

**DEVELOPMENT OF LIQUID CHROMATOGRAPHY-MASS
SPECTROMETRY FOR QUANTITATIVE DETERMINATION OF
PURINES AND URIC ACID IN THAI VEGETABLES**

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**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(PHARMACEUTICAL CHEMISTRY AND PHYTOCHEMISTRY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2015**

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ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor, Assist. Prof.Dr. ChutimaPhechkrajang for her invaluable help and constant encouragement throughout the course of this research. I am most grateful for her teaching and advice. I would not have achieved this far and this thesis would not have been not completed without all the support.

I would like to thank my co-advisor, Assist. Prof. Dr. PiyanuchRojsanga for her suggestions.

In addition, I would to thank the Bara scientific Co, Ltd. for supporting of the LC-MS/MS equipment and the training staffs.

Finally, I would like to express all my love and gratitude to my beloved parents for their support and encouragement.

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**DEVELOPMENT OF LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY
FOR QUANTITATIVE DETERMINATION OF PURINES AND URIC ACID IN
THAI VEGETABLES**

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ABSTRACT

This study was conducted to determine the amount of uric acid, and three of its precursors i.e., adenosine, guanosine and xanthine, in vegetables that were widely consumed in Thailand. An electrospray liquid chromatography triple quadrupole mass spectrometer (LC-MS/MS) was developed for this approach and caffeine was used as the internal standard. The separation was performed on a C18 column (10 cm × 2.1 mm, 2.7 μm). The mobile phase was a mixture of 0.2 % formic acid in deionized water (solvent A) and 0.1% formic acid in methanol (solvent B). Gradient elution was employed with a total run time of 8.50 minutes. The developed method was then fully validated according to USFDA guidelines. All method analytical performance characteristics were found to be acceptable. Therefore, the developed and validated LC-MS/MS method was applied to determine the amount of adenosine, guanosine, xanthine and uric acid in 18 selected Thai vegetables. The results showed that the contents of the four substances of interest in all vegetables examined in the study had less than 50 mg/100 g of fresh weight. At this concentration level, these vegetables could be classified as containing very low adenosine, guanosine, xanthine and uric acid contents. The results from this study promote valuable information for medical personal, especially hyperuricemia and gout patients, since the official report concerning uric acid and its purine precursors contents in Thai vegetables.

**KEY WORDS: PURINE / URIC ACID / LC-MS/MS TRIPLE QUADRUPOLE
MASS SPECTROMETRY**

121 pages

การพัฒนาวิธีวิเคราะห์ลิควิดโครมาโทกราฟีคู่ควบสเปกโตรเมตรีมวล เพื่อหาปริมาณพิวรีนและกรด
ยูริกในผักพื้นบ้านไทย

DEVELOPMENT OF LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY FOR
QUANTITATIVE DETERMINATION OF PURINES AND URIC ACID IN THAI
VEGETABLES

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บทคัดย่อ

การศึกษานี้จัดทำขึ้นเพื่อหาปริมาณของกรดยูริก และสารตั้งต้นของกรดยูริก 3 ชนิด คือ
อะดีโนซีน, กัวโนซีนและแซนทีน ในผักที่มีการบริโภคมากในประเทศไทย วิธีลิควิดโครมาโทกราฟีแบบอเล็ก
โตรสเปร์ย์ แมสสเปกโตรมิเตอร์ชนิดรีปเปลควอดรูโพล (LC-MS/MS) ได้ถูกพัฒนาขึ้นเพื่อวัตถุประสงค์นี้ โดย
ใช้คาเฟอีนเป็นสารมาตรฐานภายใน การแยกถูกทำบนคอลัมน์ C18 (เส้นผ่าศูนย์กลาง 2.1 มิลลิเมตร, ขนาด
พาร์ติเคิล 2.7 ไมโครเมตร) เฟสเคลื่อนที่เป็นส่วนผสมของกรดฟอร์มิก 0.2 % ในน้ำปราศจากไอออน (ตัวทำละลาย
A) และกรดฟอร์มิก 0.1 % ในเมทานอล (ตัวทำละลาย B) การจะเป็นแบบ เกรเดียนท์ ใช้เวลาในการแยกทั้งหมด
8.50 นาที วิธีที่พัฒนาแล้วถูกนำมาตรวจสอบความใช้ได้ของวิธีโดยใช้แนวทางของ US-FDA พบว่าคุณลักษณะของการ
วิเคราะห์ทั้งหมดยอมรับได้ ดังนั้นวิธีการ LC-MS/MS นำมาประยุกต์ เพื่อหาปริมาณของอะดีโนซีน กัวโนซีน
แซนทีน และกรดยูริกในผักของไทย ที่เลือกมา 18 ชนิด ผลการตรวจแสดงให้เห็นว่าปริมาณสารที่สนใจทั้ง 4
ในผักทุกชนิดที่นำมาศึกษามีค่าน้อยกว่า 50 มิลลิกรัม/100 กรัม น้ำหนักสด ด้วยระดับความเข้มข้นนี้ทำให้สามารถ
จัดผักเหล่านี้ในกลุ่มอาหารที่มี อะดีโนซีน กัวโนซีน แซนทีน และกรดยูริกต่ำมาก ผลการศึกษานี้ได้ข้อมูลที่มี
คุณค่าสำหรับบุคลากรทางการแพทย์โดยเฉพาะผู้ป่วยมีภาวะกรดยูริกสูง และผู้ป่วยเก๊าต์ และเนื่องจากยังไม่มีกร
รายงานปริมาณของกรดยูริกและสารตั้งต้นกลุ่มพิวรีนในผักของไทยนี้มาก่อน

121 หน้า

CONTENTS

	Page
ACKNOWLEDGEMENTS	iii
ABSTRACT (ENGLISH)	iv
ABSTRACT (THAI)	v
LIST OF TABLES	x
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xv
CHAPTER I INTRODUCTION	1
CHAPTER II OBJECTIVES	3
CHAPTER III LITERATURE REVIEW	
3.1 Purines metabolism	4
3.2 Hyperuricemia and gout disease	6
3.3 Analysis of purines and uric acid	7
3.4 Liquid chromatography-mass spectrometry method (LC-MS/MS)	9
3.4.1 General principle of LC-MS/MS	9
3.4.2 Ionization source	10
3.4.3 Mass spectrometry (mass analyzer)	12
3.4.4 Matrix effect in LC-MS/MS	16
3.5 Purine contents in vegetables	17
CHAPTER IV MATERIALS AND METHODS	
4.1 Materials	21
4.1.1 Chemicals and reagents	21
4.1.2 Instruments and equipment	22
4.1.3 Computer hardware and software	22
4.2 High performance liquid chromatographic method (HPLC)	23

CONTENTS (cont.)

	Page
4.2.1 Preparation of stock standard solutions	23
4.2.2 Preparation of ammonium acetate buffer	23
4.2.3 HPLC-UV method development	23
4.3 Liquid chromatography ion-trap mass spectrometry (LC-MS/MS Ion-trap mass spectrometry)	24
4.3.1 Method development	24
4.4 Liquid chromatography triple quadrupoles-mass spectrometry (LC-MS/MS) method	25
4.4.1 Method development	26
4.4.1.1 Preparation of 10 mM ammonium hydroxide	26
4.4.1.2 Preparation of stock standard solutions	26
4.4.1.3 Preparation of standard mixture solutions	26
4.4.1.4 Preparation of stock internal standard solution	26
4.4.1.5 Preparation of blank sample (onion)	26
4.4.2 Development of ultrahigh performance liquid chromatography tandem mass spectrometry (LC-MS/MS) method for determination of purines and uric acid	27
4.4.3 Method validation of triple quadrupole LC-MS/MS method	28
4.4.3.1 Preparation of mixture standards (mixture standards 1)	28
4.4.3.2 Preparation of mixture standards spiked blank vegetable (mixture standard 2)	28

CONTENTS (cont.)

	Page
4.4.3.3 Preparation of mixture standards spiked filtrate blank vegetable (mixture standard 3)	29
4.4.3.4 Specificity	29
4.4.3.5 Linearity and lower limit of quantitation (LLOQ)	30
4.4.3.6 Recovery and matrix effect	30
4.4.3.7 Accuracy and precision	30
4.4.3.8 Stability	32
4.5 Determination of purines and uric acid in Thai vegetables	32
4.5.1 Plant materials	32
4.5.2 Construction of calibration curves	33
4.5.3 Sample preparation	34
CHAPTER V RESULTS AND DISCUSSION	
5.1 High performance liquid chromatography method (HPLC)	35
5.1.1 Method development	35
5.2 Liquid chromatography ion-trap mass spectrometry method (LC-MS/MS Ion-trap mass spectrometry)	37
5.2.1 Method development	37
5.3 Liquid chromatography triple quadrupole mass spectrometry method (LC-MS/MS)	44
5.3.1 Method development of ultra-high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) method for determination of purines and uric acid	44
5.4 Method validation of triple quadrupole LC-MS/MS method	52
5.4.1 Specificity	52

CONTENTS (cont.)

	Page
5.4.2 Linearity and lower limit of quantitation (LLOQ)	53
5.4.3 Recovery and matrix effect	55
5.4.4 Accuracy and precision	
5.4.5 Stability	62
5.5 Determination of purines and uric acid in Thai vegetables	62
CHAPTER VI CONCLUSIONS	65
REFERENCES	66
APPENDICES	
Appendix A HPLC Chromatograms of standard uric acid and purine precursors	71
Appendix B Method validation results of LC-MS/MS ion-trap mass spectrometry	81
Appendix C Stability study	85
Appendix D Matrix match calibration method from LC-MS/MS triple quadrupole mass spectrometry	89
Appendix E Chromatogram of Thai vegetables samples	91
Appendix F Raw data of vegetables samples from each source	109
Appendix G Presentation and publication	114
BIOGRAPHY	121

LIST OF TABLES

Title	Page
3.1 Operation modes of triple quadrupole mass spectrometers	15
3.2 Amount of purines in vegetables	17
3.3 Classification of purines group	18
3.4 Eighteen Thai vegetables in this study	18
4.1 Mobile phase condition for HPLC-UV method	24
4.2 Electrospray ionization (ESI) parameters (ion-trap mass spectrometry)	25
4.3 Gradient condition for LC-MS/MS (ion-trap mass spectrometry)	25
4.4 Gradient program (triple quadrupole mass spectrometry)	27
4.5 Series concentrations of standards mixture solution	29
4.6 Preparation of quality control sample (QCS) for accuracy and precision	31
4.7 Thai vegetables for determine uric acid and purines compound	33
4.8 Matrix match calibration curve for determine purines and uric acid in each sample	34
5.1 Retention time of standard solution uric acid and purine precursors From HPLC-UV method	36
5.2 Electrospray ionization (ESI) parameters (ion-trap mass spectrometry)	38
5.3 The retention times, transition ions and CID energy (ion-trap mass spectrometry)	38
5.4 Precursor ions, transition ions and collision energy for all compounds (triple quadrupole mass spectrometry)	45
5.5 Summary of specific transition ions of uric acid and purines (triple quadrupole mass spectrometry)	53
5.6 Calibration curve of uric acid and purine precursors by LC-MS/MS triple quadrupole mass spectrometer	53
5.7 Summary of matrix effect by LC-MS/MS triple quadrupole mass spectrometer	56

LIST OF TABLES (cont.)

Title	Page
5.8 Summaries of extraction recovery of uric acid and purines compound by LC-MS/MS triple quadrupole mass spectrometry	59
5.9 Accuracy and precision (repeatability) of uric acid by LC-MS/MS triple quadrupole mass spectrometry	59
5.10 Accuracy and precision (repeatability) of xanthine by LC-MS/MS triple quadrupole mass spectrometry	60
5.11 Accuracy and precision (repeatability) of adenosine by LC-MS/MS triple quadrupole mass spectrometry	60
5.12 Accuracy and precision (repeatability) of guanosine by LC-MS/MS triple quadrupole mass spectrometry	60
5.13 Intermediate precision of uric acid by LC-MS/MS triple quadrupole mass spectrometry	61
5.14 Intermediate precision of xanthine by LC-MS/MS triple quadrupole mass spectrometry	61
5.15 Intermediate precision of adenosine by LC-MS/MS triple quadrupole mass spectrometry	61
5.16 Intermediate precision of guanosine by LC-MS/MS triple quadrupole mass spectrometry	62
5.17 Uric acid and purine precursors contents in eighteen selected Thai vegetables	64

LIST OF FIGURES

Title	Page
3.1 Structure of purine and pyrimidine nucleoside	4
3.2 Purine metabolism and uric acid product	5
3.3 Metabolism of purines in human body	6
3.4 The elements of an LC-MS system	10
3.5 Principle of electrospray ionization (ESI)	11
3.6 Atmospheric pressure chemical ionization (APCI) source	12
3.7 Single quadrupole mass analyzer	13
3.8 Triple quadrupole mass analyzer	14
3.9 Ion-trap mass analyzer	16
5.1 Overlaid chromatograms of uric acid and purines compounds, mobile phase 10 mM ammonium acetate buffer pH 2.5 and methanol (97 : 3, v/v); flow rate 0.3 mL/min.	36
5.2 Chromatogram and fragmentation ion of uric acid; 167>>124	39
5.3 Chromatogram and fragmentation ion of xanthine; 151 >>108	40
5.4 Chromatogram and fragmentation ion of adenosine 268>>136	41
5.5 Chromatogram and fragmentation ion of guanosine 284>>152	42
5.6 Chromatogram and fragmentation ion of dextromethorphan 272>>215	43
5.7 LC-MS/MS chromatograms of standard uric acid and purines precursors fragment ions analyzed by mass spectrometry detector triple quadrupole	46
5.8 Transition ion of uric acid analyzed by LC-MS/MS triple quadrupole mass spectrometry (m/z = 166.90 [M-H] ⁻ , 166.90 >> 124.00 and 166.90 >> 69.00)	47
5.9 Transition ion of xanthine analyzed by LC-MS/MS triple quadrupole mass spectrometry (m/z = 151.00 [M-H] ⁻ , 151.107.90 >> 124.00 and 151.00 >> 79.90)	48

LIST OF FIGURES (cont.)

Title	Page
5.10 Transition ion of adenosine analyzed by LC-MS/MS triple quadrupole mass spectrometry ($m/z = 267.95 [M+H]^+$, $267.95 \gg 135.95$ and $267.95 \gg 118.98$)	49
5.11 Transition ion of guanosine analyzed by LC-MS/MS triple quadrupole mass spectrometry ($m/z = 284.00 [M+H]^+$, $284.00 \gg 152.10$ and $284.00 \gg 135.00$)	50
5.12 Transition ion of caffeine analyzed by LC-MS/MS triple quadrupole mass spectrometry ($m/z = 195.10 \gg 138.05 [M+H]^+$ and $195.10 \gg 110.05$)	51
5.13 LC-MS/MS chromatograms of uric acid from onion analyzed by mass spectrometry detector triple quadrupole	52
5.14 Relationship between concentration and area ratio of uric acid by LC-MS/MS triple quadrupole mass spectrometer	54
5.15 Relationship between concentration and area ratio of xanthine by LC-MS/MS triple quadrupole mass spectrometer	54
5.16 Relationship between concentration and area ratio of adenosine by LC-MS/MS triple quadrupole mass spectrometer	54
5.17 Relationship between concentration and area ratio of guanosine by LC-MS/MS triple quadrupole mass spectrometer	55
5.18 Matrix effect graph of xanthine by LC-MS/MS triple quadrupole mass spectrometer	56
5.19 Matrix effect graph of uric acid by LC-MS/MS triple quadrupole mass spectrometer	56
5.20 Matrix effect graph of adenosine by LC-MS/MS triple quadrupole mass spectrometer	57
5.21 Matrix effect graph of guanosine by LC-MS/MS triple quadrupole mass spectrometer	58

LIST OF FIGURES (cont.)

Title	Page
5.22 Graph extraction recovery of uric acid by LC-MS/MS triple quadrupole mass spectrometer	58
5.23 Graph extraction recovery of xanthine by LC-MS/MS triple quadrupole mass spectrometer	58
5.24 Graph extraction recovery of adenosine by LC-MS/MS triple quadrupole mass spectrometer	58
5.25 Graph extraction recovery of guanosine by LC-MS/MS triple quadrupole mass spectrometer	58

LIST OF ABBREVIATIONS

MSU	Monosodium urate crystals
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography couple mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
API	Atmospheric pressure ionization
ESI	Electrospray ionization
m/z	Mass per charge
M.W.	Molecular weight
MRM	Multiple reactions monitoring
SRM	Selected reaction monitoring
CE	Collision energy
CID	Collision induced discociation
IS	Internal standard
US-FDA	United States Food and Drug Administration
v/v	Volume/volume
µg	Microgram
ng	Nanagram
UV	Ultraviolet
°C	Degree Celsius
mM	Millimolar
mL	Milliliter
mg	Milligram
pH	Potential of hydrogen ion
LLOQ	Lower limit of quantification
RSD	Relative standard deviation
QCS	Quality control samples

LIST OF ABBREVIATIONS (cont.)

rpm	Round per minute
min	Minute
r	Correlation coefficient

CHAPTER I

INTRODUCTION

Daily meals is a key factor of good health, controlling of foodstuffs is necessary to control the symptoms of some diseases. For example, gout causes from increasing of serum uric acid in human body. High concentrations of uric acid in blood (hyperuricemia) causes deposition of urate crystals in joints and leading to inflame, red swollen of the joint and pain [1-3]. Although, wrong metabolism of purines pathway is the major cause of elevation of uric acid in blood, purines rich foods were found to have association with hyperuricemia as well.

Purines are the common name of purine bases, nucleosides and nucleotides [4]. In human body, purines can be metabolized to uric acid by using enzyme xanthine oxidase [5]. Various vegetables especially shoot part, have been reported for high purines containing. Such vegetables should be carefully consumed in gout patients. Amount of purines and uric acid have been reported for some vegetables but official report of uric acid and purine precursors contents in Thai vegetables were not available. By these reasons, this study was conducted to contribute valuable information for controlling gout symptoms. By quantitative analyzer of uric acid and its purine precursors contents in eighteen Thai vegetables including Thai eggplant (*Solanum melongena* L.), Chinese cabbage (*Brassica pekinensis*. *pekinensis* (Lour) Olsson.), cucumber (*Cucumis sativus* L.), yardlong bean (*Vigna unguiculata* subsp. *sesquipedalis* (L.) Verdc.), winged bean (*Psophocarpus tetragonolobus* Linn.), bean sprouts (*Vigna radiata* (L.)), bamboo shoot (*Bambusa* spp.), white popinac (*Leucaena leucocephala* (Lamk.) de Wit.), stink bean (*Parkia speciosa*), melientha suavis (*Melientha suavis* Pierre), acacia pennata (*Acacia pennata* (L.) Willd. subsp. *insuavis* (Lace) I.C. Nielsen), water mimosa (*Neptunia oleracea* Lour.), water convolvulus (*Ipomoea aquatica* Forsk. Var. *reptan*), ivy gourd (*Coccinia grandis* (L.) J.Voigt), Vietnamese coriander (*Polygonum odoratum* Lour.), asiatic pennywort (*Centella*

asiatica Urban), yellow velvet leaf (*Limnocharis flava* (L.) Buchenau)) and malabar nightshade (*Basella alba* L.) by LC-MS/MS method.

There were reports for quantitative determination methods of uric acid and purine precursors contents. Liquid chromatography mass spectrophotometry (LC-MS) was used to quantify purines and uric acid in Japanese soup, beer, Chinese cabbage, potato and beef [6-9]. High performance liquid chromatography (HPLC) was also used to determine purines and uric acid in meat, alcoholic beverage, vegetarian meat, soy bean, and mushroom [4, 6, 10-13]. Although HPLC is the basic instrument in general laboratories, specific and expensive columns were required for analysis of purines and uric acid. In this research, an electrospray triple quadrupole liquid chromatography tandem mass spectrometry (LC-MS/MS) was developed and validated for determination of purines and uric acid in vegetables. The method was developed for uric acid and three purines precursors, i.e. adenosine, guanosine and xanthine by using caffeine as internal standard. Onion was selected as blank sample since it was reported as low uric acid and purine precursors content food (less than 50 mg/ 100 g of fresh weight) [4].

CHAPTER II

OBJECTIVES

There were two objectives of this study.

1. To develop and validate a rapid, sensitive and reliable a LC-MS/MS method for quantitative determination of uric acid and purine precursors.
2. To quantitatively analyze of uric acid and purine precursors contents in 18 selected Thai vegetables by using the developed LC-MS/MS method

CHAPTER III

LITERATURE REVIEW

3.1 Purines metabolism

Purines are natural substances found in all body's cells and in almost all foods. After metabolism in human's body, uric acid will be produced as the end product of purine metabolic pathway. Purines are a common name of nucleotide, nucleoside and based which are made from nucleic acids (Figure 3.1) [4, 6-7, 10, 14].

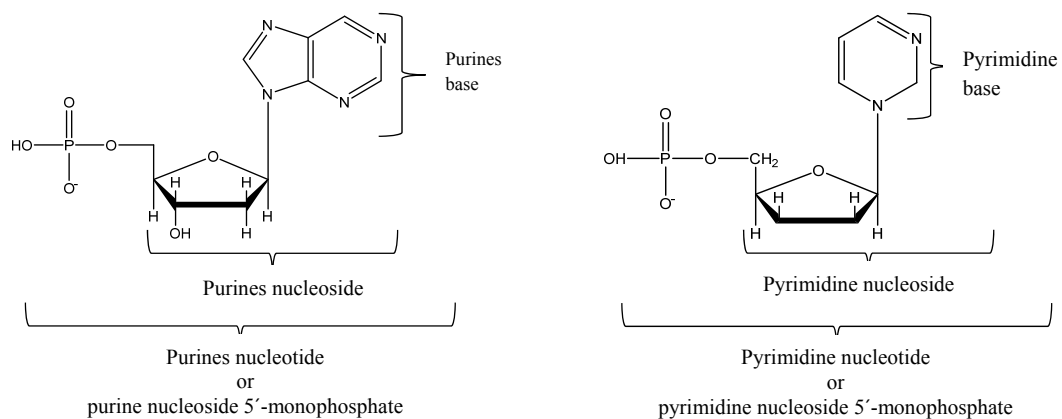


Figure 3.1 Structure of purine and pyrimidine nucleoside

Purine metabolism can be divided into two pathways, *de novo* synthesis and salvage pathway (Figure 3.2). The major pathway of purine metabolism in human body is *de novo* pathway as illustrated in figure 2. The purine salvage pathway uses purine bases i.e., guanine, hypoxanthine and adenine which produced by consumption of purines-rich foods [1, 4, 7-8, 11, 14-18]. Nucleotides are first converted to nucleosides by intracellular nucleotidases. Then, nucleosides are degraded by enzyme purine nucleoside phosphorylase (PNP) to release purines base and ribose. The PNP

products are fused into hypoxanthine and xanthine. Eventually, hypoxanthine was oxidized by xanthine oxidase to form xanthine and uric acid [2, 5, 14, 16, 19-20].

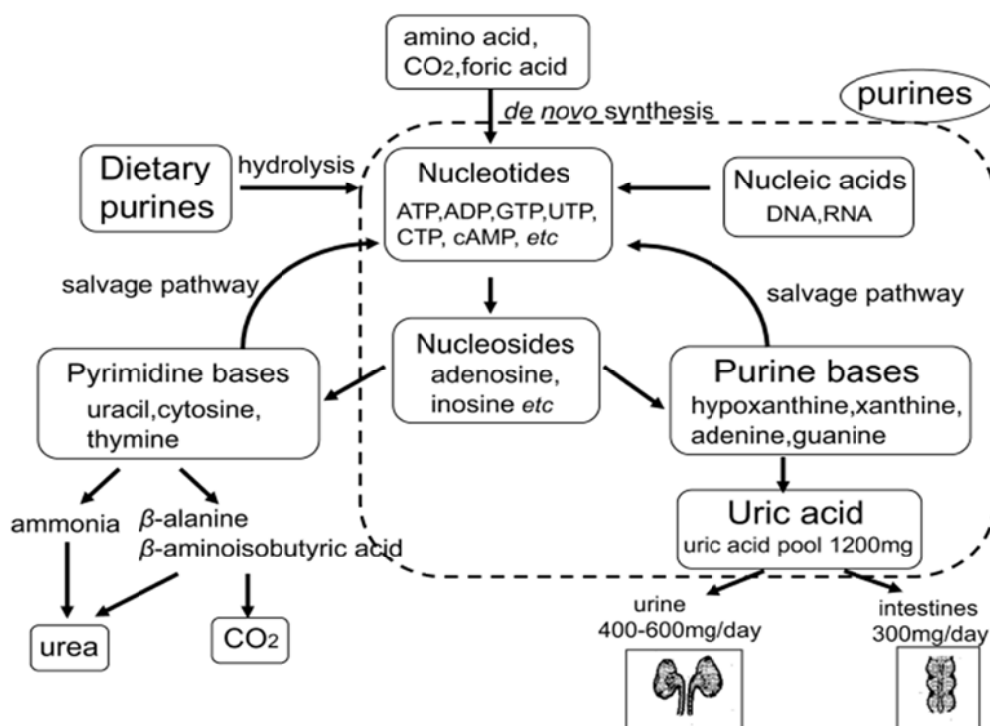


Figure 3.2 Purine metabolism and uric acid product

Normally, uric acid (2, 6, 8-trihydroxypurine) is presented in salt form of monosodium urate which can soluble in water. However, urate crystals might be formed in body if the concentration of urate exceeds the limit of solubility (7.0 mg/dL in plasma [1, 3, 15-16, 21-22]).

3.2 Hyperuricemia and gout disease

In human body, uric acid is the end product of purines metabolism as illustrated in figure 3.3. If the feature of purines metabolism syndrome is occurred, the serum uric acid level is increased and caused hyperuricemia. Hyperuricemia is demonstrated by the level of serum urea more than 0.45 mmol/L or 7.0 mg/dL in men and 0.36 mmol/L or 6.0 mg/dL in women. The case of hyperuricemia may asymptomatic but the major risk factor of gout is higher serum uric acid level for a long time and develops to gout arthritis [1-4, 9, 15-16, 19-22].

Gout is the most common case of inflammatory disease in human. Hyperuricemia will lead to the occurring of monosodium urate crystals (MSU) in around joints space and soft tissues. The symptoms of gout patients are pain and swelling around joints [1-3, 5, 12, 16, 18-19, 21-22]. The persistent of MSU crystals in joints may bring to damage of the joint structures [1-3, 12, 16, 19, 21-22].

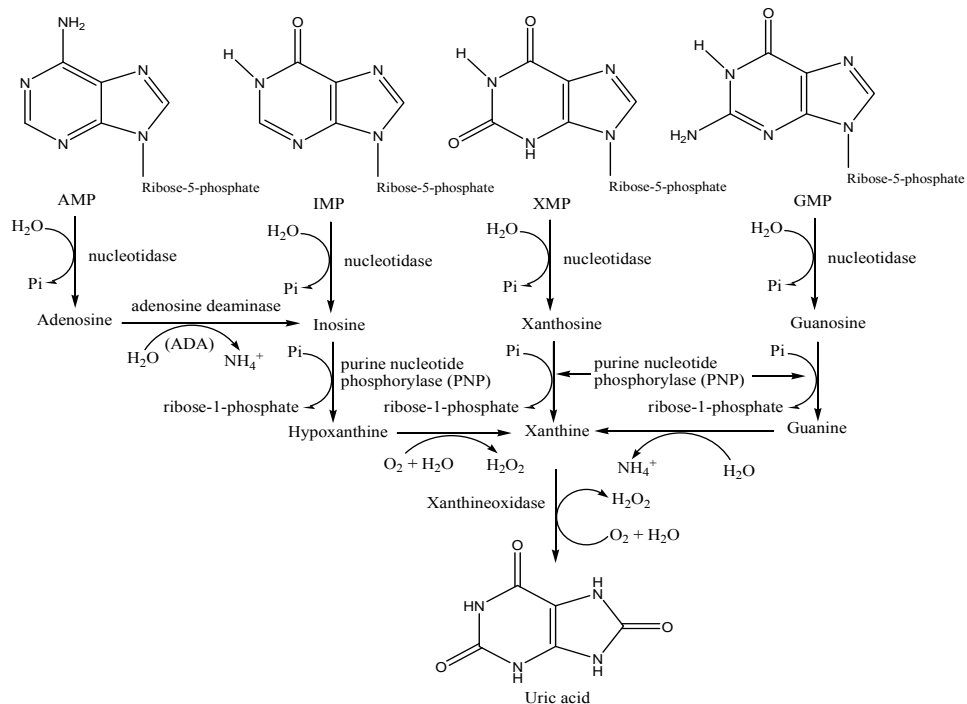


Figure 3.3 Metabolism of purines in human body

3.3 Analysis of purines and uric acid

Various determination methods have been reported for determination of uric acid, nucleotide, nucleoside and purines in several samples.

Kaneko K., et.al, [13] used high performance liquid chromatography (HPLC) with UV detector to analyze purines (adenine, guanine, hypoxanthine and xanthine) contents in alcoholic beverages intake in Japan. HPLC separation was carried out on polyvinyl alcohol Shodex Asahi Pak HQ-310 (5 mm × 300 mm) and column oven was maintained at 35°C. The mobile phase consisted of 150 mM sodium phosphate buffer (pH 2.5) and flow rate was 0.6 mL/min. UV detector was set at 260 nm.

Havlik J., et.al., [10] analyzed purines base (uric acid, adenine, guanine, hypoxanthine and xanthine) in vegetarian meat analogues by HPLC-UV. The separation was performed on Phenyl Gemini C18 column (4.6 mm × 250 mm, 5 μm) and column temperature was set at 35°C. Isocratic elution was employed by using 50 mM potassium dihydrogenphosphate buffer (pH 4.0) as mobile phase with flow rate of 0.8 mL/min. The presence of purine bases was monitored by UV detector the wavelength of 250, 260 and 285 nm for guanine and hypoxanthine, xanthine and adenine and uric acid respectively.

Kaneko K., et. al., [4] summarize the total purines in 270 various foodstuffs to facilitate the nutritional therapy for gout and hyperuricemia by HPLC-UV. Amount contents of adenine, guanine, hypoxanthine, xanthine and uric acid in there foodstuffs were performed. HPLC method was employed on polyvinyl alcohol Shodex Asahipak GS-320HQ (7.6 mm × 300 mm) column and column temperature was set at 35°C. The mobile phase was 150 mM sodium phosphate buffer (pH 2.5-2.8) and UV detector was set at the wavelength of 260 nm.

Kaneko K., et. al., [11] analyzed purine contents of soybean-derived foods, vegetables and mushrooms in Japanese foods by HPLC. Purines including adenine, guanine, hypoxanthine and xanthine were determined. HPLC condition was used as follow. Column was polyvinyl alcohol Shodex Asahi Pak HQ-310 (5 mm × 300 mm). Mobile phase was 150 mM sodium phosphate buffer (pH 2.5) and column temperature was set at 35°C. Detector wavelength was set at 260 nm.

Rong S., et.al., [12] determined purine contents in different parts of pork and beef by using HPLC method. Purine analytes composed of adenine, hypoxanthine, guanine and xanthine. Chromatographic separation was performed on reversed phase C18 Waters Atlantis T3 column (4.6 mm×250 mm, 5 µm) and column temperature was at 30°C. The mobile phase was contained 10 mM ammonium formate (pH 3.6) and methanol (99 : 1, v/v) and UV detection wavelength was 254 nm.

Yamaoka N., et.al., [4] developed method for analysis of 7 nucleosides (cytidine, uridine, inosine, guanosine, thymidine, adenosine and xanthosine) and 13 nucleotides (CMP, UMP, GMP, AMP, CDP, UDP, IDP, GDP, ADP, GTP, UTP, ATP and TTP) in Japanese soup, cauliflower and low-malt beer. The analysis was performed by using ion-pairing liquid chromatography with electrospray ionization (ESI) and a quadrupole mass detection system. The reverse phase C18 ACQUITY UPLC™ Column HSS T3 (2.1 mm×100 mm, 1.8 µm) was used. Mobile phase consisted of 1.25 mM dihexylammonium acetate (DHAA) in ammonium acetate pH 5.0 (solvent A) and 1.25 mM dihexylammonium acetate in acetonitrile (solvent B). Gradient elution was employed. The column temperature was used at 40°C.

Clariana M., et. al., [8] analyzed purines and pyrimidines (inosine, guanosine, adenosine, hypoxanthine, xanthine and uric acid) in pork meat products (dry-cured ham, cooked ham and raw meat pork). Chromatographic condition was LC system equipped with diode array detector and coupled to triple quadrupole mass spectrometer. A reverse phase C18 HSS T3 column (1.0 mm ×150 mm, 1.8 µm) was utilized and column temperature was set at 35°C. Elution was performed with 0.1% formic acid in deionized water and acetonitrile.

Zhao J., et. al., [9] assayed purines (uric acid, xanthine, hypoxanthine, adenine, adenosine, inosine, guanosine, 2'-deoxyinosine, 2'-deoxyguanosine, 2'-deoxyadenosine) in blood of gout patients. Liquid chromatograph with a mass spectrometric detector ion trap was used. The separation was performed on C18 Supelcosil™ LC-18DB column (25 cm × 4.6 mm, 5 µm) and column temperature was maintained at 25°C. The mobile phase was consisted of 10 mM ammonium acetate (pH 6.5) and methanol.

Yamaoka N., et. al., [6] analyzed 4 nucleosides and 9 nucleotides and compared their concentrations in raw and heated cauliflower. The study was performed by using LC-MS and HPLC methods. The LC-MS analysis was used for determination of each nucleosides and nucleotides. The determination was performed on reverse phase C18 HSS T3 (2.1 mm × 100 mm) column and column temperature was set at 10°C. The mobile phase was 1.25 mM dihexylammonium acetate in 10 mM ammonium acetate (pH 5.0) and acetonitrile. On the other hand, the HPLC method was also performed for quantification determination of total purines. The separation was performed on polyvinyl alcohol Shodex Asahi Pak HQ-310 (7.5 mm × 300 mm) column. The column temperature was 35°C. A 150 mM sodium phosphate buffer, pH 2.5 was used as mobile phase and UV detector was set at 260 nm.

3.4 Liquid chromatography – mass spectrometry method (LC-MS/MS)

3.4.1 General principle of LC-MS/MS

The combination of liquid chromatography with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) therefore allows more definitive identification and the quantitative determination of compounds that not are fully resolved chromatographically [23].

General liquid chromatography with UV detector, identification is carried out on the basic of the chromatographic retention characteristic. This is not sufficient to allow unequivocal identification because of the possibility of more than one analyte having virtually identical retentions. This limitation can overcome by combination of liquid chromatography with mass spectrometry (LC-MS). The most advantage of mass spectrometric detector or mass analyzer is that it can provide the absolute identification. It provides not only structural information from the molecule under investigation but also it may provide the molecular weight of the analyte. This is the powerful discrimination tool and can dramatically reduce the number of possible structures for the analyte [23]. As illustrate in figure 3.4, an LC-MS system included

the autosampler, LC system, ionization source (which interfaces the LC to MS), mass analyzer and the controlled computer.

The eluate from LC system is transferred to ionization source where molecules of interest analyte are ionized to positive or negative charge. The ions then travel through the mass analyzer and arrive at different parts of detector or different traveling times according to their mass/charge (m/z) ratio [23]. LC-MS/MS have been utilized in many area i.e., environmental, chemistry, medicine, pharmaceuticals, natural products and biochemistry for trace quantitative analysis and characterization of analyte [24].

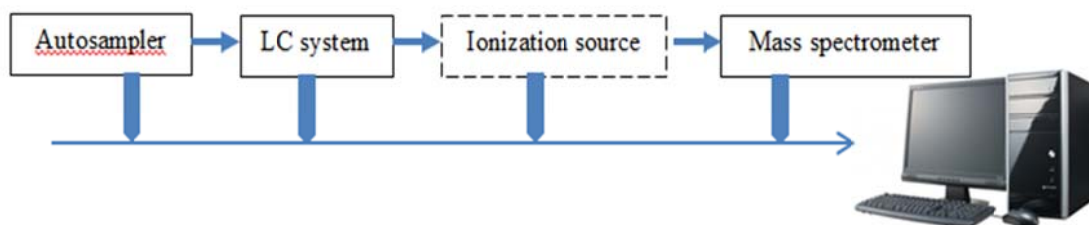


Figure 3.4 The elements of an LC-MS system

3.4.2 Ionization source

Ionization source is necessary to use as interface connection between LC and MS, because of two reasons. The first is that HPLC mobile phase is liquid, often containing a significant portion of water, which is pumped through the stationary phase at the flow rate of typically 1 mL/min. While, the mass spectrometer is normally operated at a pressure of around 10^{-6} torr. It is not possible simple to pump the eluate from HPLC column directly into the source of a mass spectrometer. Therefore, it is the function of interface to remove a significant portion of mobile solvents. The second is that the majority of analytes that are likely to be separated by HPLC are relatively involatile and stay in liquid phase. Unfortunately, any mass spectrometer allows only charge analytes in gas phase that can travel to mass detector. Therefore, it is also the function of ionization source to change neutral analyte in liquid phase to charge analyte in gas phase [23].

There are various types of ionization sources that can be used as the interface between LC system and the mass spectrometer. But the two most common sources are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). For both ESI and APCI, the ionization occurs at atmospheric pressure, so these sources are often referred to as atmospheric pressure ionization (API) [25].

Electrospray ionization used electrical energy to assist the transfer of ions from solution into gaseous phase before they are subjected to mass spectrometric analysis. A solution of analyte is passed through a capillary which is applied high voltage (typically 3-5 kV) and nebulized at the tip of capillary to form a fine spray of charged droplets [24-25]. With the aid of an elevated ESI-source temperature and/or another stream of nitrogen drying gas, the charged droplets are continuously reduced in size by evaporation of solvent, leading to an increase of surface charge density and a decrease of the droplet radius or “Coulomb explosion” as illustrate in figure 3.5 [24-28]. The emitted ions are samples by a sampling skimmer cone and then accelerated into the mass analyzer for subsequent analysis of molecular mass and measurement of ion intensity [24].

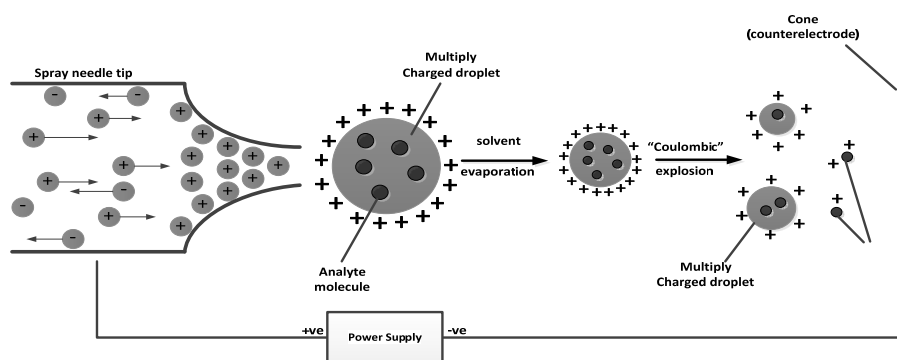


Figure 3.5 Principle of electrospray ionization (ESI)

Small molecules ($\approx < 500$ Da) with a single functional group capable of carrying charge give predominantly singly charged ions. This can involve the addition of a proton to analyte $(M+H)^+$ when ion source is operated in positive ion mode or loss of proton $(M-H)^-$ when operated in negative ion mode. Moreover, adduct of cations

(e.g. $M+NH_4^+$, $M+Na^+$ and $M+K^+$) and anions (e.g. $M + \text{formate}^-$, $M + \text{acetate}^-$) can occur when used as mobile phase buffer [26].

In atmospheric pressure chemical ionization (APCI), as with ESI, liquid is pumped through a capillary and nebulized at the tip. A corona discharge takes place near the tip of the capillary, initially ionizing gas and solvent molecules present in the ion source. These ions then react with the analyte and ionize it via charge transfer. The technique is useful for small, thermally stable molecules that are not well ionized by ESI (Figure 3.6) [26].

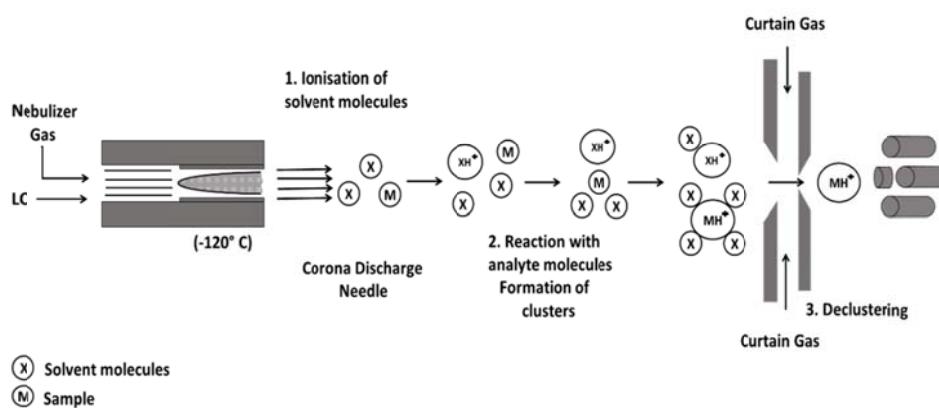


Figure 3.6 Atmospheric pressure chemical ionization (APCI) source

3.4.3 Mass spectrometry (mass analyzers)

There are many types of mass spectrometers available for interface with LC. Nowadays, the most widely used are quadrupoles and ion-trap mass analyzers.

Quadrupole is the most widely used more than other mass analyzers due to its high sensitive and specificity. Quadrupole mass analyzer consists of four parallel metal rods (Figure 3.7), the positive and negative rods are positioned 180° out of phase. Positive mass ions are attracted to negative rods while the converse is observed for negative mass ions. They have fixed direct current (DC) and varying radiofrequency (RF) potentials applied to them [23-24, 26]. Ions (usually called the precursor ion) produced in the source are focused and transferred through the middle of quadrupole mass filter. Certain ions with a distinct ratio of m/z will have stable trajectories and pass through the quadrupole and reach the detector. While all other

molecules are forced outside the quadrupole and will not reach to detector [24, 29]. Single quadrupole can be operated in scan range of m/z values, resulting in a mass spectrum. As an alternative to scanning, the quadrupole can be set to monitor specific m/z value (single monitoring mode). The single quadrupole has limitation for specific detection of target analytes [26].

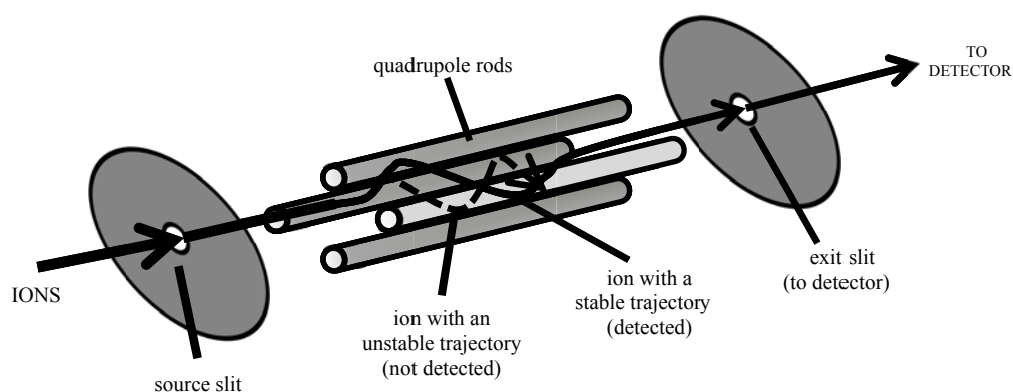


Figure 3.7 Single quadrupole mass analyzer

Quadrupoles can also be placed in tandem to perform fragmentation and alternative analysis modes. The most common set-up is the triple quadrupole (QqQ) mass spectrometer [23-24, 29].

Triple quadrupoles, composed of three sets of quadrupole arranged linearly (Figure 3.8). The first (Q1) and the third (Q3) quadrupole can be operated into two modes, scan mode and multiple reactions monitoring (MRM) mode or selected reaction monitoring (SRM) mode. The second quadrupole (Q2) is not used as a mass separation device but serves as a collision cell induced for fragmentation of the original precursor ion by applying collision energy as inert gas (helium or argon) [23-24, 26].

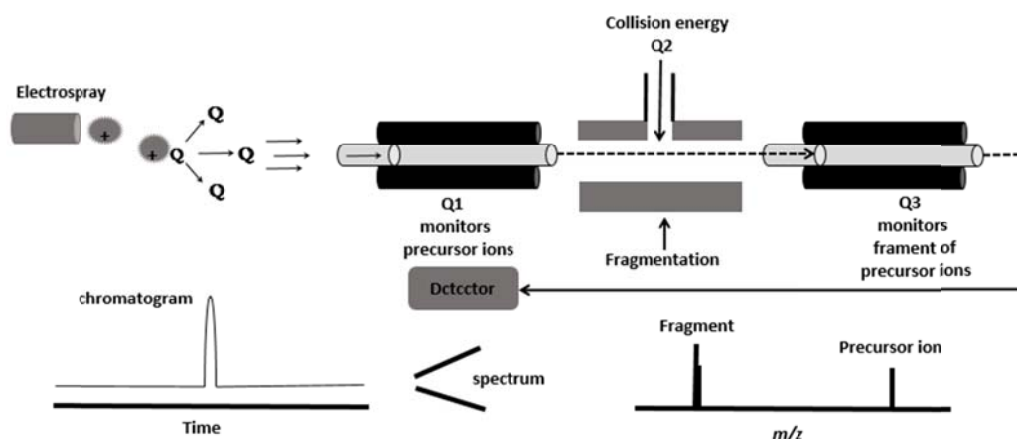


Figure 3.8 Triple quadrupole mass analyzer

Triple quadrupole is an extremely powerful analytical system that can utilize in several analytical modes as presented in Table 3.1. For product scan mode, Q1 will be fixed and only ion of interest can pass through Q2 for fragmentation. Then, product ion will be scanned in Q3. The opposite event is occurred in precursor scan mode. All mass ranges will be scanned in Q1 and passed to Q2. Then, product ion with distinct m/z will be fixed in Q3, only selected product ion can pass through Q3 and reaches to detector. However, this mode could decrease sensitivity of analyte of interest because many ions can pass through Q1 and Q2. Sensitivity of analytes can be increased in multiple reaction monitoring (MRM) mode. This mode is used for analysis of many analytes as the same time. Ions of interest will be fixed in Q1 and passed to Q2 for fragmentation. Then, all of selected fragment ions can pass through Q3 and eventually to mass analyzer. Neutral loss, both Q1 and Q3 scan together at a constant difference in m/z ratio. This is used to monitor the loss of a neutral fragment for a class of molecules from collision-induced dissociation or CID. For example, there is a neutral loss of 102 from most of the butylated amino acids [24].

The advantage of tandem MS is the greatly increased specificity of the analysis over single stage mass analysis. For example, 25-hydroxy vitamin D3 produces a major $M+H^+$ ion of 401 m/z during electrospray ionization and loses water during collision induced dissociation to produce a major 383 m/z product ion. Highly specific detection of 25-hydroxide vitamin D3 is achieved if the first and third

quadrupoles are set to transmit ions of only 401 and 383 m/z respectively. This technique is termed single reaction monitoring and fragmentation is denoted 401>383 [26].

Table 3.1 Operation modes of triple quadrupole mass spectrometers [26]

Name	First quadrupole	Third quadrupole	Application
Product scan	Fixed	Scanned	Structural studies, identification of unknowns, confirmation by spectral matching with standard
Precursor scan	Scanned	Fixed	Detection of structurally related analytes that produce a common fragment ion
Neutral loss (or gain) scan	Scanned	Scanned with fixed offset relative to first quadrupole	Detection of structurally related analytes that produce a common fragment ion
Multiple reaction monitoring	Fixed	Fixed	High sensitivity detection of a panel of targeted analytes

Ion trap mass analyzer (which normally quadrupole ion-trap), quadrupole ion-trap consists of three hyperbolic electrodes, ring electrode, the entrance end cap electrode and the exit end cap electrode (Figure 3.9). Inside the electrodes, it looks like a cavity where ions will be trapped or stored and analyzed.

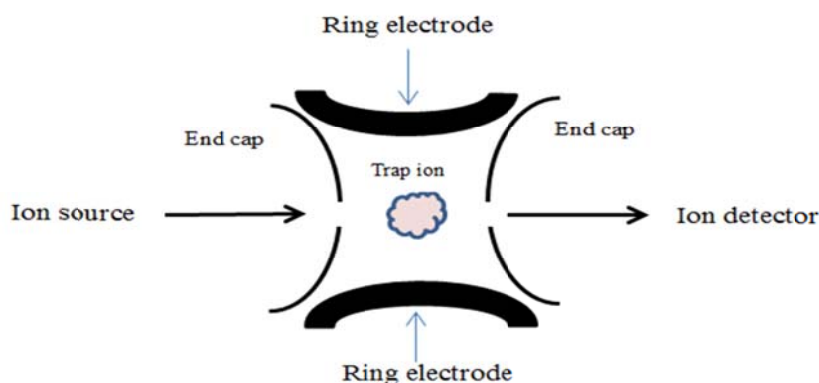


Figure 3.9 Ion-trap mass analyzer

The ions will be produced in the ionization source and then, enter the trap through the entrance end cap electrode. The ions are trapped in a cavity of the three hyperbolic electrodes. The RF potential and AC potential of constant frequency will be applied to the ring electrode to produce a 3-dimensional potential field within the trap cavity. For detection of the ions, the potentials are altered to destabilize the ion motions, resulting in ejection of the ions through the exit end cap and focussed onto the detector of the MS analyzer. When precursor ion is selected inside the trap and applied the inert gas to produce fragment ion, collision induced dissociation (CID) fragment ion will travel through mass detection.

Alternatively, the product ions can be kept inside the trap and another CID reaction can be initiated and this repeat CID reaction can continue for several times (denoted as MS^n in which n is the number of CID reactions). This can help differentiate molecules with similar structures. However, ion trap analyzers cannot provide precursor scan and neutral loss modes of data acquisition [24].

3.4.4 Matrix effect in LC-MS/MS

Matrix effect in LC-MS must be evaluated because it can experiment after analyte signal. Matrix effect will be occurred when molecules coeluting with the compound of interest after the ionization efficiency of the electrospray interface. Since the matrix effects are the results of competition between nonvolatile matrix components and analyte ions for access to the droplet surface for transferity to the gas phase. This competition may effectively decrease (also known as ions suppression) or increase (ions enhancement) the efficiency of formation of the desired analyte ions

present at the same concentrations in the interface [30-32]. There are two approaches to remove or minimize matrix effects, one is modification the sample extraction methodology, another one is improving of chromatographic separation [30].

3.5 Purine contents in vegetables

There were reports of the quantity of purines (adenine, guanine, hypoxanthine, xanthine and total amounts) in some vegetables. From the information in Table 3.2, it could be seen that total amounts of purines in example vegetables ranged from 2.3-63.3 mg/100 g of fresh weight. By using the criteria of Kaneko K. et.al. [4] (Table 3.3), these vegetables can be classified as group 1 for those contents were less than 50 mg/100 g and group 2 for those contents were between 50 and 100 mg/100 g of fresh weight.

Table 3.2 Amount of purines in vegetables [4]

Foodstuffs	Part	Adenine (mg/100 g)	Guanine (mg/100 g)	Hypoxanthine (mg/100 g)	Xanthine (mg/100 g)	Total (mg/100 g)
Bamboo shoot	Upper part	24.8	35.2	2.8	0.6	63.3
Bamboo shoot	Lower part	12.8	17.0	0.7	0.4	30.8
Bean sprouts		14.1	14.2	3.2	3.5	35.0
Cabbage		1.3	1.7	0.2	0.0	3.2
Chinese cabbage		2.6	2.9	1.2	0.3	7.0
Cucumber		4.2	5.0	0.1	0.1	9.4
Eggplant		11.0	31.6	6.6	1.6	50.7
Onion		1.0	1.1	0.1	0.0	2.3

Table 3.3 Classification of purines group [4]

Group	Purine contents group	Classified
1	Very low purines group	< 50 mg/ 100 g
2	Low group	50-100 mg/ 100 g
3	Moderate group	100-200 mg/ 100 g
4	High group	200-300 mg/ 100 g
5	Very high group	> 300 mg/ 100 g

In this study, eighteen widely consumed vegetables that in Thailand were examined for their uric acid and purine contents. The eighteen vegetables in this study were presented in Tables 3.4.

Table 3.4 Eighteen Thai vegetables in this study





No.	Vegetables samples	Common name	Scientific name	Thai name	Part use
1		Thai eggplant	<i>Solanum melongena</i> L.	Makeaoplor	Fruits
2		Chinese cabbage	<i>Brassica pekinensis</i> . Pekinesis (Lour) Olsson.	Pakgardkaw	Whole plant
3		Cucumber	<i>Cucumis sativus</i> L.	Tang-kwa	Fruits
4		Yardlong bean	<i>Vigna unguiculata</i> subsp. <i>sesquipedalis</i> (L.) Verdc.	Tuafukyaw	Whole plant

Table 3.4 Eighteen Thai vegetables in this study (cont.)















No.	Vegetables samples	Common name	Scientific name	Thai name	Part use
5		Winged bean	<i>Psophocarpus tetragonolobus</i> Linn.	Tuapoo	Whole plant
6		Bean sprouts	<i>Vigna radiata</i> (L.)	Tuangok	Whole plant
7		Bamboo shoot	<i>Bambusa</i> spp.	Nor-mai	Shoots
8		White popinac	<i>Leucaena leucocephala</i> (Lamk.) de Wit	Yodkratin	Shoots
9		Stink Bean	<i>Parkia speciosa</i>	Sator	Fruits
10		Melientha suavis	<i>Melientha suavis</i> Pierre	Pakwanpa	Shoots
11		Acacia pennata	<i>Acacia pennata</i> (L.) Willd. subsp. insuavis (Lace) I.C. Nielsen	Chaom	Shoots
12		Water minosa	<i>Neptunia oleracea</i> Lour.	Pakkrached	Shoots
13		Water Convolvulus	<i>Ipomoea aquatica</i> Forsk. Var. reptan	Pakbung	Shoots

Table 3.4 Eighteen Thai vegetables in this study (cont.)

No.	Vegetables samples	Common name	Scientific name	Thai name	Part use
14		Ivy Gourd	<i>Coccinia grandis</i> (L.) J.Voigt	Tumlung	Shoots
15		Vietnamese coriander	<i>Polygonum odoratum</i> Lour.	Pakpeaw	Leaves
16		Asiatic Pennywort	<i>Centella asiatica</i> Urban	Buabok	Leaves
17		Yellow velvet leaf	<i>Limnocharis flava</i> (L.) Buchenau)	Pakkangong	Shoots
18		Malabar nightshade	<i>Basella alba</i> L.	Pakprung	Leaves

CHAPTER IV

MATERIALS AND METHODS

All materials and procedures used for development and validation of liquid chromatographic methods for determination of eighteen Thai vegetables were presented in this chapter. Chemicals, reagents and instruments were also described.

4.1 Materials

4.1.1 Chemicals and reagents

Name	Grade	Source/Supplier
Acetonitrile	HPLC	Burdick and Jackson, Korea
Ammonium hydroxide	Analytical	Sigma-Aldrich, USA
Ammonium acetate	Analytical	Sigma-Aldrich, USA
Adenosine	Analytical	Sigma-Aldrich, China
Caffeine	Analytical	Sigma-Aldrich,
Deionized water	Sterile water for injection	V&VBangkok, Co. Ltd., Thailand
Dextrometophan HBr	Analytical	Sigma-Aldrich, USA
Formic acid	Analytical	Sigma-Aldrich, Germany
Guanosine	Analytical	Sigma-Aldrich, Slovakia
Hypoxanthine	Analytical	Sigma-Aldrich, China
Methanol	HPLC	Burdick and Jackson, Korea
Uric acid	Analytical	Sigma-Aldrich, Hungary
Xanthine	Analytical	Sigma-Aldrich, USA

4.1.2 Instruments and equipment

Name	Source/Supplier
Analytical balance	Shimadzu, Japan
High performance liquid chromatography (LC-10AD)	Shimadzu, Japan
HPLC column (Chromolith [®] C18, 4.6×100 mm)	Merck, Germany
HPLC column (Symmetry [®] C18, 4.6×150 mm, 5µm)	
HPLC column (Ascentis [®] Express C18, 2.1×100 mm, i.d. 2.7 µm)	Sigma-Aldrich, USA
HPLC guard column (Ascentis [®] Express C18, 0.5 × 2.1 mm, i.d. 2.7 µm)	Sigma-Aldrich, USA
HPLC guard cartridge holder (Ascentis [®] Express)	Sigma-Aldrich, USA
LCQ Fleet Ion trap LC/MS	Thermo Scientific, USA
Mass spectrometry (LC-MS 8030)	Shimadzu, Japan
Micropipette	Trefflab, Germany
Nylon membrane filter (47 mm, 0.2 µm)	Filtrex, Thailand
Nylon syringe filter (13 mm, 0.22 µm)	National scientific, USA
Sorvall RC5 plus super speed centrifuge	N.Y.R. Co., Ltd., USA
Ultra high performance liquid chromatography (LC-30AD)	Shimadzu, Japan
Ultrasonic bath	D.S.C. group, Thailand
Vacuum grade filtration set	Schott Duran, USA
Vortex mixer (VTX-3000L)	Uzusio, Japan
Electronic blender	WARING, USA

4.1.3 Computer hardware and software

Name	Supplier
Class-LC10 version 1.60	Shimadzu Corporation, Japan
LCQ Fleet MS 2.5.0	Thermo LCQ Fleet
Xcalibur 2.0.7 SP1	Thermo LCQ Fleet
Lab solution version 5.72	Shimadzu Corporation, Japan

4.2 High performance liquid chromatographic method (HPLC)

High performance liquid chromatography model LC-10AD (Shimadzu, Japan) with UV detector model SPD-10A (Shimadzu, Japan) was used to develop a separation method for adenosine, guanosine, hypoxanthine, xanthine and uric acid. Types and concentration of organic solvents were studied. Types of mobile phase buffer solution and appropriate pH condition were also evaluated. UV detector was set at 284 nm through studies.

4.2.1 Preparation of stock standard solutions

Stock standard solutions of 100 µg/mL for adenosine, guanosine, xanthine, hypoxanthine and uric acid were prepared by dissolving amount of powder in 10 mM ammonium hydroxide to achieve the required concentration.

4.2.2 Preparation of ammonium acetate buffer

Ten millimolars ammonium acetate was prepared by dissolving 770.80 mg of solid in deionized water 1000 mL. The pH of buffer solution was adjusted with glacial acetic acid to achieve the required pH buffer.

4.2.3 HPLC-UV method development

HPLC method was performed on Chromolith[®]C18 column (4.6× 100 mm). The mobile phase was the mixture of 10 mM ammonium acetate buffer (solvent A) and organic solvent (solvent B), acetonitrile or methanol. The pH of buffer was adjusted to 4.5 and 2.5. Organic solvent ratios were studied between 3% - 40% (v/v). The developed mobile phase conditions were illustrated in Table 4.1.

Table 4.1 Mobile phase condition for HPLC-UV method

Mobile phase		Ratio
Solvent A (buffer solution)	Solvent B (organic solvent)	Solvent A : Solvent B
10 mM ammonium acetate buffer, pH 4.5	Acetonitrile	95:5
10 mM ammonium acetate buffer, pH 4.5	Acetonitrile	60:40
10 mM ammonium acetate buffer, pH 2.5	Acetonitrile	95:5
10 mM ammonium acetate buffer, pH 2.5	Methanol	95:5
10 mM ammonium acetate buffer, pH 2.5	Methanol	97:3

4.3 Liquid chromatography ion-trap mass spectrometry (LC-MS/MS Ion-trap mass spectrometry)

Ion-trap mass spectrometry (Thermo LCQ Fleet) with electrospray ionization (ESI) was optimized for determination of purines and uric acid. Tuning of each standard solution (adenosine, guanosine, hypoxanthine, xanthine and uric acid) was performed. Varying collision induced dissociation (CID) energy for fragmentation of each compound was carried out.

4.3.1 Method development

The appropriated mobile phase condition from HPLC-UV was transferred to LC-MS/MS instrument. MS/MS parameters were optimized via direct injection and tuning each standard solution at 1000 µg/mL. The mass spectrometer was utilized in the positive and negative electrospray ionization mode. Collision induced dissociation (CID) energy to achieve transition ion of each standard was varied between 20 and 45 eV. The first transition ion was used for quantitation and the second one was used for confirmation of intend compound. The MS parameters were showed in Table 4.2. To reduce MS noise and interferences mobile phase condition was further adjusted by using 0.2 % formic acid in deionized water (solvent A) which pH of this mobile phase was about 2.5 used instead of 10 mM ammonium acetate buffer (pH 2.5). In addition, 0.1 % formic acid was added in methanol (solvent B) to promote solvent

volatilization. Gradient elution was used instead of isocratic to make sure that all of matrices in vegetables will be eluted before the next injection. The mobile phase flow rate was maintained at 0.3 mL/min. The gradient condition for LC-MS/MS ion trap was showed in Table 4.3.

Table 4.2 Electrospray ionization (ESI) parameters (ion-trap mass spectrometry)

Spray voltage	5.00 kV
Capillary temperature	350C°
Capillary voltage	26.00 V
Tube lens	70.00 V

Table 4.3 Gradient condition for LC-MS/MS (ion-trap mass spectrometry)

Time	% Solvent A (0.2% formic acid in DI water)	% Solvent B (0.1% formic acid in methanol)
0.00	98.0	2.0
8.00	98.0	2.0
8.10	10.0	90.0
13.00	10.0	90.0
13.10	98.0	2.0
15.00	98.0	2.0

4.4 Liquid chromatography triple quadrupoles-mass spectrometry (LC-MS/MS) method

Triple quadrupole mass spectrometry method was developed and validated. Then, the validated LC-MS/MS method was applied to determine purines and uric acid in sixteen Thai vegetables.

4.4.1 Method development

4.4.1.1 Preparation of 10 mM ammonium hydroxide

A 10 mM ammonium hydroxide was prepared by diluting 15.6 mL of 25% ammonium hydroxide solution with deionized water and adjusted to 100 mL to obtain 1 M solution. The further 100 fold dilution was performed to obtain 10 mM concentration.

4.4.1.2 Preparation of stock standard solutions

Stock standard solutions 100 µg/mL for adenosine, guanosine, xanthine and uric acid were separately prepared by dissolving amount of powder in 10 mM ammonium hydroxide to achieve the required concentration.

4.4.1.3 Preparation of standard mixture solutions

Mixture standard solution of adenosine and guanosine was prepared by diluting 100 µg/mL stock standard solutions with 10 mM ammonium hydroxide to obtain mixture standard solution of 10 µg/mL. For uric acid and xanthine, standard mixture solution was prepared by diluting 100 µg/mL stock standard solutions with 10 mM ammonium hydroxide until the final concentration of 20 µg/mL was obtained.

4.4.1.4 Preparation of stock internal standard solution

Caffeine was used as internal standard (IS). Stock internal standard solution was prepared by dissolving and diluting caffeine standard with 10 mM ammonium hydroxide until the final concentration of 10 µg/mL was obtained.

4.4.1.5 Preparation of blank sample (onion)

About 20 g of chopped onion was accurately weighed and mixed with 10 mM ammonium hydroxide 200 mL then homogenized in blender for 5 minutes. A 10.6 g of blender blank sample was weighed into 50 mL centrifuge tube and spiking of standard mixture solution used for linearity, recovery, accuracy and precision. The blended blank sample was centrifuged at 10,000 rpm for 10 minutes used for matrix effect. A vegetable without uric acid and purine precursors was used as blank sample. Therefore, onion was used as blank sample for method development and validation [4].

4.4.2 Development of ultrahigh performance liquid chromatography tandem mass spectrometry (LC-MS/MS) method for determination of purines and uric acid

Ultra-high performance liquid chromatography (UPLC) was performed on a Shimadzu LC-30AD (Japan) equipped with CBM-20A controller, LC-30AD binary pump, DGU-20A 5R degasser, SIL 30-AC auto sampler and CTO-30A a column oven. The separation was achieved on an Ascentis[®] Express C18-column (10 cm × 2.1 mm, 2.7 μm) with Ascentis[®] Express C₁₈-guard column (0.5 cm × 2.1 mm, 2.7 μm). The elution type was performed on gradient elution systems using solvent A (0.2 % formic acid in deionized water) and solvent B (0.1 % formic acid in methanol). Gradient condition was illustrated in Table 4.4.

Table 4.4 Gradient program (triple quadrupole mass spectrometry)

Time (min)	% Solvent A (0.2% formic acid in DI water)	% Solvent B (0.1% formic acid in methanol)
0.50	98	2
1.50	98	2
3.10	10	90
4.00	10	90
5.50	98	2
8.50	98	2

The column oven temperature was maintained at 40°C, injection volume was 5 μL and the mobile phase flow rate was maintained at 0.2 mL/min. The LC-MS/MS quantitative analysis of analyte was calculated area ratio of selection transition ion per area of internal standard.

Triple quadrupole mass spectrometer was performed on a Shimadzu (Japan) LCMS-8030 with an electrospray ionization (ESI) source. A 1,000 μg/mL of each standard solution was used for mass tuning. Mass spectrometric detection was performed in full scan and MRM modes. The collision energy (CE) was optimized to obtained transition ions. Two transition ions were utilized, one for quantitation and another one for identification of intend compound. The instrument was set as follow;

Nebulizing gas N ₂ flow:	3.0 L/min
Drying gas N ₂ flow:	12.0 L/min
Temperature:	350°C
Heat block temperature:	500°C

4.4.3 Method validation of triple quadrupole LC-MS/MS method

Validation of the developed triple quadrupole LC-MS/MS method was performed by using onion as blank sample. Validation parameters i.e., linearity, recovery, accuracy, precision, matrix effect, stability, lower limit of quantitation (LLOQ) and specificity were evaluated according to United States Food and Drug Administration (US-FDA) guideline [33].

4.4.3.1 Preparation of mixture standards linearity (mixture standards 1)

A one milligram of standard uric acid, xanthine, adenosine and guanosine was separately weighed and dissolved in 10 mM ammonium hydroxide to obtain stock solution of 100 µg/mL. The stock solutions of 100 µg/mL were further diluted to obtain the mixture stock solution of uric acid and xanthine at 20 µg/mL. For the mixture stock solution of adenosine and guanosine the stock solutions of 100 µg/mL were further diluted until the final concentration of 10 µg/mL was obtained. A series concentrations mixture standards solution were prepared according to table 4.4, by using stock solution of 20 µg/mL for uric acid and xanthine and 10 µg/mL for adenosine and guanosine. A 1.0 mL of 10 µg/mL internal standard caffeine was added into all solutions. Then, each tube was mixed by using vortex mixer and filtered through 0.22 µm membrane before injection to LC-MS/MS instrument. A series concentration of standard mixture solution was presented in Table 4.5.

4.4.3.2 Preparation of mixture standards spiked blank vegetable (mixture standard 2)

Twenty grams of chopped onion was homogenized with 200 mL of 10 mM ammonium hydroxide for 5 minute. Then, a portion 10.6 g of homogenized sample (weight of 1 g onion + 10 mL of 10 mM ammonium hydroxide) was separately weighed into 50 centrifuge tubes. Stock standards solutions, internal standard and 10 mM ammonium hydroxide were added according to Table 4.5. Then,

each tube was mixed by using vortex mixer then centrifuged at 10,000 rpm for 10 minute and filtered through 0.22 μm membrane before injection to LC-MS/MS instrument.

Table 4.5 Series concentrations of standards mixture solution

Solution number	Uric acid (ng/mL)	Xanthine (ng/mL)	Adenosine (ng/mL)	Guanosine (ng/mL)	Caffeine (IS) (ng/mL)
1	-	-	-	-	-
2	-	-	-	-	500
3	2,000	2,000	1,000	1,000	500
4	1,500	1,500	1,500	1,500	500
5	1,000	1,000	1,000	1,000	500
6	750	750	800	800	500
7	500	500	600	600	500
8	400	400	400	400	500
9	300	300	200	200	500
10	200	200	100	100	500
11	100	100	10	10	500

4.4.3.3 Preparation of mixture standards spiked filtrate blank vegetable (mixture standard 3)

Twenty grams of chopped onion was homogenized with 200 mL of 10 mM ammonium hydroxide for 5 minute. The homogenized sample then was centrifuged at 10,000 rpm for 10 minute. The filtrate was used to prepare a series of mixtures standards according to table 4.4. Then, each tube was mixed by using vortex mixer and filtered through 0.22 μm membrane before injection to LC-MS/MS.

4.4.3.4 Specificity

For specificity of the developed LC-MS/MS method, two specific transition ions were employed for each compound. One was used for quantitation another one was used for identification.

4.4.3.5 Linearity and lower limit of quantitation (LLOQ)

Linearity and LLOQ were performed by the set of mixture standard 2. Standard curve of each compound was separately plotted between concentrations (x-axis) and peak area ratios of specific transition ion and internal standard (y-axis).

Acceptance criteria:

Linearity; $r \geq 0.99$ and $\% \text{RSD} \leq 15.0 \%$, except LLOQ

LLOQ; The lowest concentration with $\% \text{RSD} \leq 20.0 \%$ and accuracy within 80.0 – 120.0 %.

4.4.3.6 Recovery and matrix effect

Here, recovery was the recovery of sample preparation of the method. Recovery was obtained by plotting the graph between peak area ratios obtained from mixture standard 3 (x-axis) and peak area ratios from mixture standard 2 (y-axis). Equation of the graph was obtained by least square regression method. The slope of the graph was used to evaluation method recovery (%) as follow,

$$\text{Recovery \%} = \text{Slope} \times 100$$

Matrix effect was obtained by plotting the graph between peak area ratios obtained from mixture standard 1 set (x-axis) and peak area ratios of mixture standard 3 set (y-axis). Equation of the graph was obtained by least square regression method. The slope of the graph was used to evaluate matrix effect as follow,

$$\text{Matrix effect (\%)} = (1 - \text{slope}) \times 100$$

Interpretation: Slope < 1 = Ion suppression

Slope > 1 = Ion enhancement

4.4.3.7 Accuracy and precision

Quality control samples (QCS) at 4 concentrations (100 - 2,000 ng/mL for uric acid and xanthine and 10 - 1,000 ng/mL for adenosine and guanosine) were used to evaluate accuracy and precision of the developed LC-MS/MS method.

Twenty grams of chopped onion was homogenized with 200 mL of 10 mM ammonium hydroxide for 5 minute. Then, homogenized sample was centrifuged at 10,000 rpm for 10 minute.

A 10.6 g portion of filtrate was transferred to 4 test tubes. Stock standards solutions, internal standard and 10 mM ammonium hydroxide were added according to table 4.5. Then, each tube was mixed by using vortex mixer and filtered through 0.22 μm membrane before injection to LC-MS/MS instrument.

Three replicates were performed for each concentration to evaluate repeatability (intra-day precision). Fully determination was repeated in another two different days to evaluate intermediate-precision. Accuracy of the method was expressed in term of percent recovery (% recovery). Whereas, repeatability and intermediate precision were expressed in term of percent of relative standard deviation (% RSD). Percent recovery and % RSD were calculated as follow,

$$\% \text{ Recovey} = \frac{\text{Found concentration}}{\text{Nominal concentration}} \times 100$$

$$\% \text{ RSD} = \frac{\text{SD}}{\text{Average}} \times 100$$

Acceptance criteria: % Recovery = 85.0 – 115.0 % (at LLOQ = 80.0 – 120.0)

% RSD \leq 15 % (at LLOQ \leq 20.0 %)

Table 4.6 Preparation of quality control sample (QCS) for accuracy and precision

Sample	Conc. of uric acid and xanthine (ngmL^{-1})	Conc. of adenosine and guanosine (ngmL^{-1})	mL of uric acid and xanthine $20 \mu\text{gmL}^{-1}$	mL of adenosine and guanosine $10 \mu\text{gmL}^{-1}$	mL of caffeine $10 \mu\text{gmL}^{-1}$	mL of $10 \text{ mM NH}_4\text{OH}$
QCS 0	0	0	-	-	1.00	9.00
QCS 1	100	10	2.00	2.00	1.00	5.00
QCS 2	200	50	0.50	0.60	1.00	7.90
QCS 3	500	300	0.20	0.10	1.00	8.70
QCS 4	2000	1000	0.10	0.02	1.00	8.88

4.4.3.8 Stability

Three stability studies were performed. The first one was stability of standards spiked samples. Spiking was performed at low, middle and high concentrations. The spiked samples were stored at -20°C. Three freeze-thaw cycles were performed. The second one was stability study of mixture standards solution at -20°C. The last one was stability study of mixture standards solution at room temperature.

4.5 Determination of purines and uric acid in Thai vegetables

The developed and validated LC-MS/MS method was used to assay for uric acid and purines contents in eighteen Thai vegetables.

4.5.1 Plant materials

Eighteen widely consumed Thai vegetables were bought from three different markets in Bangkok, Thailand in November 2014 - January 2015. Plant samples were identified by Assist. Prof. Piyanuch Rojsanga, (Faculty of Pharmacy Mahidol University). All samples were cleaned and dried in room temperature for 2 hours. The edible part used of each vegetable was separated and chopped into small pieces, using electronic blender. The fresh vegetables were utilized without drying in the oven or heating. Eighteen Thai vegetables used in the study were show in Table 4.7.

Table 4.7 Thai vegetables for determine uric acid and purines compound

Common name	Scientific name	Part use
Yardlong bean	<i>Vigna unguiculata</i> Hc.	Whole plant
Cucumber	<i>Cucumis Sativus</i> Linn.	Fruits
Chainese cabbage	<i>Brassica pekinensis</i>	Whole plant
Bean Sprouts	<i>Vigna radiate</i> (L.)	Whole plant
Water Convolvulus	<i>Ipomoea aquatic</i> Forsk. Var. reptan	Shoots
Winged bean	<i>Psophocarpus tetragonolobus</i>	Whole plant
Melientha suavis	<i>Melientha suavis</i> Pierre	Shoots
Asiatic pennywort	<i>Centella asiatica</i> (Linn.) Urban	Leaves
Water mimosa	<i>Neptunia oleracea</i> Lour. FL.	Shoots
Acacia pennata	<i>Acacia Insuavis</i> , Lace	Shoots
Bitter bean or twisted cluster bean	<i>Parkia speciosa</i> Hassk.	Fruits
Bamboo shoots	<i>Bambusa</i> spp.	Shoots
White popinac or wild tamarind	<i>Leucaena glauca</i> Benth.	Shoots
Thai Eggplant	<i>Solanum xanthocarpum</i> Schrad.	Fruits
Malabar Nightshade or Ceylon Spinach	<i>Basella alba</i> Linn.	Leaves
Ivy Gourd	<i>Coccinia grandis</i> (L.) Voigt	Shoots
Yellow Velvet Leaf	<i>Limnocharis flava</i> (L.) Buch.	Shoots
Vietnamese Coriander	<i>Polygonatum odoratum</i>	Leaves

4.5.2 Construction of calibration curves

Matrix match calibration method was employed for quantitative determination of purines and uric acid in Thai vegetables. Calibration curve of each vegetable was performed by using pooled - sample from 3 different sources. About 10 g of pooled and chopped – sample was accurately weighed and mixed with 300 mL of 10 mM ammonium hydroxide. Then, mixed sample was homogenized in the blender for 5 minutes. To prepare a series concentrations of matrix match calibration curves, 10.6 g of blender sample was mixed with certain volume of stock standards and internal standard solutions and 10 mM ammonium hydroxide according to Table 4.8.

Table 4.8 Matrix match calibration curve for determine purines and uric acid in each sample.

Solution number	Uric acid (ng/mL)	Xanthine (ng/mL)	Adenosine (ng/mL)	Guanosine (ng/mL)	Caffeine (IS) (ng/mL)
1	-	-	-	-	-
2	-	-	-	-	500
3	2,000	2,000	2.00	1,000	500
4	1,500	1,500	1.50	1,500	500
5	1,000	1,000	1.00	1,000	500
6	750	750	0.80	800	500
7	500	500	0.60	600	500
8	400	400	0.40	400	500
9	300	300	0.20	200	500
10	200	200	0.10	100	500
11	100	100	0.02	20	500

4.5.3 Sample preparation

Ten grams of each chopped vegetable sample from 4.5.1 was accurately weighed and mixed with 100 mL of 10 mM ammonium hydroxide using electronic blender for 5 minutes. A 10.6 g of blended sample (weight of 1 g sample and 10 mL of 10 mM ammonium hydroxide) was weighed into 50 mL-centrifuge tube. One milliliter of internal standard caffeine solution (10 µg/mL) and 9.00 mL of 10 mM ammonium hydroxide solution were added respectively. The mixture was mixed by vortexes centrifuged at 10,000 rpm at 25°C for 10 minutes. The supernatant was filtered through 0.22 µm nylon membrane filter before injected into LC-MS/MS instrument. Uric acid and each purine precursors contents were calculated from the ratio of the area of quantitated transition ion of analyte per the area of transition ion of caffeine internal standard. Each sample was analyzed in triplicate. The average uric acid and purine precursors contents were calculated and expressed with matrix match calibration method.

CHAPTER V

RESULTS AND DISCUSSION

All results of the method development for quantitative analysis of uric acid and purine precursors contents in eighteen selected Thai vegetables were presented in this chapter.

5.1 High performance liquid chromatography method (HPLC)

5.1.1 Method development

The separation of uric acid and purine precursors (xanthine, hypoxanthine, adenosine and guanosine) was achieved on Chromolith C18 (4.6 x 100 mm) column. The wavelength of UV detector was set at 284 nm and mobile phase flow rate was maintained at 0.3 mL/min. The starting mobile phase was the mixture of 10 mM ammonium acetate buffer (pH 4.5) and acetonitrile under isocratic flow. Then, the ratio of acetonitrile was varied from 5 to 40 % (v/v). However, in these mobile phase conditions, each standard compound promoted the peak with retention time in the same range around 1-10 minute, as show in Appendix A. The retention time of each compound was showed in Table 5.1. This might be caused by the inappropriate pH of the mobile phase. Then, the pH of the mobile phase was adjusted to 2.5 and the concentration of acetonitrile was fixed at 5 % (v/v). The resulting HPLC chromatograms with retention time were improved. However, the acceptable resolutions of all compounds were not achieved yet. Next, methanol was used instead of acetonitrile, since elution strength of acetonitrile higher than methanol. The separation was improved but not completely with 5% methanol. Finally, methanol was reduced to 3% (v/v) and acceptable results were achieved in this condition. The overlaid chromatograms obtained from the optimum condition were show in Figure

5.1 and retention time of standard uric acid and purine precursors compound was showed in Table 5.1.

Table 5.1 Retention time of standard solution uric acid and purine precursors from HPLC-UV method

Mobile phase condition	Retention time (minute)				
	Uric acid	Xanthine	Hypoxanthine	Adenosine	Guanosine
10 mM ammonium acetate, pH 4.5: acetonitrile; (95:5)	1.75	3.4	3.3	3.1	1.6
10 mM ammonium acetate, pH 4.5: acetonitrile; (60:40)	1.5	2.8	2.9	2.9	1.3
10 mM ammonium acetate, pH 2.5: acetonitrile; (95:5)	3.7	3.9	3.6	4.2	4.3
10 mM ammonium acetate, pH 2.5: methanol; (95:5)	3.7	4.2	3.5	4.6	4.9
10 mM ammonium acetate, pH 2.5: methanol; (97:3)	4.2	5.2	4.9	7.9	8.9

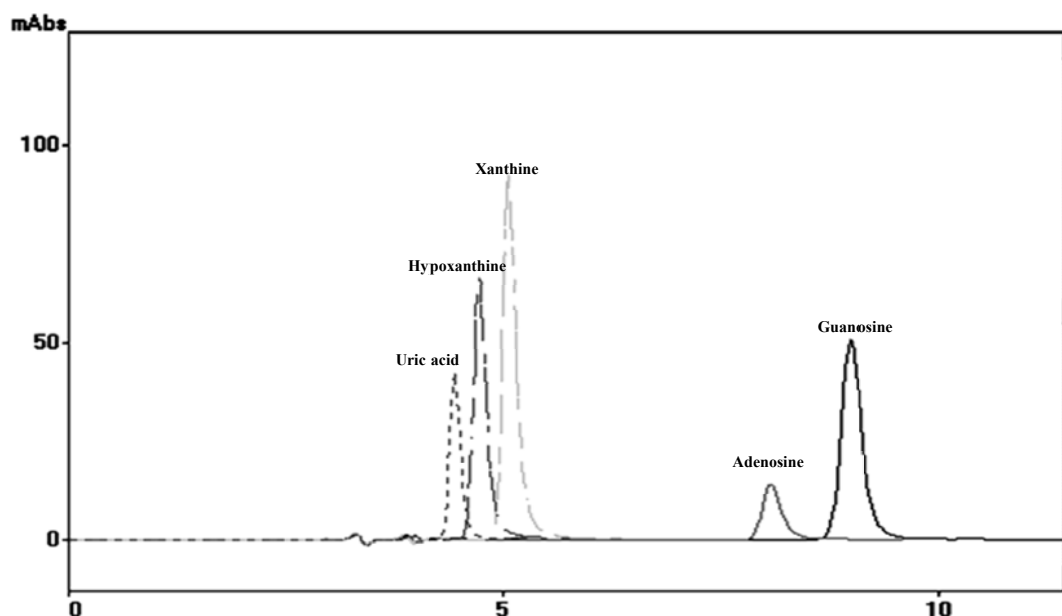


Figure 5.1 Overlaid chromatograms of uric acid and purines compounds, mobile phase 10 mM ammonium acetate buffer, pH 2.5 and methanol (97 : 3, v/v); flow rate 0.3 mL/min.

5.2 Liquid chromatography ion-trap mass spectrometry method (LC-MS/MS Ion-trap mass spectrometry)

5.2.1 Method development

Mobile phase condition from HPLC-UV system, the mixture of 10 mM ammonium acetate, pH 2.5 and methanol, was transferred to LC-MS/MS ion-trap mass spectrometer. Unfortunately, MS noise signal was found to be interfered from ammonium acetate buffer, therefore 0.2 % formic acid in deionized water (solvent A) was used instead of 10 mM ammonium acetate buffer pH 2.5. The pH of 0.2 % formic acid in deionized water was 2.5 (solvent A). In addition, 0.1 % formic acid was added in methanol mobile phase solvent to promote solvent volatilization. An HPLC column was symmetry C18 (4.6 x 150 mm, 5 μ m) column and mobile phase flow rate was maintained at 0.3 mL/min. Gradient elution was used instead of isocratic elution to make sure that all of matrices in vegetables will be eluted before the next injection.

The collision induced dissociation energy or CID was adjusted to obtain two transition ions of each compound. Unfortunately, the study failed to find the major transition ion of hypoxanthine. This may be due to the fact that molecular weight of hypoxanthine is too low (MW 135) so it was hard to get the major transition ion after fragmentation. From theory of ion trap mass analyzer, the ions are created, trapped and accumulated in a chamber then RF potential is applied to the trap. The ions were oscillatory excited and collision between each analyte may be occur. This may be resulted to the lowering signal of analytes. Therefore, only four compounds, uric acid, xanthine, adenosine and guanosine, were employed for LC-MS/MS method development. In fact, caffeine was firstly selected as internal standard but the signal of caffeine can not monitor in LC-MS/MS ion-trap instrument. Therefore, dextromethorphan was used as internal standard. For ionization mode, it was found that uric acid and xanthine were capable ionized in negative mode while adenosine, guanosine and dextromethorphan were capable ionized in positive mode. Mass analyzer was set up into two segments. Time interval between 0 to 7 minutes was set as the first segment and negative mode was employed to detect xanthine and uric acid. The second segment was from 7 to 15 minute and the instrument was switched to positive mode for detection of adenosine, guanosine and dextromethorphan.

Electrospray ionization parameters were showed in Table 5.2. The retention times, transition ions and CID energy were showed in Table 5.3. The LC-MS/MS chromatograms and transition ions of uric acid and purine precursors were showed in figure 5.2 - 5.5. The peak area ratio of parent ion and internal standard was used for quantitation. The presence of fragmentation ion of each analytical compound was used for confirmation of desired analyte.

Table 5.2 Electrospray ionization (ESI) parameters (ion-trap mass spectrometry)

Spray voltage	5.00 kV
Capillary temperature	350°C
Capillary voltage	26.00 V
Tube lens	70.00 V

Table 5.3 The retention times, transition ions and CID energy (ion-trap mass spectrometry)

Compound	Retention time (min)	Transition ion	CID (eV)
Uric acid	5.07	167 >> 124	33
Xanthine	6.39	151 >> 108	30
Adenosine	7.89	268 >> 136	28
Guanosine	10.78	284 >> 152	22
Dextromethorphan (IS)	12.37	272 >> 215	40

However, LC-MS/MS ion-trap method cannot be used due to the non-acceptable precision values obtained from the method validation process. Therefore, LC-MS/MS triple quadrupole instrument was applied. The results of validation method LC-MS/MS ion-trap were showed in Appendix B.

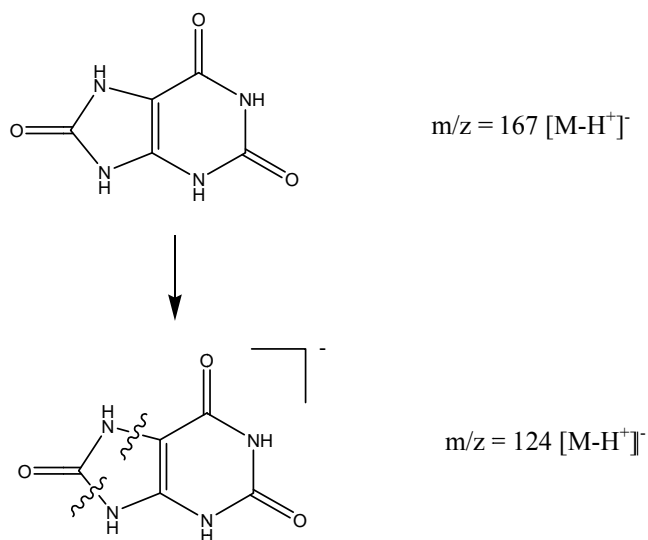
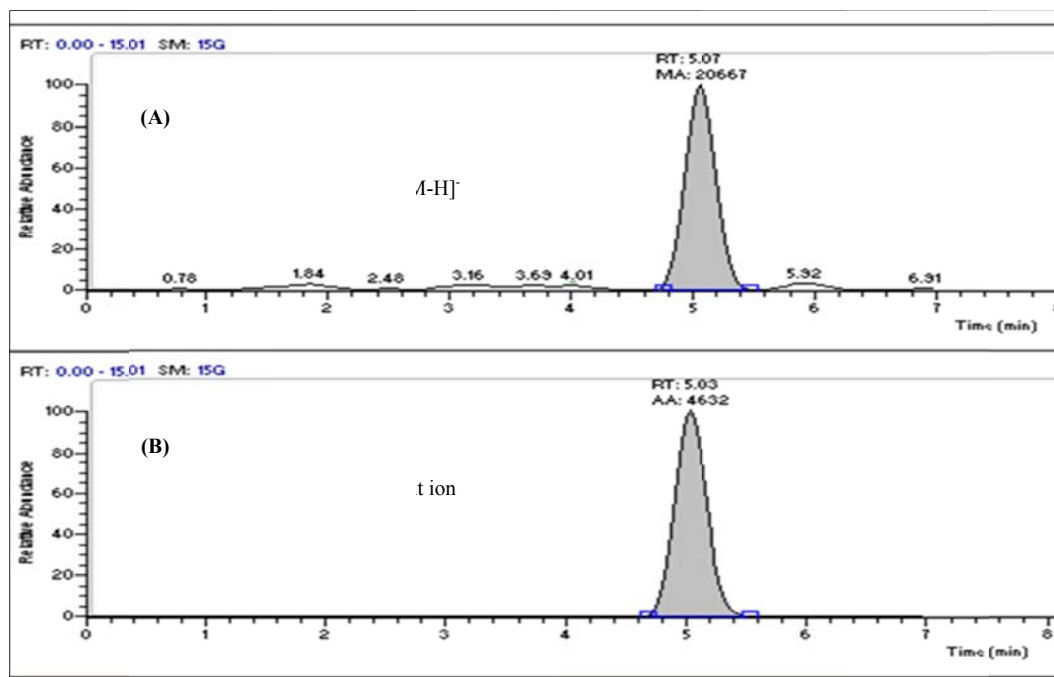


Figure 5.2 LC-MS/MS chromatogram of uric acid (A) and its fragmentation ion (B) analyzed by LC-MS/MS ion-trap

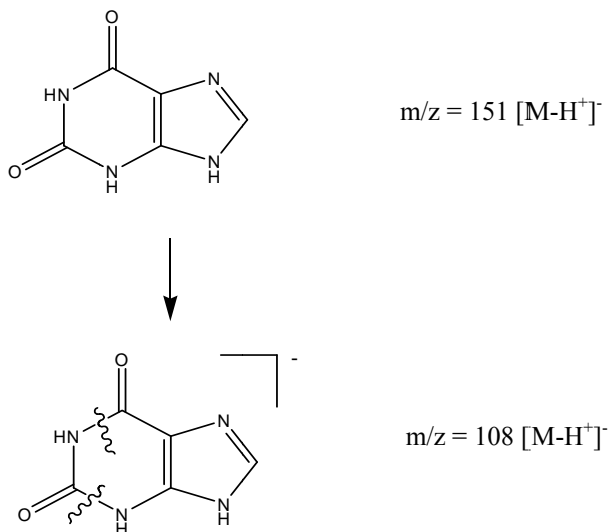
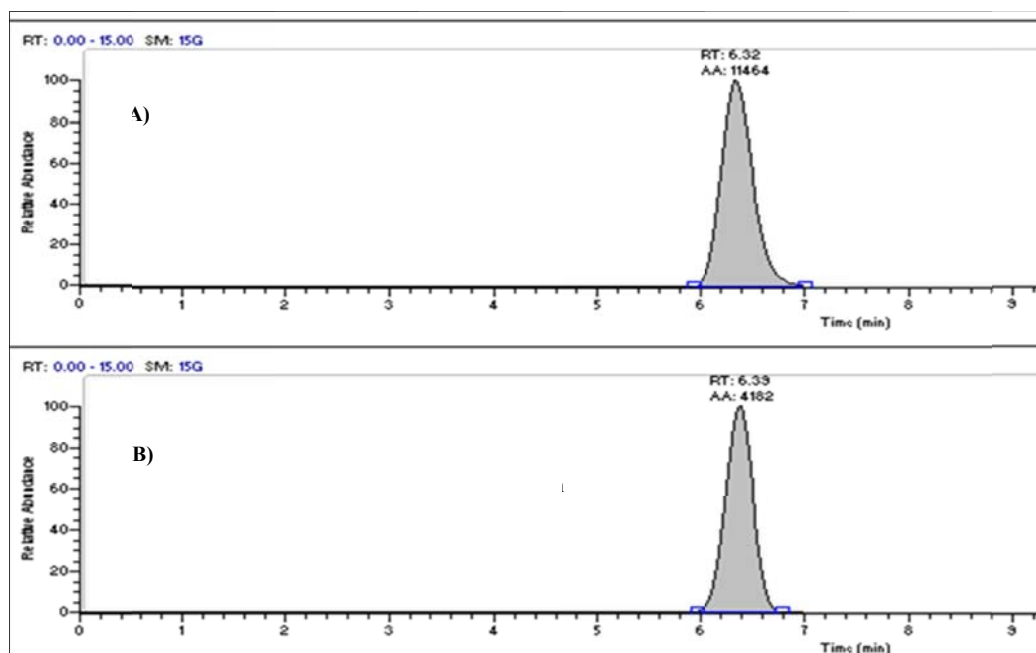


Figure 5.3 LC-MS/MS chromatogram of xanthine (A) and its fragmentation ion (B) analyzed by LC-MS/MS ion-trap

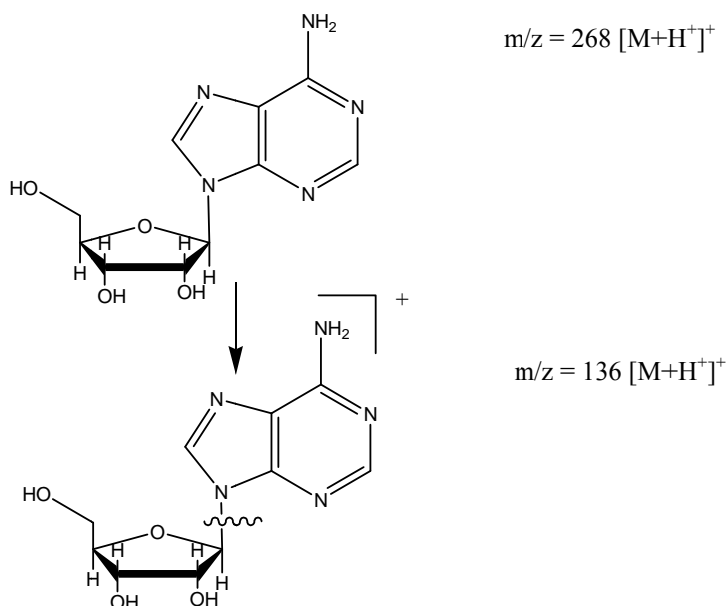
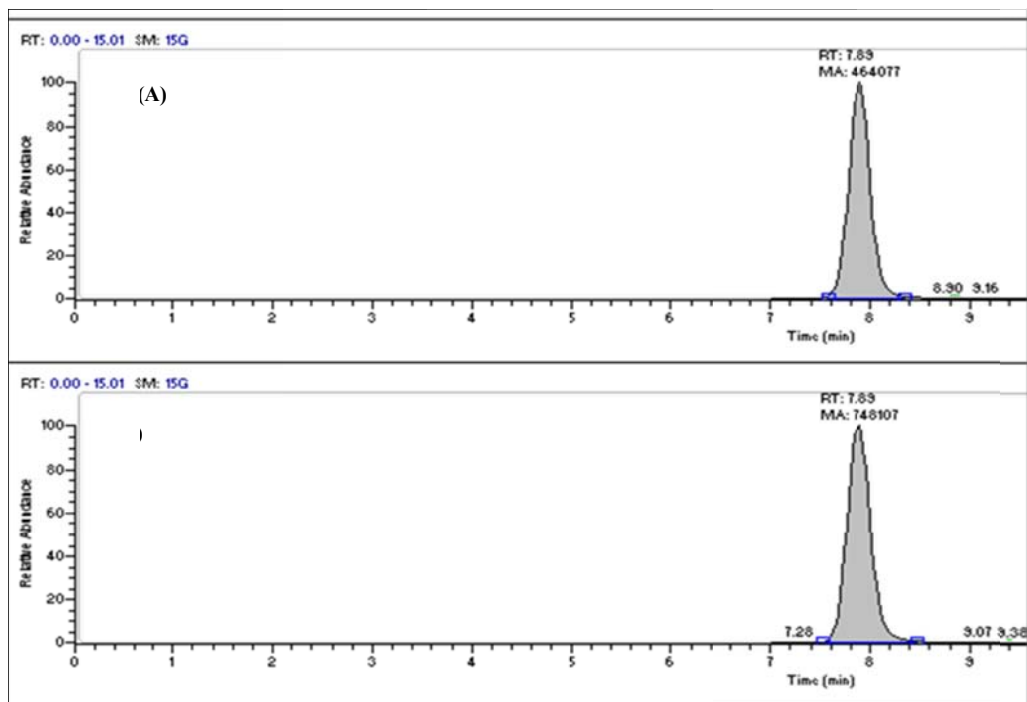


Figure 5.4 LC-MS/MS chromatogram of adenosine (A) and its fragmentation ion (B) analyzed by LC-MS/MS ion-trap

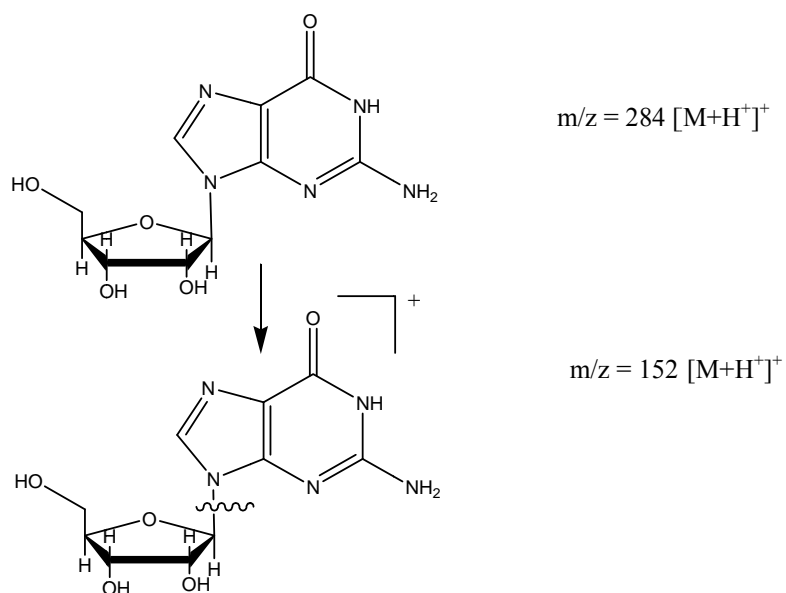
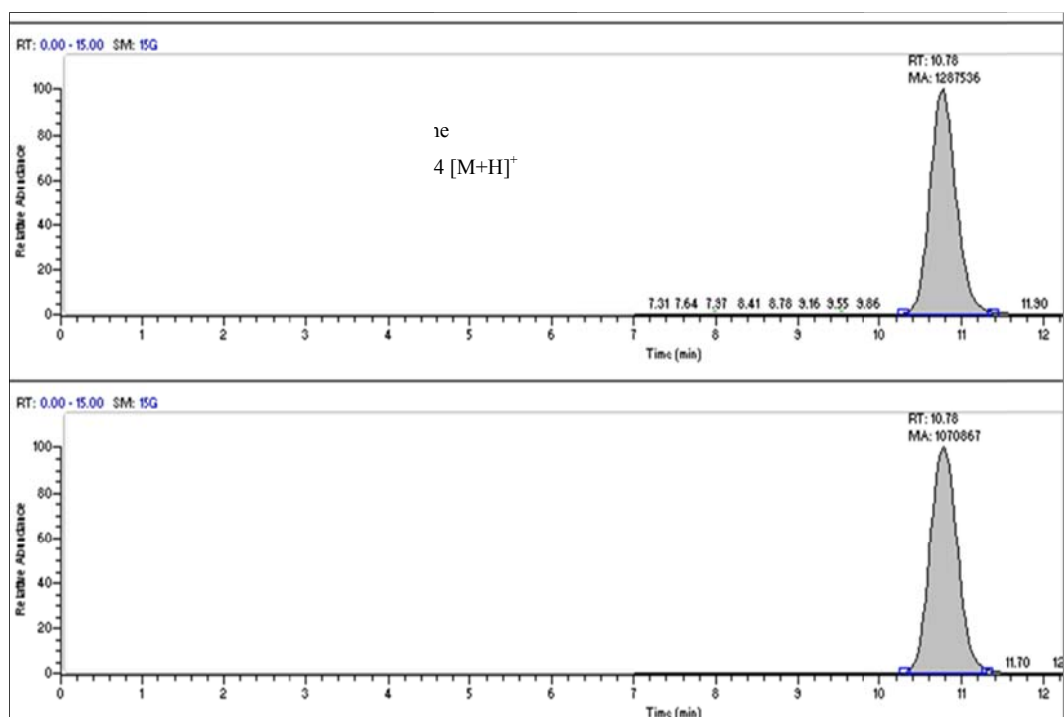


Figure 5.5 LC-MS/MS chromatogram of guanosine (A) and its fragmentation ion (B) analyzed by LC-MS/MS ion-trap

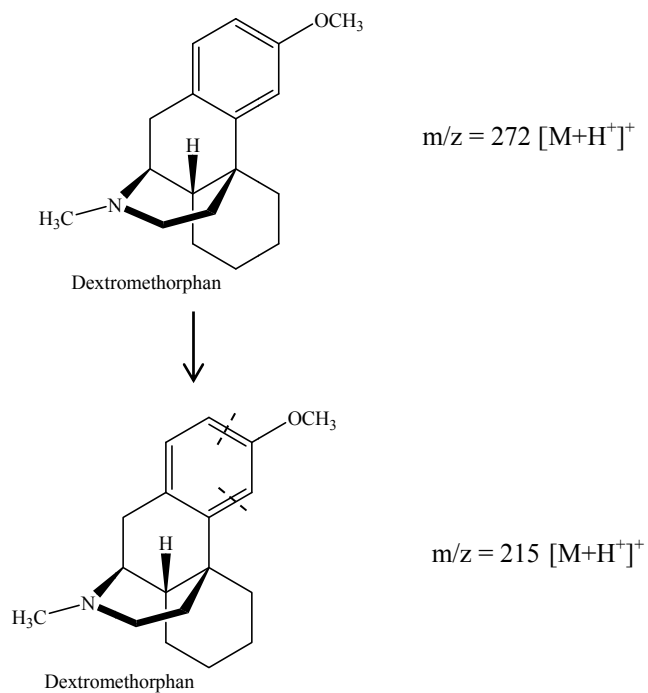
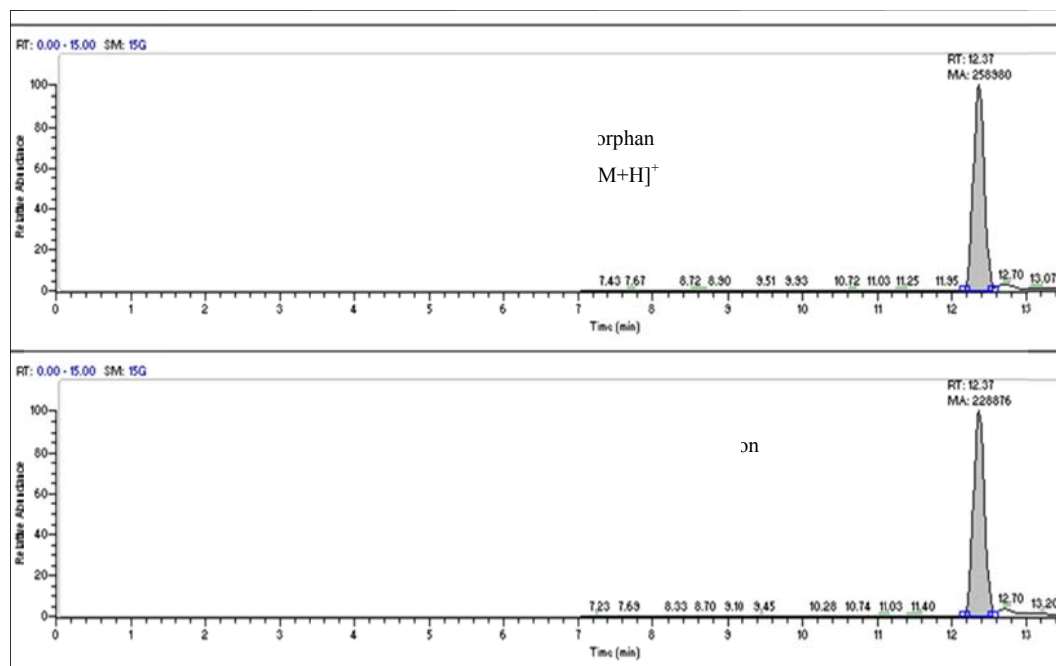


Figure 5.6 LC-MS/MS chromatogram of internal standard dextromethorphan (A) and its 9-fragmentation ion (B) analyzed by LC-MS/MS ion-trap

5.3 Liquid chromatography triple quadrupole mass spectrometry method (LC-MS/MS)

5.3.1 Method development of ultrahigh performance liquid chromatography tandem mass spectrometry (LC-MS/MS) method for determination of purines and uric acid

Optimization of LC conditions was performed to obtain the best separation and peak intensities of uric acid and purines compound. In this part, HPLC column was changed to increase the speed, resolution, improve peak sharp and sensitivity by decreasing particle size of stationary phase. The separation was achieved on an Ascentis[®] Express C18 column (10 cm × 2.1 mm, 2.7 μm) with Ascentis[®] Express C18 guard column (0.5 cm × 2.1 mm, 2.7 μm). Furthermore, the mobile phase from LC-MS ion trap mass spectrometry was transferred to the current LC-MS/MS instrument. The transferred mobile phase was consisted of 0.2 % formic acid in deionized water (mobile phase A) and 0.1 % formic acid in methanol (mobile phase B). Mobile phase flow rate was maintained at a 0.2 μL/min for all run and 5 μL of sample was injected. The elution type was performed on gradient elution system. The column oven temperature was maintained at 40°C.

Optimization of MS parameters was performed to achieve the maximum specificity and sensitivity of intended compounds. MS parameters of five compounds (adenosine, guanosine, uric acid and xanthine) including caffeine (IS) was optimized by tuning analyte in full scan mode. Then, multiple reactions monitoring (MRM) mode was optimized to obtain high sensitivity and selectivity of fragmentation or transition ions. Optimization results showed that appreciate sensitivity of uric acid and xanthine were achieved in negative ionization mode $[M-H]^-$. For adenosine, guanosine and caffeine, the signal from positive ionization mode $[M+H]^+$ showed better sensitivity than negative ionization mode. For each target analyte, two transitions ions were determined. The highest intensity transition ion from each precursor ion was selected and optimized the condition to obtain the highest intensity of the instrumental response for quantitation. One another high sensitivity transition ion was selected and used for identification. The optimum collision energy (CE) for each precursor ion to

obtain high sensitivity transition ions was presented in Table 5.4. The ion chromatograms were shown in figure 5.7 and transition ion of each analyte were showed in figure 5.8 - 5.12. Electrospray ionization (ESI) parameters for ionized the solvent and promote gas phase ions were set as follow;

Nebulizing gas N ₂ flow:	3.0 L/min
Drying gas N ₂ flow:	12.0 L/min
Temperature:	350°C
Heat block temperature:	500°C

Table 5.4 Precursor ions, transition ions and collision energy for all compounds

Compound	Precursor ion (m/z)	Transition ion (m/z)	Collision energy (V)
Uric acid	166.90	124.00	19.2
		69.00	19.0
Xanthine	151.00	107.90	9.0
		79.90	25.2
Adenosine	267.95	135.95	-14.7
		118.98	-43.1
Guanosine	284.00	152.10	-12.1
		135.00	-32.1
Caffeine	195.10	138.05	-18.2
		110.05	-22.1

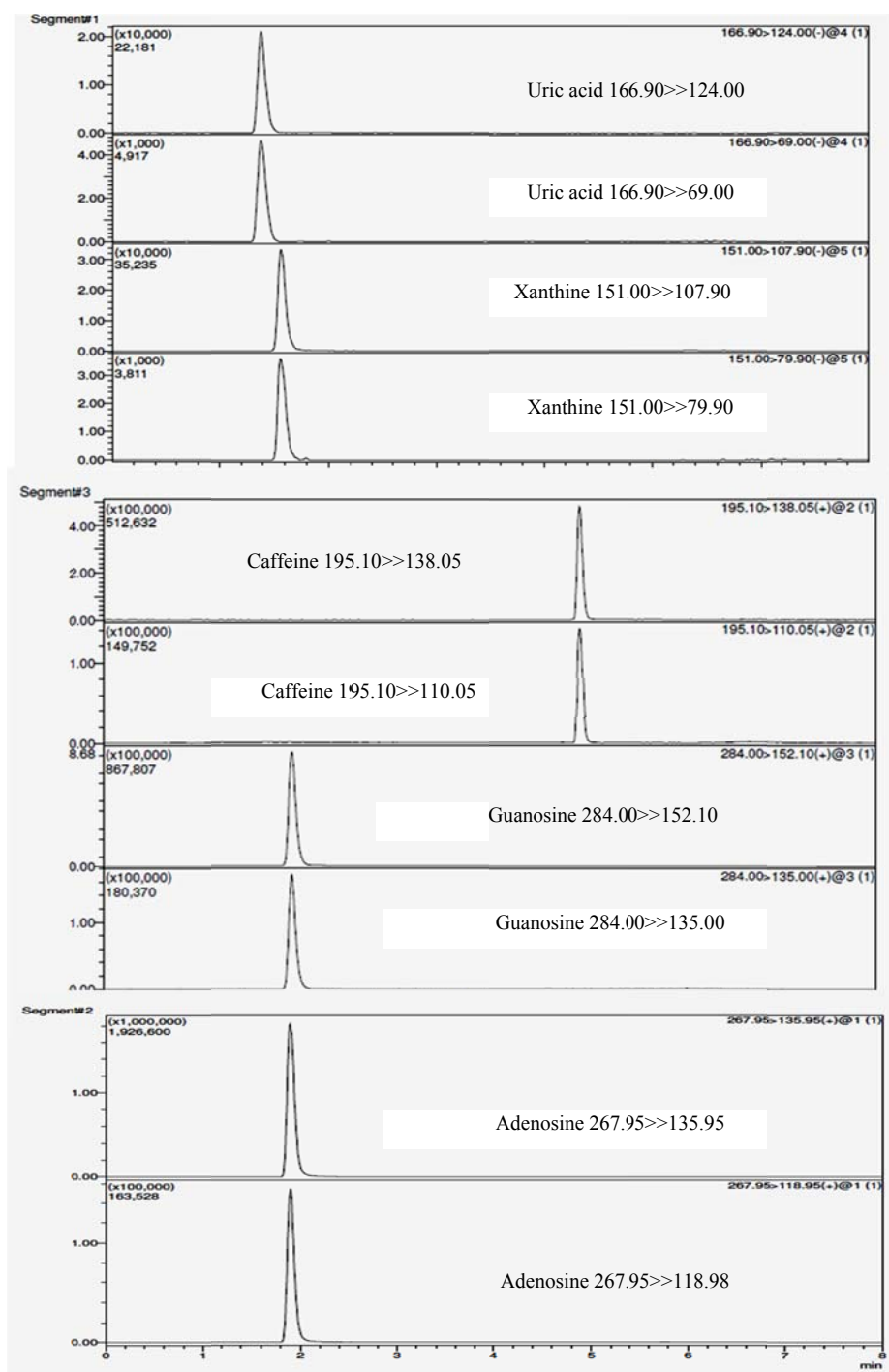
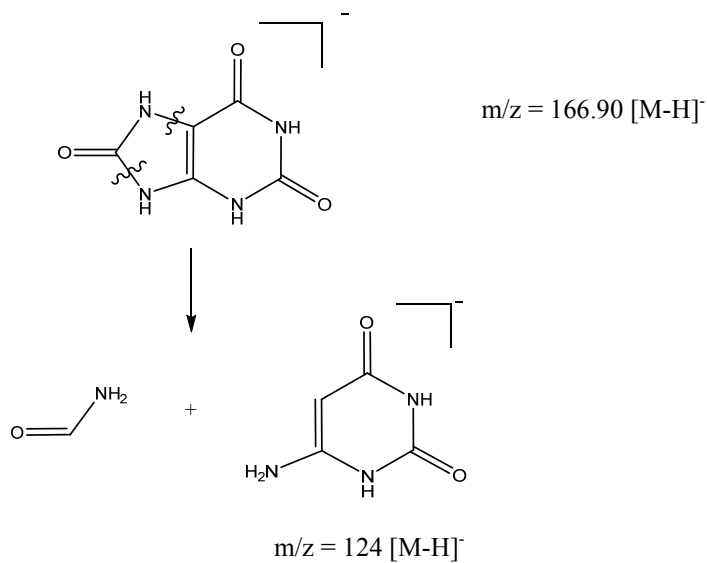


Figure 5.7 LC-MS/MS chromatograms of standard uric acid and purines precursors fragment ions analyzed by mass spectrometry detector triple quadrupole

Uric acid; 166.90>>124.00



Uric acid; 166.90>>69.90

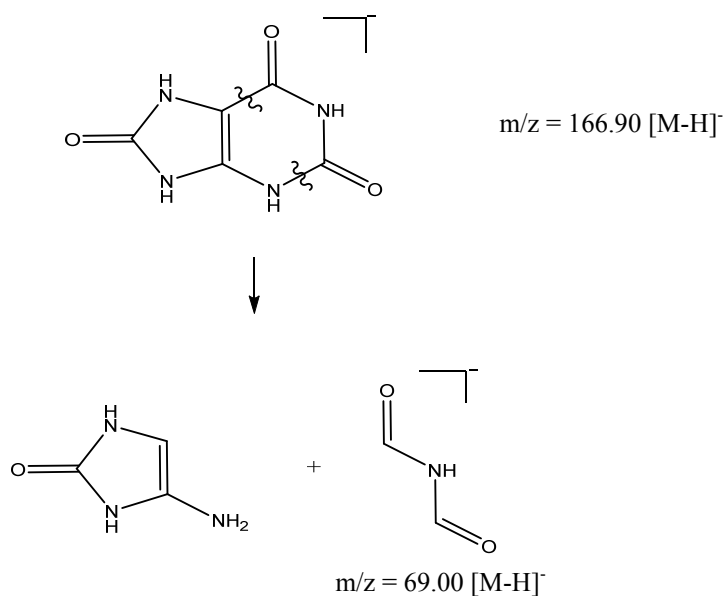


Figure 5.8 Transition ion of uric acid analyzed by LC-MS/MS triple quadrupole mass spectrometry ($m/z = 166.90 [M-H]^-$, $166.90 \gg 124.00$ and $166.90 \gg 69.00$)

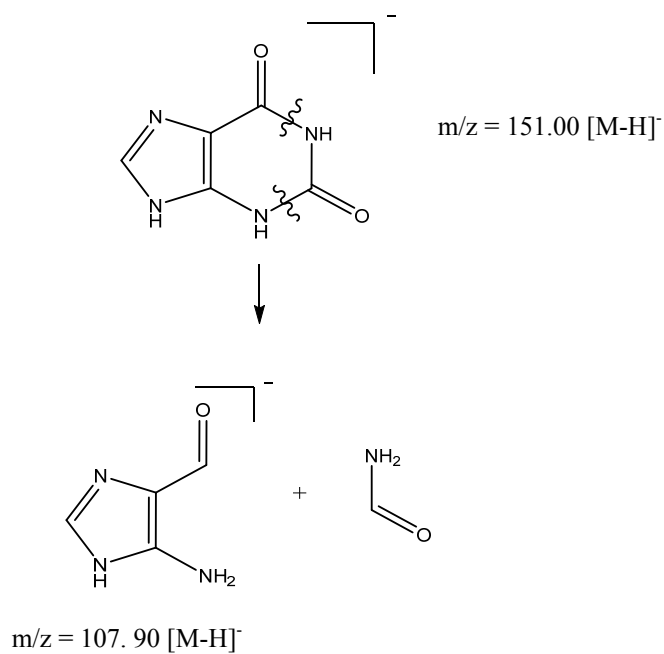
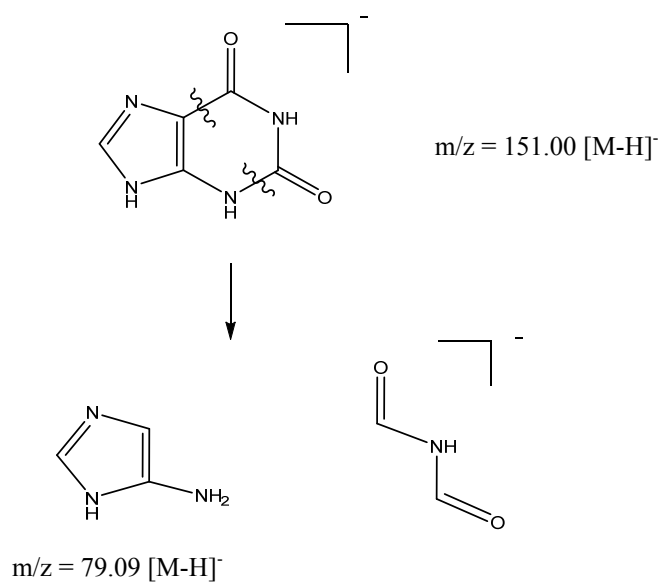
Xanthine; 151.00 >> 107.90**Xanthine; 151.00 >> 79.90**

Figure 5.9 Transition ion of xanthine analyzed by LC-MS/MS triple quadrupole mass spectrometry ($m/z = 151.00$ [M-H]⁻, $151.107.90 >> 124.00$ and $151.00 >> 79.90$)

Adenosine; 267.95 >> 135.95

267.95 >> 118.98

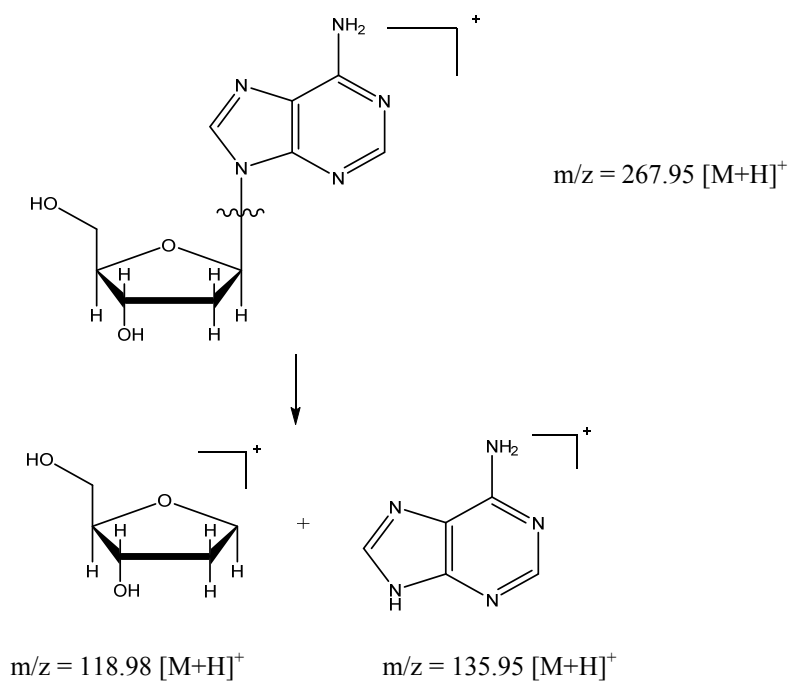


Figure 5.10 Transition ion of adenosine analyzed by LC-MS/MS triple quadrupole mass spectrometry ($m/z = 267.95 [M+H]^+$, $267.95 \gg 135.95$ and $267.95 \gg 118.98$)

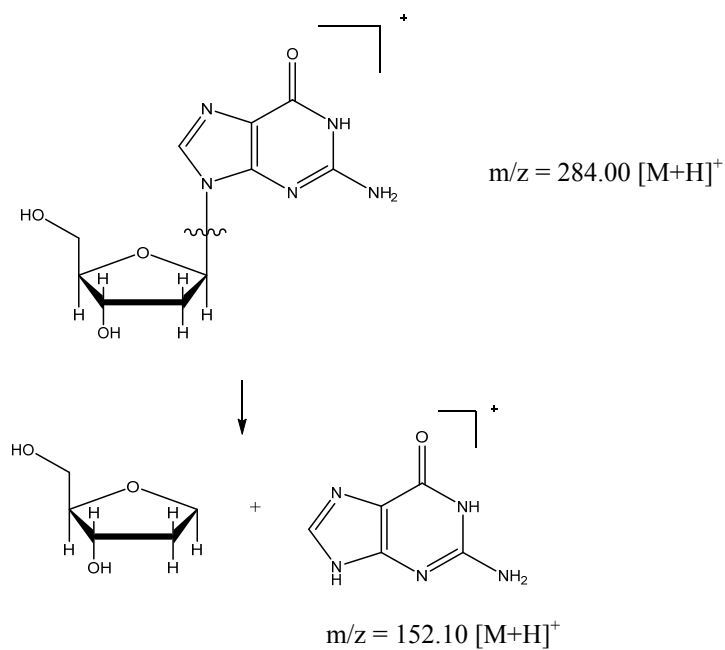
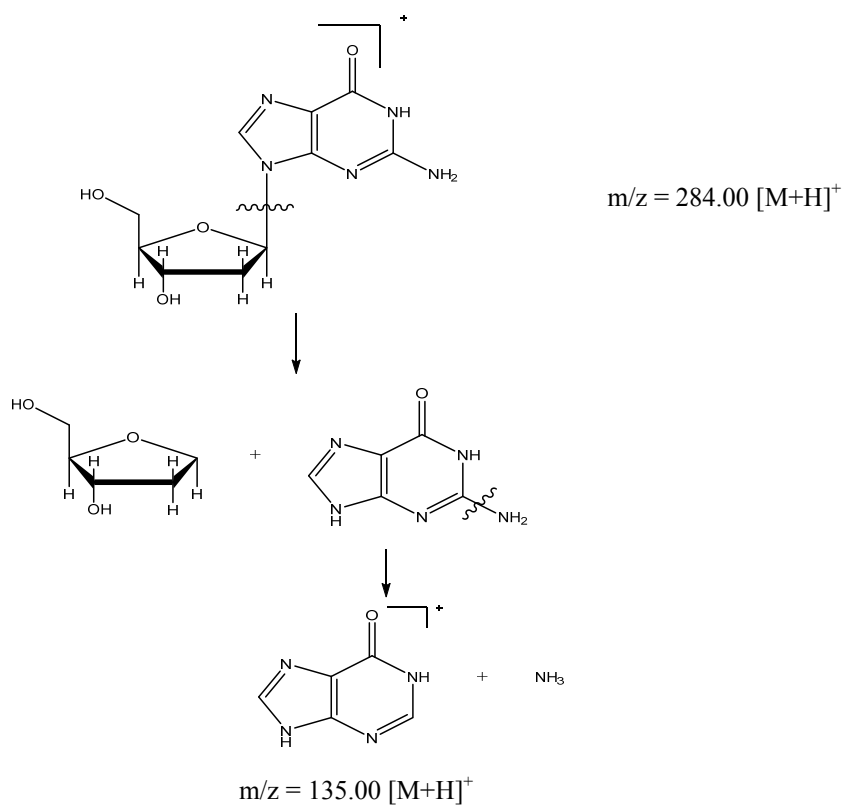
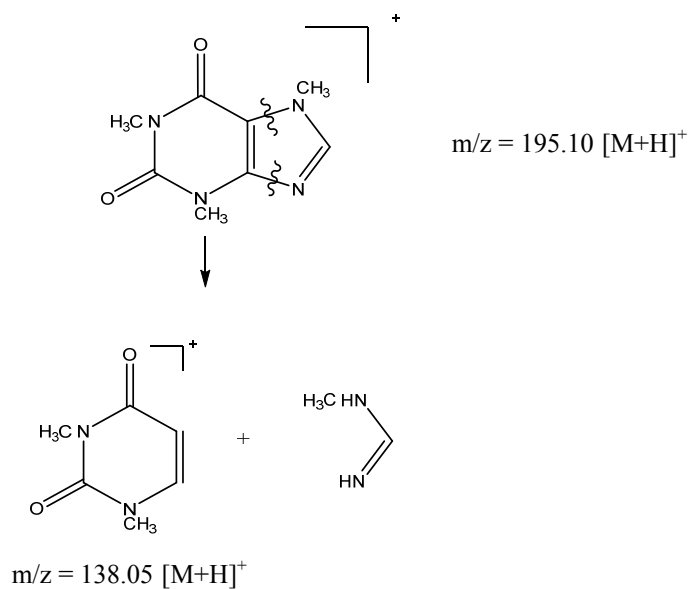
Guanosine; 284.00 >> 152.10**Guanosine; 284.00 >> 135.00**

Figure 5.11 Transition ion of guanosine analyzed by LC-MS/MS triple quadrupole mass spectrometry ($m/z = 284.00 [M+H]^+$, $284.00 >> 152.10$ and $284.00 >> 135.00$)

Caffeine; 195.10 >> 138.05



Caffeine; 195.10 >> 110.05

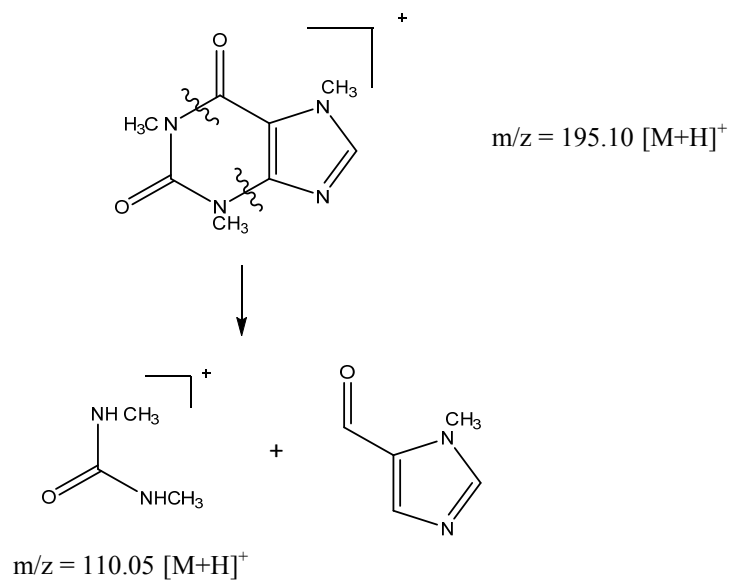


Figure 5.12 Transition ion of caffeine analyzed by LC-MS/MS triple quadrupole mass spectrometry ($m/z = 195.10 >> 138.05 [M+H]^+$ and $195.10 >> 110.05$)

5.4 Method validation of triple quadrupole LC-MS/MS method

The developed triple quadrupole LC-MS/MS method was fully validated according to US FDA guideline [33]. The performance characteristics i.e., linearity, recovery, accuracy, precision, matrix effect, stability, lower limit of quantitation (LLOQ) and specificity were evaluated.

In this study, onion was selected as blank because it was reported as non-uric acid content vegetable [4]. Moreover, onion was tested before using. As shown in figure 5.13, it could be seen that there was not uric acid in onion.

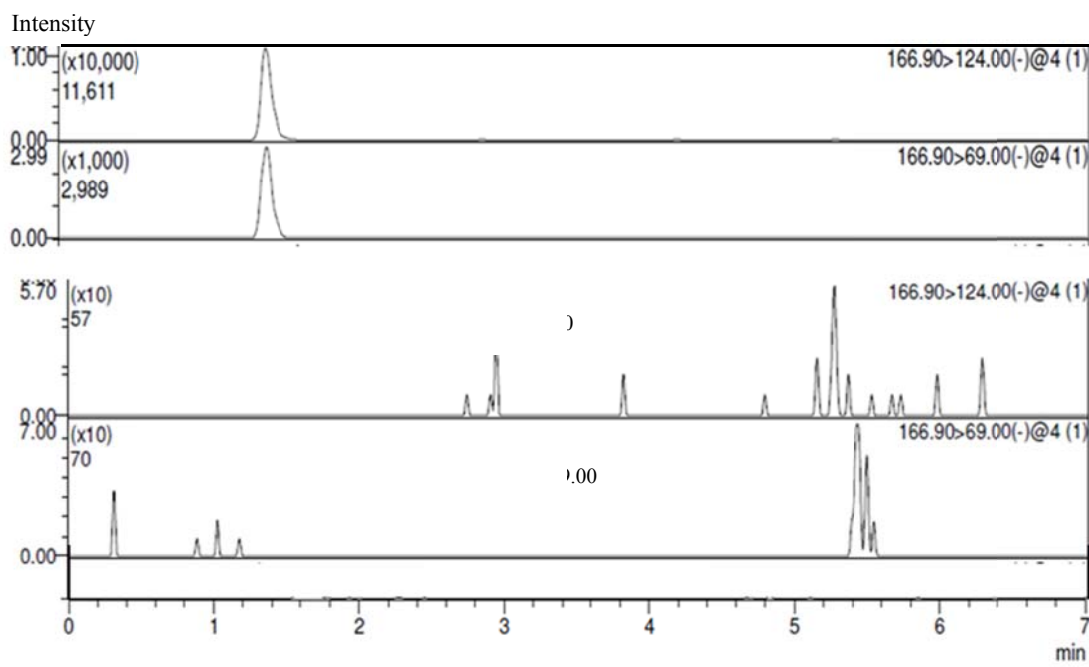


Figure 5.13 LC-MS/MS chromatograms of uric acid from onion analyzed by mass spectrometry detector triple quadrupole

5.4.1 Specificity

Specificity of the method was achieved by comparing of the retention time of analyte in sample and its standard. Analyte in the sample must be presented at the same retention time of its standard. Furthermore, two specific transition ions were utilized, one for quantitation and another one for identification. The two specific transition ions of each compound were summarized in Table 5.5. Moreover, it had not interferences presented at the same retention time of interest ions.

Table 5.5 Summary of specific transition ions of uric acid and purines

Compound	Parent ion	Quantitation ion	Identification ion
Uric acid	166.90	124.00	69.00
Xanthine	151.00	107.90	79.90
Adenosine	267.95	135.95	118.98
Guanosine	284.00	152.10	135.00

5.4.2 Linearity and lower limit of quantitation (LLOQ)

Linearity of the methods were evaluated in the concentration ranges of 100 to 2,000 ng/mL for uric acid and xanthine and 10 to 1,000 ng/mL for adenosine and guanosine. The graphs represent linearity were plotted between concentrations (x-axis) and their corresponding peak area ratios of selected transition ion and internal standard (Y-axis). Least-square regression method was employed. The linearity of the method was accepted with the correlation coefficient (r) ranged from 0.9878-0.9991. The lowest concentration of linearity range was set as lower limit of quantitation (LLOQ).

Table 5.6 Calibration curve of uric acid and purine precursors by LC-MS/MS triple quadrupole mass spectrometer

Substance	Equation	r
Uric acid	$Y = 0.0031x + 0.3348$	0.9878
Xanthine	$Y = 0.0032x - 0.1038$	0.9974
Adenosine	$Y = 0.8471x + 37.18$	0.9974
Guanosine	$Y = 0.302x + 1.4408$	0.9991

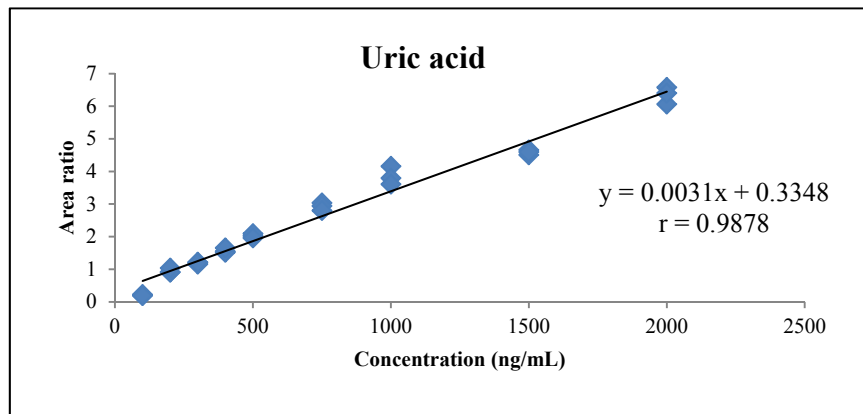


Figure 5.14 Relationship between concentration and area ratio of uric acid by LC-MS/MS triple quadrupole mass spectrometer

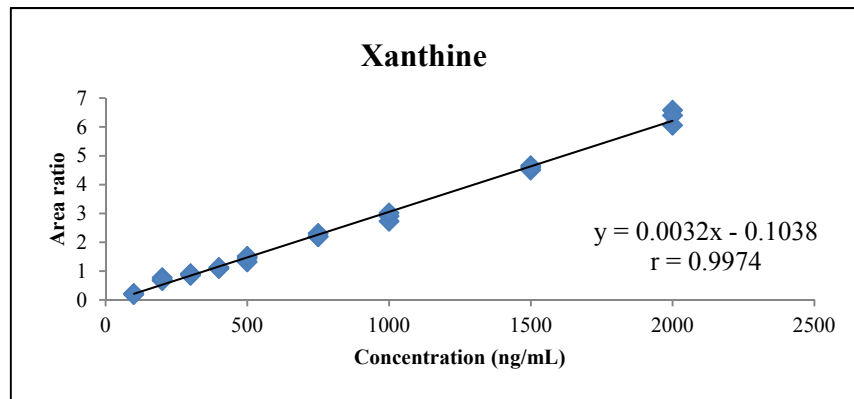


Figure 5.15 Relationship between concentration and area ratio of xanthine by LC-MS/MS triple quadrupole mass spectrometer

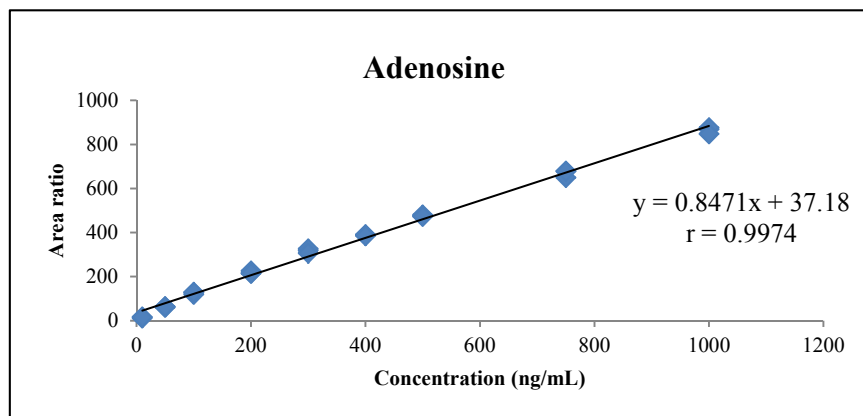


Figure 5.16 Relationship between concentration and area ratio of adenosine by LC-MS/MS triple quadrupole mass spectrometer

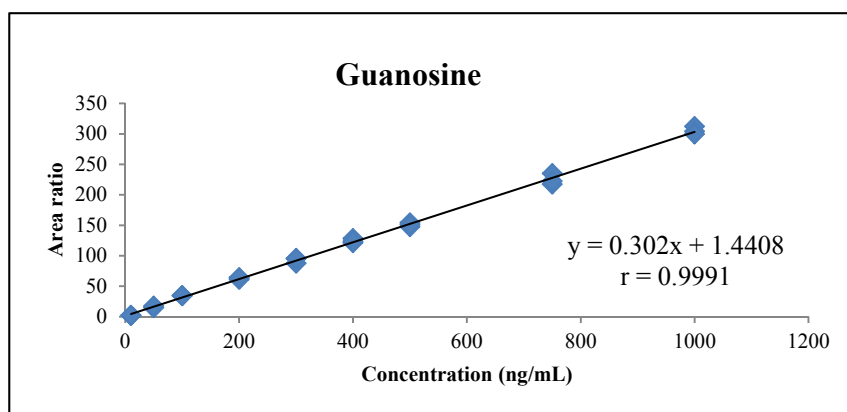


Figure 5.17 Relationship between concentration and area ratio of guanosine by LC-MS/MS triple quadrupole mass spectrometer

5.4.3 Recovery and matrix effect

The recovery from blank vegetable was evaluated by plotting the peak areas of mixture standards spiked to the filtrate of blank vegetable and then injected directly (x-axis) with mixture standards spiked to blank vegetable and passed sample preparation process before injection (y-axis). Matrix effect was evaluated by comparing the peak areas of mixture standards and then injected directly (x-axis) with mixture standards spiked to the filtrate of blank vegetable and then injected directly (y-axis). Recovery and matrix effect was evaluated from correlation equation, $y = mx$. For recovery, percent recovery was expressed as $\text{slope } m \times 100$ and matrix effect was expressed as $(m-1) \times 100$. Positive value presented to ion enhancement and negative value presented to ion suppression. Percent recovery of the method was ranged from 88-116 % while matrix effect was up to 58%. To compensate the interferences from various vegetable samples, matrix-matched calibration was utilized in real samples analysis.

Table 5.7 Summary of matrix effect

Substance	Equation	Matrix effect	Percent matrix effect
Uric acid	$Y = 1.3821x - 0.1471$	Ion enhancement	38.2
Xanthine	$Y = 1.0832x - 0.0683$	Ion enhancement	8.3
Adenosine	$Y = 1.5825x + 27.266$	Ion enhancement	58.2
Guanosine	$Y = 1.5646x + 5.4401$	Ion enhancement	56.5

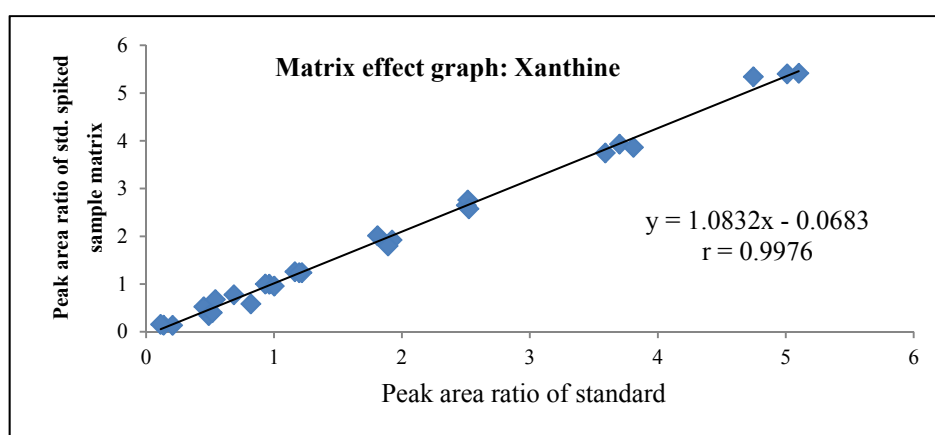


Figure 5.18 Matrix effect graph of xanthine by LC-MS/MS triple quadrupole mass spectrometer

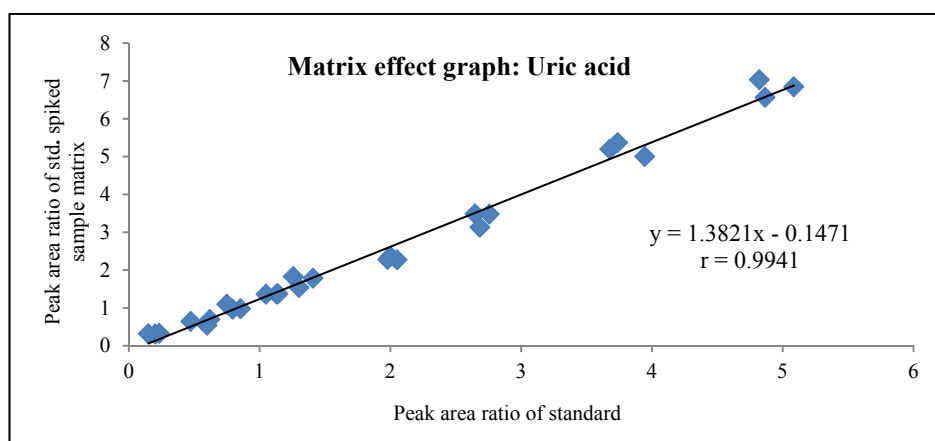


Figure 5.19 Matrix effect graph of uric acid by LC-MS/MS triple quadrupole mass spectrometer

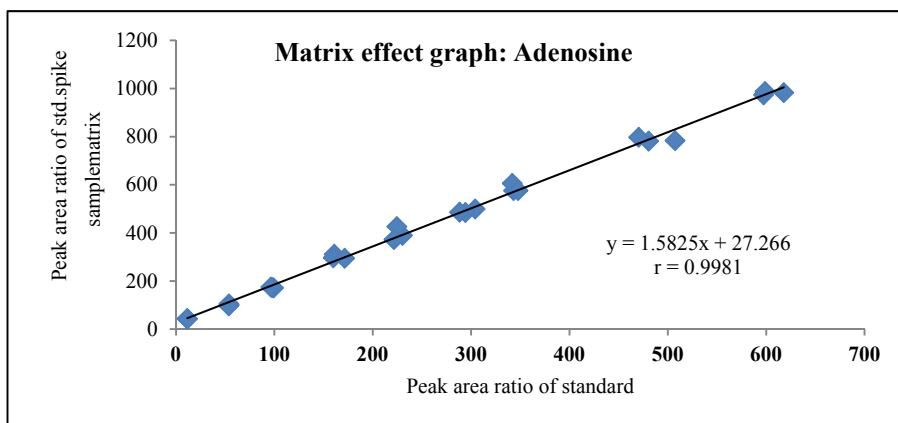


Figure 5.20 Matrix effect graph of adenosine by LC-MS/MS triple quadrupole mass spectrometer

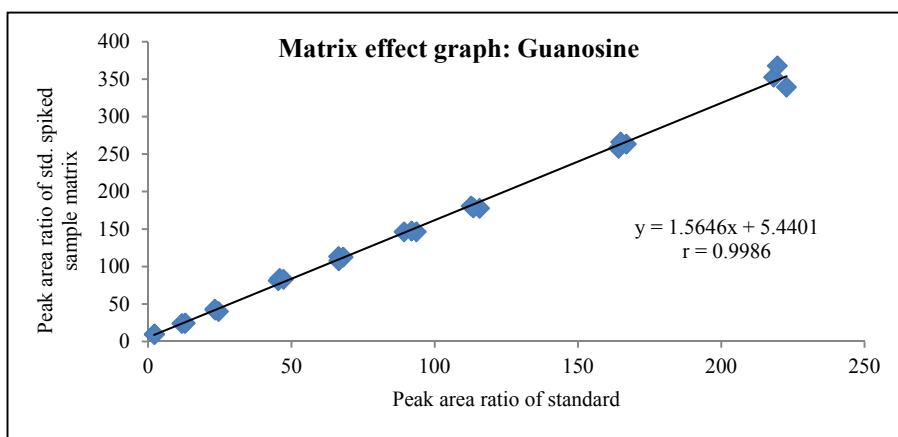


Figure 5.21 Matrix effect graph of guanosine by LC-MS/MS triple quadrupole mass spectrometer

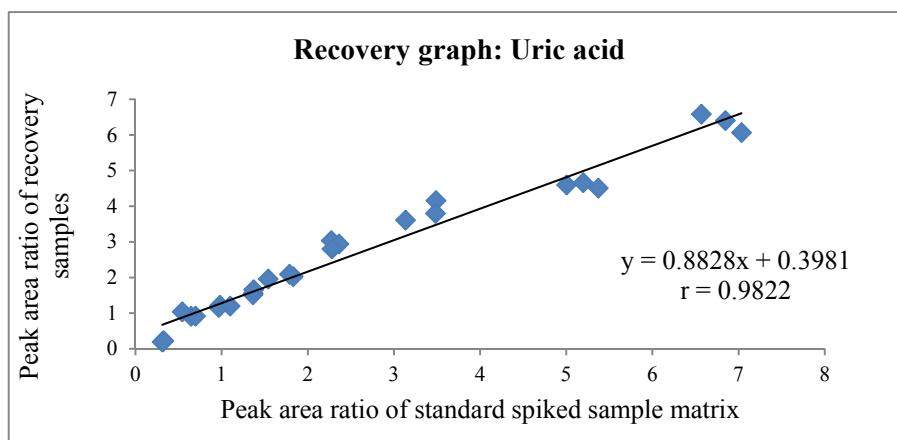


Figure 5.22 Graph extraction recovery of uric acid by LC-MS/MS triple quadrupole mass spectrometer

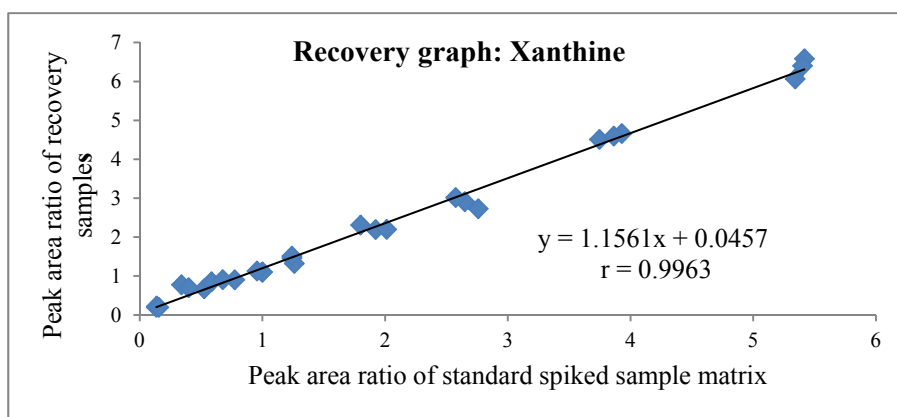


Figure 5.23 Graph extraction recovery of xanthine by LC-MS/MS triple quadrupole mass spectrometer

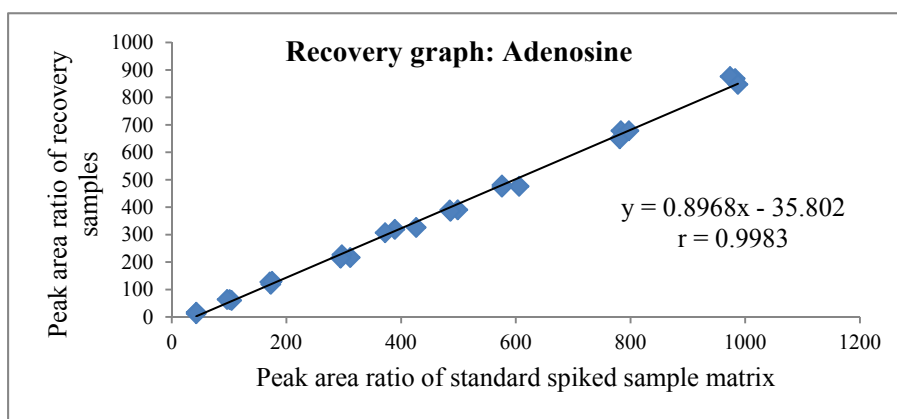


Figure 5.24 Graph extraction recovery of adenosine by LC-MS/MS triple quadrupole mass spectrometer

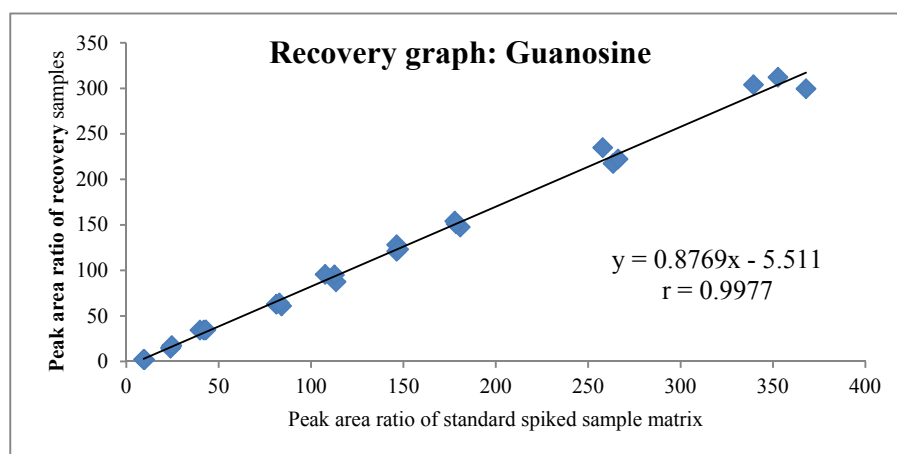


Figure 5.25 Graph extraction recovery of guanosine by LC-MS/MS triple quadrupole mass spectrometer

Table 5.8 Summaries of extraction recovery of uric acid and purines compound by LC-MS/MS triple quadrupole mass spectrometry

Substance	Equation	Recovery (%)
Uric acid	$Y = 0.8828x + 0.0004$	88.3
Xanthine	$Y = 1.1561x + 5E-05$	115.6
Adenosine	$Y = 0.8968x - 0.0358$	89.7
Guanosine	$Y = 0.8769x - 0.0055$	87.7

5.4.4 Accuracy and precision

The accuracy of the method was accepted with the recovery values of QCS were within 85.0-115.0 %. The %RSD of replicated samples in the same day (repeatability) and of three different days were within 15% except the lowest concentration that was set as lower limit of quantitation (LLOQ) was within 20%.

Table 5.9 Accuracy and precision (repeatability) of uric acid by LC-MS/MS triple quadrupole mass spectrometry

Uric acid Nominal conc. (ng/mL)	% Recovery #1	% Recovery #2	% Recovery #3	Average (n=3)	%RSD (n=3)
100 (QCS1)	103.6	84.6	83.4	90.5	12.5
200 (QCS1)	99.8	95.8	89.5	95.1	5.4
500 (QCS1)	100.7	97.5	102.7	100.3	2.6
1000 (QCS1)	107.0	98.9	106.6	104.2	4.4

Table 5.10 Accuracy and precision (repeatability) of xanthine by LC-MS/MS triple quadrupole mass spectrometry

Xanthine Nominal conc. (ng/mL)	% Recovery #1	% Recovery #2	% Recovery #3	Average (n=3)	%RSD (n=3)
100 (QCS1)	106.1	101.7	102.3	103.4	2.3
200 (QCS1)	109.0	100.9	102.6	104.2	4.1
500 (QCS1)	107.3	112.0	109.3	109.5	2.1
1000 (QCS1)	101.6	111.0	105.9	106.2	4.4

Table 5.11 Accuracy and precision (repeatability) of adenosine by LC-MS/MS triple quadrupole mass spectrometry

Adenosine Nominal conc. (ng/mL)	%Recovery #1	%Recovery #2	%Recovery #3	Average (n=3)	%RSD (n=3)
10 (QCS1)	116.2	113.1	81.0	103.4	18.9
50 (QCS1)	106.1	89.4	92.3	95.9	9.3
300 (QCS1)	100.5	104.7	104.6	103.3	2.3
1000 (QCS1)	91.9	99.5	100.5	97.3	4.8

Table 5.12 Accuracy and precision (repeatability) of guanosine by LC-MS/MS triple quadrupole mass spectrometry

Guanosine Nominal conc. (ng/mL)	% Recovery #1	% Recovery #2	% Recovery #3	Average (n=3)	%RSD (n=3)
10 (QCS1)	108.2	83.1	114.7	102.0	16.4
50 (QCS1)	94.4	93.5	96.6	94.8	1.7
300 (QCS1)	93.5	104.8	97.4	98.6	5.9
1000 (QCS1)	85.5	112.5	99.0	99.0	13.6

Table 5.13 Intermediate precision of uric acid by LC-MS/MS triple quadrupole mass spectrometry

Uric acid Nominal conc. (ng/mL)	% Recovery day 1	% Recovery day 2	% Recovery day 3	Average (n=3)	%RSD (n=3)
100 (QCS1)	97.1	99.3	103.6	100.0	3.3
200 (QCS1)	104.6	99.9	99.8	101.4	2.7
500 (QCS1)	101.9	97.4	100.7	100.0	2.3
2000 (QCS1)	99.6	98.0	107.0	107.0	4.7

Table 5.14 Intermediate precision of xanthine by LC-MS/MS triple quadrupole mass spectrometry

Xanthine Nominal conc. (ng/mL)	% Recovery day 1	% Recovery day 2	% Recovery day 3	Average (n=3)	%RSD (n=3)
100 (QCS1)	83.5	82.7	102.3	89.5	12.4
200 (QCS1)	90.5	88.7	102.6	93.9	8.1
500 (QCS1)	107.8	101.5	109.3	106.2	3.9
2000 (QCS1)	95.0	97.8	105.9	99.6	5.7

Table 5.15 Intermediate precision of adenosine by LC-MS/MS triple quadrupole mass spectrometry

Adenosine Nominal conc. (ng/mL)	% Recovery day 1	% Recovery day 2	% Recovery day 3	Average (n=3)	%RSD (n=3)
10 (QCS1)	83.5	82.7	102.3	89.5	12.4
50 (QCS1)	90.5	88.7	102.6	93.9	8.1
300 (QCS1)	107.8	101.5	109.3	106.2	3.9
1000 (QCS1)	95.0	97.8	105.9	99.6	5.7

Table 5.16 Intermediate precision of guanosine by LC-MS/MS triple quadrupole mass spectrometry

Guanosine Nominal conc. (ng/mL)	% Recovery day 1	% Recovery day 2	% Recovery day 3	Average (n=3)	%RSD (n=3)
10 (QCS1)	95.2	86.3	83.1	88.2	7.1
50 (QCS1)	105.9	101.8	93.5	100.4	6.3
300 (QCS1)	102.3	103.6	104.8	103.6	1.2
1000 (QCS1)	103.1	103.4	112.5	106.3	5.0

5.4.5 Stability

Three experiments were performed for testing stability. The first one was mixture standards spiked blank vegetable and kept at -20°C. Three freeze-thaw weekly cycles was performed. The second one was the stability of mixture standard solution at -20°C. The last one was the stability of mixture standard solution at room temperature (25°C). Peak area of each desired substance at the experiment time point was plotted as in Appendix C. The results at room temperature showed that adenosine and guanosine were stable than xanthine and uric acid. Adenosine and guanosine were stable at room temperature at least 72 h, whereas concentrations of uric acid and xanthine were less than 90% in 24 h. For the two experiments at -20°C, adenosine and guanosine were stable at least 2 weeks or 2 freeze-thaw cycles, whereas degradation of uric acid and xanthine were found higher than 10% in two weeks. The graphs of stability study were illustrated in Appendix C.

5.5 Determination of purines and uric acid in Thai vegetables

Eighteen daily consumed Thai vegetables were determined for their purines and uric acid contents. Each vegetable samples were obtained from three different sources from markets in Bangkok. Amount of uric acid and purines (adenosine, guanosine and xanthine) were quantitated by using matrix-matched

calibration method as described in experimental section. The representative graphs of matrix match calibration curve of sample were showed in Appendix D. Concentrations of uric acid and purines were reported in unit of “mg/100 g” fresh weigh and “less than LLOQ” in the case that amount found was less than LLOQ. The determination results as presented in Tables 5.18. Showed that amount of uric acid and purines in all vegetable samples were less than 50 mg/100 g. This concentration level could be classified as low purines and uric acid content meal [4]. From the current results, it was seen that vegetables invented in this study can be consumed in hyperuricemia and gouty patients. The representative chromatograms and raw data of purines and uric acid determination in vegetables samples were presented in Appendix E and F respectively.

Table 5.17 Uric acid and purine precursors contents in eighteen selected Thai vegetables

Sample	Content of uric acid and purine precursors in sample (mg/100 g)* **			
	Uric acid	Xanthine	Adenosine	Guanosine
Yardlong bean	0.2 - 0.2	3.4 - 4.9	1.1 - 1.2	0.4 - 0.6
Cucumber	ND	ND	0.1	0.1
Chinese cabbage	ND	< LLOQ	0.2 - 0.7	0.1 - 0.2
Bean sprouts	ND	< LLOQ	0.8 - 2.7	0.4 - 0.9
Water Convolvulus	0.2 - 0.5	ND	3.3 - 5.0	0.2
Winged bean	< LLOQ	0.2	1.4 - 2.9	0.2 - 0.4
Melientha suavis	0.6 - 1.3	< LLOQ - 0.2	1.1 - 2.6	0.8 - 1.8
Water minosa	ND	ND	0.4 - 0.7	1.3 - 1.5
White popinac	0.2 - 0.5	< LLOQ	0.1 - 0.3	1.6 - 6.2
Acacia pennata	ND	0.0 - 0.8	0.1 - 0.2	1.8 - 3.5
Ivy Gourd	ND	0.1	0.9 - 4.7	0.6 - 1.5
Asiatic Pennywort	ND	ND	1.2 - 2.1	0.2 - 0.3
Malabar nightshade	7.8 - 10.1	<LLOQ	0.6 - 1.2	0.2 - 0.4
Yellow velvet leaf	7.8 - 10.1	0.2	1.2 - 2.0	0.6 - 0.7
Vietnamese coriander	<LLOQ	< LLOQ	0.1	0.1 - 0.2
Thai eggplant	<LLOQ	ND	< LLOQ	0.4 - 0.5
Stink Bean	ND	ND	0.8-1.6	0.3-1.3
Bamboo shoot	ND	0.2-0.3	0.4-0.9	0.4-2.9

*ND: not detected

**LLOQ: adenosine and guanosine 0.02 mg/100 g and uric acid and xanthine 0.2 mg/100 g of fresh weight

CHAPTER VI

CONCLUSIONS

In this study, an electrospray ionization LC-MS/MS triple quadrupole method was successfully developed for determination of purines (xanthine, adenosine and guanosine) and uric acid in vegetables using caffeine as an internal standard. The successive LC-MS/MS condition was achieved by using a mobile phase mixture of 0.2% formic acid in deionized water (solvent A) and 0.1% formic acid in methanol (solvent B). The gradient elution was utilized with the total run time of 8.50 minute. Both negative and positive ionization modes were employed. Adenosine, guanosine and caffeine were ionized and detected in positive mode while, xanthine and uric acid were ionized and detected in negative mode.

The developed an LC-MS/MS method was validated to prove for its intended purpose. The validation parameter i.e., linearity, accuracy, precision, matrix effect, stability, lower limit of quantitation (LLOQ) and specificity were evaluated and accepted according to US-FDA guideline.

Finally, the developed and validated LC-MS/MS method was applied to quantitative analyze of uric acid and purine precures contents in eighteen selected widely consumed Thai vegetables. The results showed that all vegetables samples contained uric acid and purine precursors less than 50 mg/100 g of fresh weight, which could be classified as very low uric acid and purine precursors contents.

The results from this study were useful information to hyperuricemia and gouty patients. In addition, this is the first official study of purines and uric acid determination in Thai vegetables.

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APPENDICES

APPENDIX A

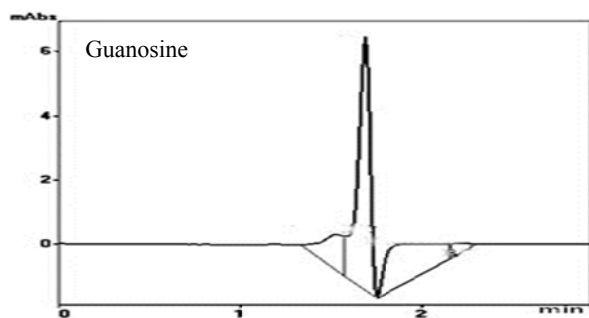
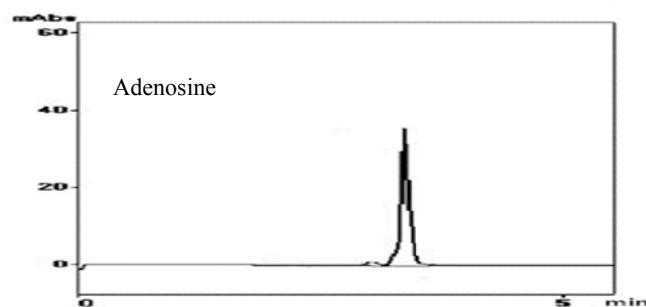
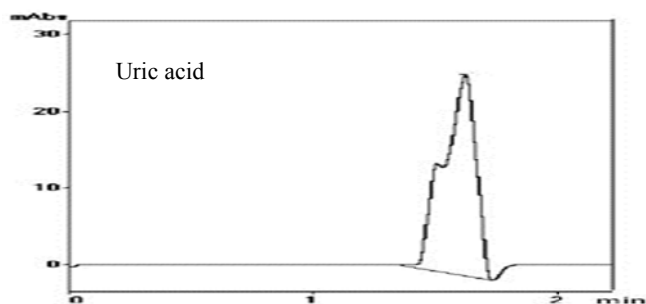
HPLC CHROMATOGRAMS OF STANDARD URIC ACID AND PURINE PRECURSORS

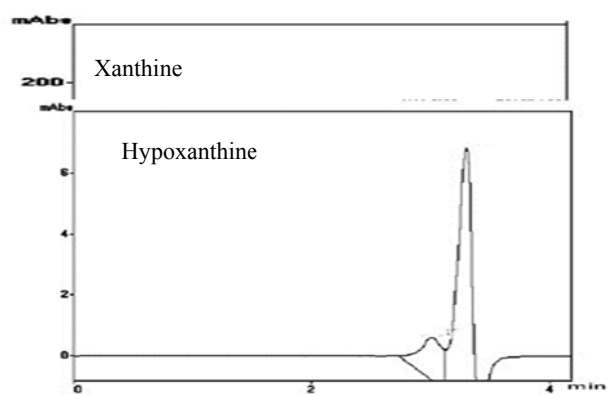
Mobile phase condition 1

A: 10 mM ammonium acetate buffer, pH 4.5; (95%)

B: Acetonitrile; (5%)

HPLC-UV chromatogram of standard uric acid and purine precursors



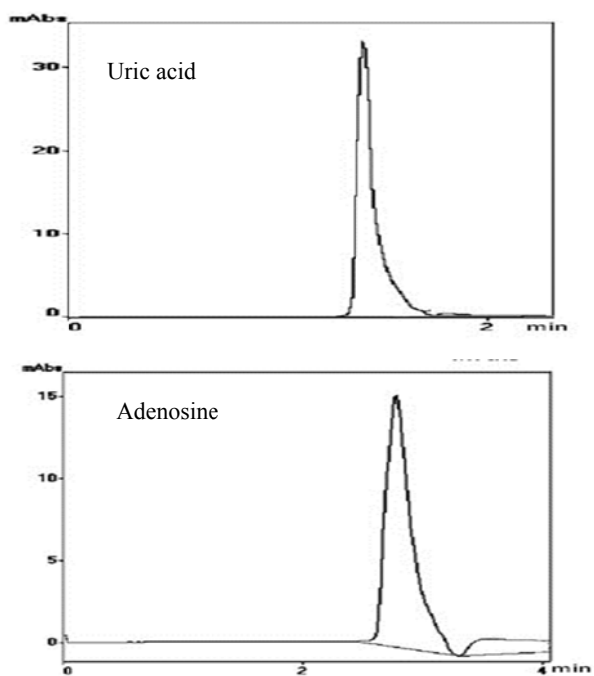


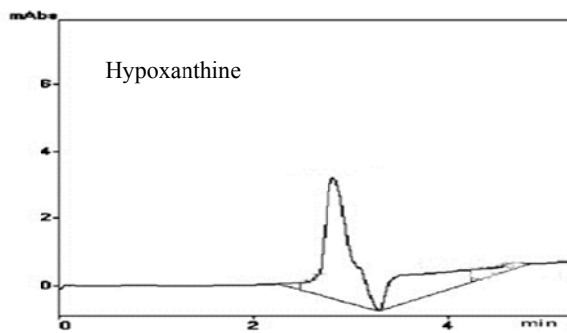
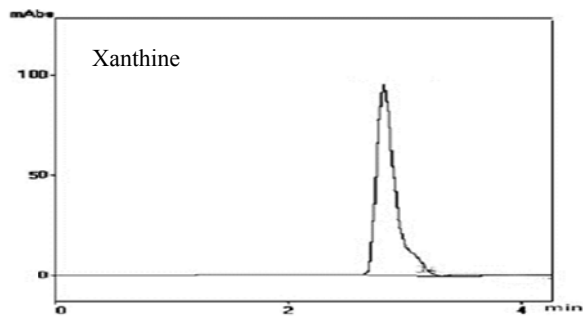
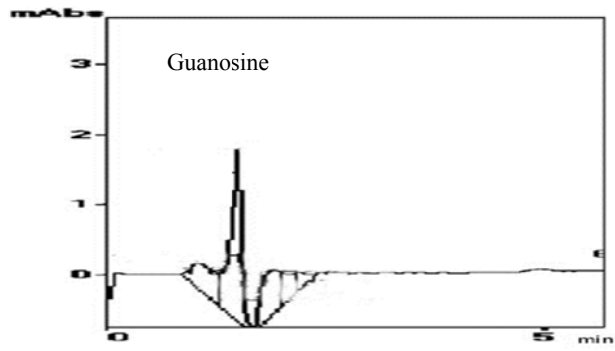
Mobile phase condition 2

A: 10 mM ammonium acetate buffer, pH 4.5; (60%)

B: Acetonitrile; (40%)

HPLC-UV chromatogram of standard uric acid and purine precursors



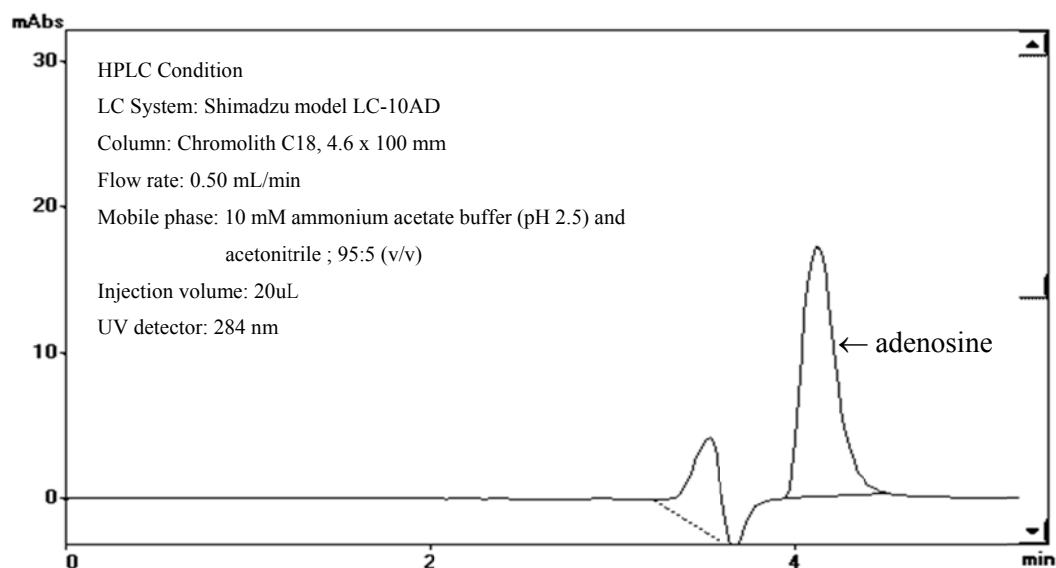
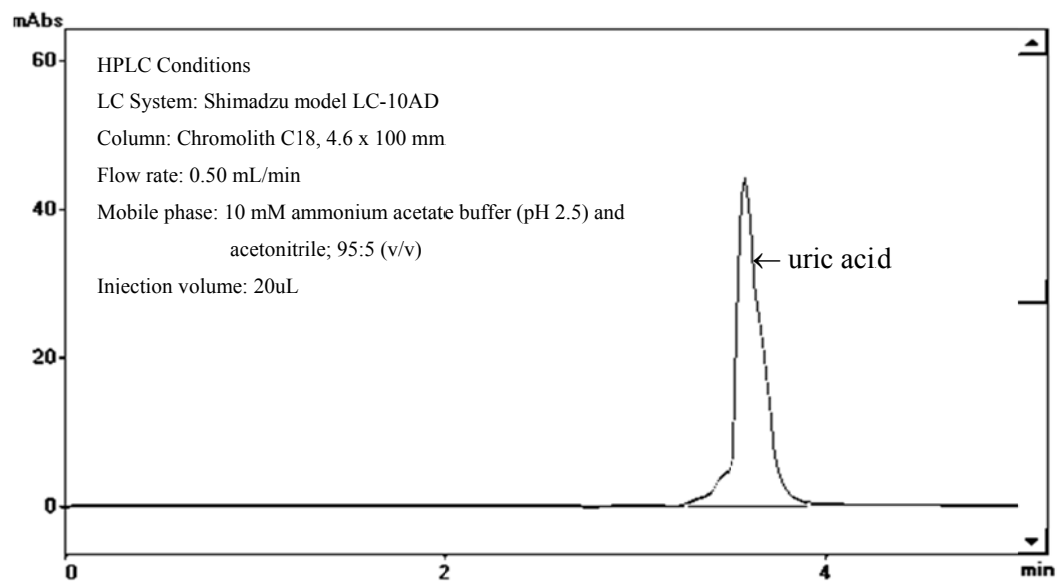


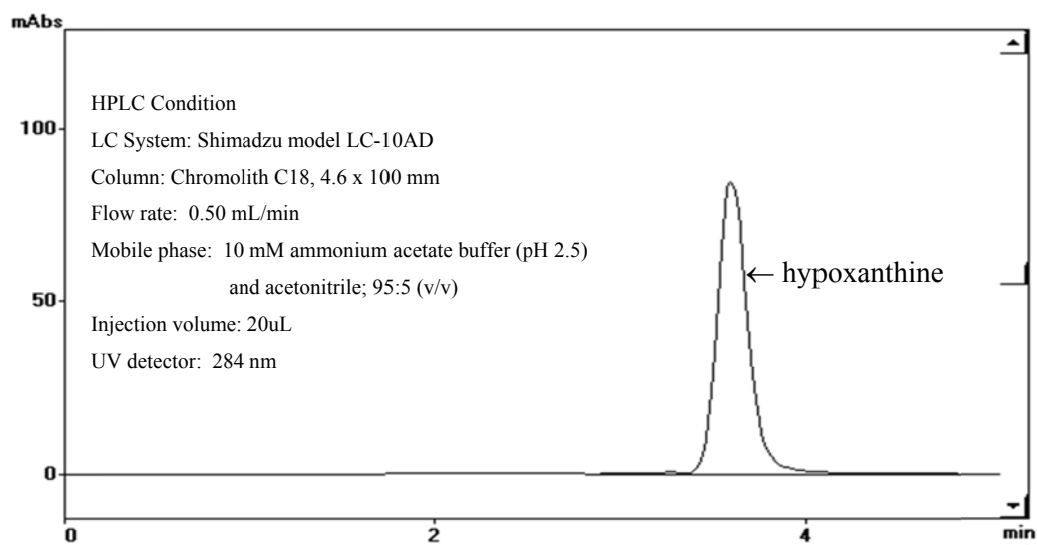
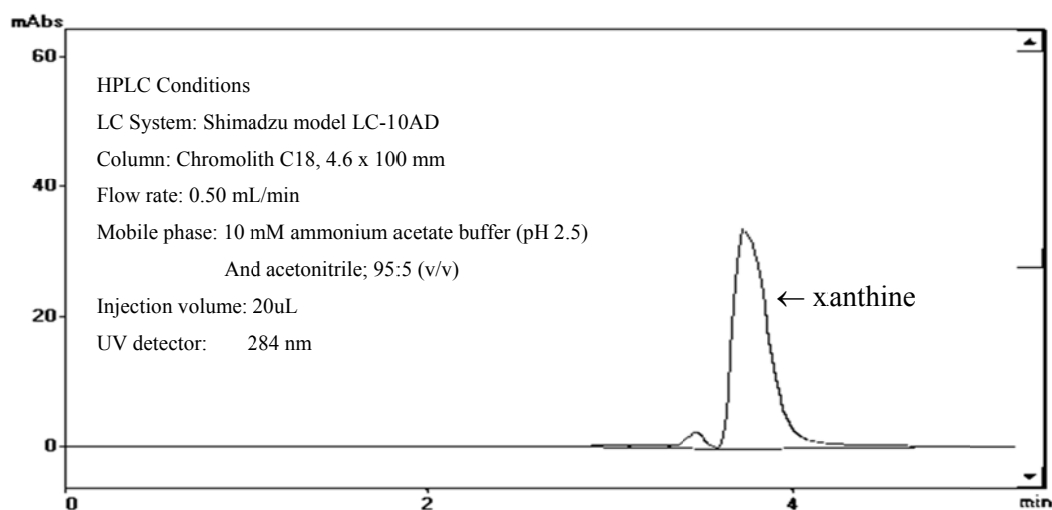
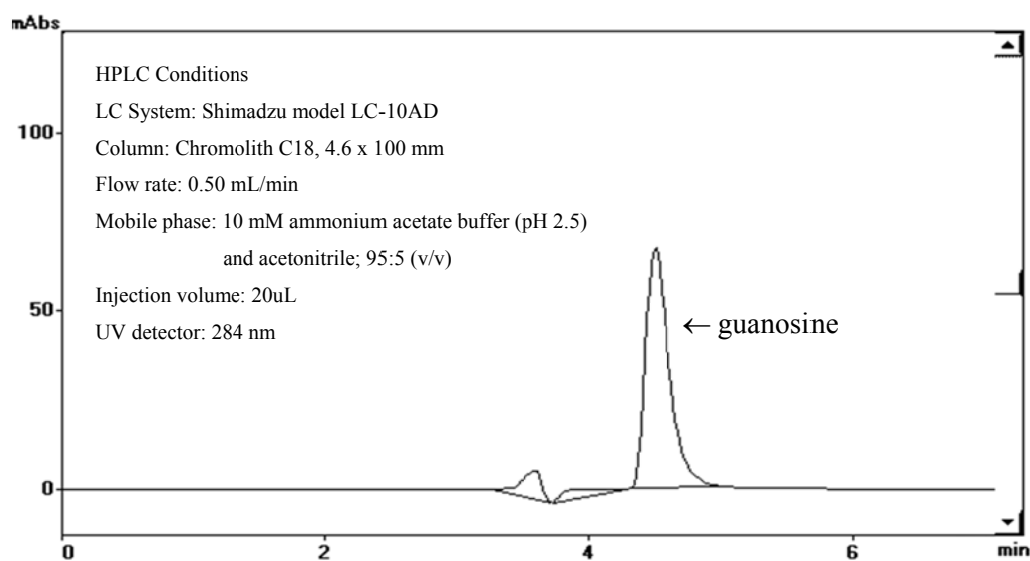
Mobile phase condition 3

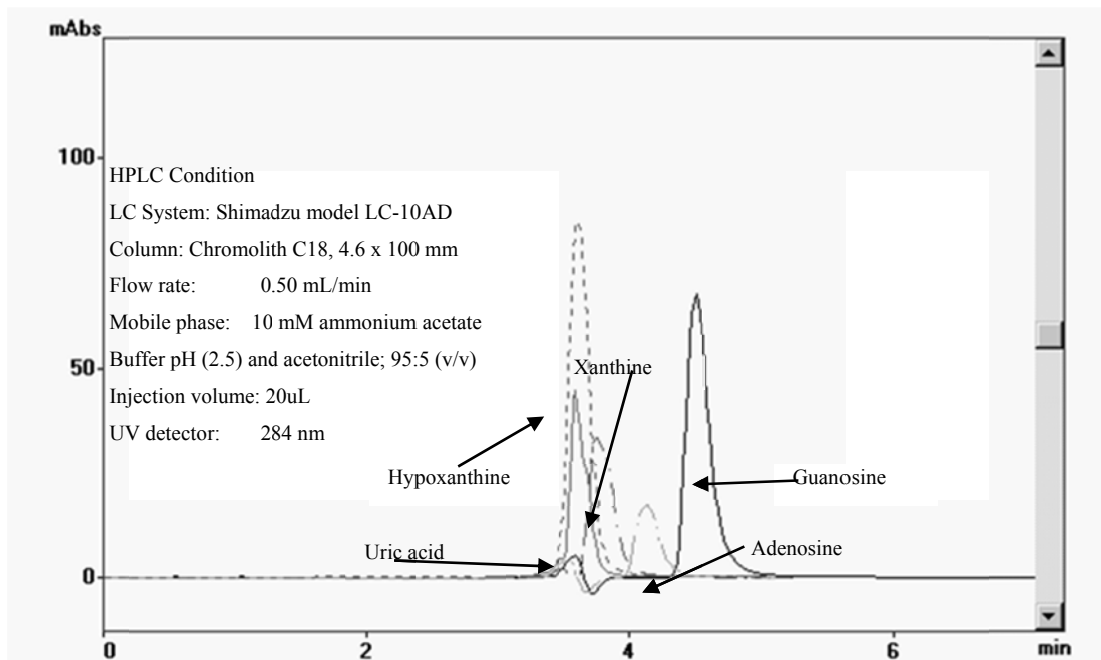
A: 10 mM ammonium acetate buffer, pH 2.5; (95%)

B: Acetonitrile; (5%)

HPLC-UV chromatogram of standard uric acid and purine precursors







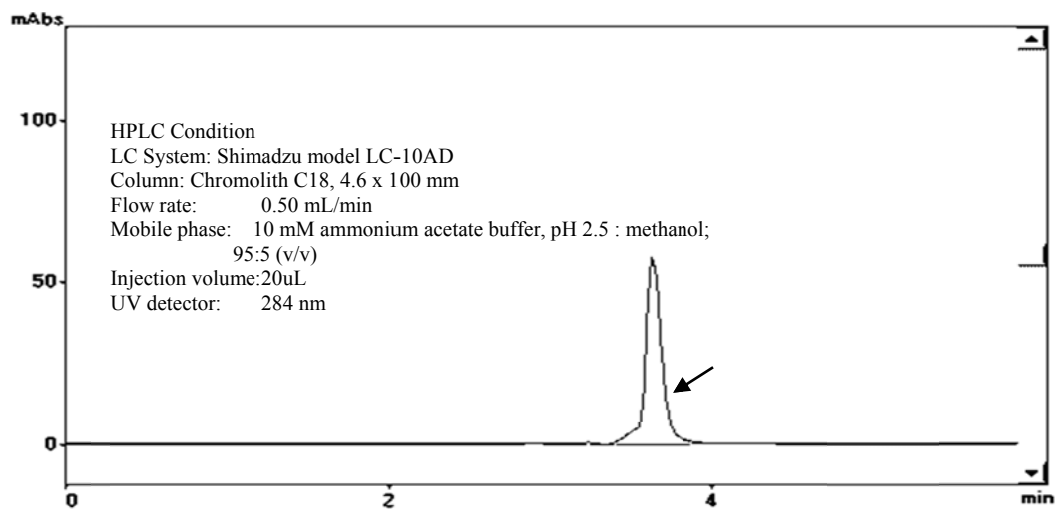
Overlaid chromatogram of standard uric acid and purine precursors in 10 mM ammonium acetate (pH2.5) : acetonitrile; (95:5, v/v)

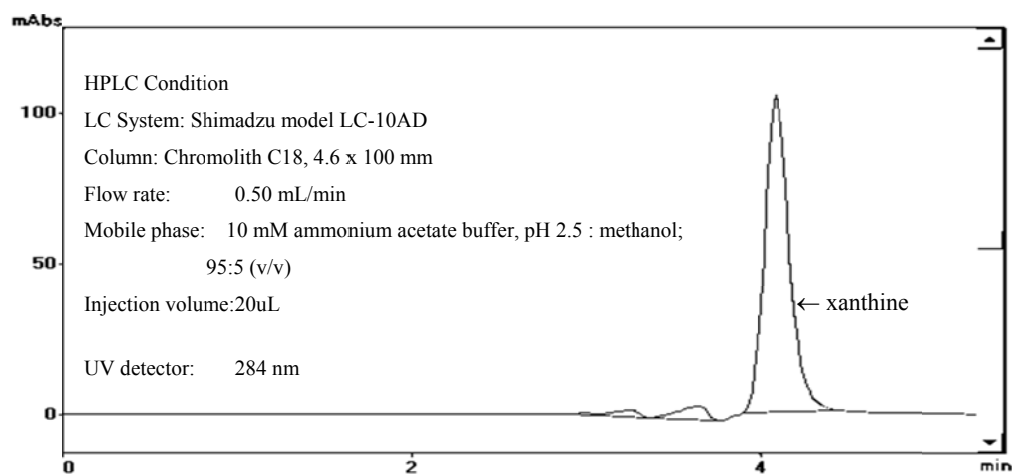
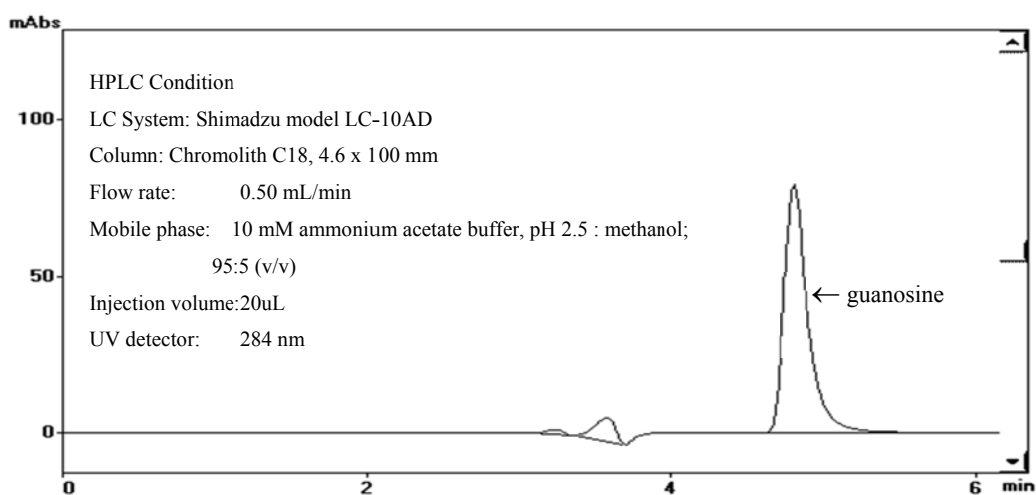
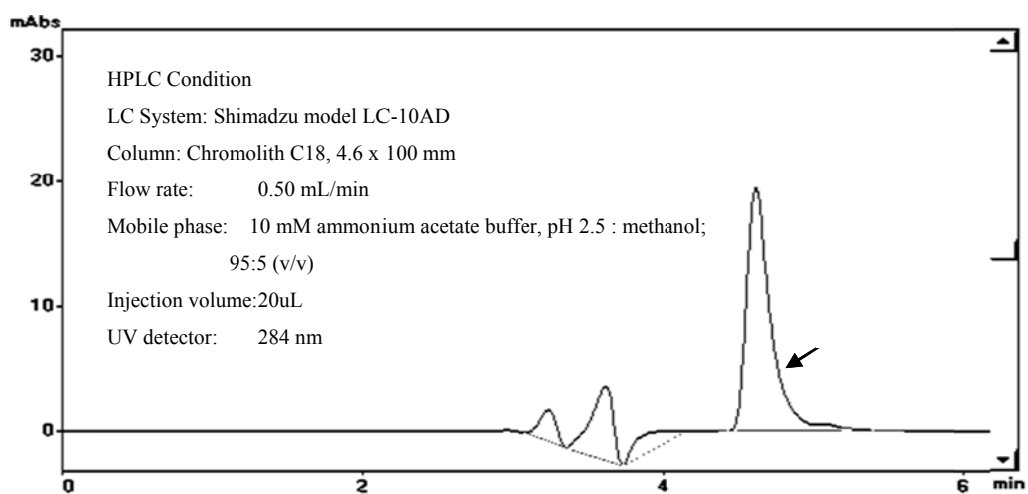
Mobile phase condition 4

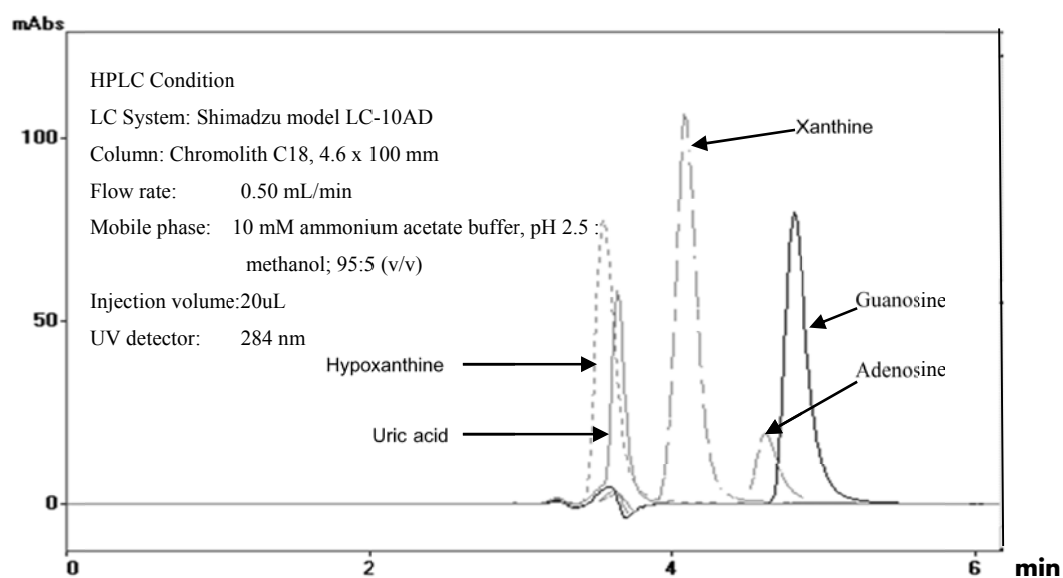
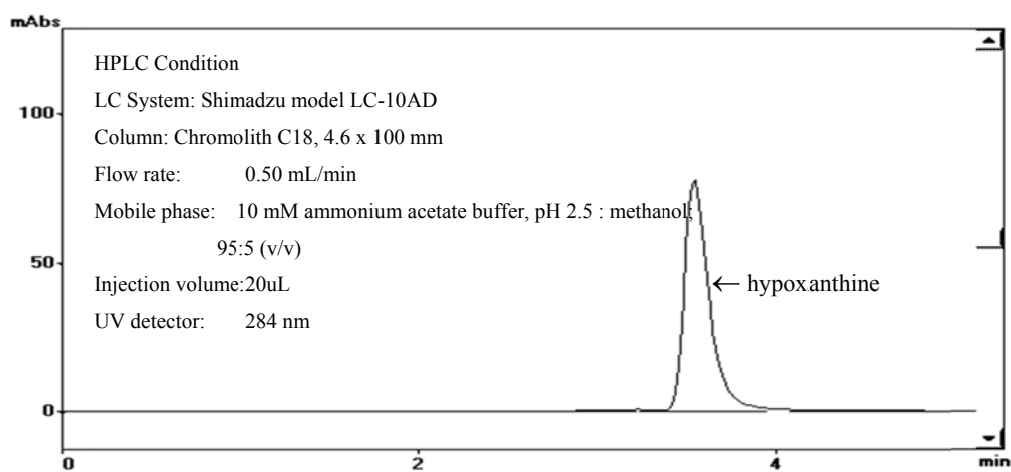
A: 10 mM ammonium acetate buffer, pH 2.5; (95%)

B: Methanol; (5%)

HPLC-UV chromatogram of standard uric acid and purine precursors







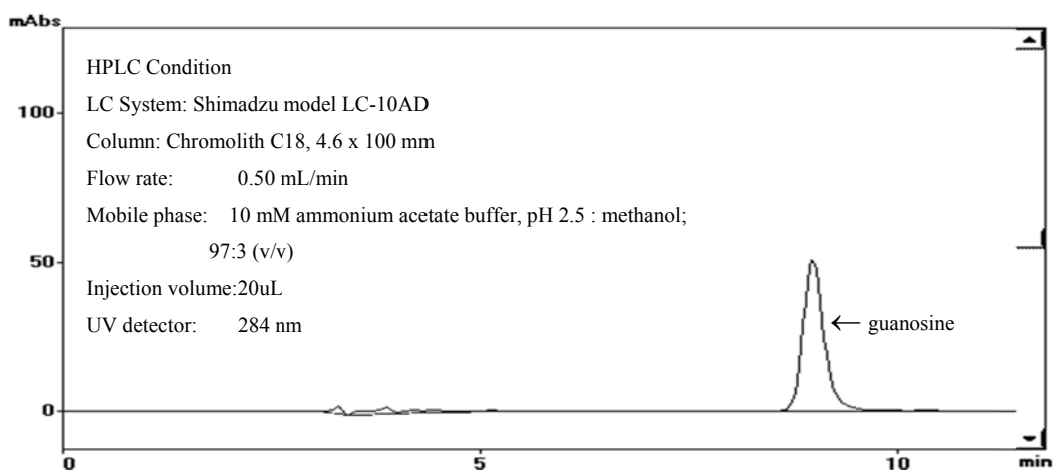
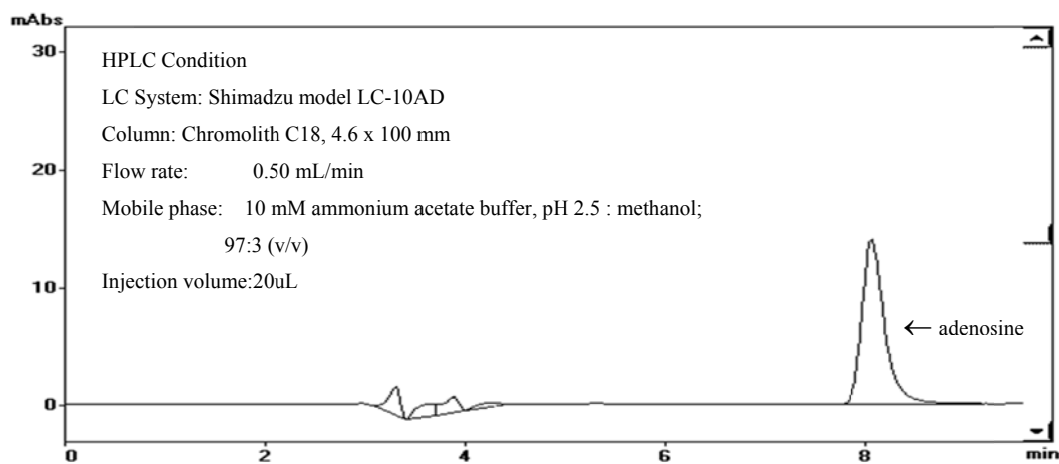
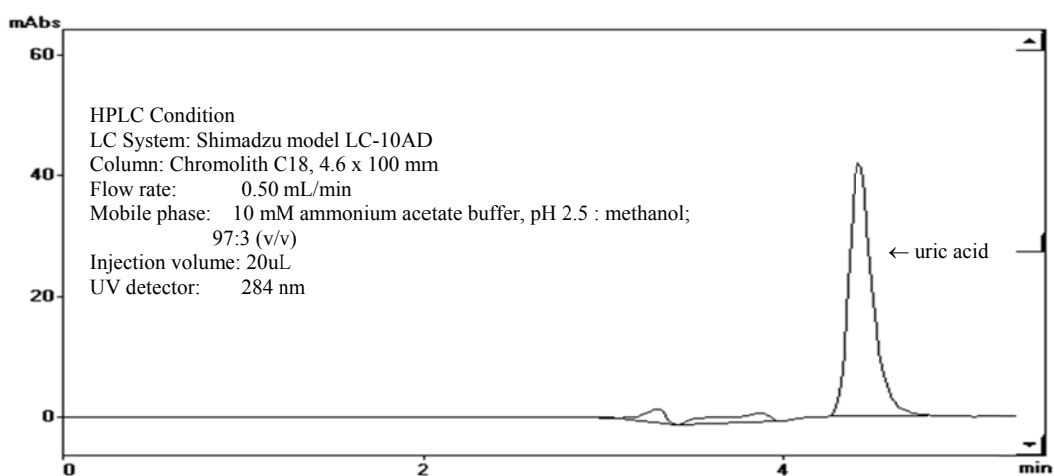
Overlaid chromatograms of uric acid and purines compound in 10 mM ammonium acetate (pH2.5) : methanol; (95:5, v/v)

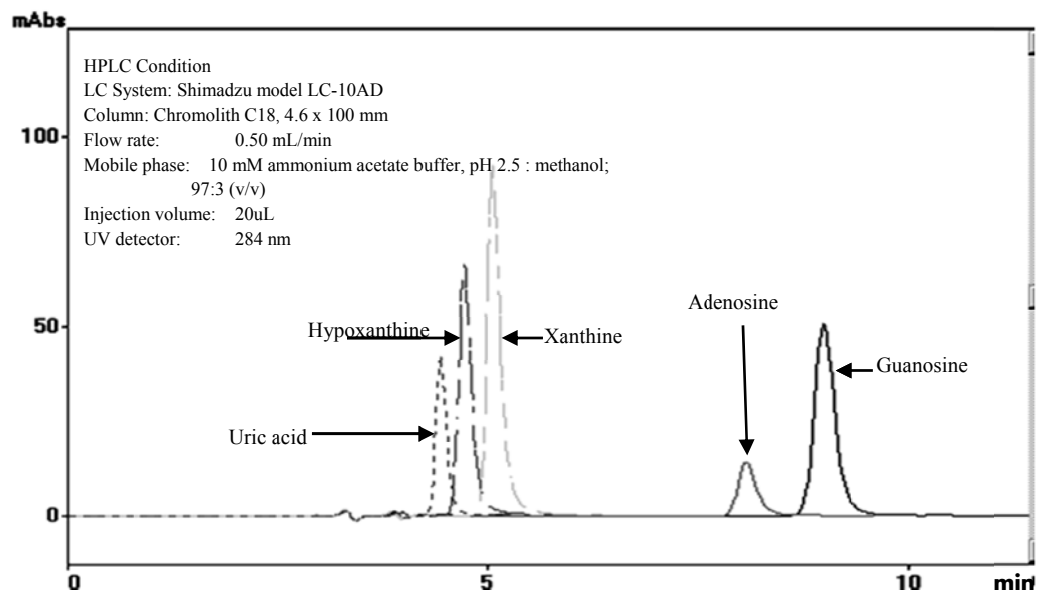
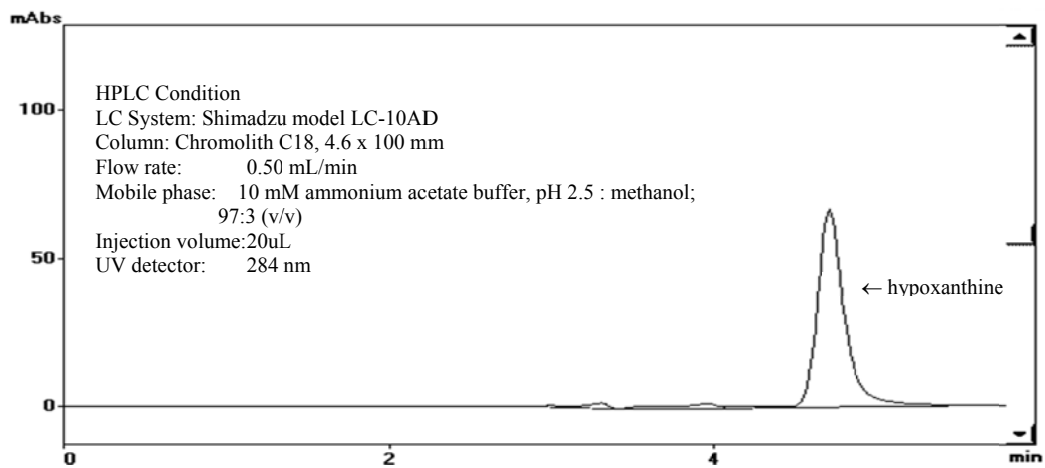
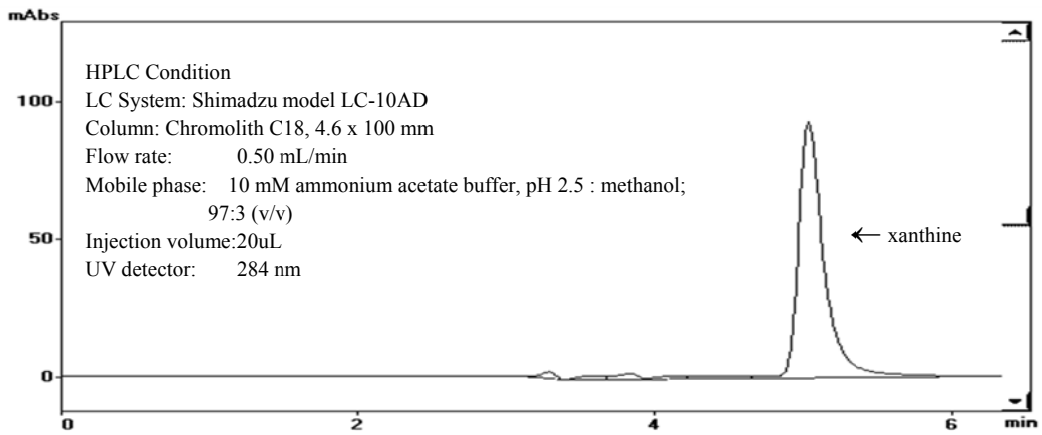
Mobile phase condition 5

A: 10 mM ammonium acetate buffer, pH 2.5; (97%)

B: Methanol; (3%)

HPLC-UV chromatogram of standard uric acid and purine precursors





Overlaid chromatograms of uric acid and purines compound in 10 mM ammonium acetate (pH2.5): methanol; (97:3, v/v)

APPENDIX B
METHOD VALIDATION RESULTS OF LC-MS/MS ION-TRAP
MASS SPECTROMETRY

1. Specificity

Specificity of the method was obtained by comparing the retention time of analyte and its standard. Analyte must be presented at the same retention time of its corresponding standard.

Compound	Retention time (min)	Transition ion	CID (eV)
Uric acid	5.07	167 >> 124	33
Xanthine	6.39	151 >> 108	30
Adenosine	7.89	268 >> 136	28
Guanosine	10.78	284 >> 152	22
Dextromethorphan (IS)	12.37	272>>215	40

2. Linearity

Substance	Equation	r
Uric acid	$Y=0.0033X+0.0018$	0.9996
Xanthine	$Y=0.0002X+0.0011$	0.9974
Adenosine	$Y=0.0575X-0.1636$	0.9918
Guanosine	$Y=0.0551X-0.1354$	0.9746

Linearity graph was plotted between concentrations (x-axis) and their corresponding peak area ratios of total ion chromatography (TIC) and internal standard (IS) (y-axis). Least-square regression method was employed.

3. Recovery and matrix effect

Recovery and matrix effect was evaluated from correlation equation, $y = mx$. For recovery, percent recovery was expressed as $\text{slope (m)} \times 100$ and matrix effect was expressed as $(m - 1) \times 100$. Positive value presented to ion enhancement and negative value presented to ion suppression.

Matrix effect was evaluated by comparing the peak areas of mixture standards and then injected directly (x-axis) with mixture standards spiked to filtrate of blank vegetable and then injected directly (y-axis).

Recovery from blank vegetable was evaluated by plotting the peak areas of mixture standards spiked to the filtrate of blank vegetables and then injected directly (x-axis) with mixture standards spike to blank vegetable and passed sample preparation process before injection (y-axis).

Matrix effect

substance	Equation	Matrix effect	Percent matrix effect
Uric acid	$Y=0.9634X-0.0007$	Ion suppression	3.66
Xanthine	$Y=1.0448X-0.0003$	Ion enhancement	4.48
Adenosine	$Y=1.0094X-0.0535$	Ion enhancement	0.94
Guanosine	$Y=1.0615X+0.1442$	Ion enhancement	6.16

Recovery

Substance	Equation	Percent recovery
Uric acid	$Y=0.3831X-0.0023$	38.3
Xanthine	$Y=0.3798X-0.0005$	38.0
Adenosine	$Y=0.4838X-0.1040$	48.4
Guanosine	$Y=0.4464+0.3071$	44.6

4. Accuracy and precision (repeatability)

The precision of method validation was calculated in term of relative percent difference (% RPD).

Where, $\% \text{ RPD} = ((\text{max}-\text{min})/\text{mean}) \times 100$

The % RPD must be within 15%, except at LLOQ level that % RPD must be within 20%.

The accuracy of the method was expressed in term of % bias.

Where, $\% \text{ Bias} = [(\text{Found conc.} / \text{Nominal conc.}) - 1] \times 100$

The % RPD must be within 15%, except at LLOQ level that % RPD must be within 20%.

Accuracy and precision of uric acid

Uric acid Nominal conc. ($\mu\text{g/mL}$)	Sample 1 Found conc ($\mu\text{g/mL}$)	% Bias	Sample 2 Found conc ($\mu\text{g/mL}$)	% Bias	% RPD
5.0	4.75	-5.06	4.66	-6.84	1.90
10.0	8.69	-13.12	8.95	-10.48	2.99
20.0	19.45	-2.77	19.88	-0.59	2.22
40.0	38.88	-2.80	41.48	3.71	6.48

Accuracy and precision of xanthine

Xanthine Nominal conc. ($\mu\text{g/mL}$)	Sample 1 Found conc ($\mu\text{g/mL}$)	% Bias	Sample 2 Found conc ($\mu\text{g/mL}$)	% Bias	% RPD
5.0	5.58	11.50	5.10	2.07	8.83
10.0	10.21	2.08	11.82	18.20	14.64
20.0	16.88	-15.58	16.11	-19.47	4.72
40.0	35.65	-10.88	42.69	6.73	17.98

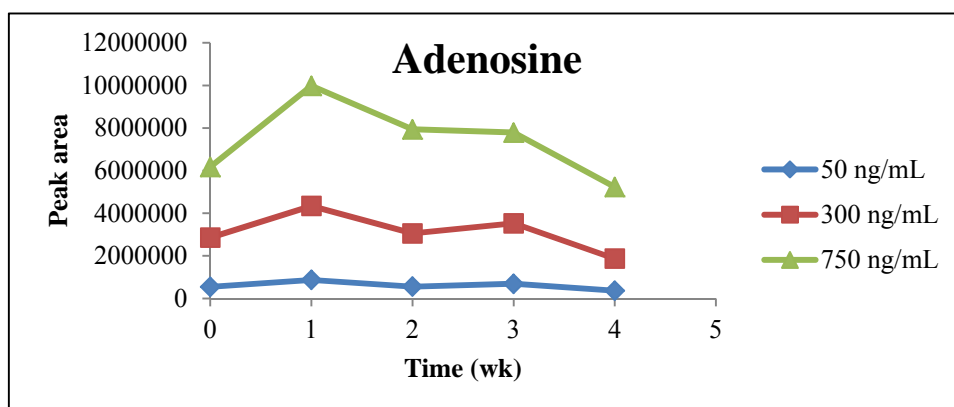
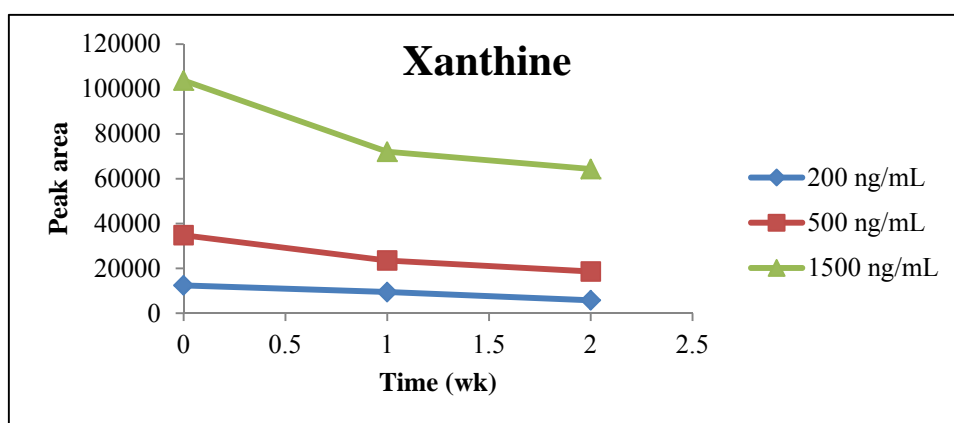
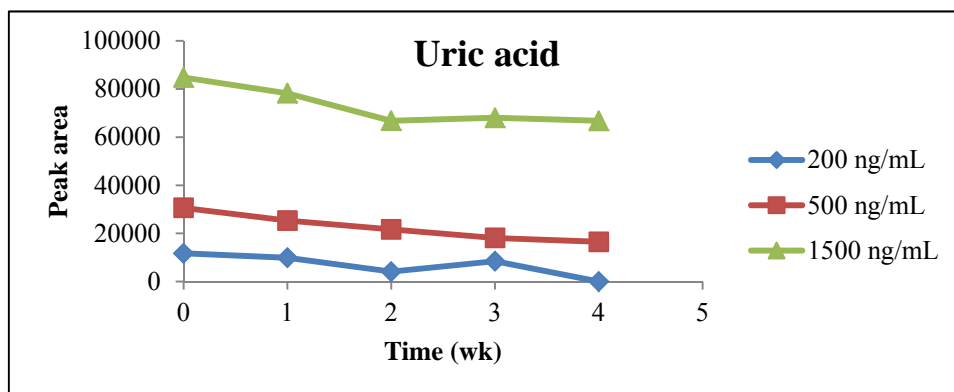
Accuracy and precision of guanosine

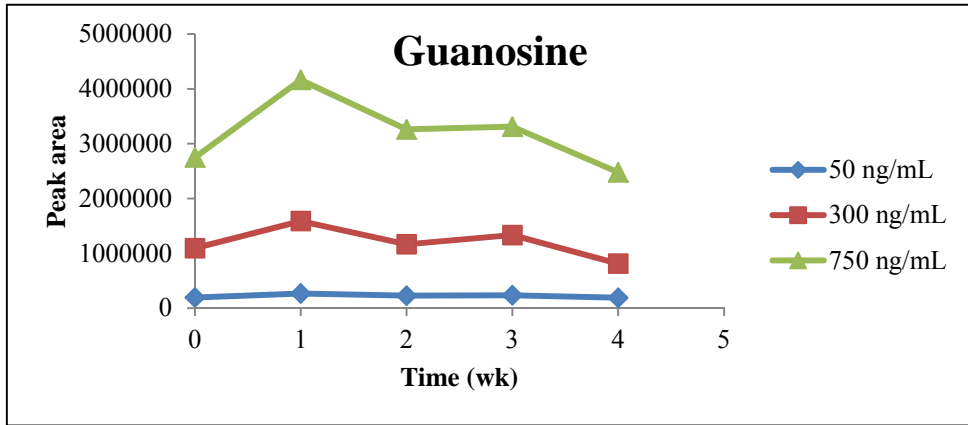
Guanosine Nominal conc. ($\mu\text{g/mL}$)	Sample 1 Found conc. ($\mu\text{g/mL}$)	% Bias	Sample 2 Found conc.($\mu\text{g/mL}$)	% Bias	% RPD
5.0	3.39	-32.17	6.18	23.54	58.22
10.0	9.38	-6.16	12.70	27.00	30.03
20.0	18.91	-5.46	22.49	12.43	17.29
40.0	39.30	-1.75	39.43	-1.42	0.34

APPENDIX C

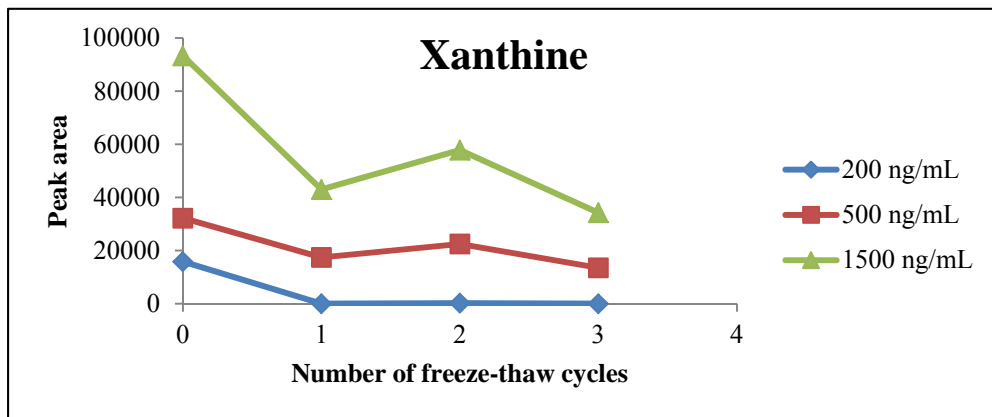
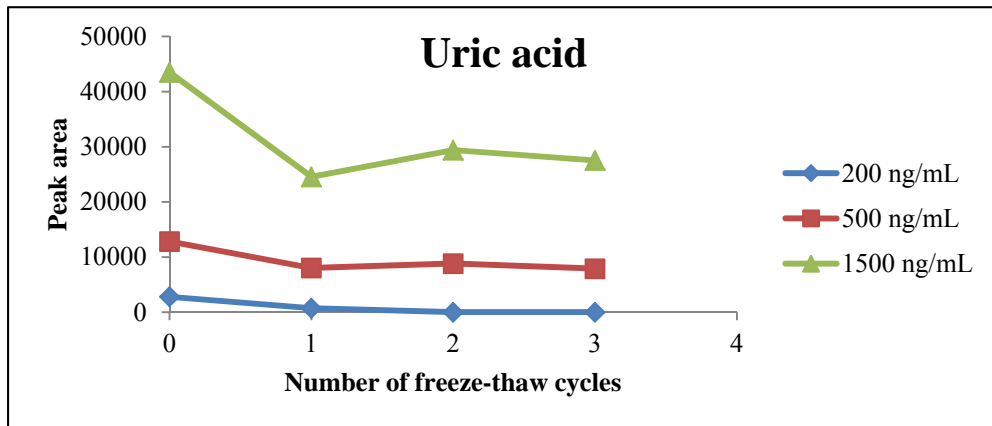
STABILITY STUDY

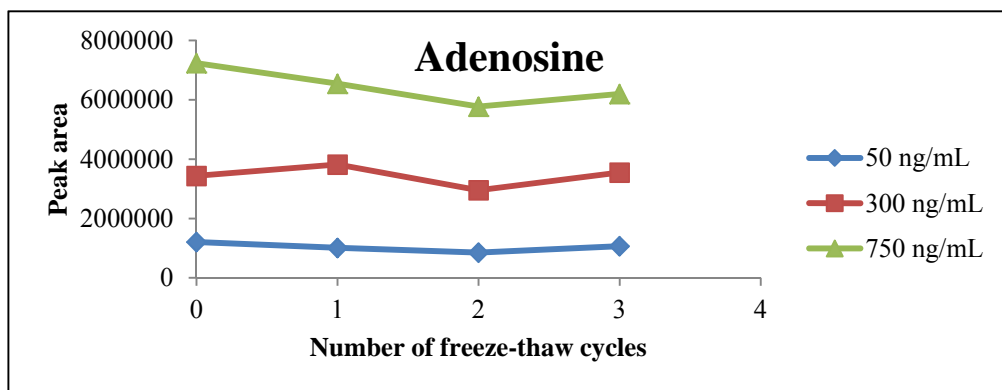
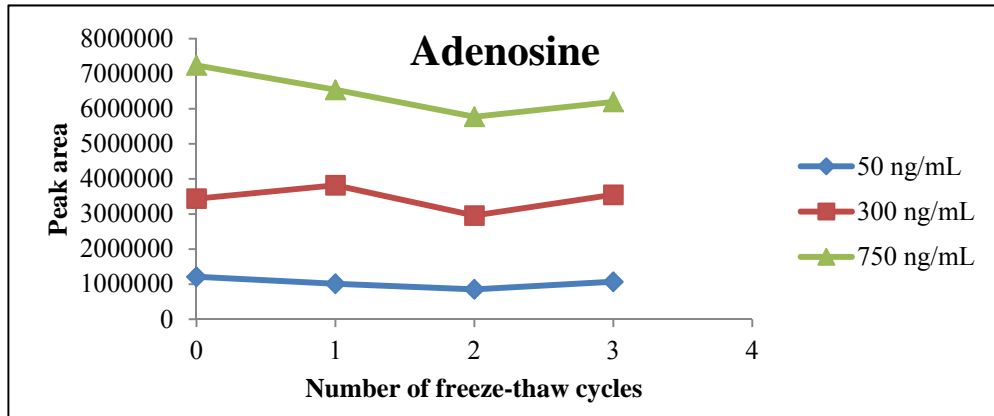
1. Stability of standard mixture solution at -20°C for 1 month



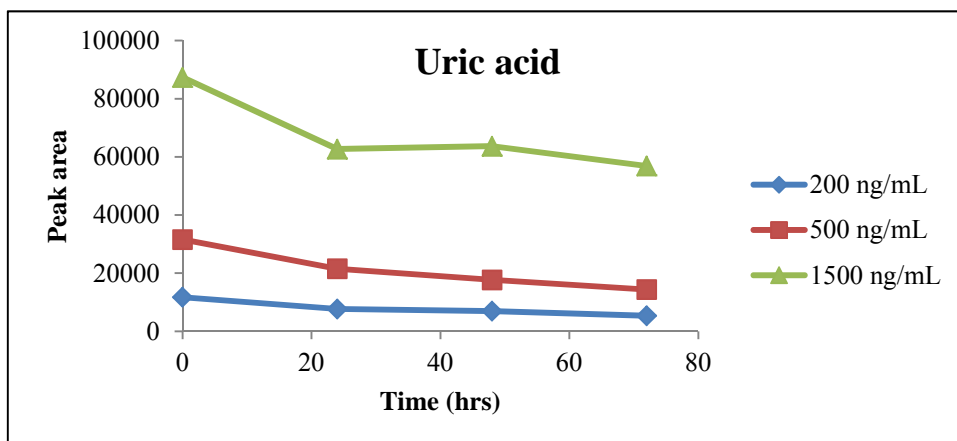


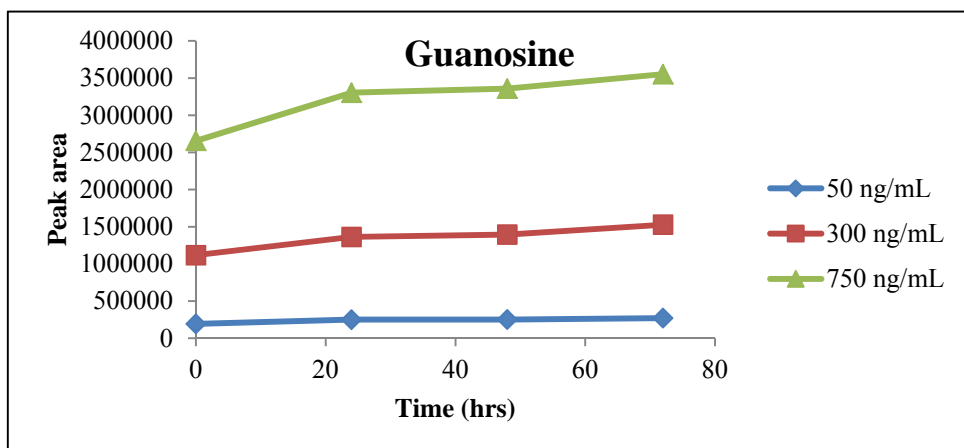
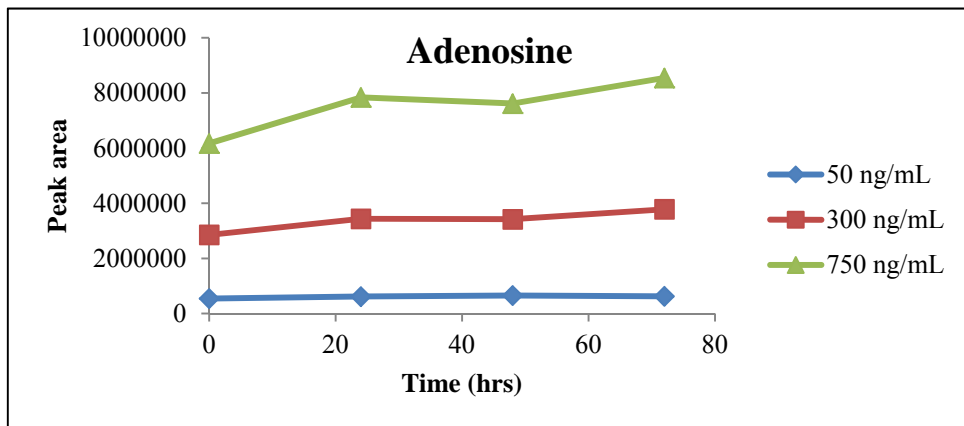
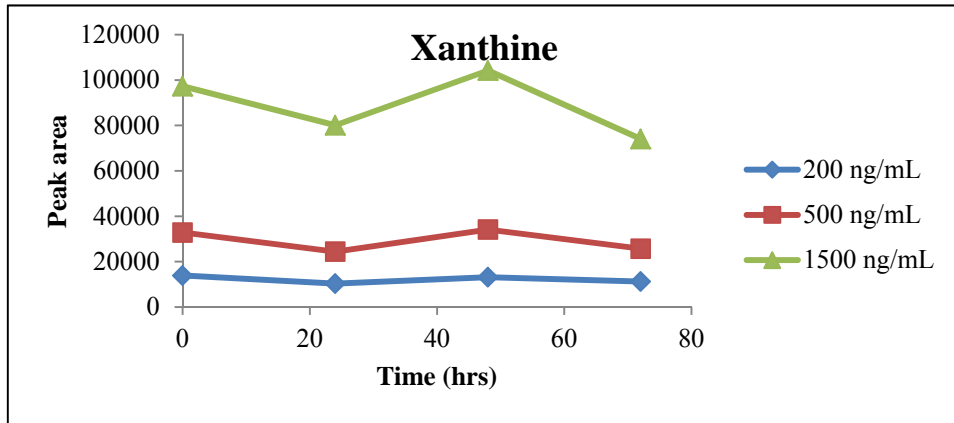
2. Stability of standard mixture spike blank vegetable at -20°C (three freeze thaw cycle)





3. Stability of standard mixture solution at room temperature (25°C)



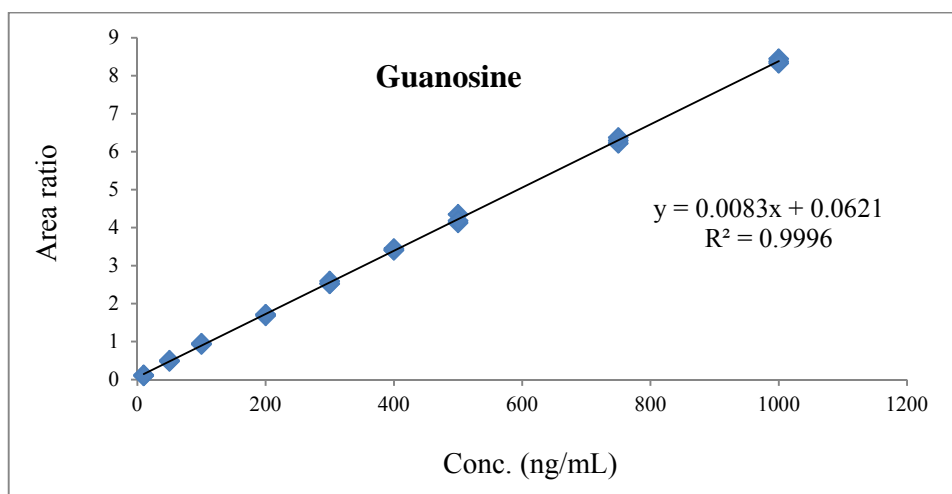
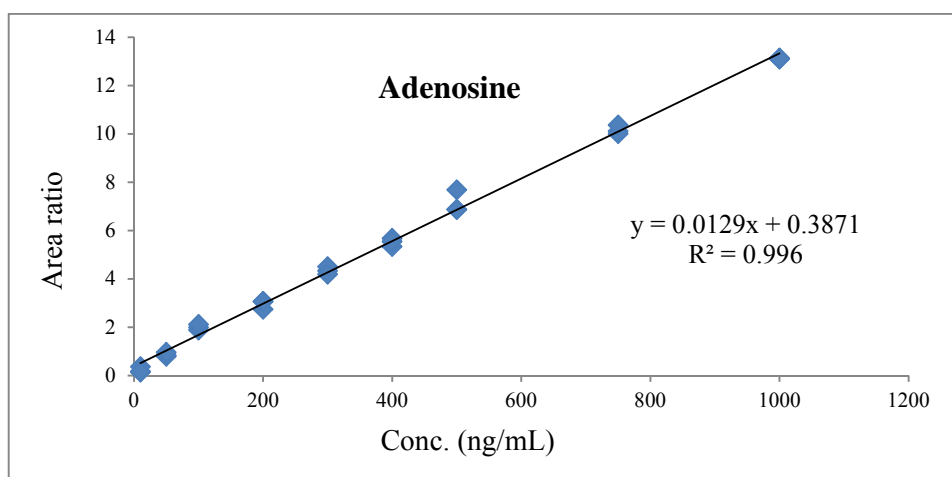


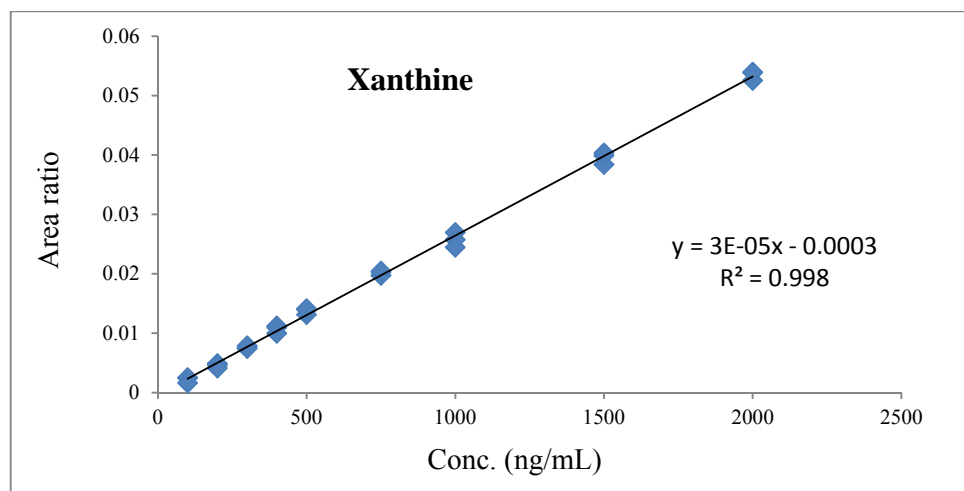
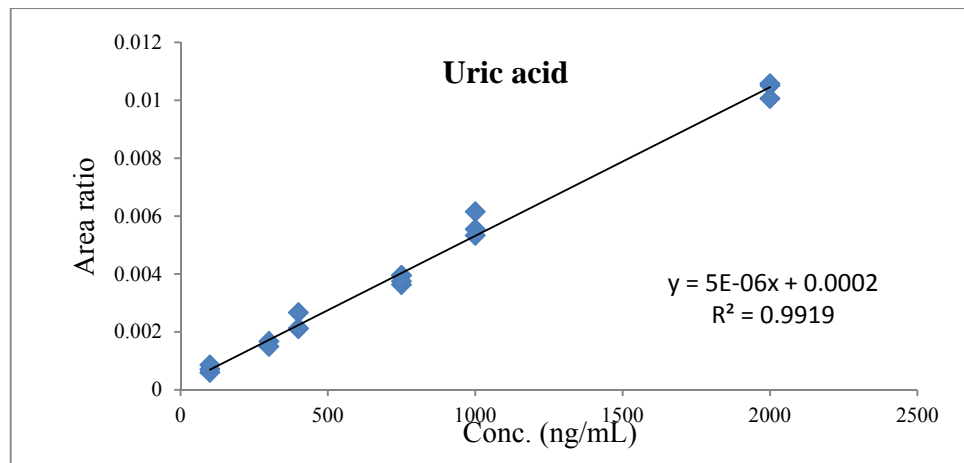
APPENDIX D

MATRIX MATCH CALIBRATION METHOD FROM LC-MS/MS

TRIPLE QUADRUPOLE MASS SPECTROMETRY

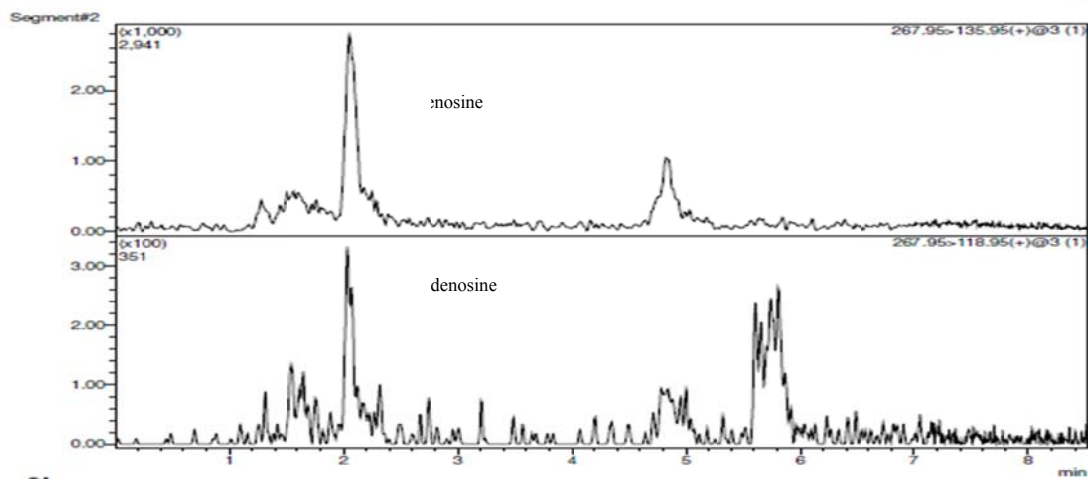
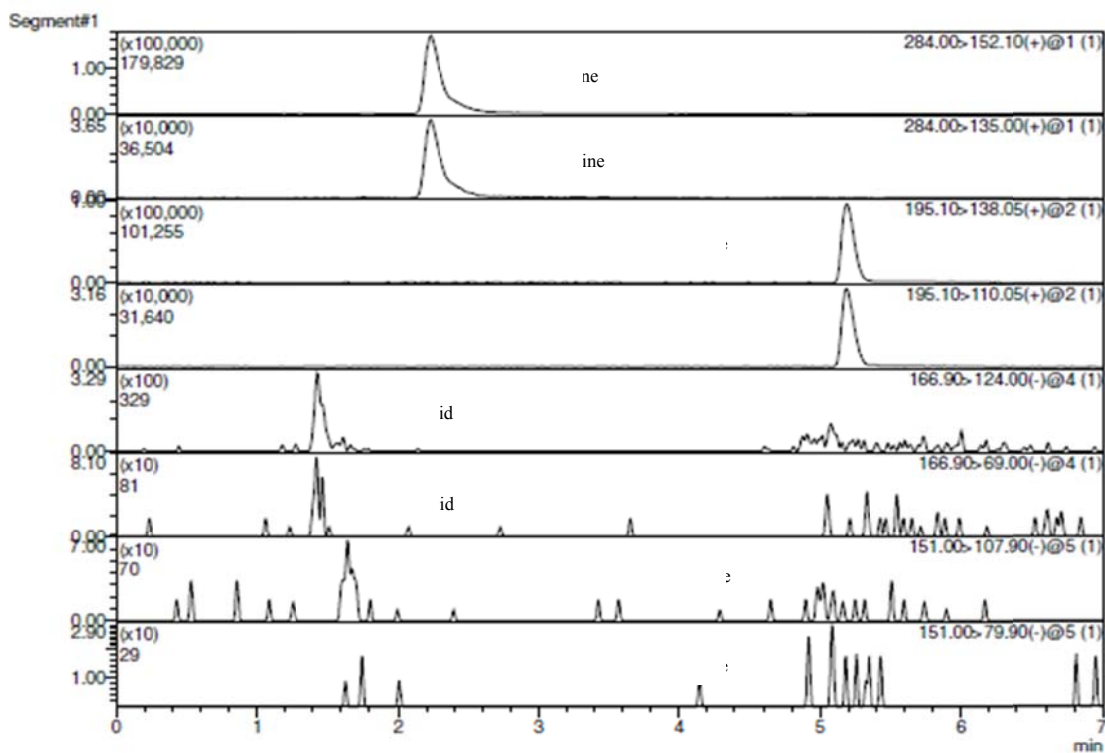
Matrix match calibration curve of Asiatic pennywort (*Centella asiatica* Urban)



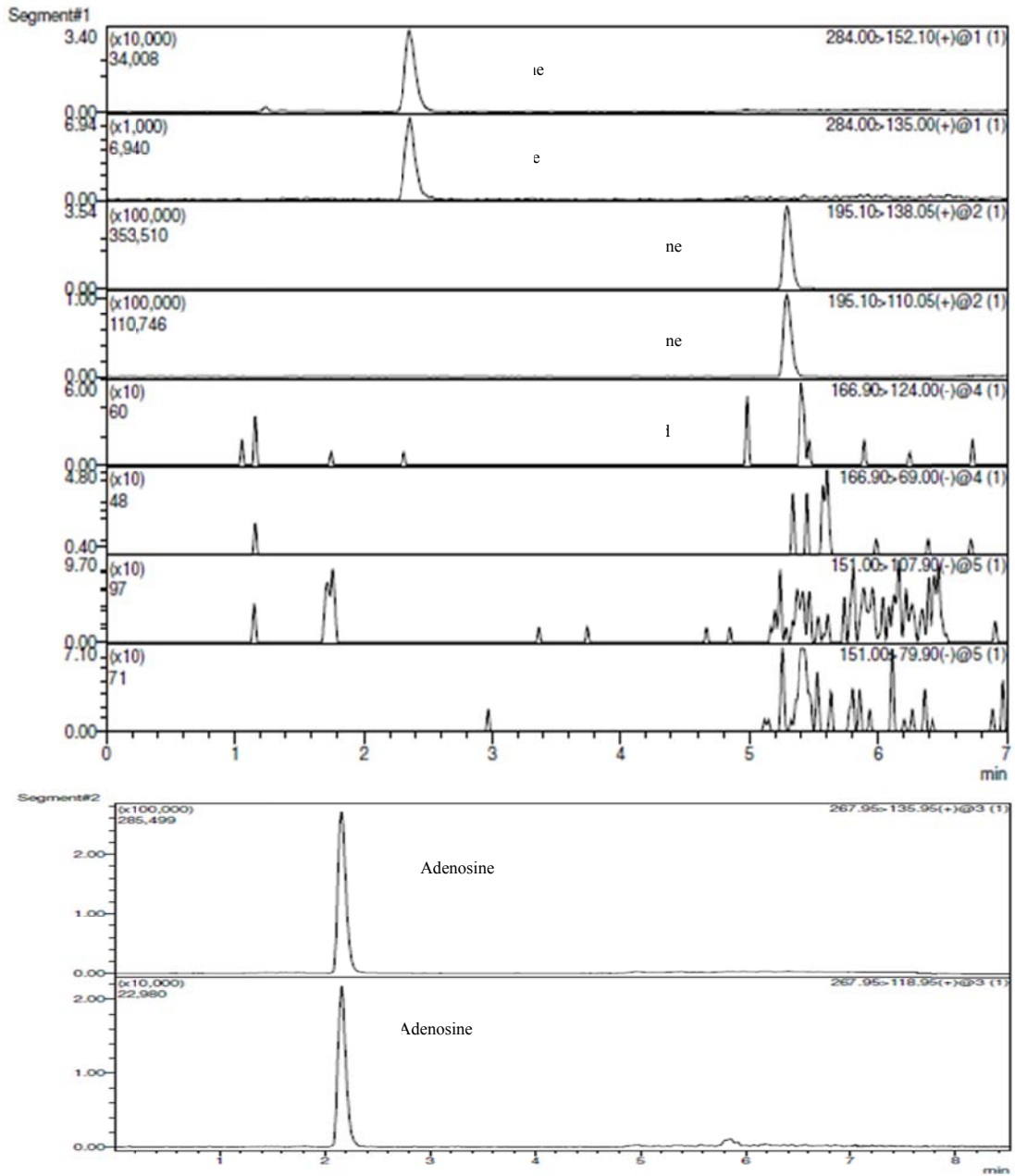


APPENDIX E
CHROMATOGRAMS OF THAI VEGETABLES SAMPLES

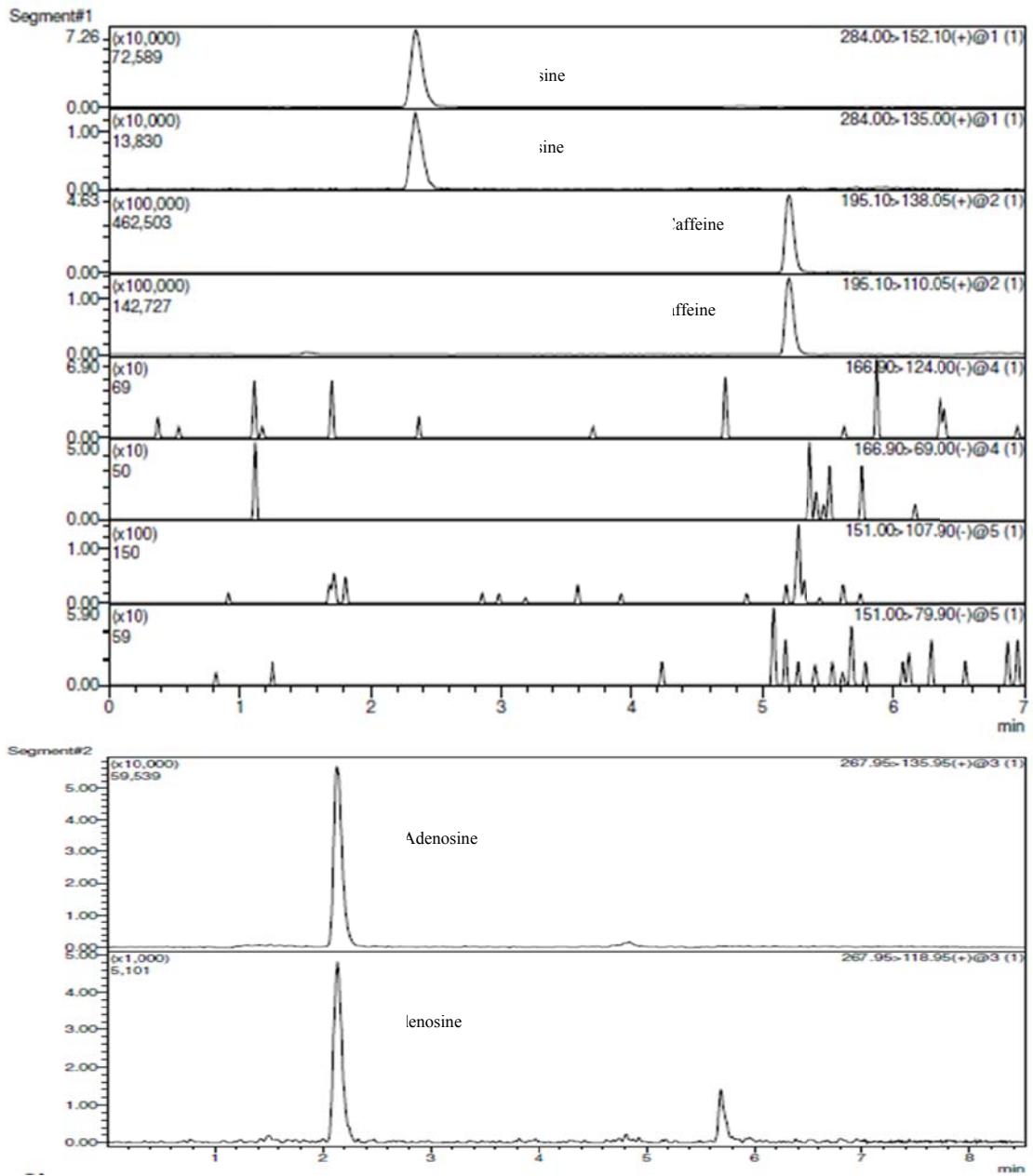
Thai eggplant (*Solanum melongena* L.)



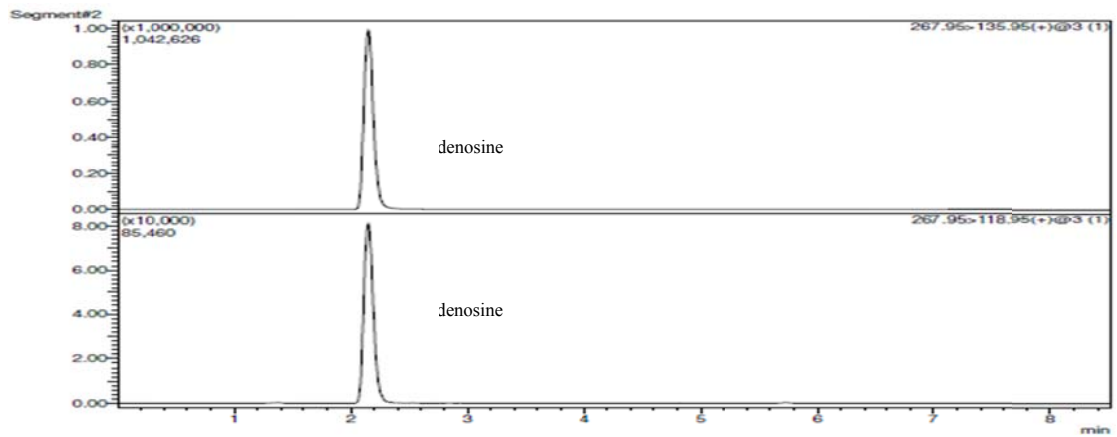
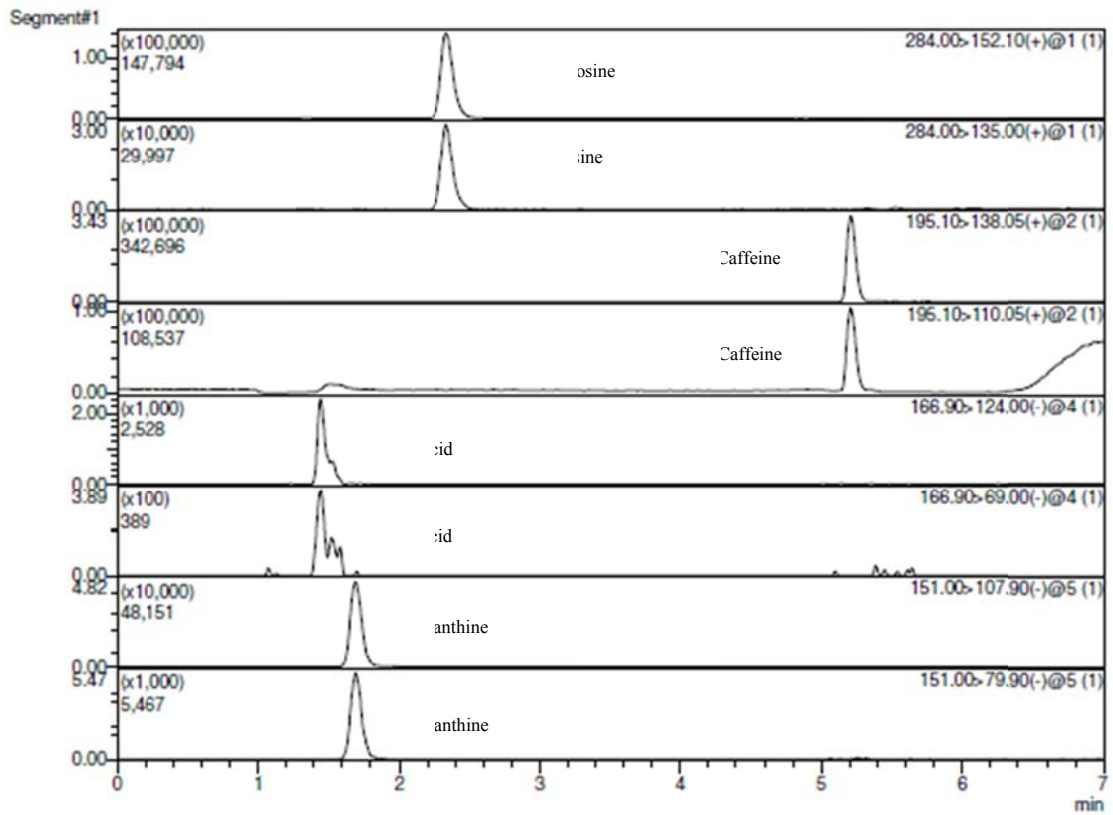
Chinese cabbage (*Brassica pekinensis*. Pekinesis (Lour) Olsson



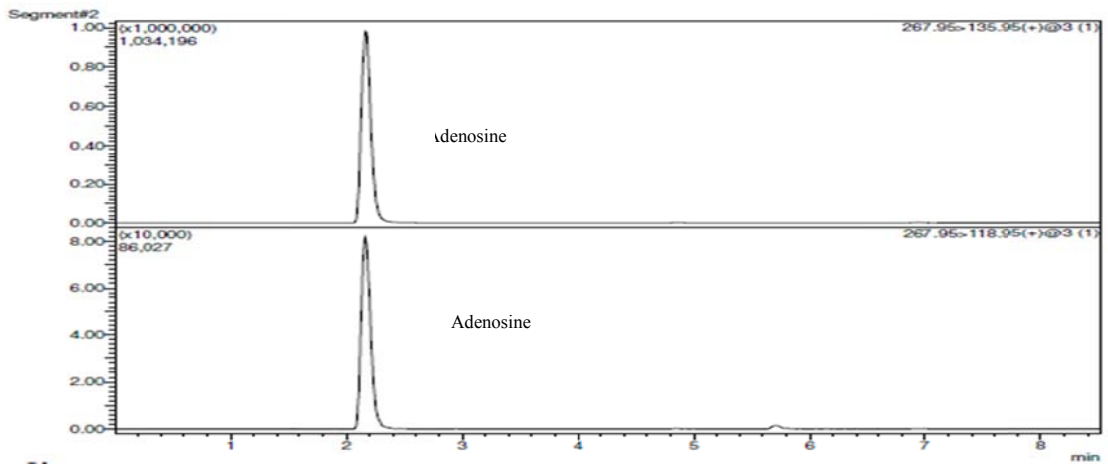
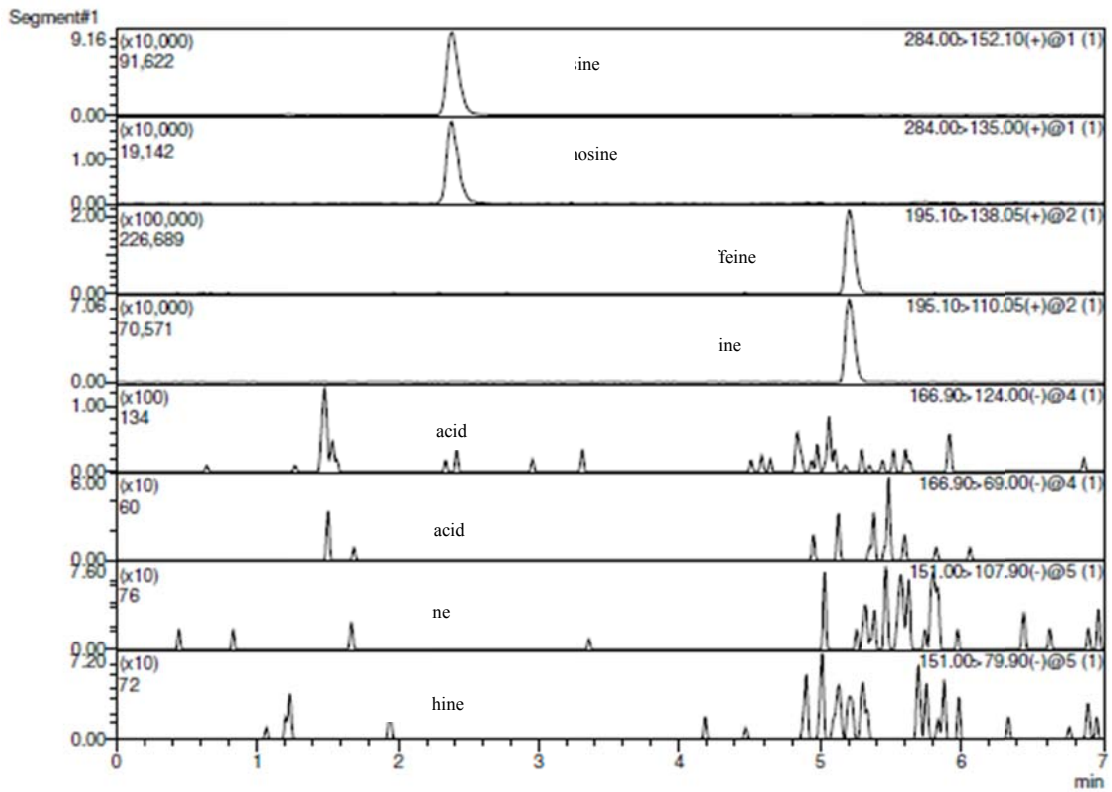
Cucumber (*Cucumis sativus* L.)



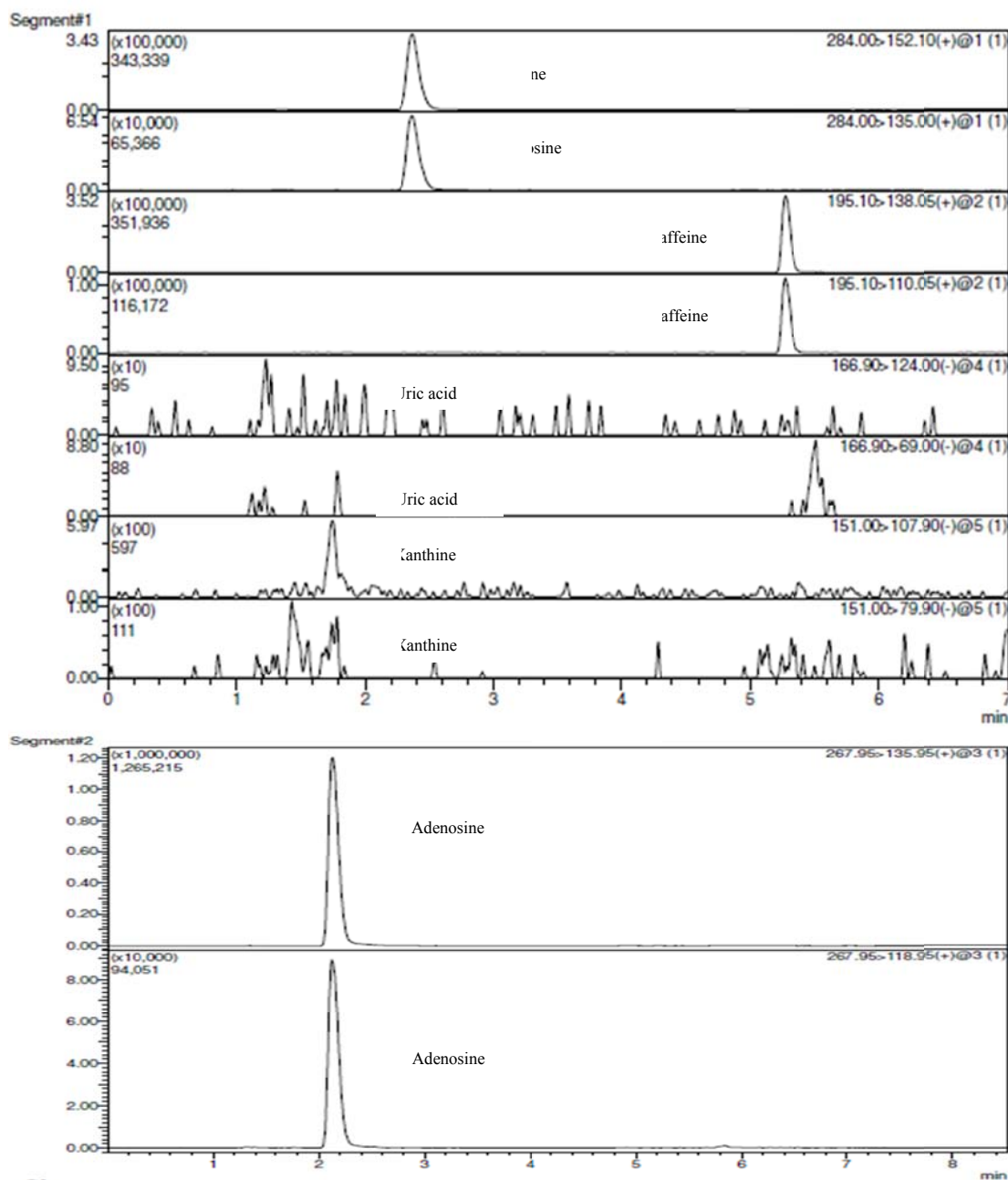
Yardlong bean (*Vigna unguiculata* subsp. *sesquipedalis* (L.) Verdc.)



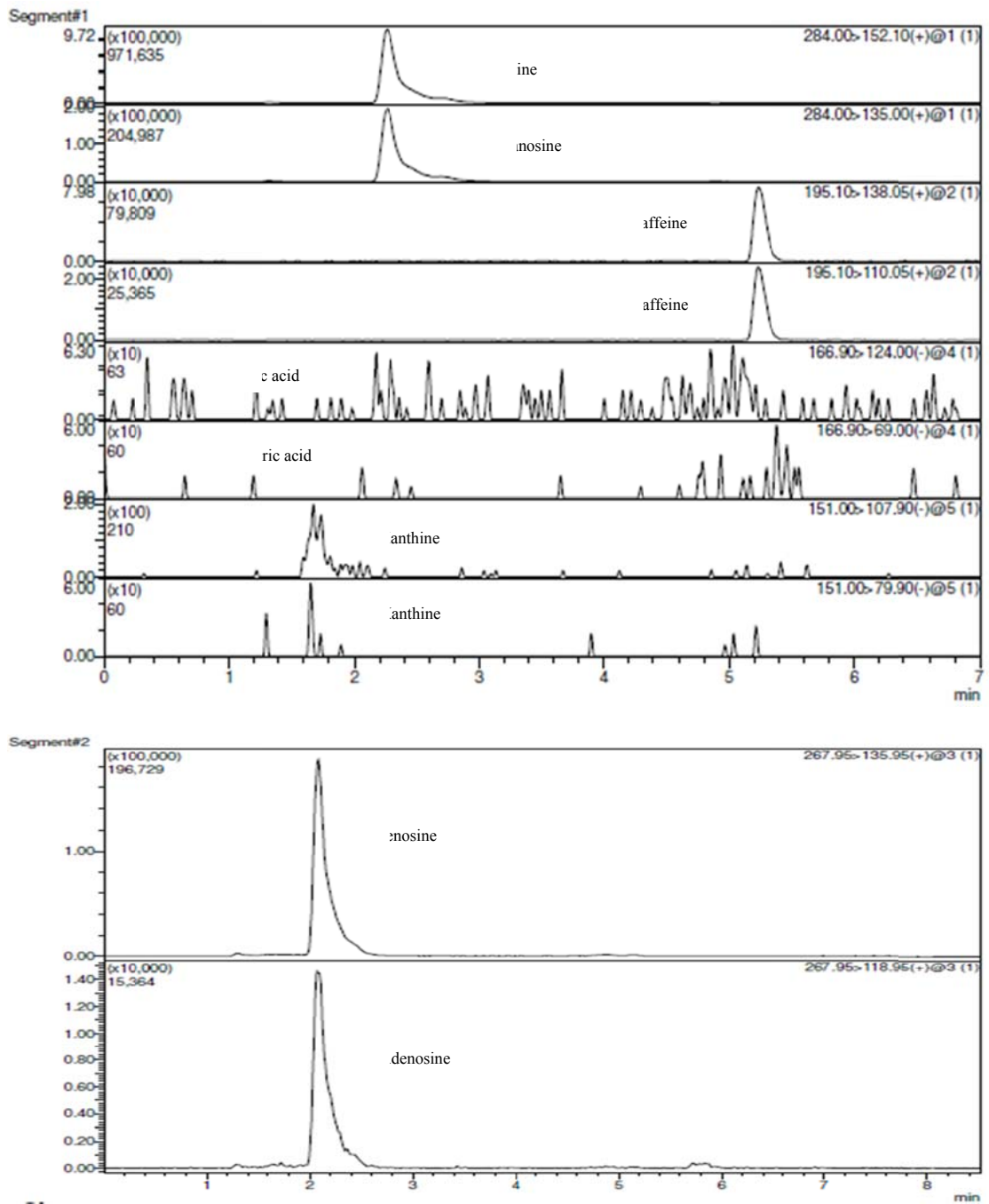
Winged bean (*Psophocarpus tetragonolobus* Linn.)



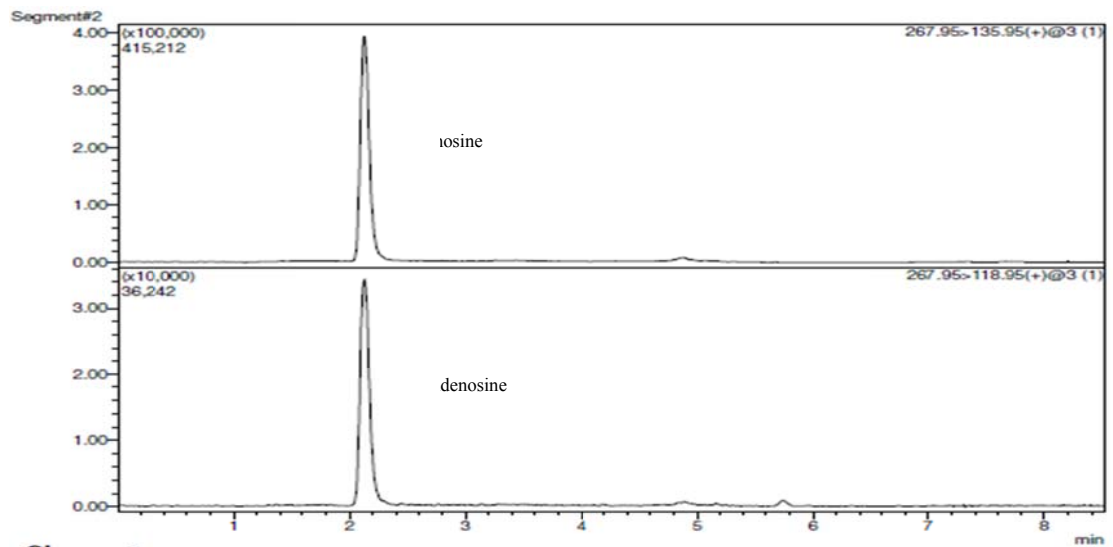
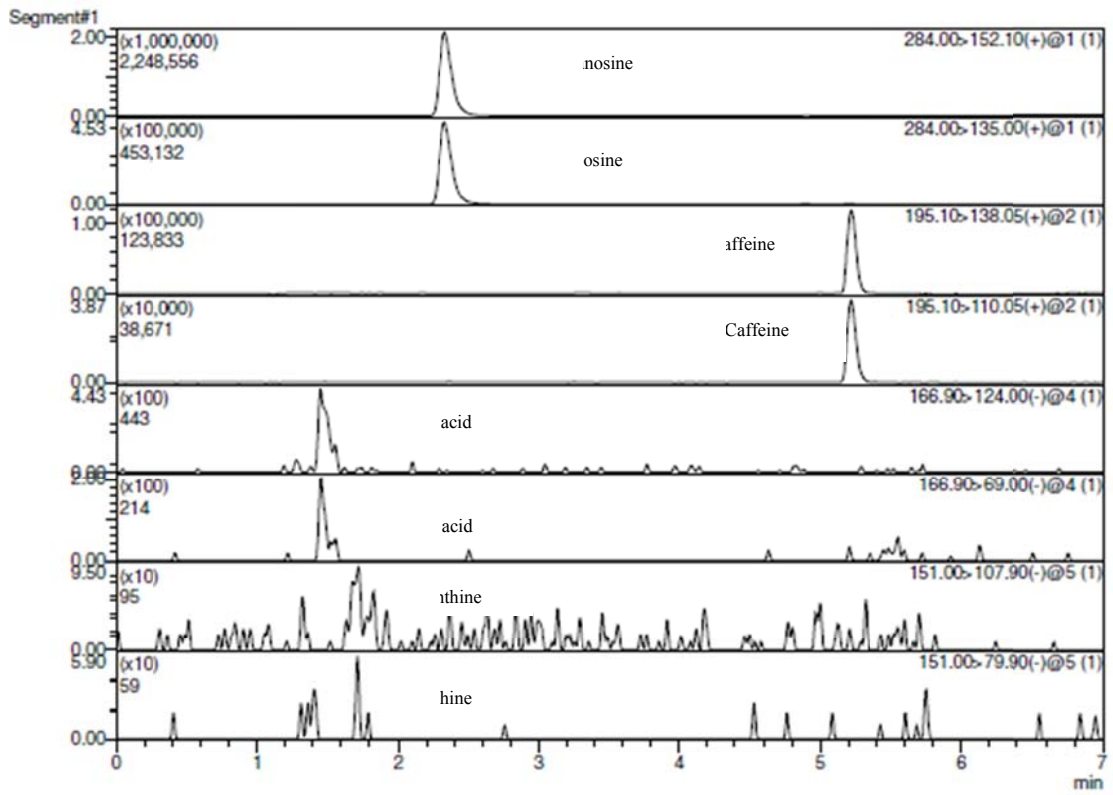
Bean sprouts (*Vigna radiata* (L.))



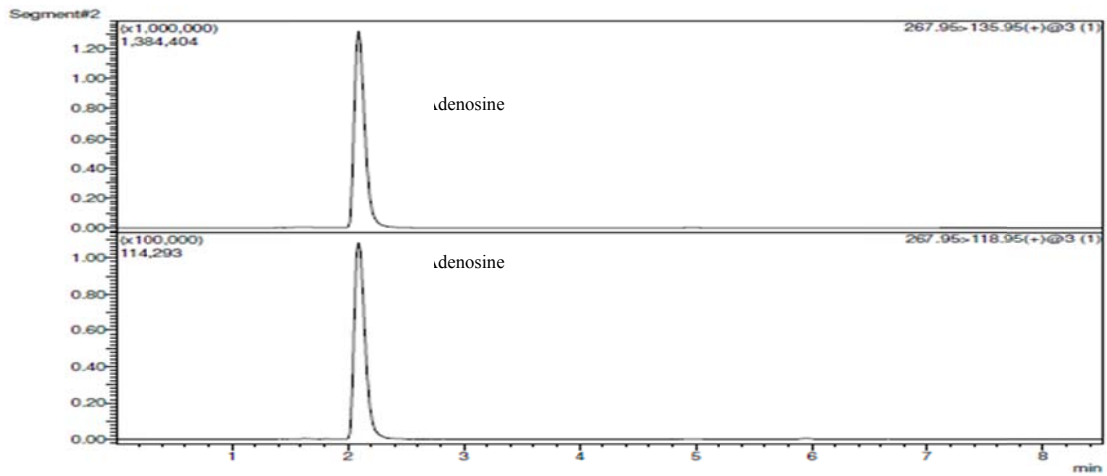
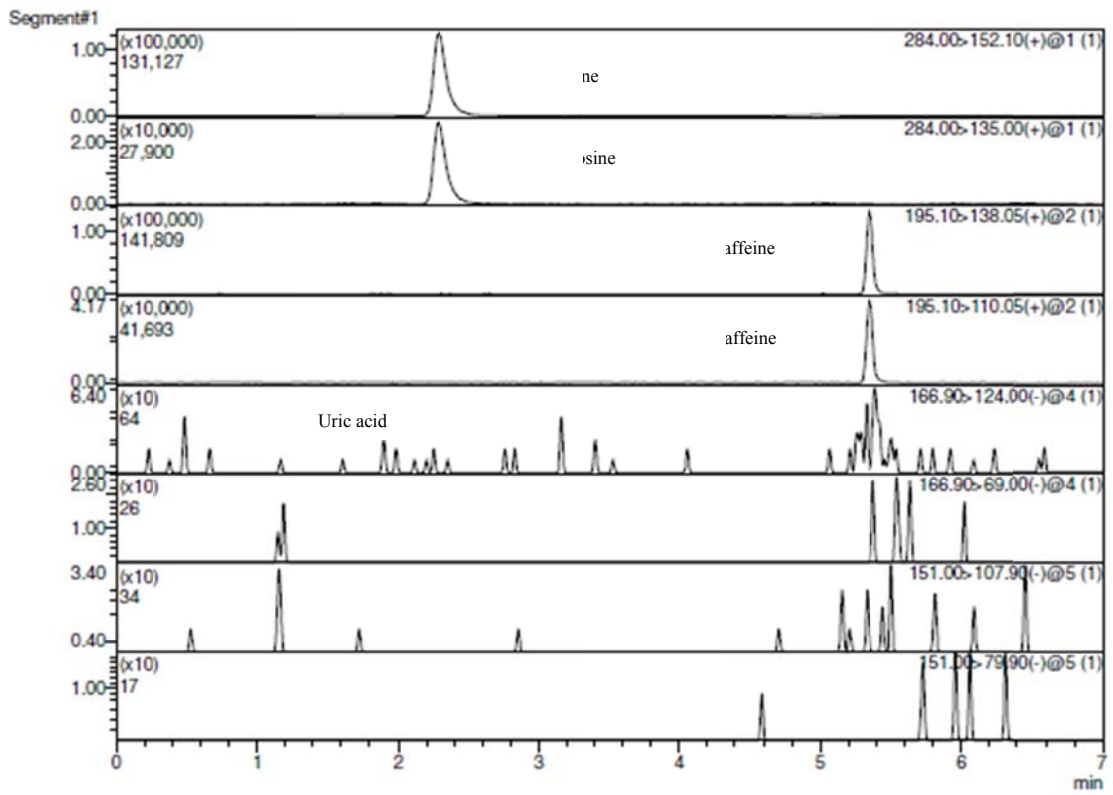
Bamboo shoot (*Bambusa* spp.)



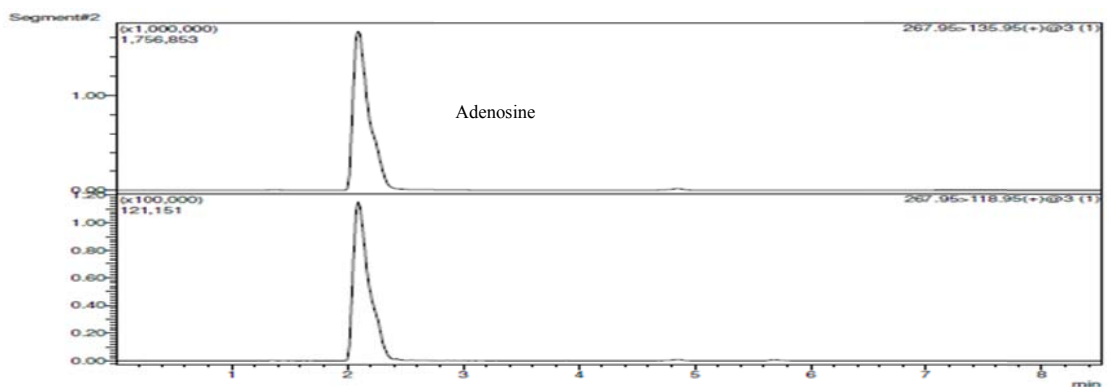
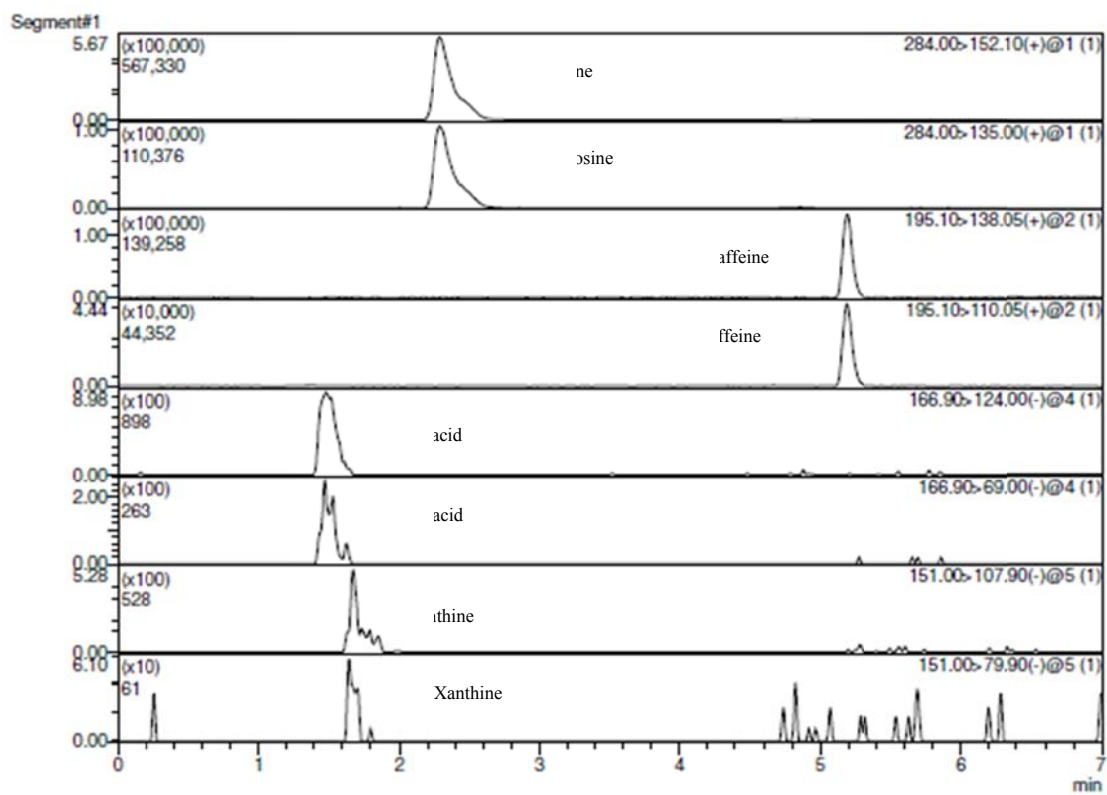
White popinac (*Leucaena leucocephala* (Lamk.) de Wit)



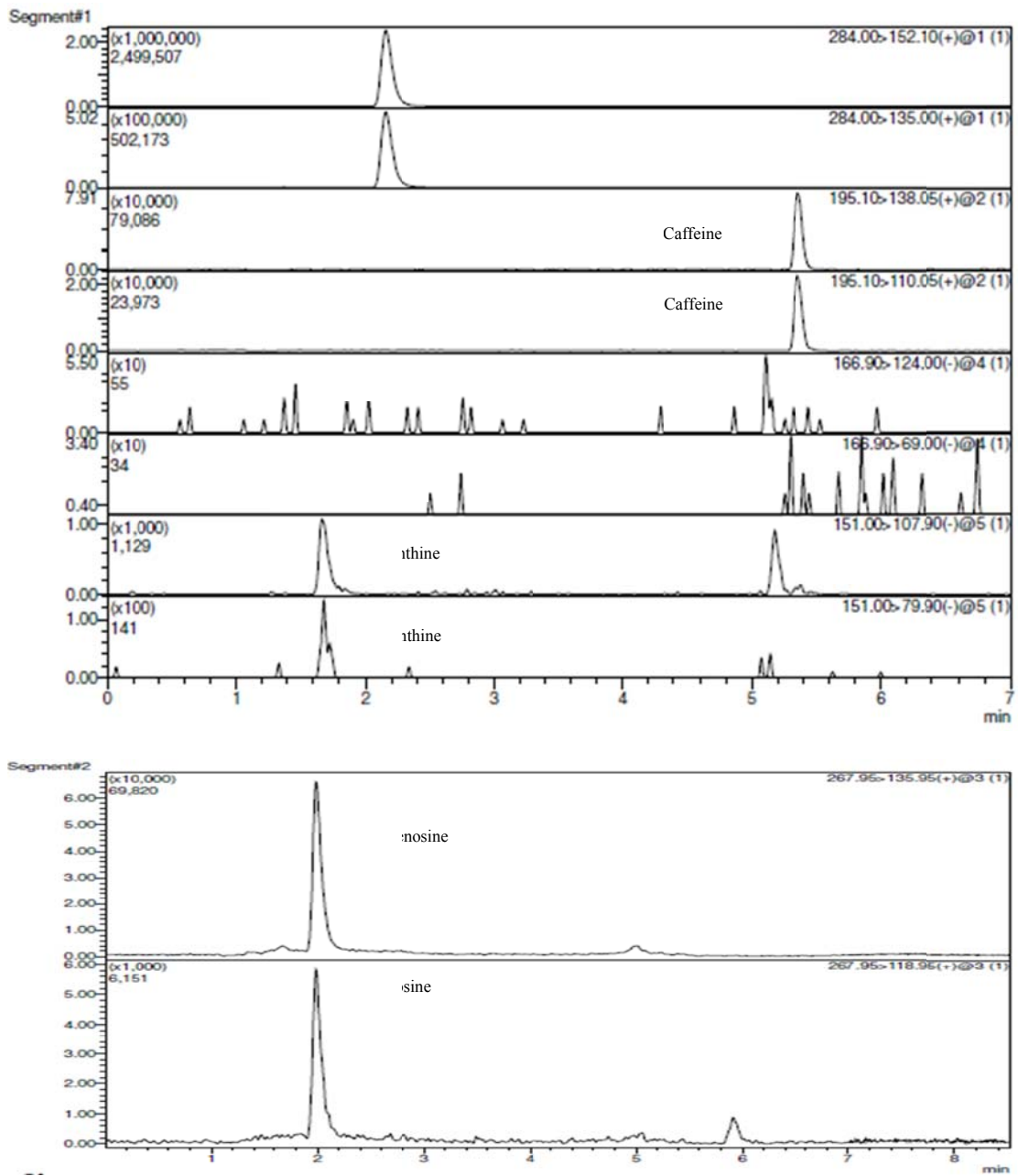
Stink Bean (*Parkia speciosa*)



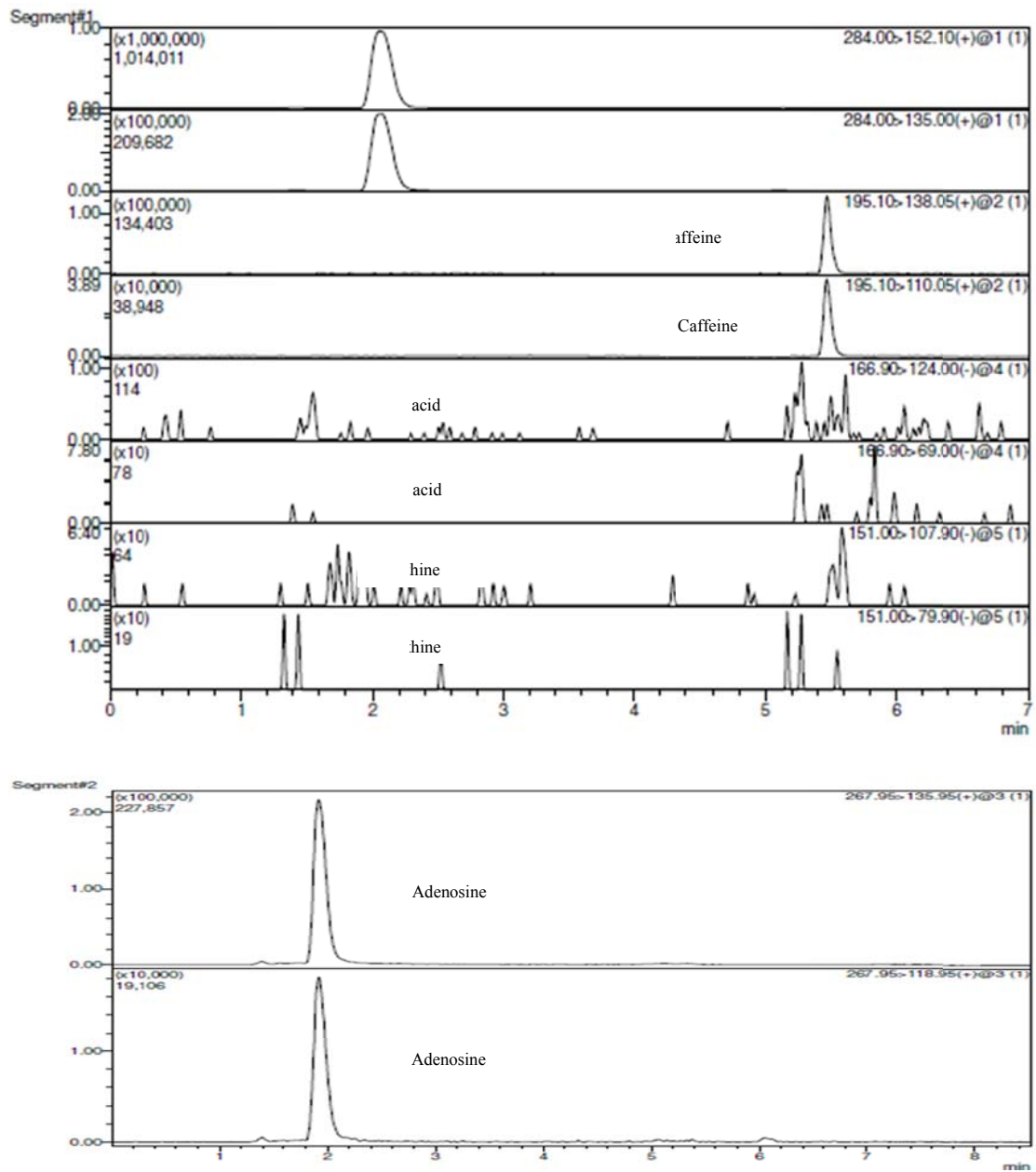
Melientha suavis (*Melientha suavis* Pierre)



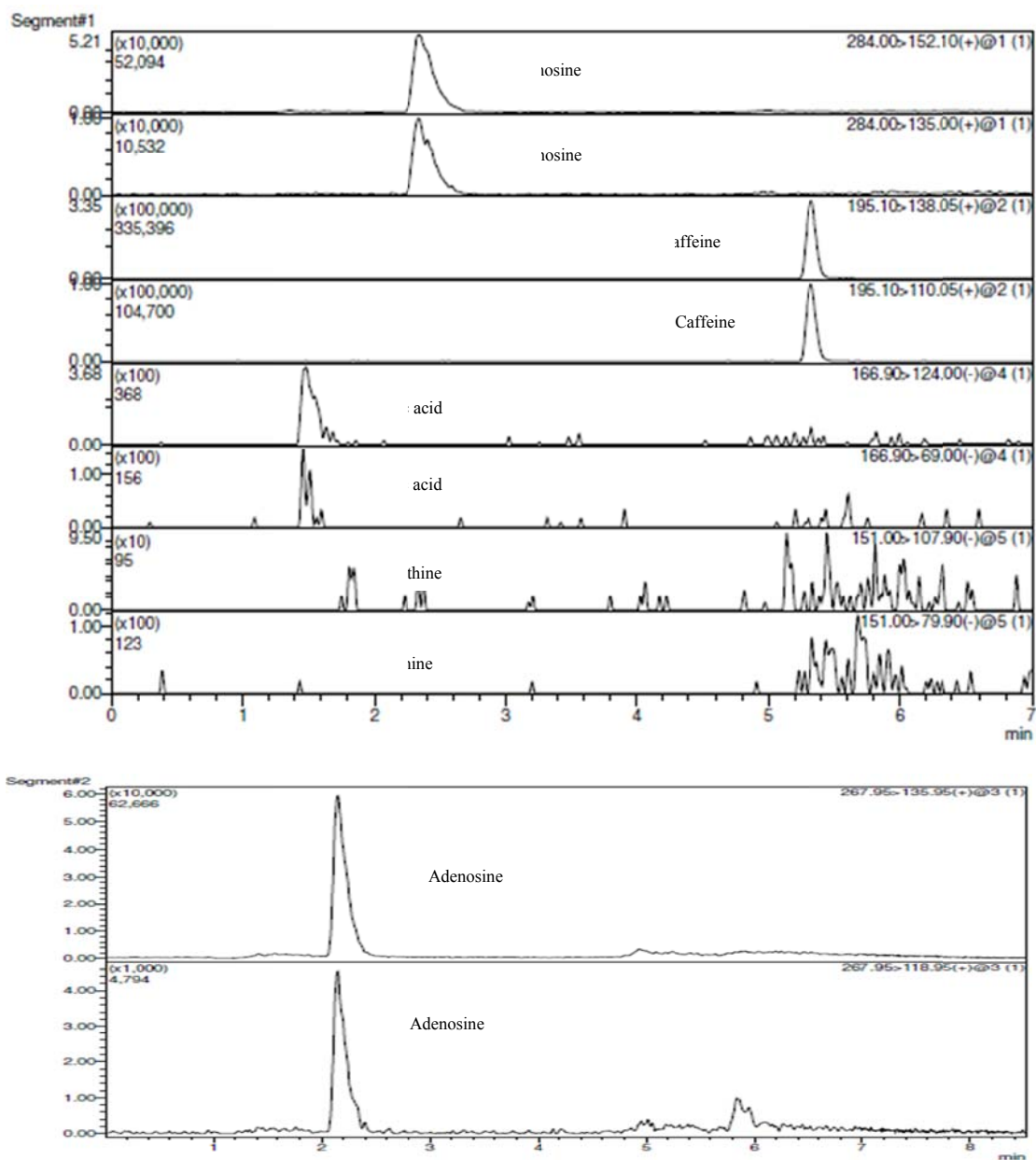
Acacia pennata (*Acacia pennata* (L.) Willd. subsp. *insuavis* (Lace) I.C. Nielsen)



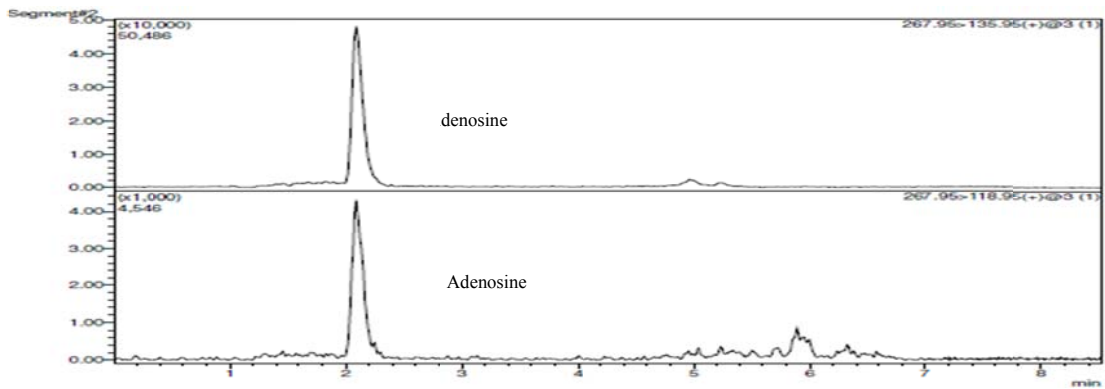
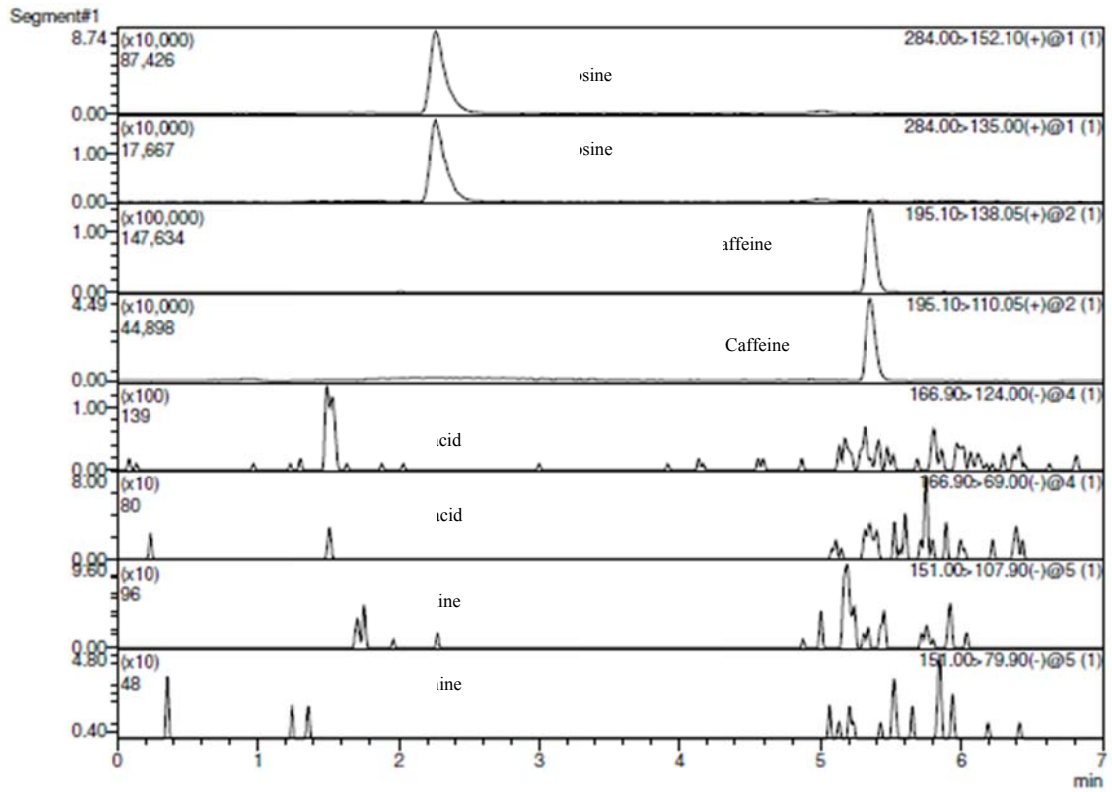
Water mimosa (*Neptunia oleracea* Lour.)



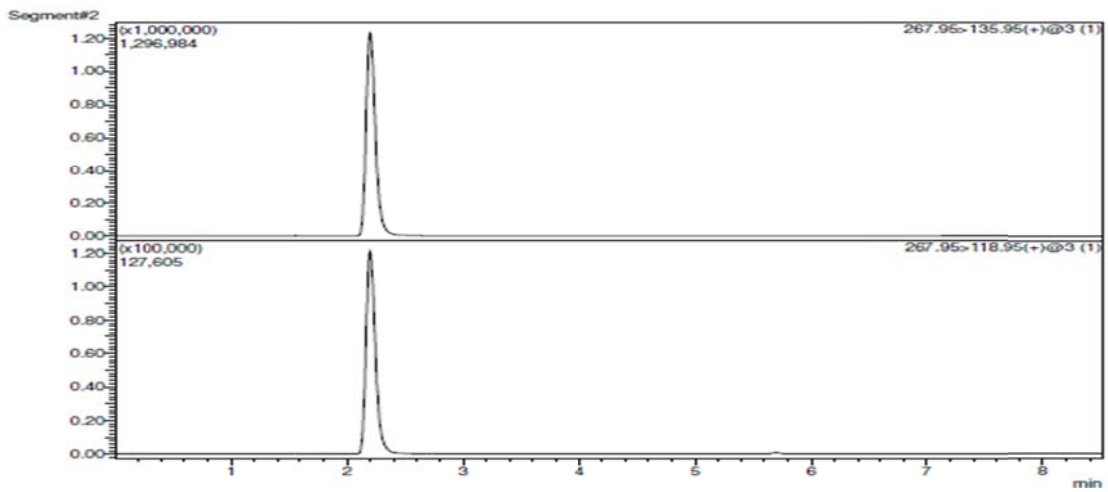
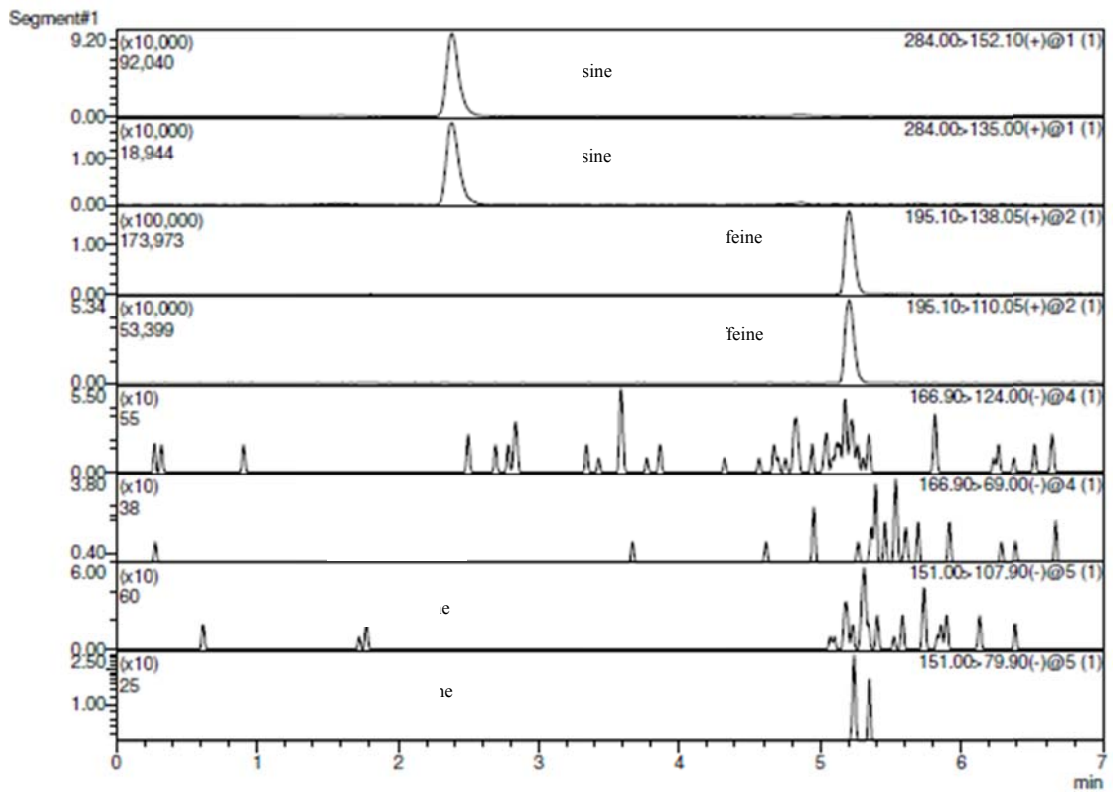
Water Convolvulus (*Ipomoea aquatica* Forsk. Var. reptan)



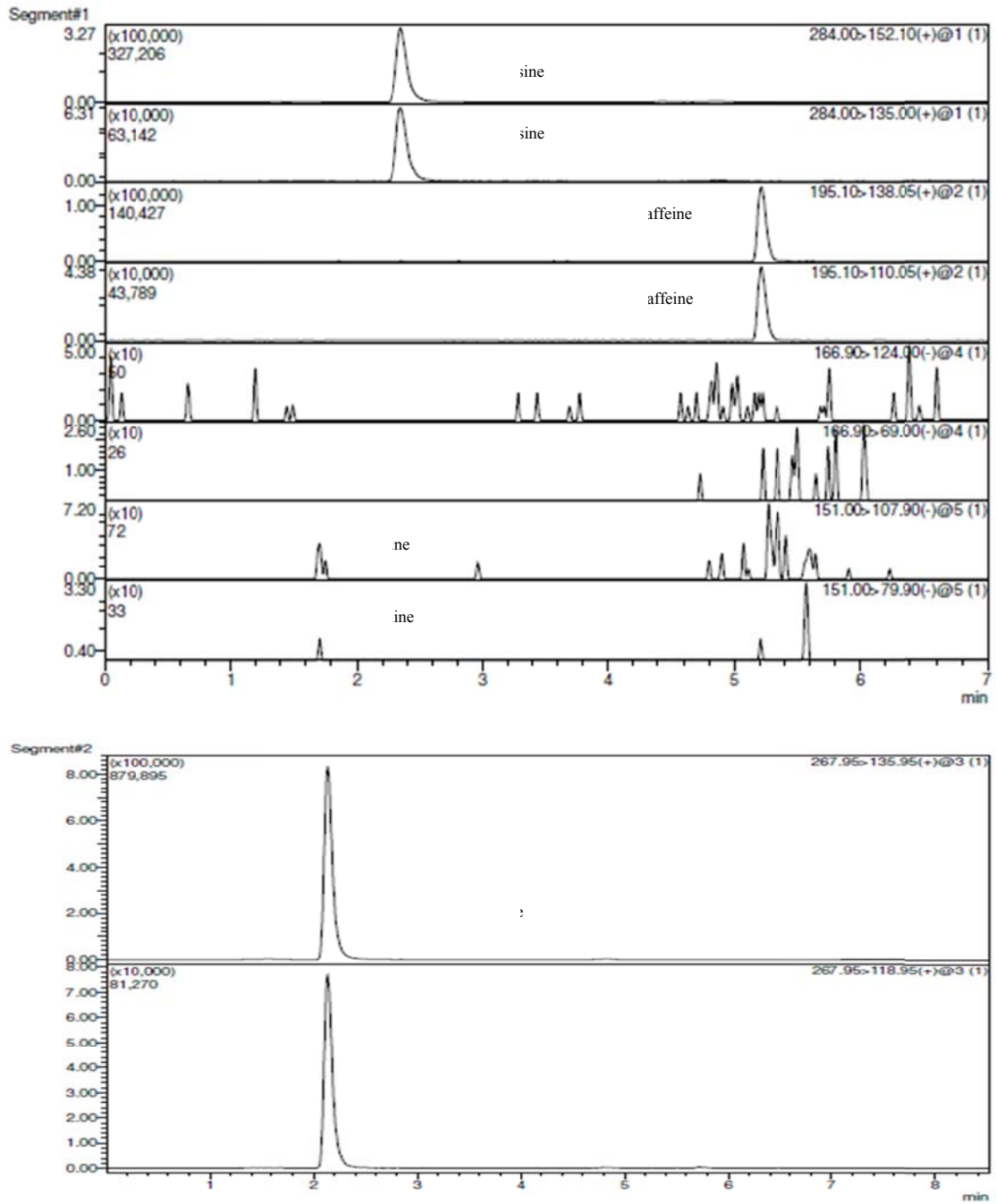
Vietnamese coriander (*Polygonum odoratum* Lour.)



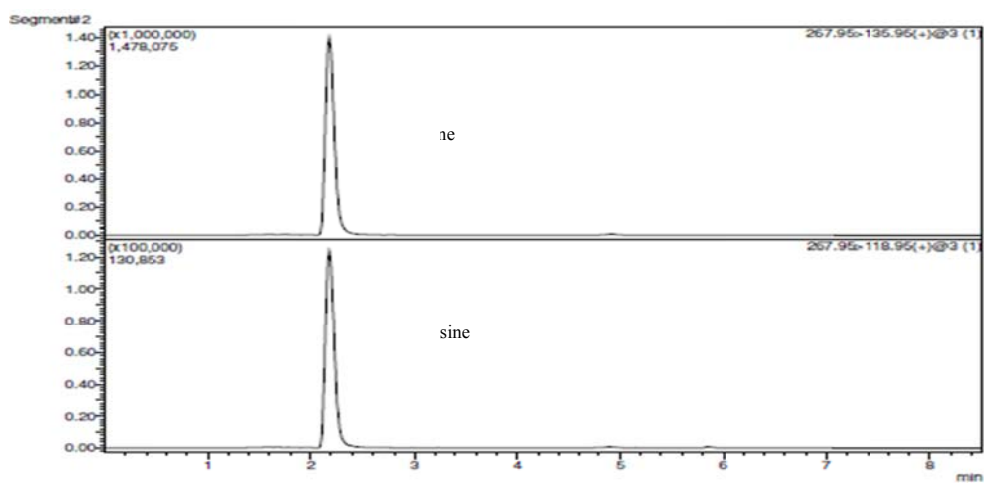
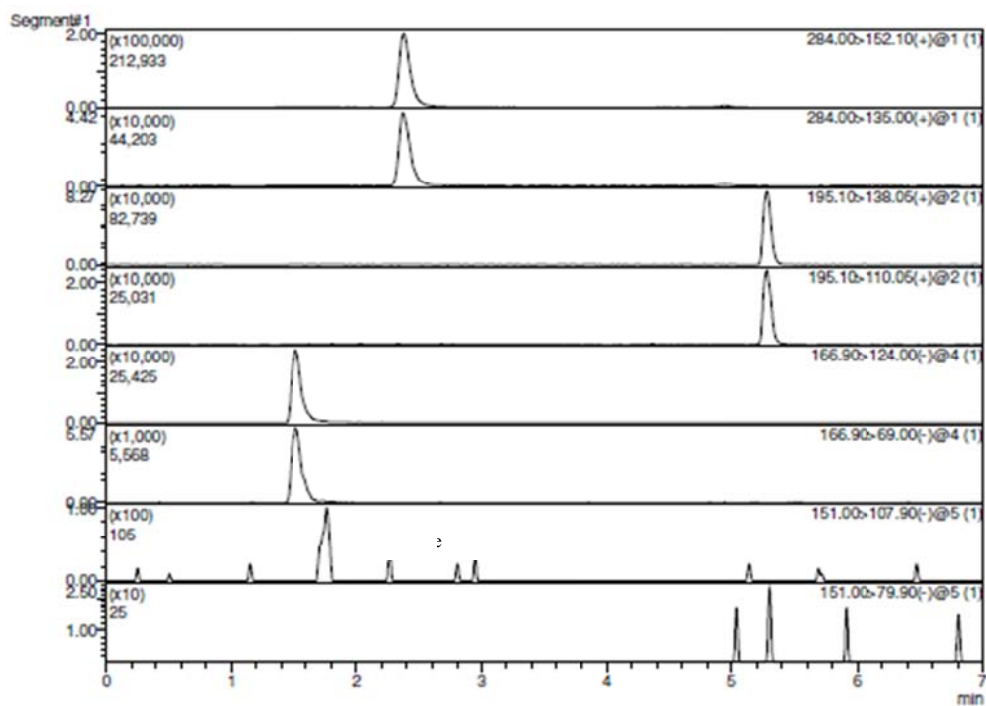
Asiatic Pennywort (*Centella asiatica* Urban)



Yellow velvet leaf (*Limnocharis flava* (L.) Buchenau)



Malabar nightshade (*Basella alba* L.)



APPENDIX F
RAW DATA OF VEGETABLES SAMPLES FROM EACH
SOURCE

Content of purines and uric acid in eighteen Thai vegetable

1. Yardlong bean (*Vigna unguiculata* Hc.)

Store	Amount (ng/g)			
	Uric acid	Xanthine	Adenosine	Guanosine
1	2,349 ± 94	48,303 ± 836	12,387 ± 155	4,430 ± 14
2	1,107 ± 26	34,246 ± 110	11,066 ± 24	5,753 ± 2
3	2,299 ± 126	49,380 ± 5	10,967 ± 209	4,442 ± 17

2. Cucumber (*Cucumis Sativus* Linn.)

Store	Amount (ng/g)*			
	Uric acid	Xanthine	Adenosine	Guanosine
1	ND	ND	1,020 ± 45	597 ± 20
2	ND	ND	797 ± 3	586 ± 11
3	ND	ND	830 ± 13	636 ± 26

*ND: not detected

3. Chainese cabbage (*Brassica pekinensis*)

Store	Amount (ng/g)**			
	Uric acid	Xanthine	Adenosine	Guanosine
1	ND	Less than LLOQ	1,861 ± 20	763 ± 2
2	ND	Less than LLOQ	7,198 ± 38	1,637 ± 33
3	ND	Less than LLOQ	3,165 ± 31	939 ± 17

*ND: not detected

**LLOQ: adenosine and guanosine 0.02 mg/100 g and uric acid and xanthine 0.2 mg/100 g of fresh weight

4. Bean Sprouts (*Vigna radiate* (L.)

Store	Amount (ng/g) ^{***}			
	Uric acid	Xanthine	Adenosine	Guanosine
1	ND	Less than LLOQ	26,113 ± 738	9,209 ± 60
2	ND	Less than LLOQ	27,494 ± 612	7,167 ± 128
3	ND	Less than LLOQ	8,269 ± 230	4,078 ± 27

*ND: not detected

**LLOQ: adenosine and guanosine 0.02 mg/100 g and uric acid and xanthine 0.2 mg/100 g of fresh weight

5. Water Convolvulus (*Ipomoea aquatica* Forsk. Var. reptan)

Store	Amount (ng/g) [*]			
	Uric acid	Xanthine	Adenosine	Guanosine
1	4,862 ± 17	ND	36,622 ± 590	2,076 ± 20
2	2,657 ± 19	ND	33,300 ± 418	2,095 ± 50
3	1,515 ± 34	ND	50,216 ± 656	2,237 ± 94

*ND: not detected

6. Winged bean (*Psophocarpus tetragonolobus*)

Store	Amount (ng/g) ^{**}			
	Uric acid	Xanthine	Adenosine	Guanosine
1	Less than LLOQ	2,404 ± 53	29,056 ± 215	3,602 ± 43
2	Less than LLOQ	2,335 ± 93	25,964 ± 52	3,226 ± 98
3	Less than LLOQ	2,306 ± 52	13,690 ± 294	2,142 ± 14

**LLOQ: adenosine and guanosine 0.02 mg/100 g and uric acid and xanthine 0.2 mg/100 g of fresh weight

7. Melientha suavis (*Melientha suavis* Pierre)

Store	Amount (ng/g) ^{**}			
	Uric acid	Xanthine	Adenosine	Guanosine
1	11,144 ± 102	2,497 ± 158	11,489 ± 440	8,093 ± 285
2	5,511 ± 48	Less than LLOQ	11,324 ± 29	8,173 ± 19
3	13,010 ± 280	Less than LLOQ	25,792 ± 75	17,954 ± 42

**LLOQ: adenosine and guanosine 0.02 mg/100 g and uric acid and xanthine 0.2 mg/100 g of fresh weight

8. Asiatic pennywort (*Centella asiatica* (Linn.) Urban)

Store	Amount (ng/g)*			
	Uric acid	Xanthine	Adenosine	Guanosine
1	ND	ND	18,765.69 ± 18.62	3,080.01 ± 4.20
2	ND	ND	12,185.91 ± 168.77	1,643.07 ± 0.89
3	ND	ND	21,144.98 ± 91.20	2,799.00 ± 20.08

*ND: not detected

9. Water mimosa (*Neptunia oleracea* Lour. FL.)

Store	Amount (ng/g)*			
	Uric acid	Xanthine	Adenosine	Guanosine
1	ND	ND	4,383.56 ± 7.39	14,586.90 ± 10.44
2	ND	ND	7,297.15 ± 22.19	15,016.98 ± 47.59
3	ND	ND	6,490.30 ± 18.39	12,798.67 ± 16.66

*ND: not detected

10. Acacia pennata (*Acacia Insuavis*, Lace)

Store	Amount (ng/g)*			
	Uric acid	Xanthine	Adenosine	Guanosine
1	ND	483.83 ± 3.54	985.38 ± 1.11	18,321.77 ± 11.31
2	ND	1,984.33 ± 2.35	2,006.36 ± 0.09	19,527.79 ± 137.98
3	ND	8,400.85 ± 17.52	748.69 ± 5.28	34,704.00 ± 17.28

*ND: not detected

11. Bitter bean or twisted cluster bean (*Parkia speciosa* Hassk.)

Store	Amount (ng/g)*			
	Uric acid	Xanthine	Adenosine	Guanosine
1	ND	ND	8,074.32 ± 159.09	2,928.49 ± 1.55
2	ND	ND	26,272.13 ± 7.87	12,707.89 ± 79.18
3	ND	ND	16292.39 ± 83.16	4,281.35 ± 11.30

*ND: not detected

12. Bamboo shoots (*Bambusa* spp.)

Store	Amount (ng/g)*			
	Uric acid	Xanthine	Adenosine	Guanosine
1	ND	3,052.97 ± 7.54	9,299.58 ± 3.66	4,114.46 ± 1.42
2	ND	1,748.03 ± 11.46	3,698.61 ± 2.18	14,654.88 ± 94.79
3	ND	2,228.71 ± 18.04	6,684.92 ± 7.90	28,751.69 ± 5.31

*ND: not detected

13. White popinac or wild tamarind (*Leucaena glauca* Benth.)

Store	Amount (ng/g)**			
	Uric acid	Xanthine	Adenosine	Guanosine
1	5,372.55 ± 9.72	601.88 ± 13.25	11,933.38 ± 70.46	62,486.25 ± 34.73
2	3,100.68 ± 6.95	321.24 ± 30.62	3,083.17 ± 4.62	16,017.00 ± 110.78
3	2,262.41 ± 5.56	Less than LLOQ	2,501.56 ± 4.00	45,407.86 ± 16.23

**LLOQ: adenosine and guanosine 0.02 mg/100 g and uric acid and xanthine 0.2 mg/100 g of fresh weight

14. Thai Eggplant (*Solanum xanthocarpum* Schrad. & Wendl.)

Store	Amount (ng/g)***			
	Uric acid	Xanthine	Adenosine	Guanosine
1	646.91 ± 2.72	ND	Less than LLOQ	5,177.37 ± 3.72
2	445.30 ± 1.14	ND	Less than LLOQ	4,056.039 ± 8.09
3	643.98 ± 1.34	ND	Less than LLOQ	4,537.706 ± 9.69

*ND: not detected

**LLOQ: adenosine and guanosine 0.02 mg/100 g and uric acid and xanthine 0.2 mg/100 g of fresh weight

15. Malabar Nightshade or Ceylon Spinach (*Basella alba* Linn.)

Store	Amount (ng/g)***			
	Uric acid	Xanthine	Adenosine	Guanosine
1	102,494.30 ± 53.61	Less than LLOQ	12,460.33 ± 8.19	2,835.94 ± 12.85
2	78,780.22 ± 107.88	Less than LLOQ	5,871.94 ± 16.01	2,106.73 ± 0.60
3	87,275.65 ± 98.40	ND	7,655.41 ± 575.86	4,051.23 ± 130.54

*ND: not detected

**LLOQ: adenosine and guanosine 0.02 mg/100 g and uric acid and xanthine 0.2 mg/100 g of fresh weight

16. Ivy Gourd (*Coccinia grandis* (L.) Voigt)

Store	Amount (ng/g)* **			
	Uric acid	Xanthine	Adenosine	Guanosine
1	ND	ND	43,982.50 ± 203.62	13,602.68 ± 304.08
2	ND	2,019.81 ± 47.90	13,141.09 ± 66.07	14,931.04 ± 18.65
3	ND	Less than LLOQ	9,007.34 ± 7.45	6,130.16 ± 15.70

*ND: not detected

**LLOQ: adenosine and guanosine 0.02 mg/100 g and uric acid and xanthine 0.2 mg/100 g

17. Yellow Velvet Leaf (*Limnocharis flava* Buch.)

Store	Amount (ng/g)*			
	Uric acid	Xanthine	Adenosine	Guanosine
1	ND	ND	19,904.25 ± 891.31	7,008.46 ± 85.32
2	ND	2,453.96 ± 15.83	12,047.81 ± 92.34	6,989.41 ± 9.17
3	ND	ND	17,702.07 ± 8.16	6,280.11 ± 1.30

*ND: not detected

18. Vietnamese Coriander (*Polygonum odoratum*)

Store	Amount (ng/g)* **			
	Uric acid	Xanthine	Adenosine	Guanosine
1	Less than LLOQ	ND	801.49 ± 29.26	1,579.44 ± 3.37
2	ND	ND	1,067.90 ± 39.59	1,049.11 ± 7.16
3	Less than LLOQ	ND	872.80 ± 0.71	862.99 ± 10.51

*ND: not detected

**LLOQ: adenosine and guanosine 0.02 mg/100 g and uric acid and xanthine 0.2 mg/100 g

APPENDIX G

PRESENTATION AND PUBLICATION

This paper was presented as poster presentation and published as a full paper in Pure and Applied Chemistry International Conference 2015 (PACCON 2015) in analytical chemistry; on January 21-23, 2015 in Bangkok, Thailand.

DEVELOPMENT OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY FOR DETERMINATION OF PURINES AND URIC ACID IN THAI VEGETABLES

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Abstract

Purines and uric acid are causative of gout disease. This study is aimed at developing new and rapid methods for determining purines and uric acid in Thai vegetables using a high-performance liquid chromatography with UV detector (HPLC-UV) and a liquid chromatography ion-trap mass spectrophotometry (LC-MS). For HPLC-UV method, Chromolith C18 column (4.6 × 100 mm) and UV detector setting at 284 nm were used throughout the study. Type and concentration of organic solvents were evaluated to obtain an appropriate mobile phase condition. Accordingly, the optimum separation was achieved from the mixture of 10 mM ammonium acetate, pH 2.5 and methanol (97:3, v/v). Using ion-trap LC-MS technique, a mixture of 0.1% formic acid and acetonitrile with gradient elution was exploited as mobile phase condition. Five intended compounds (adenosine, guanosine, hypoxanthine, xanthine and uric acid)

were well separated and detected under this condition. Results obtained from this study would be enormously valuable to the treatment of gout disease.

Keywords Purines; Uric acid; Gout disease

Introduction

Daily meal is an important factor of health. In addition, when human is subjected to some diseases, controlling of foods is necessary to control the symptoms. For example, gout disease causes from increasing of uric acid in human body. Elevating of uric acid causes from wrong metabolism of purines pathway, exceed synthesized uric acid will deposit in joints and leading to inflame, red swollen of the joint and pain [1-2].

Purine is the common name of purine bases, nucleotides and nucleosides. In human body, purines can be metabolized to uric acid by using enzyme xanthine oxidase. Various Thai vegetables, especially the shoot part, have been reported for high containing of purines. Such vegetables should be limit consumed in gout patients. However, there was not official report of purines and uric acid in Thai vegetables. By these reasons, this study was conducted to contribute valuable information for controlling gout symptoms.

There were reports for quantitative determination methods of purines and uric acid. Liquid-chromatography mass spectrophotometry (LC-MS) was used to quantify purines and uric acid in Japanese soup, beer, Chinese cabbage, potato and beef [3-6]. High-performance liquid chromatography (HPLC) was also used to determine purines and uric acid in meat, alcoholic beverage, vegetarian meat, soy bean, and mushroom [7-10]. Although HPLC is the basic instrument in general laboratories, certain and expensive columns were used in these studies. In this research, a simple HPLC-UV method was developed for determination of purines and uric acid by using C18 column. An ion-trap LC-MS method was also developed to determine such compounds. The optimum HPLC-UV and LC-MS methods will be further validated and used to quantify purines and uric acid in Thai vegetables.

Materials and Methods

A Shimadzu LC-10AD system equipped with UV detector was employed for chromatographic separation on a Chromolith C18 column (4.6 x 100 mm). A mixture of 10 mM ammonium acetate buffer, pH 2.5 and methanol (97:3, v/v) was used as a mobile phase. The flow rate of mobile phase was 0.5 mL/min. The UV detector wavelength was set at 284 nm. Standards purines (adenosine, guanosine, hypoxanthine, xanthine), uric acid, dextrometoprophan HBr and ammonium acetate were purchased from Sigma Chemical, methanol (HPLC grade) was purchased from Labscan (Thailand). For LC-MS ion-trap system, a Thermo LCQ Fleet equipped with Symmetry C18 column (4.6 x 150 mm, 5 μ m) was used. A gradient program was applied consisting of A) 0.2 % formic acid in deionized water; B) 0.1% formic in methanol (HPLC grade). The gradient started with a 8 min initial equilibration with 2% of solvent B , then increased to 90% in 0.1 min, and held for 4.9 min to elute strongly retained analytes and clean the column. Finally, solvent B was decreased to 2% within 0.1 min and held for 1.9 min. Flow rate was set at a constant 300 μ L/min for the whole run and 10 μ L of sample was injected. Electrospray ionization (ESI) was utilized, spray voltage was 5.00kV, capillary temperature was 350.0 $^{\circ}$ C, capillary voltage was 26.00V and tube lens was set at 70.00V. Dextrometoprophan HBr was used as an internal standard for LC-MS experiment.

3. Results and Discussion

3.1 Development of HPLC-UV method

Chromolith C18 column and UV detector setting at 284 nm were utilized to develop HPLC-UV method for determination of four purines and uric acid (Figure 1). The starting mobile phase was the mixture of 10 mM ammonium acetate, pH 4.5 and acetonitrile. The ratio of acetonitrile was varied between 5 and 40 % (v/v). The separation was performed using isocratic elution. It was found that the interest compounds were not completely separated, and the retention times of five compounds were almost the same in these mobile phase conditions.

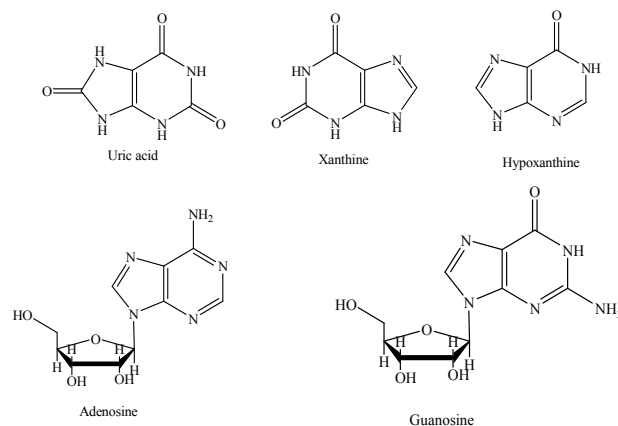


Figure 1. Chemical structures of purines and uric acid

This might be due to the pH of mobile phase was not allowed an appropriate separation. The pH of mobile was further adjusted to 2.5 and concentration of acetonitrile was 5% by volume. The resulting chromatograms were improved, but acceptable resolutions of all compounds were not achieved yet. Methanol was then used instead of acetonitrile. The separation was better but the separation was still not complete with 5% methanol by volume. Finally, methanol was reduced to 3% by volume and acceptable results were achieved in this condition. The chromatograms obtained from the optimum condition are illustrated in Figure 2.

3.2 Development of Ion-trap LC-MS method

Mobile phase from HPLC-UV system, 10 mM ammonium acetate, pH 2.5 and methanol, was transferred to LC-MS condition. However, MS noise signal was found to be interfered with ammonium acetate, therefore deionized water adjusted to pH 2.5 was used instead of ammonium acetate. In addition, formic acid was added in both mobile solvent to promote solvent volatilization. The collision induced dissociation or CID was adjusted to obtain the dominate transition ions of each compound because the transition ions will be used for quantitative determination. Unfortunately, the study failed to find the major transition ion of hypoxanthine. This may be due to the fact that molecular weight of hypoxanthine is too low (MW.135) so it was hard to get the major transition ion after fragmentation.

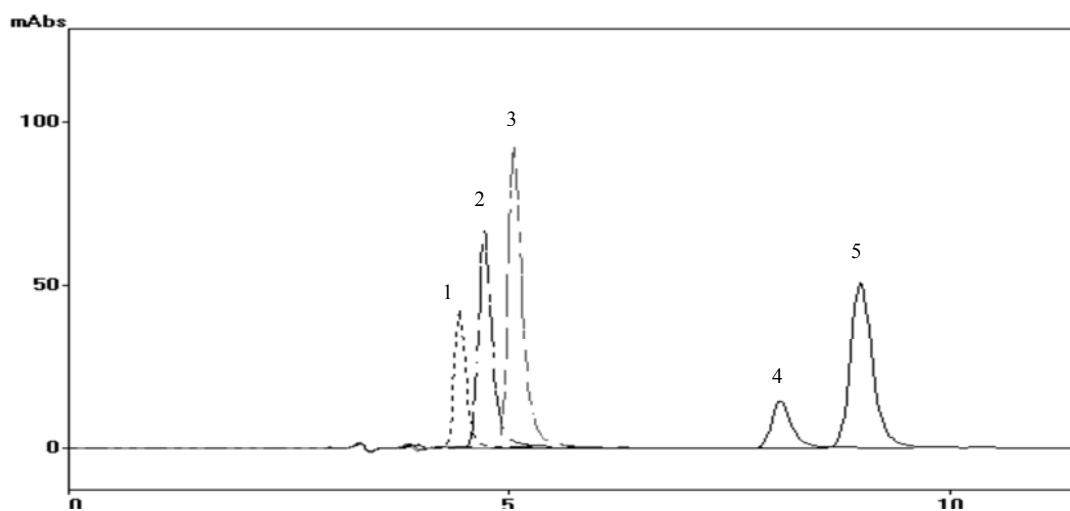


Figure 2. Overlaid chromatograms of uric acid and purine compounds. Column Chromolith C18, (4.6 x 100 mm); mobile phase, 10 mM ammonium acetate buffer, pH 2.5 : methanol (97:3, v/v); flow rate, 0.5 mL/min; injection volume, 20 μ L; detection, 284 nm. Peak 1, uric acid; peak 2, hypoxanthine; peak 3, xanthine; peak 4, adenosine; peak 5, guanosine.

Therefore, only four compounds namely, uric acid, xanthine, adenosine and guanosine, were investigated for LC-MS method development. Dextromethorphan was used as internal standard. For ionization mode, it was found that uric acid and xanthine might be ionized in negative ion mode while adenosine, guanosine and dextromethorphan were ionized in positive ion mode. By using the condition described above, five compounds were eluted between 5.0-12.5 min. The retention times, transition ions and CID energy are summarized in Table 1. Chromatograms of transition ions are shown in Figure 3.

Table 1. The retention times, transition ions and CID energy

Compound	Retention time	Transition	CID (eV)
Uric acid	5.07	167>>124	33
Xanthine	6.39	151>>108	30
Adenosine	7.89	268>>136	28
Guanosine	10.78	284>>152	22
Dextromethorphan	12.37	272>>215	40

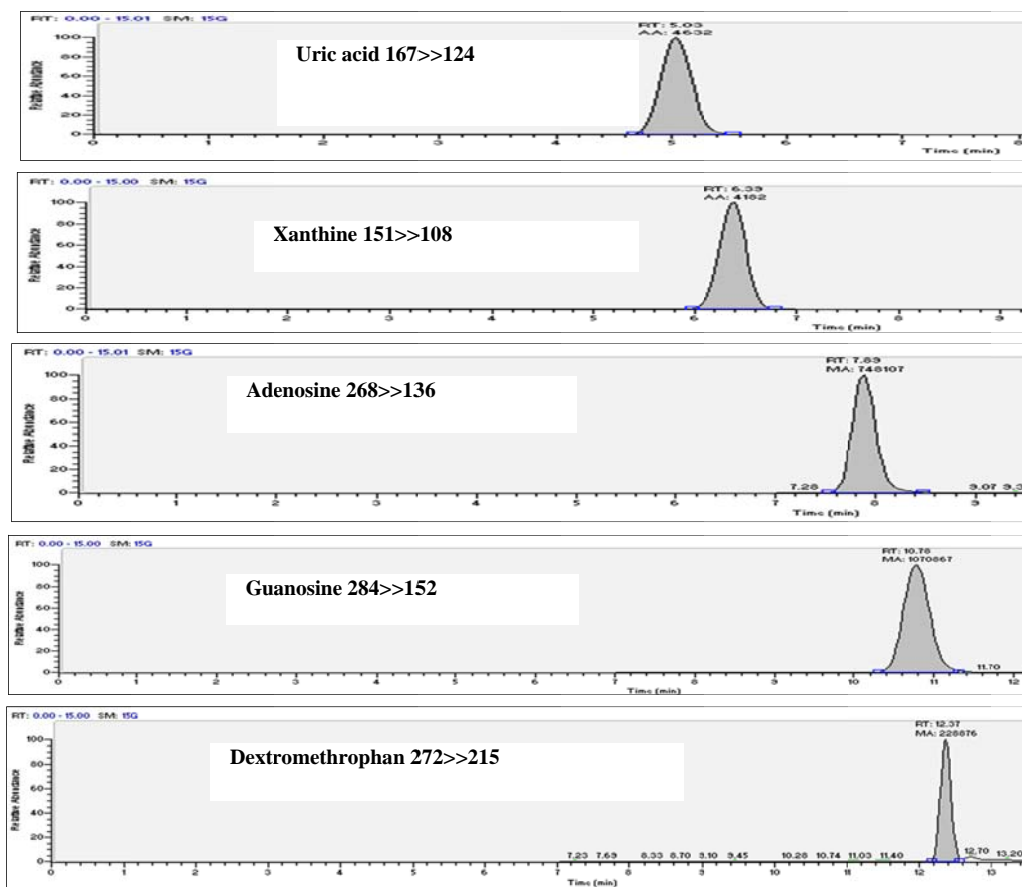


Figure 3. Chromatograms of transition ions.

4. Conclusions

A simple HPLC-UV and ion-trap LC-MS were successfully developed for the determination of purines (xanthine, adenosine and guanosine) and uric acid. The developed methods will be validated for intend purpose and further applied to quantify purines and uric acid in Thai vegetables. The information from the study will contribute to the treatment of gout disease.

Acknowledgements

The authors would like to thank The Thailand Research Fund (RDG 5620011) and the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University for research facilities.

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