: galangal, LG*: lemongrass, C*: chili, FS*: fish sauce, LJ*: lime juice, Expected TYS*: expected Tom Yum soup,

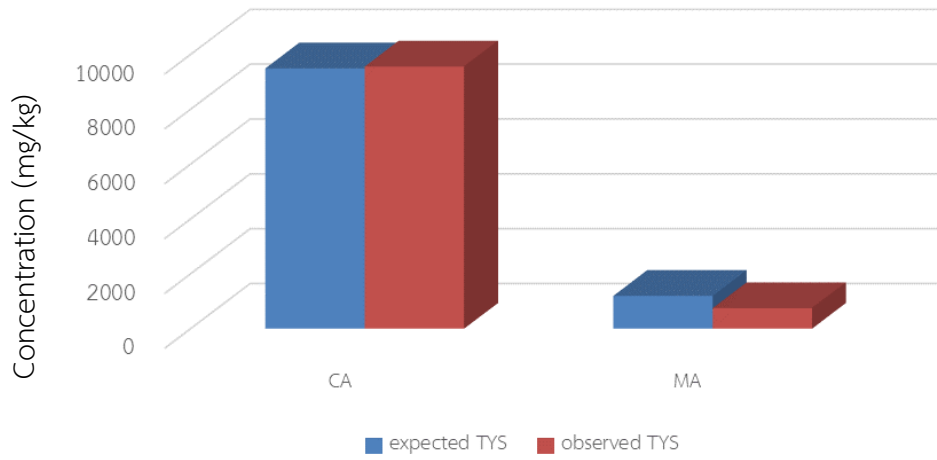Observed TYS*: observed Tom Yum soup

Figure 4.9 The amounts of organic acids in observed Tom Yum soup compared with expected Tom Yum Soup
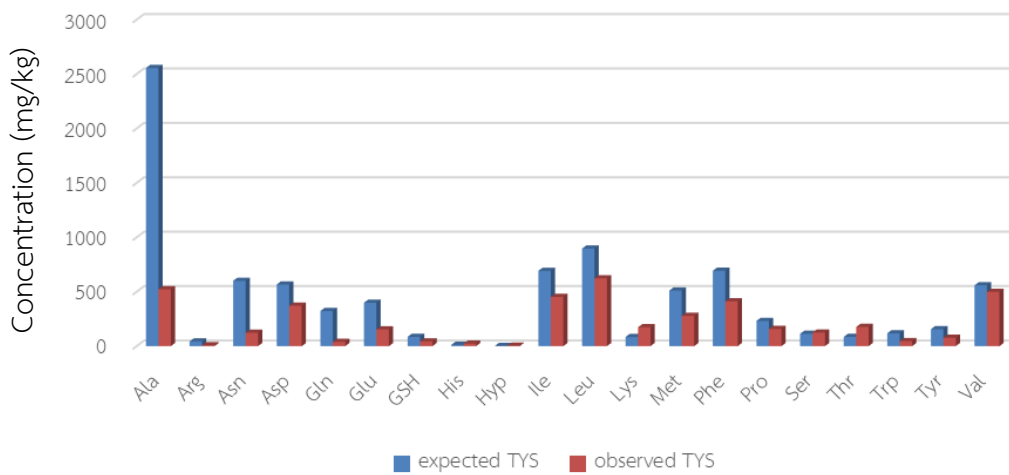

Figure 4.10 The amounts of amino acids in observed Tom Yum soup compared with expected Tom Yum Soup
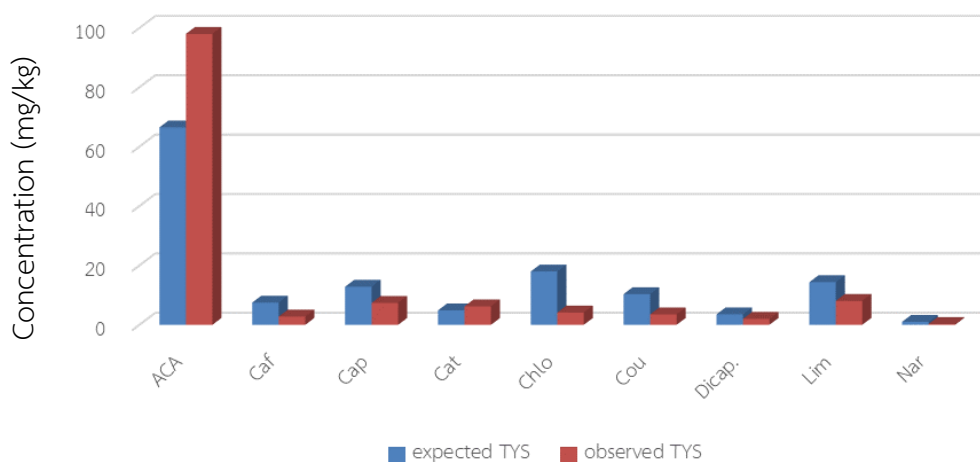
Figure 4.11 The amounts of phenolic compounds in observed Tom Yum soup
compared with expected Tom Yum Soup

4.5   Method validation

4.5.1   Analytical limits

Currently, The modern detection limit (limit of detection) is the smallest amount or concentration of analyte in the test sample that can be reliably distinguished from zero following IUPAC recommendation, involve a risk of false positives detection [58], express by Equation 4.1

$$\text{LOD} = \frac{3.3 S_{x/y}}{A} \sqrt{1 + h_0 + \frac{1}{I}} \qquad \textbf{4. 1}$$

A is the slope of calibration graph, plot between the signal and the concentration. $S_{x/y}$ is the residual standard deviation. I is the number of calibration samples and h_0 is the leverage for the blank sample:

$$h_0 = \frac{\bar{c}_{cal}^2}{\sum_{i=1}^{I} (c_i - \bar{c}_{cal})^2} \qquad \textbf{4. 2}$$

where $\bar{c}_{cal}$ is the mean calibration concentration and c_i is each of calibration concentration values. The calculation of LOQ apply 10 instead of 3.3 in Equation 4.1, as shown in Equation 4.3:

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

Table 4.5 shows results of calibration plot data obtained from four triplicate concentration levels of each analyte (*l* of 12 in Equation 4.1) with sample preparation for 10 g of sample and 50 mL of hot water extraction. This experiment can be concluded that LOD and LOQ value of pooled Tom Yum soup in ranges 0.010 – 0.62 mgL^{-1} and 0.032 – 1.9 mgL^{-1}

Table 4.5   Analytical limits of pooled ingredients sample set obtained from the internal standard calibration results: LOD, LOQ

Analytes	Conc. range (mgL^{-1})	Calibration plot			LOD (mgL^{-1})	LOQ (mgL^{-1})
		Slope	Intercept	R^2		
Group I: amino acids						
Alanine	0.05-0.5	1.82×10^{-7}	9.22×10^{-5}	0.994	0.12	0.36
Arginine	0.05-0.5	1.26×10^{-4}	-3.23×10^{-3}	0.9999	0.62	1.9
Asparagine	0.05-0.5	7.54×10^{-7}	1.54×10^{-4}	0.9918	0.17	0.50
Aspartic acid	0.05-0.5	8.29×10^{-7}	2.62×10^{-4}	0.9922	0.17	0.50
Glutamic acid	0.05-0.5	2.52×10^{-6}	5.03×10^{-4}	0.9967	0.15	0.48
Glutamine	0.05-0.5	9.30×10^{-6}	1.77×10^{-3}	0.9975	0.044	0.13
Glutathione	0.05-0.5	5.74×10^{-6}	-3.23×10^{-4}	0.9995	0.16	0.50
Histidine	0.01-0.1	6.82×10^{-5}	2.32×10^{-3}	0.9985	0.048	0.15
Hydroxyproline	0.01-0.1	3.21×10^{-5}	4.49×10^{-3}	0.9936	0.015	0.044
Isoleucine	0.05-0.5	2.83×10^{-6}	2.64×10^{-4}	0.9965	0.066	0.20
Leucine	0.05-0.5	1.48×10^{-5}	2.82×10^{-4}	0.9999	0.060	0.18
Lysine	0.05-0.5	3.87×10^{-5}	6.04×10^{-5}	0.9993	0.28	0.82

Methionine	0.01-0.1	8.59×10^{-6}	9.48×10^{-5}	0.9999	0.015	0.046
Phenylalanine	0.05-0.5	6.92×10^{-5}	2.53×10^{-3}	0.9999	0.11	0.34
Proline	0.05-0.5	2.81×10^{-6}	4.62×10^{-4}	0.9963	0.14	0.44
Serine	0.05-0.5	7.80×10^{-7}	1.77×10^{-4}	0.9942	0.24	0.76
Threonine	0.05-0.5	1.88×10^{-6}	4.74×10^{-4}	0.9919	0.17	0.50
Tryptophan	0.01-0.1	7.81×10^{-5}	1.22×10^{-3}	0.9999	0.030	0.088
Tyrosine	0.05-0.5	7.81×10^{-5}	1.22×10^{-3}	0.9999	0.030	0.088
Valine	0.05-0.5	4.28×10^{-6}	3.95×10^{-5}	0.9988	0.15	0.44
Group II: Phenolic compounds						
ACA	0.01-0.1	1.89×10^{-4}	1.74×10^{-2}	0.9989	0.010	0.032
Caffeic acid	0.01-0.1	3.85×10^{-4}	1.26×10^{-2}	0.9998	0.012	0.038
Capsaicin	0.01-0.1	5.70×10^{-5}	-1.8×10^{-3}	0.9996	0.010	0.032
Cathechin	0.01-0.1	1.35×10^{-5}	3.43×10^{-4}	0.9984	0.020	0.060
Chlorogenic acid	0.01-0.1	1.76×10^{-6}	1.76×10^{-4}	0.9984	0.013	0.040
Dihydrocapsaicin	0.01-0.1	1.08×10^{-4}	5.60×10^{-3}	0.9989	0.036	0.11
Limonin	0.01-0.1	7.48×10^{-6}	1.60×10^{-4}	0.9999	0.011	0.034
Naringin	0.01-0.1	1.36×10^{-5}	-1.13×10^{-3}	0.999	0.011	0.032
p-Coumaric acid	0.01-0.1	2.64×10^{-4}	2.16×10^{-2}	0.9982	0.052	0.16
Group III: Organic acids						
Malic acid	0.05-0.5	4.28×10^{-5}	-3.94×10^{-3}	0.9977	0.062	0.19
Citric acid	0.1-1.0	5.70×10^{-5}	-3.72×10^{-2}	0.9943	0.54	1.6

4.5.2 Standard calibration curve

A standard calibration curves were analyzed from the relationship between the response and the analyte concentration with seven concentration levels and three replicates for each level, calibration plot shown in Table 4.6. The linear regression plots were performed using the relative response of the ratio of peak area of analyte and internal standard, against the analyte concentration of each analyte.

The results demonstrated a good linearity and coefficients of determination (R^2) always higher than 0.99 in the studied. The calibration curve of targeted non-volatile standards in this studied express at appendix.

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Table 4.6   Calibration parameter of standard calibration curve

Analytes	Conc. range (µg/L)	Calibration plot		
		Slope	Intercept	R^2
Group I: amino acids				
Alanine	0.05-5.0	1.04×10^{-7}	3.26×10^{-5}	0.9933
Arginine	0.05-5.0	1.11×10^{-4}	-7.28×10^{-3}	0.9979
Asparagine	0.05-5.0	8.18×10^{-7}	1.84×10^{-4}	0.9939
Aspartic acid	0.05-5.0	6.74×10^{-7}	2.23×10^{-4}	0.9930
Glutamic acid	0.05-5.0	2.17×10^{-5}	7.15×10^{-4}	0.9901
Glutamine	0.05-5.0	5.96×10^{-6}	1.12×10^{-3}	0.9959
Glutathione	0.05-5.0	7.27×10^{-6}	-4.27×10^{-3}	0.9935
Histidine	0.01-1.0	1.39×10^{-4}	-2.08×10^{-3}	0.9967
Hydroxyproline	0.01-1.0	8.36×10^{-5}	1.63×10^{-3}	0.9933
Isoleucine	0.05-5.0	1.65×10^{-5}	1.25×10^{-3}	0.9972
Leucine	0.05-5.0	1.89×10^{-5}	9.97×10^{-4}	0.9989
Lysine	0.05-5.0	2.91×10^{-5}	-6.72×10^{-3}	0.9986
Methionine	0.01-1.0	7.21×10^{-6}	-9.63×10^{-5}	0.9993
Phenylalanine	0.05-5.0	5.64×10^{-5}	9.12×10^{-4}	1.0000
Proline	0.05-5.0	2.64×10^{-6}	5.86×10^{-4}	0.9905
Serine	0.05-5.0	5.82×10^{-7}	1.59×10^{-4}	0.9911
Threonine	0.05-5.0	5.12×10^{-7}	1.36×10^{-4}	0.9909
Tryptophan	0.01-1.0	4.58×10^{-5}	4.80×10^{-5}	0.9996
Tyrosine	0.05-5.0	1.94×10^{-5}	-1.52×10^{-4}	0.9998
Valine	0.05-5.0	1.26×10^{-6}	9.84×10^{-5}	0.9981
Group II: Phenolic compounds				
ACA	0.01-1.0	1.67×10^{-4}	1.64×10^{-2}	0.9913
Caffeic acid	0.01-1.0	3.56×10^{-4}	-3.43×10^{-3}	0.9994
Capsaicin	0.01-1.0	9.87×10^{-5}	-1.89×10^{-3}	0.9996
Cathechin	0.01-1.0	7.78×10^{-5}	-3.33×10^{-4}	0.9988
Chlorogenic acid	0.01-1.0	1.07×10^{-5}	4.42×10^{-4}	0.9984
Dihydrocapsaicin	0.01-1.0	9.40×10^{-4}	4.93×10^{-3}	0.9984
Limonin	0.01-1.0	5.06×10^{-5}	-1.65×10^{-4}	0.9993

Naringin	0.01-1.0	1.92×10^{-4}	-8.21×10^{-4}	0.9993
p-Coumaric acid	0.01-1.0	3.39×10^{-4}	7.59×10^{-4}	0.9911
Group III: Organic acids				
Malic acid	0.05-5.0	2.35×10^{-5}	-1.38×10^{-4}	0.9989
Citric acid	0.10-10.0	2.82×10^{-5}	-4.98×10^{-3}	0.9957

4.5.3   Accuracy and Precision

The accuracy and precision of standards spiked in Tom Yum soups are expressed by recovery and %RSD, respectively. According to Section 3.6.3, a pooled sample was obtained from three batches of Tom Yum soup, and spiked with targeted non-volatile standards at three concentration levels, and each analyte for three days. The amounts of analytes before and after spiking with standards were determined by HPLC-MS/MS with six runs using the ratio of peak area of analyte and internal standard. For each day and each concentration level, the determined amount of standards spiked samples was obtained from the different amounts of analyte before and after spiking with standard, where the amount of before spiking used in this case is the average value from six runs. Therefore, % recovery is calculated using Equation 4.4.

$$\% \text{ Recovery} = \frac{C_{total} - C_{sample}}{C_{spiked}} \times 100 \qquad \textbf{Equation} \quad \textbf{4. 4}$$

where C_{spiked} is the amount of standard spiked in a sample

C_{sample} is the average amount of analyte in a sample from six runs

C_{total} is the amount of analyte after spiking with standards

Over 3.0-150 mg/kg of spiked standard in sample, it can be seen Table 4.4 that the recovery values for intraday precision are in the range of 62-115%, and within the criteria for acceptable recovery [59] for all data. It should be noted that the acceptable recovery were calculated using Horwitz Equation: %RSD < 0.67 x 2 $^{(1-0.5 \log C)}$, where C is the analyte concentration.

Table 4.7   Accuracy and precision of standards spiked in Tom Yum soups made from fresh ingredients at three level

Analytes	Concentration Spiked std.		% Recovery (% RSD)			
	a (mg/L)	b (mg/kg)	Acceptable criteria	Day 1	Day 2	Day 3
Group I: amino acids						
Alanine	0.60	30	80-110(12)	81(12)	97(12)	107(13)
	1.00	50	80-110(11)	104(12)	115(13)	92(8)
	3.00	150	80-110(9)	84(15)	85(16)	102(10)
Arginine	0.40	20	80-110(12)	84(9)	81(7)	106(4)
	0.50	25	80-110(12)	108(9)	104(7)	104(4)
	2.00	100	80-110(10)	109(10)	108(7)	81(5)
Asparagine	0.60	30	80-110(12)	85(15)	85(18)	88(19)
	1.00	50	80-110(11)	110(12)	104(9)	92(17)
	3.00	150	80-110(9)	83(16)	104(10)	107(13)
Aspartic acid	0.60	30	80-110(12)	81(12)	82(10)	84(8)
	1.00	50	80-110(11)	109(12)	90(9)	86(10)
	3.00	150	80-110(9)	83(12)	86(9)	81(7)
Caffeic acid	0.060	3	80-110(16)	86(15)	96(5)	97(11)
	0.080	4	80-110(16)	97(9)	94(9)	90(9)
	0.60	30	80-110(12)	97(9)	85(9)	90(9)
Glutamic acid	0.60	30	80-110(12)	105(9))	89(12)	90(12)
	1.00	50	80-110(11)	88(11)	109(12)	109(13)
	3.00	150	80-110(9)	107(9)	87(11)	97(13)
Glutamine	0.60	30	80-110(12)	95(20)	97(25)	101(22)
	1.00	50	80-110(11)	107(20)	94(18)	99(19)
	3.00	150	80-110(9)	86(20)	88(25)	88(19)
Glutathione	0.40	20	80-110(12)	88(15)	94(6)	92(2)
	0.50	25	80-110(12)	108(13)	94(13)	92(12)
	2.00	100	80-110(10)	82(14)	80(5)	80(6)
Histidine	0.060	3	80-110(16)	62(16)	69(16)	97(16)
	0.080	4	80-110(16)	96(17)	90(13)	91(14)
	0.60	30	80-110(12)	88(17)	82(13)	83(13)

Hydroxyproline	0.060	3	80-110(16)	88(18)	88(21)	89(18)
	0.080	4	80-110(16)	88(21)	8915)	90(13)
	0.60	30	80-110(12)	89 (21)	99(9)	96(6)
Isoleucine	0.60	30	80-110(12)	107(9)	85(5)	114(4)
	1.00	50	80-110(11)	102(5)	99(8)	105(4)
	3.00	150	80-110(9)	85(5)	83(5)	103(7)
Leucine	0.60	30	80-110(12)	82(2)	90(5)	92(4)
	1.00	50	80-110(11)	101(5)	83(5)	110(3)
	3.00	150	80-110(9)	110(9)	95(18)	107(11)
Lysine	0.60	30	80-110(12)	106(7)	107(20)	94(8)
	1.00	50	80-110(11)	87(7)	88(20)	93(8)
	3.00	150	80-110(9)	103(7)	104(20)	109(8)
Methionine	0.40	20	80-110(12)	84(7)	84(4)	82(13)
	0.50	25	80-110(12)	89(16)	89(4)	107(13)
	2.00	100	80-110(10)	106(6)	93(4)	83(13)
Phenylalanine	0.60	30	80-110(12)	90(3)	102(3)	103(9)
	1.00	50	80-110(11)	96(3)	106(8)	106(8)
	3.00	150	80-110(9)	87(9)	109(2)	108(12)
Proline	0.60	30	80-110(12)	84(13)	86(8)	83(8)
	1.00	50	80-110(11)	107(15)	85(8)	92(6)
	3.00	150	80-110(9)	95(20)	109(9)	104(12)
Serine	0.60	30	80-110(12)	92(9)	89(11)	94(7)
	1.00	50	80-110(11)	102(13)	99(8)	107(7)
	3.00	150	80-110(9)	110(10)	98(7)	99(7)
Throrine	0.60	30	80-110(12)	81(11)	83(9)	92(9)
	1.00	50	80-110(11)	98(12)	100(6)	106(6)
	3.00	150	80-110(9)	105(11)	108(8)	106(11)
Tryptophan	0.40	20	80-110(12)	85(14)	87(22)	87(17)
	0.50	25	80-110(12)	89(15)	89(16)	89(16)
	2.00	100	80-110(10)	84(5)	109(5)	101(19)
Tyrosine	0.40	20	80-110(12)	100(8)	100(19)	84(11)
	0.50	25	80-110(12)	109(8)	105(3)	98(14)
	2.00	100	80-110(10)	109(3)	108(11)	108(9)
Valine	0.60	30	80-110(12)	99(6)	103(5)	106(11)
	1.00	50	80-110(11)	108(6)	110(9)	109(4)

	3.00	150	80-110(9)	89(6)	99(4)	106(7)
Group II: Phenolic compounds						
ACA	0.60	30	80-110(12)	95(2)	93(7)	93(7)
	1.00	50	80-110(11)	99(2)	95(5)	93(7)
	3.00	150	80-110(9)	85(2)	80(10)	106(7)
Capsaicin	0.40	20	80-110(12)	92(2)	93(2)	96(13)
	0.50	25	80-110(12)	80(1)	98(2)	101(10)
	2.00	100	80-110(10)	81(2)	84(4)	81(6)
Cathechin	0.060	3	80-110(16)	75(6)	70(10)	81(10)
	0.080	4	80-110(16)	80(6)	88(10)	88(10)
	0.60	30	80-110(12)	97(5)	92(10)	94(10)
Chlorogenic acid	0.60	30	80-110(12)	88(7)	94(9)	96(10)
	1.00	50	80-110(11)	83(3)	81(8)	104(10)
	3.00	150	80-110(9)	108(21)	110(14)	107(13)
Coumaric acid	0.060	3	80-110(16)	67(3)	78(2)	82(3)
	0.080	4	80-110(16)	84(3)	104(2)	106(3)
	0.60	30	80-110(12)	94(3)	82(2)	110(3)
Dihydrocapsaicin	0.060	3	80-110(16)	67(3)	77(3)	68(5)
	0.080	4	80-110(16)	99(3)	103(3)	100(5)
	0.60	30	80-110(12)	103(3)	108(3)	107(5)
Limonin	0.060	3	80-110(16)	67(9)	77(7)	72(9)
	0.080	4	80-110(16)	92(9)	104(7)	97(7)
	0.60	30	80-110(12)	102(9)	98(7)	89(9)
Naringin	0.060	3	80-110(16)	74(12)	88(11)	79(11)
	0.080	4	80-110(16)	104(12)	99(11)	99(10)
	0.60	30	80-110(12)	110(12)	86(10)	103(11)
Group III: Organic acids						
Malic acid	0.60	30	80-110(12)	82(2)	86(8)	83(8)
	1.00	50	80-110(11)	107(2)	106(8)	109(10)
	3.00	150	80-110(9)	109(2)	105(7)	85(8)
Citric acid	0.60	30	80-110(12)	94(4)	86(7)	90(8)
	1.00	50	80-110(11)	107(4)	105(7)	103(8)
	3.00	150	80-110(9)	86(4)	91(6)	86(8)

a: Concentration of spiked standard in a solution prior to LC-MS/MS injection

b: Concentration of spiked standard in mixed ingredients of Tom Yum

4.6 Application in Tom Yum pastes

It can be seen in Table 4.8, the quantitative analysis was also applied in four commercial Tom Yum pastes, at Gourmet market paragon. The non-volatile compounds in Tom Yum soup were made in laboratory followed the recipe compared with four pastes. In the procedure about made Tom Yum soup from paste followed by the description on the other pastes.

Table 4.8 The amounts of targeted non-volatile compounds in four commercial paste from the department store in Bangkok.

Analytes	The amount of analytes (mg/kg)				
	TYS	Paste1	Paste2	Paste3	Paste4
Organic acids					
CA	6.79×10^3	1.04×10^4	7.46×10^3	1.10×10^4	3.52×10^3
MA	531	822	494	282	766
Amino acids					
Ala	371	408	31.8	112	43.0
Arg	6.30	5.10	44.1	19.6	46.1
Asn	87.6	321	319	0.900	110
Asp	264	384	72.0	nd	74.5
Gln	28.4	56.9	2.40	nd	nd
Glu	110	159	68.1	56.4	40.9
GSH	30.8	38.6	2.00	2.80	1.80
His	16.4	nd	nd	nd	nd
Hyp	2.70	0.100	nd	nd	nd
Ile	321	327	21.5	75.8	22.4
Leu	442	439	36.1	120	37.9
Lys	124	133	10.2	5.30	7.80
Met	198	204	3.80	24.0	4.70
Phe	293	307	30.2	90.1	47.4
Pro	113	117	194	84.3	321
Ser	88.5	102	56.0	1.70	31.8
Thr	125	41.6	27.4	nd	13.6
Trp	32.6	51.3	nd	nd	nd

Tyr	nd	61.3	40.6	65.7	47.1
Val	354	417	37.4	105	43.5
Phenolic compounds					
ACA	69.6	87.8	6.30	nd	nd
Caf	2.00	1.30	1.10	0.100	nd
Cap	5.30	10.1	2.00	5.50	5.70
Cat	4.40	nd	nd	nd	nd
chlo	2.90	nd	nd	nd	nd
Cou	2.50	1.60	1.00	nd	nd
Dicap	1.40	0.600	nd	nd	nd
lim	5.70	0.800	nd	nd	nd
Nar	0.200	2.30	nd	nd	nd

nd refers to "not detected"
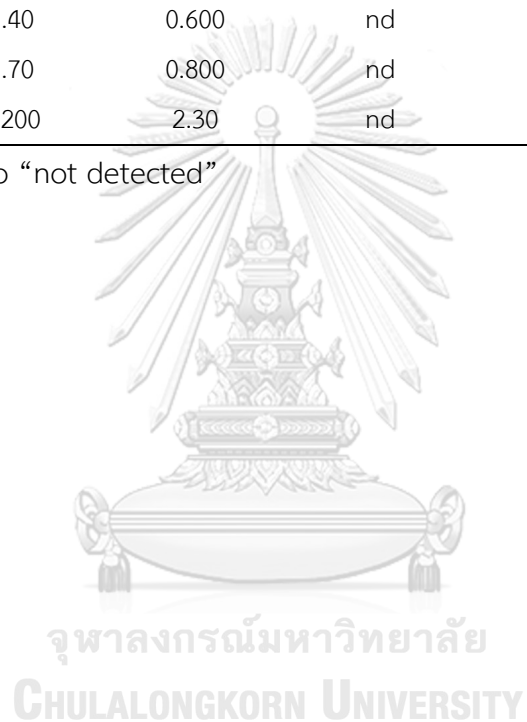
CHAPTER V

CONCLUSION

In this work, HPLLC-MS/MS was successfully applied to analyze following targeted non-volatile compounds in Tom Yum ingredient and Tom Yum soup: amino acids, organic acids and phenol compounds that contribute to the basic taste compounds. The following HPLC-MS/MS conditions for simultaneous separation and detection of all of these compounds were used, a PoroShell C18 column (4.6 x 100 mm, 2.7 μm), column temperature of 30 °C and a gradient elution of A:B mobile phase at a flow-rate of 0.3 mL/min, where A consists of 0.1%v/v formic acid in water and B consists of 0.1%v/v formic acid in methanol. The triple quadrupoles mass analyzer with electrospray ionization (ESI) interface was performed in both positive and negative modes under multiple reaction monitoring (MRM). The MS/MS conditions were set as a defaults follows: capillary voltage of 3000 V, nozzle pressure of 20 psi, sheath gas flow of 11 L/min, sheath gas temperature of 400 °C, fragmentor of 380 V and dwell time of 50 ms.

For method validation, the following parameters were evaluated for HPLC-MS/MS analysis: limit of detection (LOD), limit of quantitation (LOQ), standard calibration curve, accuracy and precision. LOD and LOQ values were obtained in ranges 0.010 – 0.62 mgL^{-1} and 0.032 – 1.9 mgL^{-1}, respectively. Acceptable linearity of internal standard calibration curve was found with R^2 > 0.99. By spiking the known concentration standards in the diluted Tom Yum soup at three levels, satisfactory accuracy, that is the recovery in a range of 62-115%, was obtained, with 98.6% of the recovery data being within 80-110% for the analytes concentration in the range of 0.06-3 ppm. An accepted level of precision intraday and interday were also obtained with RSD of <16%.

Hot water extraction was used for extracting non-volatile compounds in ingredients at 100±5 °C as comparable temperature as a cooking process in order to determine the targeted non-volatile compounds dissolved in hot water. Using the key targeted non-volatile compounds including organic acids, amino acids and phenolic acids were determined in the individual ingredients and observed/expected Tom Yum soup.

The main sources were obtained: two organic acids including citric acids and malic acid from lime juice, fourteen amino acids from fish sauce, another six amino acids from lime juice, where the former fourteen amino acids (alanine, glutamine, histidine, hydroxyproline, tryptophan, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tyrosine and valine), while the latter six amino acids (arginine, asparagine, aspartic acid, glutamic acid, glutathione and serine). Moreover, the main source of these phenolics were obtained in other ingredients include acetoxy chavicol acetate (galangal), caffeic acid (lemongrass), capsaicin (chili), cathechin (galangal), chlorogenic acid (chili), coumaric acid (lemongrass), dihydrocapsaicin (chili), limonin (lime juice) and naringin (lime juice).

In the future work, the hot water extraction and chromatographic research can be extended to HPLC-MS/MS determination of non-targeted and targeted non-volatile compounds in another food ingredients and soup. Moreover, the analytical technique may be applied to sensory analysis for proving the chemicals that contribute to the taste in food products and also can be extended to food industry.

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APPENDIX

Standard calibration curve
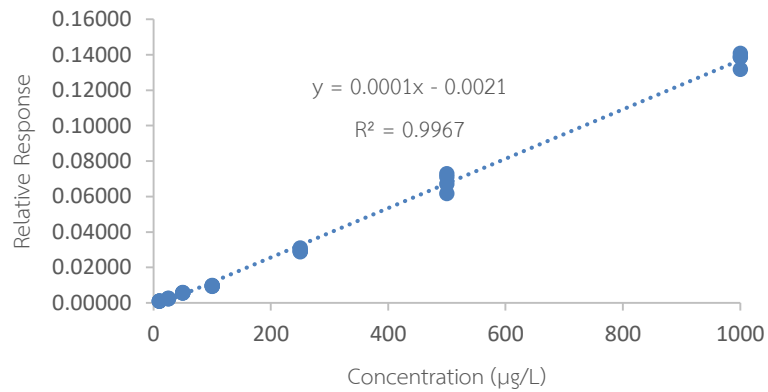
Figure A.1   Standard calibration curve of histidine
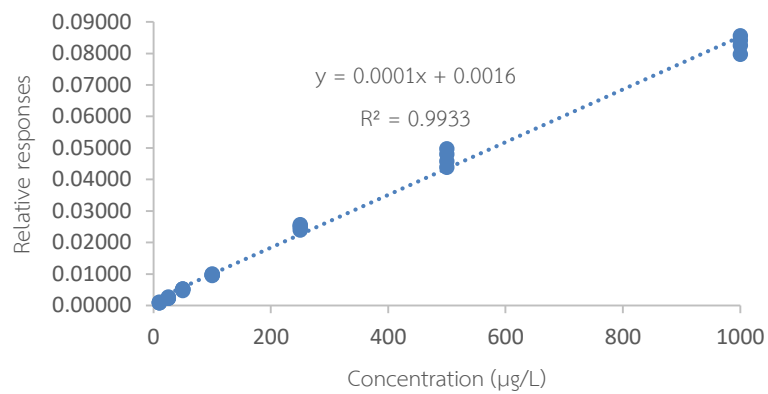
Figure A.2   Standard calibration curve of hydroxyproline
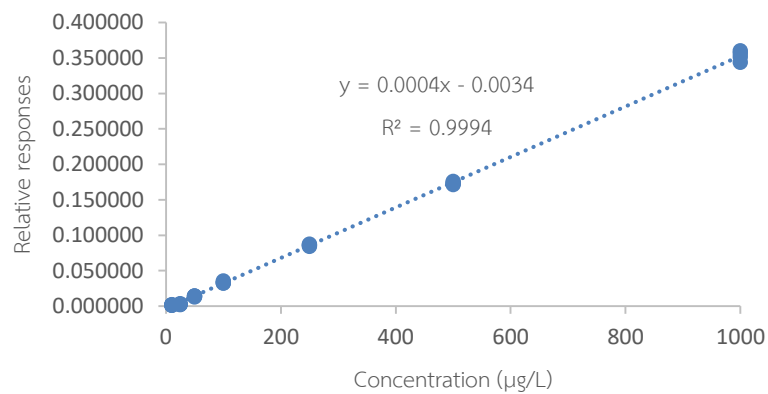
Figure A.3   Standard calibration curve of caffeic acid
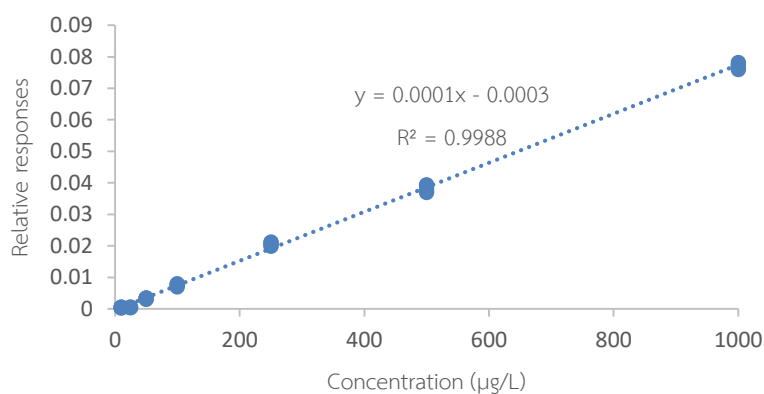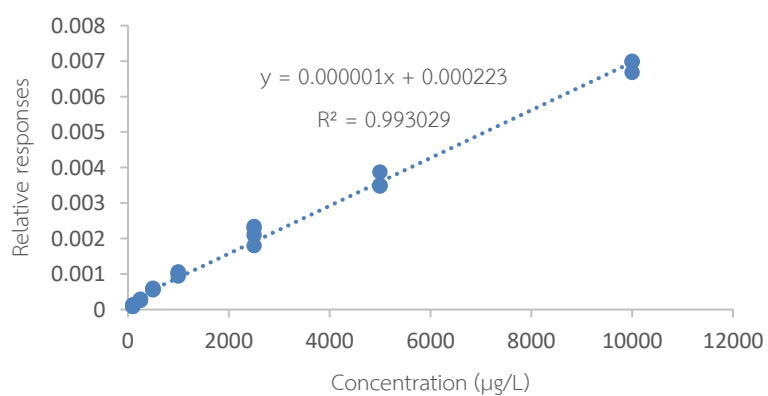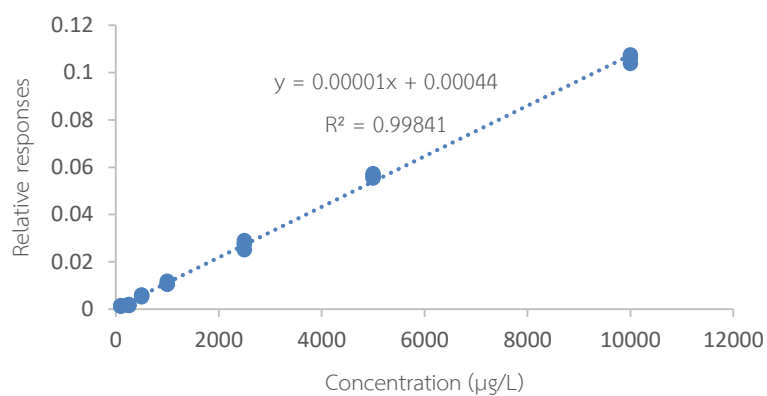
Figure A.4   Standard calibration curve of cathechin
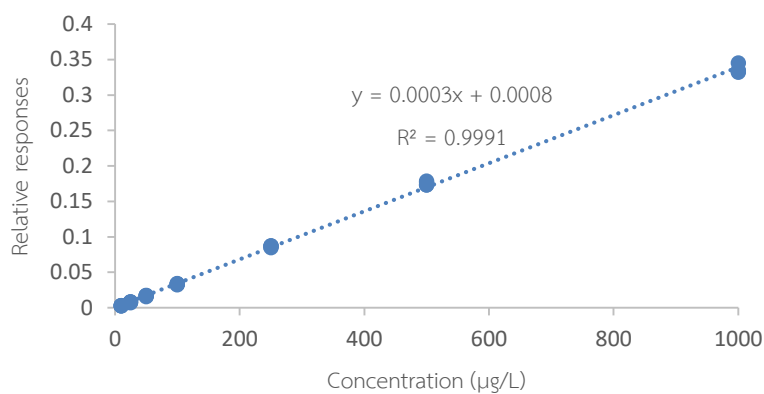
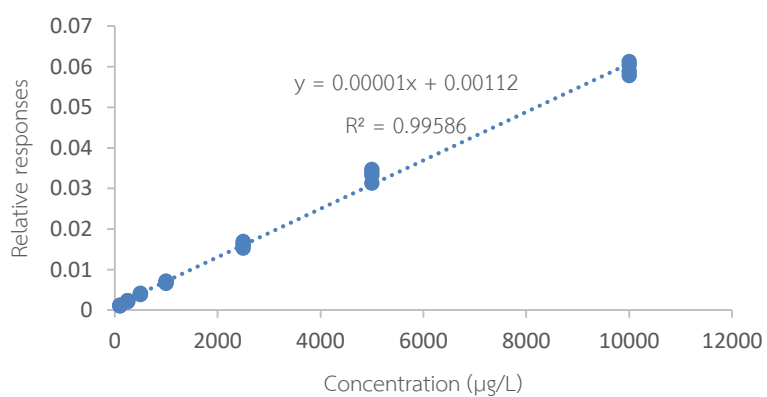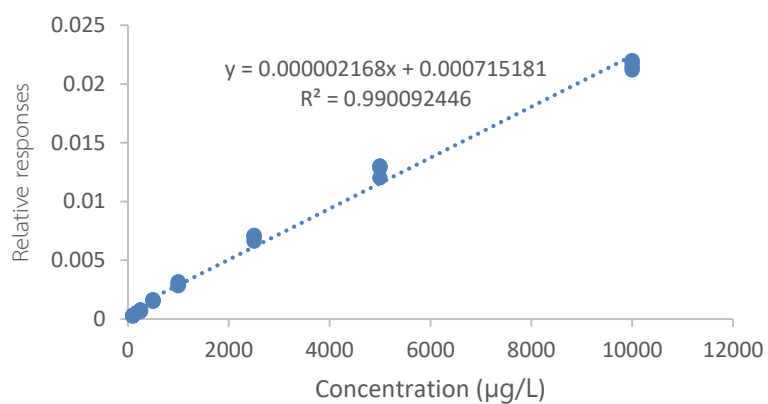**Figure A.5**   Standard calibration curve of coumaric acid
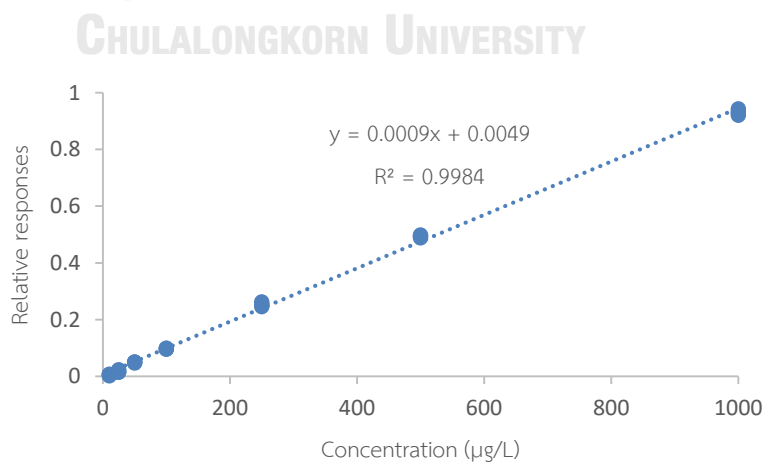
Figure A.6   Standard calibration curve of dihydrocapsaicin
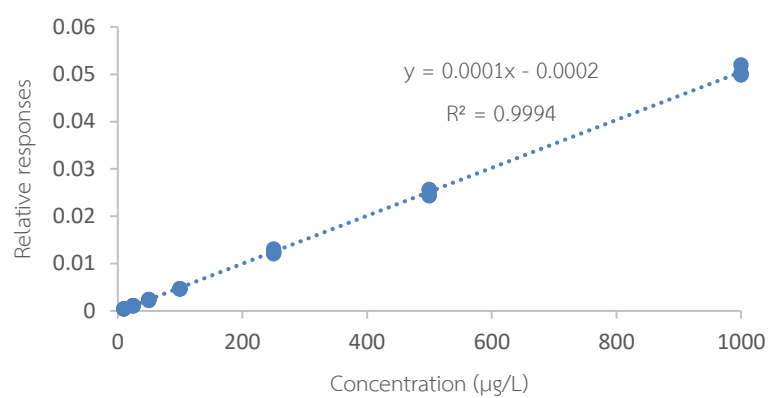
Figure A.7   Standard calibration curve of limonin
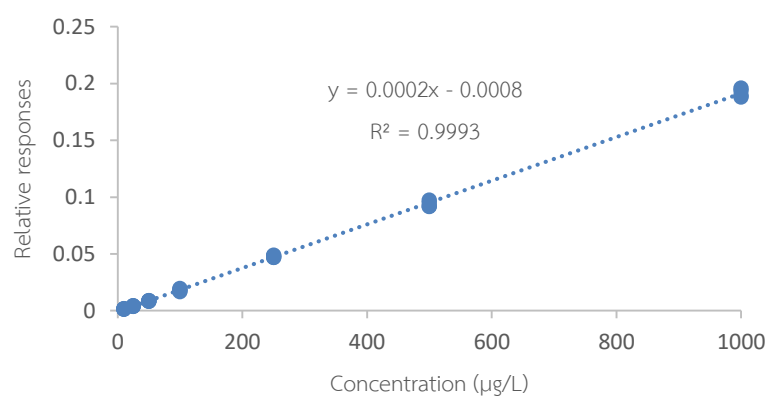
Figure A.8   Standard calibration curve of naringin

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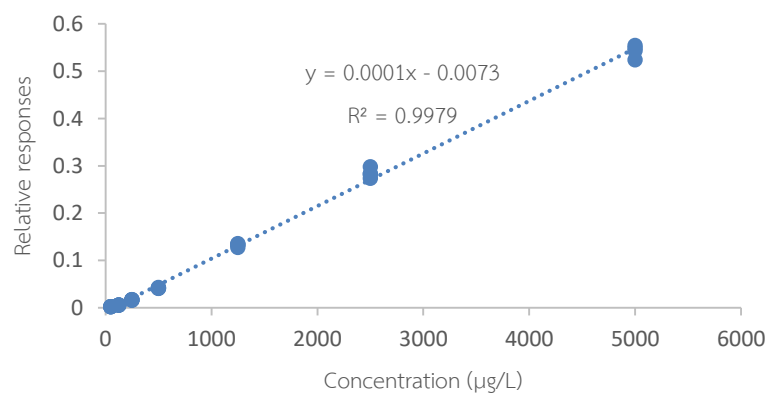
Figure A.9   Standard calibration curve of arginine
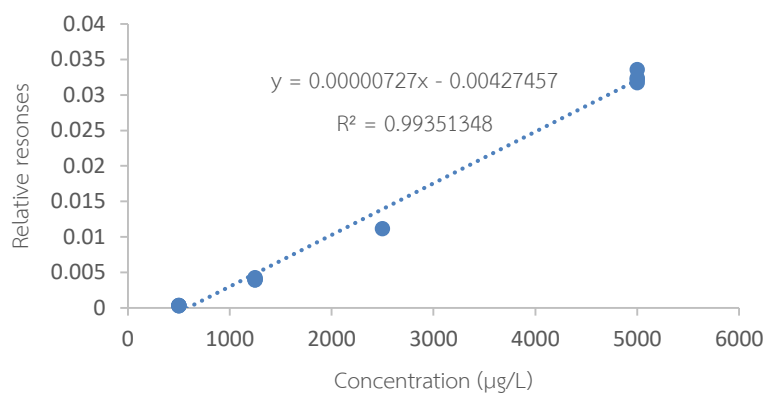
Figure A.10 Standard calibration curve of glutathione
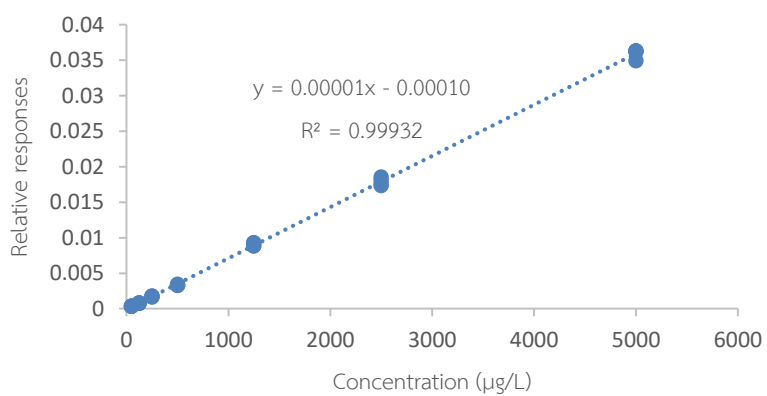
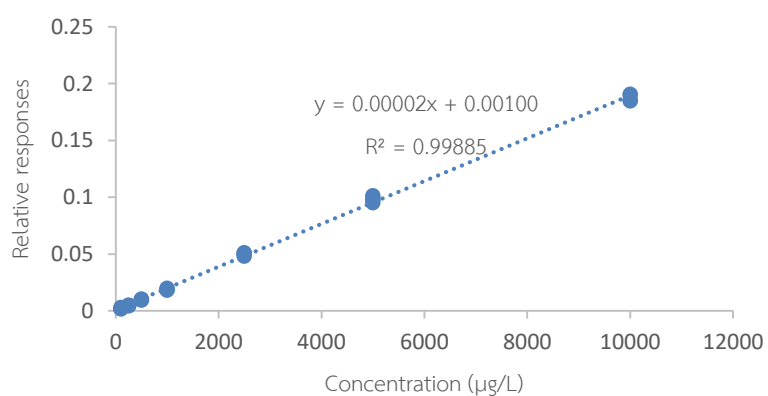**Figure A.11** Standard calibration curve of methionine
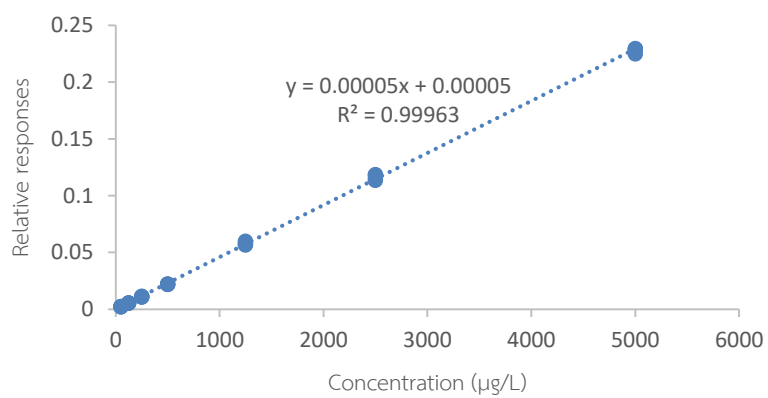
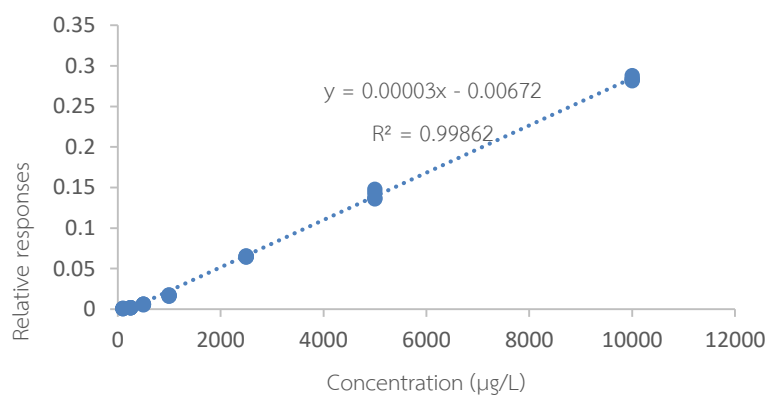**Figure A.12** Standard calibration curve of tryptophan
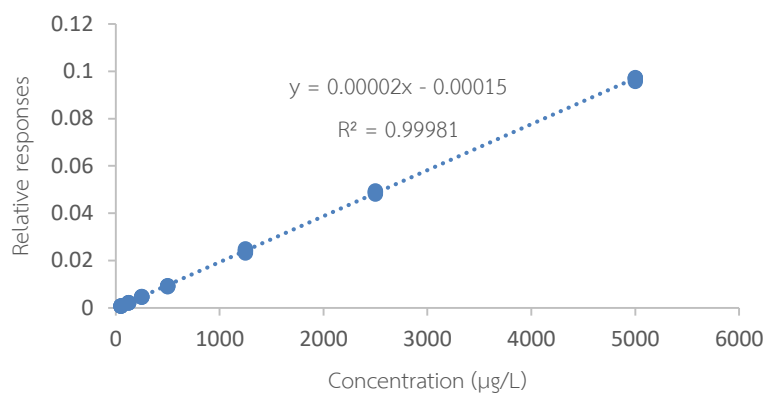
Figure A.13   Standard calibration curve of tyrosine
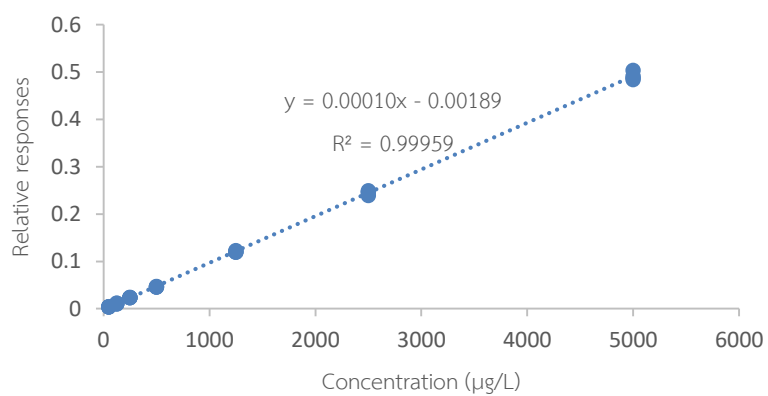
Figure A.14   Standard calibration curve of capsaicin
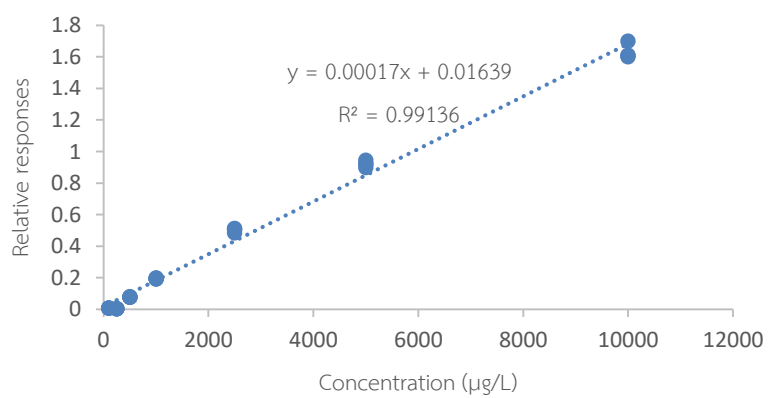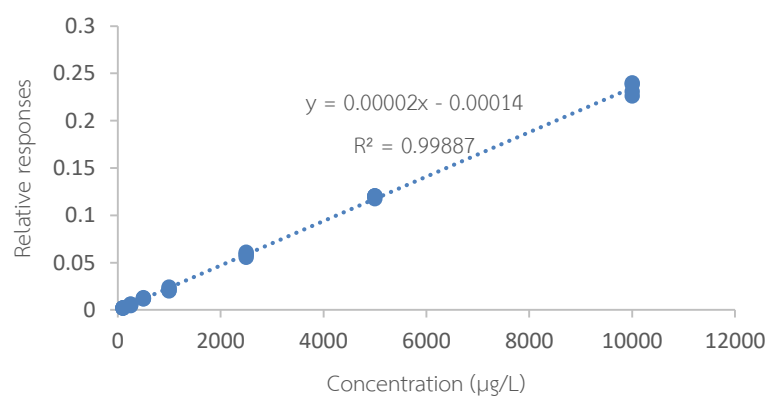

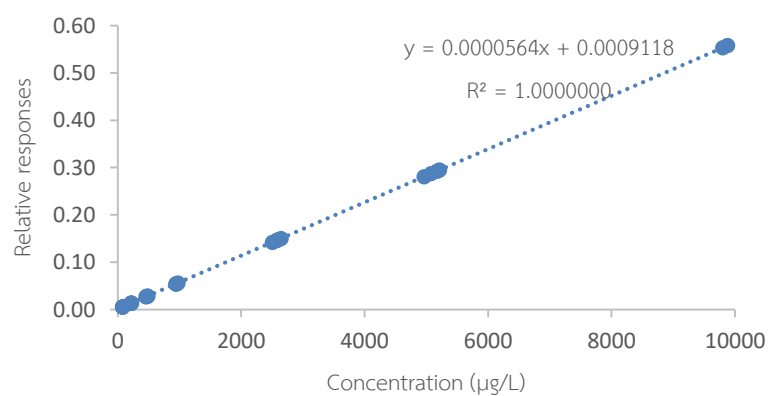Figure A.15   Standard calibration curve of acetoxy chavicol acetate
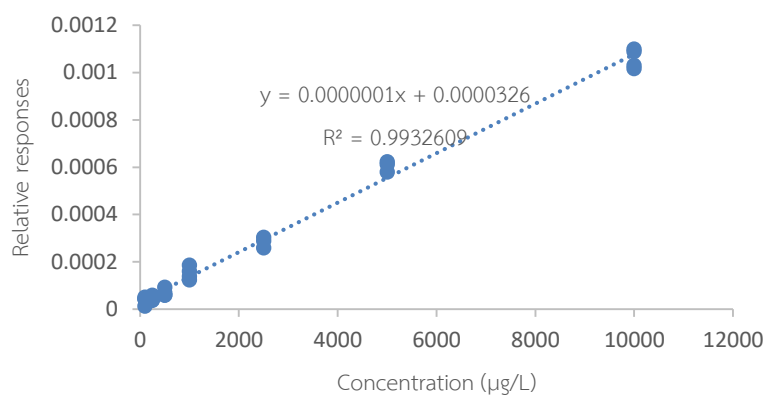
Figure A.16   Standard calibration curve of alanine
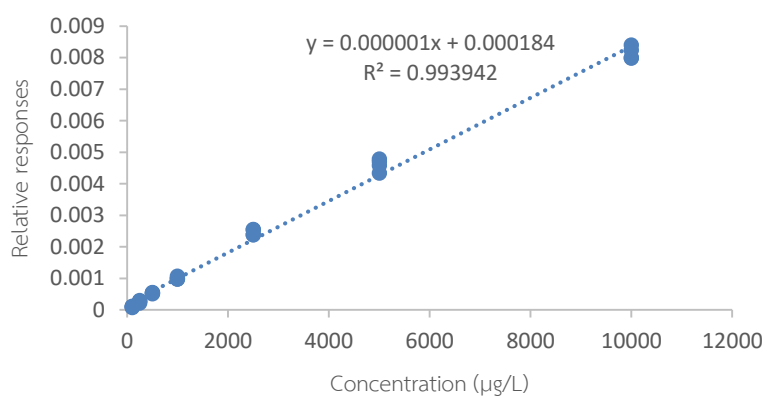
Figure A.17   Standard calibration curve of asparagine

Figure A.18   Standard calibration curve of aspartic acid

Figure A.19   Standard calibration curve of chlorogenic acid
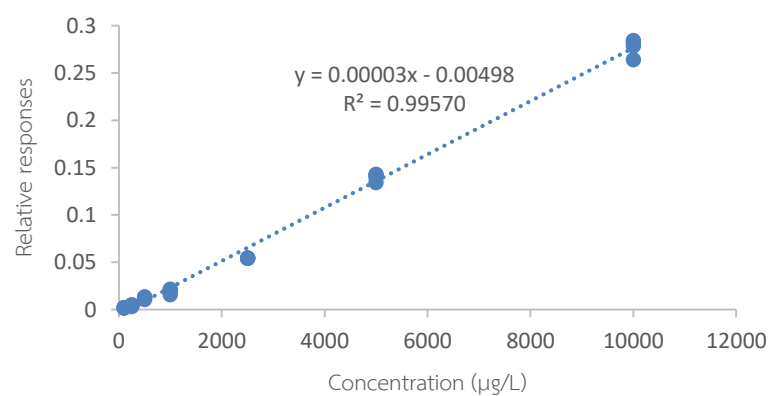
Figure A.20   Standard calibration curve of citric acid

Figure A.21   Standard calibration curve of glutamine

Figure A.22   Standard calibration curve of glutamic acid
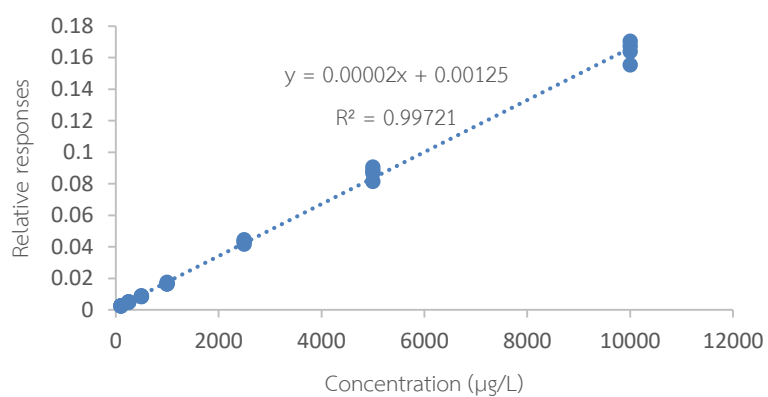
Figure A.23   Standard calibration curve of isoleucine

Figure A.24   Standard calibration curve of leucine

Figure A.25   Standard calibration curve of lysine

Figure A.26   Standard calibration curve of malic acid

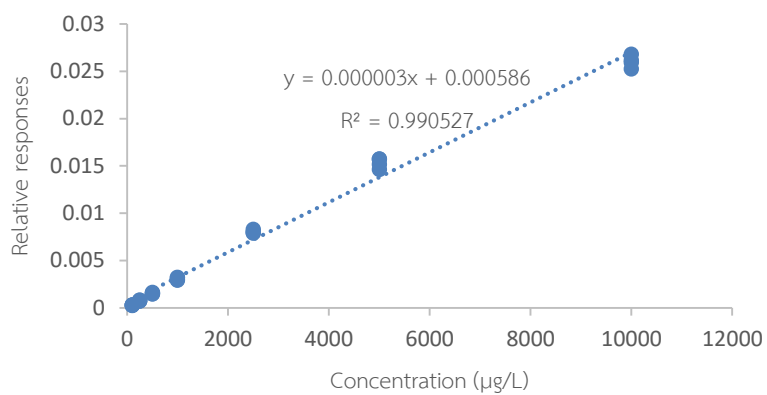
Figure A.27   Standard calibration curve of phenylalanine

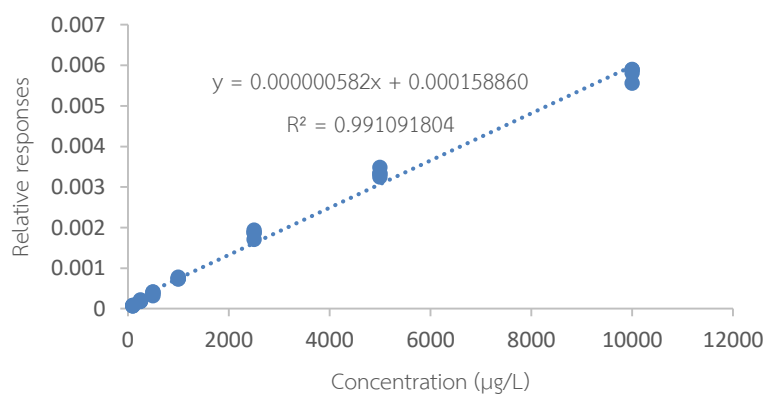**Figure A.28**   Standard calibration curve of proline

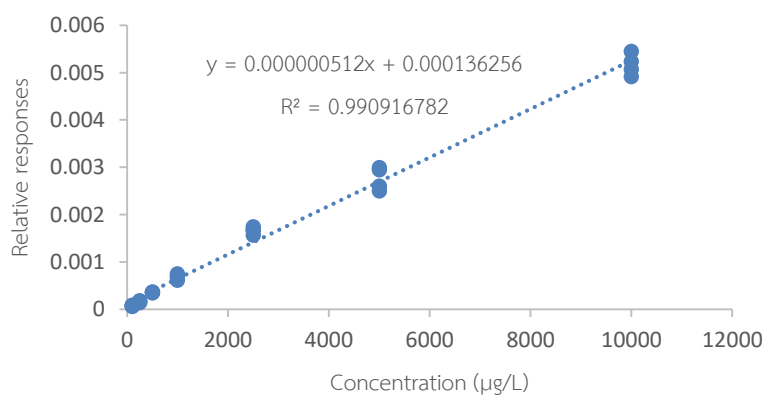**Figure A.29**   Standard calibration curve of serine

Figure A.30   Standard calibration curve of threonine

The chart shows a standard calibration curve with the following:
- y-axis: Relative responses (0 to 0.014)
- x-axis: Concentration (μg/L) (0 to 12000)
- Equation: y = 0.000001x - 0.000098
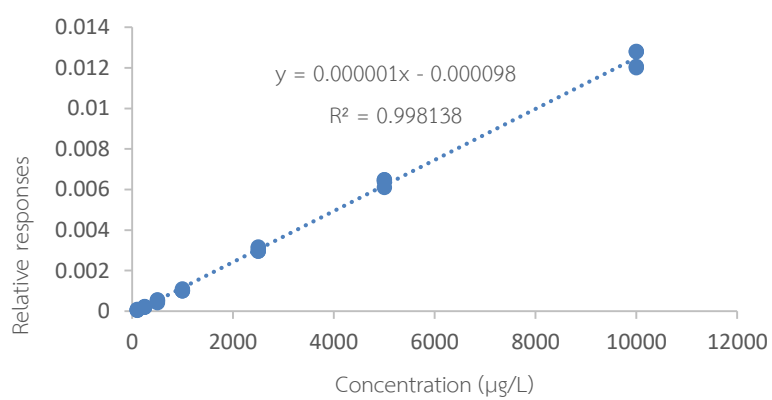- R² = 0.998138

Figure A.31   Standard calibration curve of valine

VITA

Miss Wanabud Wongbubpha was born on 22nd July 1991, in Rayong, Thailand. She graduated with a Bachelor's degree in chemistry, Faculty of Science, Srinakharinwirot University in 2014. After graduated, she continued her academic education with Master's degree in Department of Chemistry majoring in Analytical Chemistry, Faculty of Science, Chulalongkorn University and graduated with a Master's degree in Analytical Chemistry in October 2018.

Poster presentation and proceeding

- "Comparative quantification of non-volatile compounds in Tom Yum soup and Tom Yum pastes using high performance liquid chromatography-tandem mass spectrometry" at the 23rd International Symposium on Separation Sciences (ISSS 2017), Vienna, Austria.

- Wanabud Wongbubpha, Chadin Kulsing, Thumnoon Nhujak "Comparative determination of non-volatile compounds in Tom Yum paste using ultra-high performance liquid chromatography-tandem mass spectrometry." 2nd Nation Graduate Research Conference and Creative Innovation Competition, Empress Convention Center, Chiang Mai, Thailand, May 17-18, 2018, pp 972-984.

Publication

- P. Muangthai, W. Wongbubpha, R. Ouyporn. Analysis of total phenolic compound and inhibition power in extracted substance from Kai algae (Cladophoraspp). Asian j. basic appl. sci. (2015) 2:55-60.