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METHOD DEVELOPMENT FOR THE DETERMINATION OF SULFONYLUREA HERBICIDES RESIDUE IN RICE USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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งานวิจัยนี้เป็นการพัฒนาวิธีวิเคราะห์สารตกค้างยาฆ่าวัชพืชกลุ่มซัลโฟนิลยูเรีย รวม 7 ชนิด ในข้าวโดยใช้เทคนิคลิควิดโครมาโทกราฟี-แมสสเปกโทรเมทรี แบบ electrospray ionization และตรวจวัดในรูปของประจุบวก วิธีการเตรียมตัวอย่างได้พัฒนาขึ้นโดยแบ่งเป็น 2 ส่วนคือ สกัดสารตกค้างออกจากข้าวก่อนโดยการสกัดด้วยตัวทำละลาย (Liquid Liquid Extraction) หลังจากนั้นนำสารละลายที่สกัดได้ไปสกัดต่อด้วยวัฏภาคของแข็งดูดซับ (Solid Phase Extraction) Octadecyl (C18) ต่อกับ aminopropyl (NH_2) cartridge มีการศึกษาหาสภาวะที่เหมาะสมต่อการสกัดสาร พบว่าวิธีการสกัดที่พัฒนามีประสิทธิภาพดีให้ค่าร้อยละการคืนกลับของสารแต่ละชนิดอยู่ในช่วง 70 ถึง 131 เปอร์เซ็นต์ ขีดจำกัดต่ำสุดของวิธีการตรวจวิเคราะห์และขีดจำกัดต่ำสุดของการวิเคราะห์หาปริมาณมีค่าต่ำสุดอยู่ที่ระดับ 0.91 และ 1.55 มิลลิกรัมต่อกิโลกรัม ตามลำดับ กราฟเทียบมาตรฐานสำหรับวิเคราะห์หาปริมาณมีความเป็นเส้นตรงอยู่ในช่วงความเข้มข้น 1.00 ถึง 100 มิลลิกรัมต่อกิโลกรัม ความถูกต้องและความเชื่อถือได้ของวิธีการวิเคราะห์นั้นทำการทดสอบโดยใช้ข้าวอินทรีย์เป็นตัวแทนของข้าวตัวอย่าง ความแม่นและความเที่ยงของวิธีวิเคราะห์ทั้งในวันเดียวกันและต่างวันกัน พบว่ามีประสิทธิภาพดีให้ผลการวิเคราะห์อยู่ในช่วงที่ยอมรับได้ วิธีการสกัดสารที่ได้พัฒนาขึ้นนั้นมีความแกร่งเป็นที่ยอมรับได้จากการทดสอบโดย Placket-Burman Experimental Design

ภาควิชา.....เคมี..... ลายมือชื่อนิสิต.....ดวงกมล ภิญโญ.....
สาขาวิชา.....เคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....Patchanun Leepipatpaibun.....
ปีการศึกษา.....2548..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....Puttha V.....

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DUANGKAMOL PINYO: METHOD DEVELOPMENT FOR THE DETERMINATION OF SULFONYLUREA HERBICIDES RESIDUE IN RICE USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY. THESIS ADVISOR: ASST. PROF. NATCHANUN LEEPIPATPIBOON, Dr.,rer.nat, THESIS CO-ADVISOR: PUTTARAKSA VARANUSUPAKUL, Ph.D., 195 pp. ISBN 974-53-2559-7

Sulfonylurea herbicides (SUHs) are widely used for controlling weeds in paddy. The residue of these herbicides may contaminate to rice. Thus, an analysis of Sulfonylurea herbicide residue was conducted. A determination method of Sulfonylurea herbicides was developed and validated seven compounds. Trace determination level was based on liquid liquid extraction and followed by solid phase extraction as a sample preparation step. Octadecyl (C18) was coupled with aminopropyl (NH_2) cartridge which isolated and cleaned up the analytes from the rice sample. Liquid Chromatography/Mass Spectrometry was optimized and developed for multiresidual analysis of seven sulfonylurea herbicides. Reversed-phase LC/ESI/MS in positive ion modes was used to separation and analysis. MS data acquisition was performed by selected ion monitoring (SIM) mode. The average recoveries of seven compounds were 79-131% in the rice sample. Method detection limits and method quantitation limits were reduced to 0.91 and 1.55 ppb, respectively. Method validation was studied in an organic rice sample. Linearity for quantitative analysis was shown a linear dynamic range 1.00 to 100 ppb. The accuracy and intra- and inter-day precision were reported in an acceptable range. Method robustness was developed from the method Placket-Burman Experimental Design.

Department.....Chemistry.....Student's signature.....Duangkamol Pinyo.....
Field of study.....Chemistry..... Advisor's signature.....Natchanun Leepipatpiboon.....
Academic year.....2005.....Co-advisor's signature.....Puttaraksa V.....

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LIST OF ABBREVIATIONS AND SYMBOLS

SUHs	Sulfonylurea herbicides
ALS	Acetolactate syntheses
GC	Gas chromatography
CE	Capillary electrophoresis
ELISA	Enzyme linked immuno-sorbent assay
HPLC	High Performance Liquid Chromatography
UV	Ultraviolet
LC/MS	Liquid Chromatography/Mass Spectrometry
MS/MS	Mass Spectrometry /Mass Spectrometry
EI	Electron impact ionization
CI	Chemical ionization
HED	High energy dynode detector
cf-FAB	Continuous flow fast atom bombardment
TSP	Thermospray
API	Atmospheric pressure ionization
ESI	Electrospray Ionization
APCI	Atmospheric pressure chemical ionization
LLE	Liquid Liquid Extraction
SPE	Solid Phase Extraction
I.D.	internal diameter
MDL	Method Detection Limit
MQL	Method Quantitation Limit
RSD	relative standard deviation
ppm	part pet million
ppb	part per billion
mL	milliter (s)
g	gram (s)
cm	centimeter
mm	millimeter

μm	micrometer
nm	nanometer
M.W.	molecular weight
t_R	retention time
R^2	correlation coefficient

CHAPTER I

INTRODUCTION

1.1 Problem Definition

Large portions of the world's wheat and corn crops go toward feeding livestock whereas rice is produced mainly for human consumption. Rice is the main staple food of the world’s population. In most the Asian countries people consume rice at every meal. However, it is not only a staple food in many developing countries in Asia and Africa, it is also becoming more and more popular in developed countries-especially long-grain rice. The human population now grows faster than ever before and food production is forced to increase at the same rate. It is still the primary means of livelihood among cultivated areas. Rice is also an important crop to millions of small farmers and to many landless workers who derive income from these farms.

A new era in the Thai economy appears to be starting with a transition to the nonagricultural sector. The growth of the urban economy has forced rice-farming areas to change. Reducing farmland is allocated to serve the growth of industry. To meet this demand, the escalating agricultural food production must make use of modern technology and use new types of pesticides as part of this development. These developments force the young blood tiller farmers to solve this situation by changing their farming practices. Consequently, the use of agricultural chemicals in the world is increasing. It is providing protection against damages caused by insects, fungi, weeds, etc. Among various agricultural chemicals, pesticides can be classified into four major categories depending on mode of action. These four groups are insecticide, fungicide, herbicides and rodenticides.

Table 1.1 Category of pesticides

Types	Properties
Insecticide	Substances that repel or kill insects.
Fungicide	Substances that prevent, destroy, or inhibit the growth of fungi in crops.
Herbicide	Substances that are used to prevent, inhibit or kill growth of weeds.
Rodenticides	Substances that are use not only to inhibit, prevent or destroy rodents, but also kill prudential species.

The total Thai consumption of pesticides is made up of imported insecticides, fungicides and herbicides. Each year, herbicides are the highest imported agricultural chemical by value. This has promoted their excessive use. In many situations, weeds growth prolific and weeds are a major constraint on crop yield. The loss in rice production due to weeds is of particular importance. Rice and weeds emerge at the same time, and weed control by flooding is difficult in seeding rice. Herbicides are being used more to control weeds, and pollution is an emerging issue in direct-seeded systems. The usage of herbicides to control weeds depends on the types of weeds, crop factors, activity of herbicides and so on. These rising trends in agrochemical consumption are making herbicides interesting chemicals to study.

1.2 Sulfonylurea Herbicides

According to weed control effectiveness, a new herbicide is presented everyday to the market. Most of these newly introduced herbicides are in the sulfonylurea family. Du Pont developed sulfonylurea herbicides (SUHs) in 1975 and the first commercial products, produced in 1982, were metsulfuron and sulfometuron. These herbicides efficiently control a variety of weeds without harming the major crops. Broadleaf and broad-spectrum weeds are controlled by SUHs. These agricultural chemicals are a highly specific catalysis acetolactate syntheses (ALS) in weeds. ALS works as an

enzyme in the 1 st step of branched-chain amino acid synthesis. The 3 essential amino acid-valine, isoleucine and leucine-are not synthesized. The SUHs actions are primarily absorbed by shoots and roots and translocated to actively growing meristematic tissue where they inhibit cell division. SUHs are highly specific; the usage of application rates is quite low, in the range of 10-40 g/ha. After applying herbicides, weeds cannot grow due to the rapid cessation of plant cell division and growth. This causes the weeds' death. The general structure of sulfonylurea herbicides is shown in figure 1.1, and consists of 3 parts.

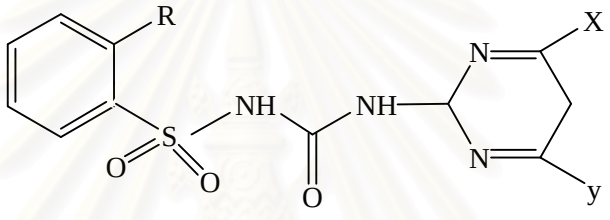
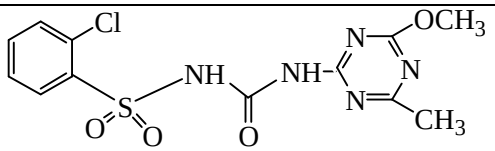
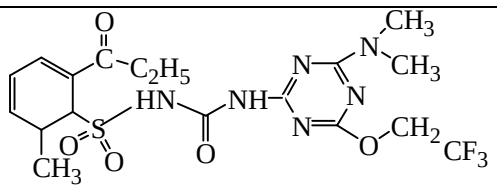


Figure1.1 The general structure of sulfonylurea herbicides (SUHs)

In general, 1 is a phenolic ring or pyridinic ring and 2 is a triazine symmetric ring or a pyrimidinyl ring. Between 1 and 2 is sulfonylurea bridge. Table 1.2 illustrates molecular weight and structure of analytes.

Table 1.2 Sulfonylurea characterized by a phenylic ring in R_1, and a triazinic symmetric ring (1, 2, 3 triazine) in R_2

Compound	Mw	Structure
Chlorsulfuron	357.7709	
Triflusulfuron methyl	492.4289	

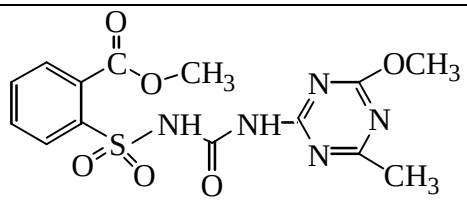
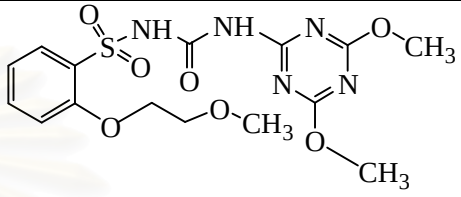
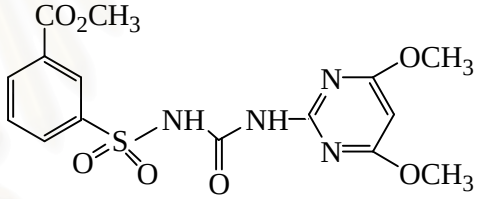
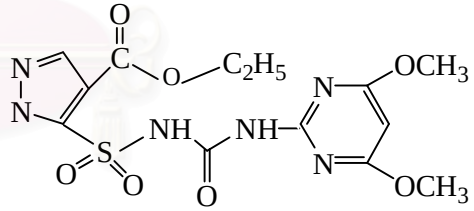
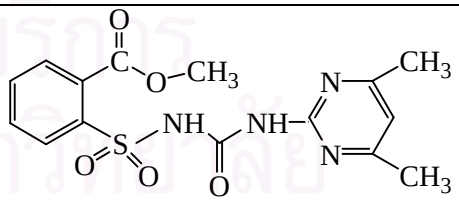
Compound	Mw	Structure
Metsulfuron	381.3624	
Cinosulfuron	413.4044	

Table 1.3 Sulfonylurea herbicides (SUHs) categorized by pyrimidinyl

Compound	Mw	Structure
Bensulfuron methyl	410.4088	
Pyrazosulfuron ethyl	414.3922	
Sulfometuron	364.3752	

1.2.1 Degradation

Sulfonylurea degrades via two main processes: chemical hydrolysis and microbial breakdown. Hydrolysis is a process in which the sulfonylurea molecules react with water, resulting in the break down of the sulfonylurea bridge. One or more atoms or groups of atoms are replaced by hydroxyl ions (-OH) from water.

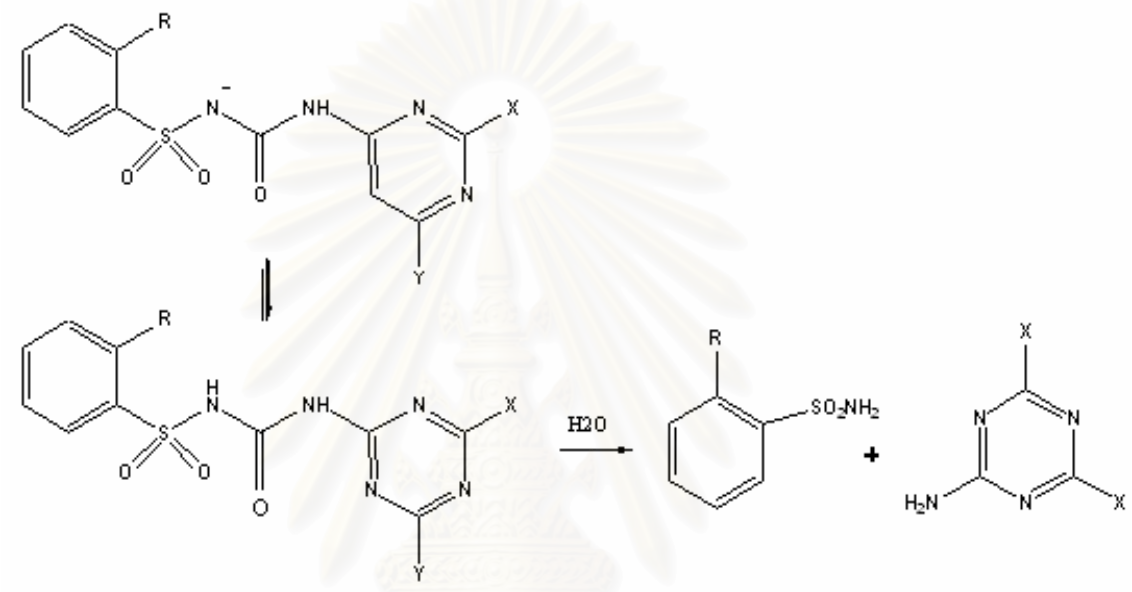


Figure 1.2 Typical hydrolysis of sulfonylurea herbicides

In field conditions sulfonylurea degradation also depends on environmental conditions, moisture content, temperature and so on. Chlorsulfuron ethyl *(1)* and sulfometuron *(2)* were hydrolyzed in low pH (4.0). Tribenuron methyl, chlorsulfuron and imazamethabenz methyl degraded in acidic conditions when studied in soil and water samples. *(3)* Sulfonylureas exhibit pH-dependent hydrolysis/degradation, which degrade more rapidly at acidic pH level than under neutral to alkaline conditions. *(4)*

Microbial decomposition is one of the methods through which sulfonylureas are decomposed, especially in soil. Microorganisms in soils consume herbicide molecules and utilize them as a source of energy and nutrients for growth and reproduction. The population levels and activity of microorganisms depend on food supply, soil pH, moisture and organic matter content. Trukey et al (1998) reported that sulfometuron methyl degraded rapidly under actual field conditions. The degradation process could be through microbial activity and chemical hydrolysis. *(2)* Cinosulfuron methyl

residue was investigated in rice paddies by ^{14}C labeled. The study was found possible chemical hydrolysis and microbial degradation due to $^{14}CO_2$ present in soil. *(5)* In 2002, there was a report on the influence of abiotic to sulfonylurea decomposition. *(4)* The organic soil matter is important to microbial activity. Jean et al (1997) reported that green manure slowed the rimsulfuron degradation. The organic matter from pigs metabolized faster in soil than that of cows. The metabolism proceeds via two pathways. The first was rimsulfuron which decomposed into 2 and 3 further transformed into 4. The second pathway was broken after nucleophilic substitution by –OH and generated 4 and 5. *(6)*

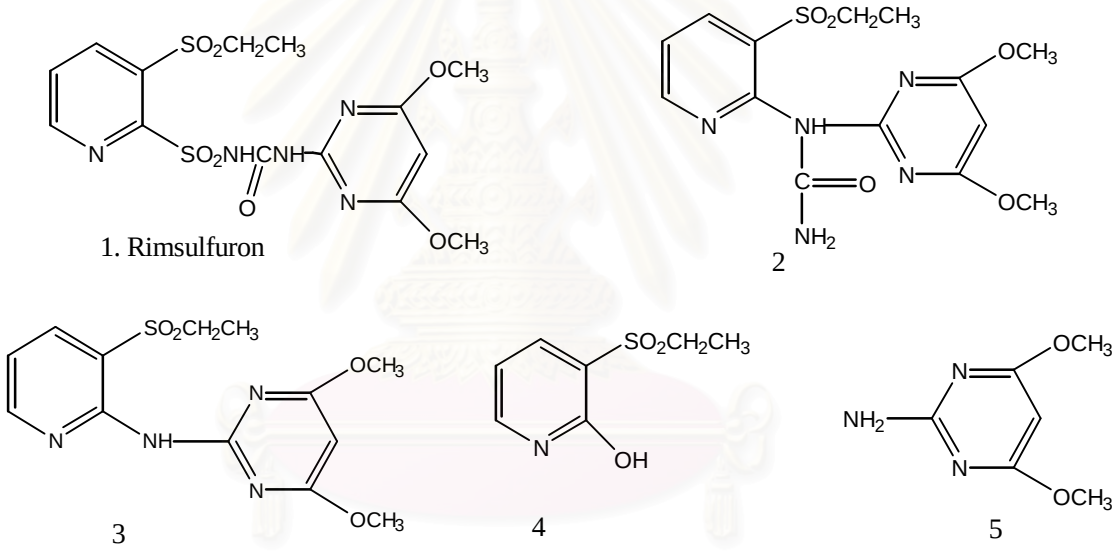


Figure 1.3 Rimsulfuron and its metabolites 2-5

The photochemical behavior was also reported. Cinosulfuron and triasulfuron degraded faster in acidic medium and at > 290 nm carbon-sulfur bond cleavage whereas short wavelength (220 nm) nitrogen-sulfur degraded. *(7)* In conclusion, sulfonylureas degrade due to microbial metabolism, pH-dependent hydrolysis, light, etc. The hydrolysis is higher in acidic conditions. *(1, 3, 4, and 7)* The appropriate preparation solution is acetonitrile and the optimum temperature must be below 40 ^0C. *(4)*

1.2.2 Toxicology and Regulation

Sulfonylureas acts upon a specific enzyme in plants, which is not found in mammals or other animals. For this reason, SUHs have very low acute and chronic toxicity. However, there is not any research to confirm that hypothesis. The handbook of pesticides reported the mammalian toxicology, which is presented in Table 1.4. Other properties are illustrated in appendix A.

Table 1.4 Toxicology of sulfonylurea herbicides

Compound	Toxicology		Other
	LD_{50}(rat)	LD_{50}(rabbits)	
Bensulfuron	Acute oral >500 mg/kg	Acute percutaneous >2000 mg/kg	Not a skin irritant or a sensitizer to guinea pigs, nor eye irritant to rabbits.
Metsulfuron	Acute oral > 5000 mg/kg	Acute percutaneous >2000 mg/kg	Mild skin irritant to guinea pigs, but not a skin sensitizer; moderate but reversible eye irritant.
Pyrazosulfuron ethyl	Acute oral > 5000 mg/kg	Acute percutaneous male rats 2079-2349 mg/kg, for females 1052 mg/kg	No irritant eyes or skin of rabbits, and not skin sensitive in guinea pigs.
Sulfometuron	Acute oral > 5000 mg/kg	Acute percutaneous >2000 mg/kg	Mild skin irritation but no sensitization occurs with guinea pigs, and temporary mild eye irritation with rabbits.
Trifulsulfuron	Acute oral > 5000 mg/kg	Acute percutaneous >2000 mg/kg	No skin irritation or sensitizer to guinea pigs, mild reversible eyes irritant to rabbits.

Thus, there is establishing of sulfonylurea regulation of tolerance. Environmental Protection Agency (US-EPA) has considered available information about the tolerance of combined residues of metsulfuron methyl and its metabolite in or on sorghum, grain, grain at 0.1 mg/kg; sorghum, grain, forage and sorghum grain, stove at 0.2 mg/kg in 2002. In the same year, the US-EPA also set up the tolerances for chlorsufuron residue in or on barley straw 0.5 mg/kg, barley grain at 0.1 mg/kg, wheat grain 0.1 mg/kg and wheat straw 0.5 mg/kg etc. Moreover, the increasing concerns regarding the cause and effects of crop protection chemicals in food and diet. A ministration of health and labour welfare of Japan (MHLW) defined the Maximum Residual Limits (MRLs) of pesticide contaminant in rice for bensulfuron-methyl at 0.1 mg/kg and for chlorsufuron and metsulfuron-methyl at the same level (0.05 mg/kg).

1.3 Literature Review

1.3.1 Sulfonylurea analysis by Gas Chromatography (GC)

GC is a separation technique for volatile and semi-volatile components of a mixture based on the difference in distribution or partitions of substances between a stationary liquid phase and mobile gas phase. GC is limited to compounds that are thermally stable, non reactive and volatile at typical operating temperature. Fortunately, most herbicides are amenable to GC separation. Thermally stable *N, N'* dimethyl derivatives for the GC analysis of chlorsulfuron and metsulfuron was studied by Peter et al. *(8, 9)* In this work, the various methylation processes and methylation of chlorsulfuron and metsulfuron-methyl by diazomethane in ethyl acetate solution produced *N, N'* dimethyl derivative in high yield. These approaches have not become widely accepted, because of poor performance. Derivative products were not only thermally stable forms (*N, N'* dimethyl) but also *N*-monomethyl which decomposes under spilt/spiltlless injection condition. Using the solid phase extraction (SPE), the C18 disk is used to determine the chlorsulfuron and metsulfuron methyl in the environmental soil and water samples. Detection limits were below 0.1 ppb (μg/L) for water and below 1.0 ppb (μg/L) for soils. GC-MS was used for confirmation. *(9)*

GC/MS was applied to quantification of flupyrasulfuron in wheat crop soils during 1997-1998. Soil was extracted with $NaHCO_3$ and cleaned up with TLC before methylation. LOD was 0.5 ppb (μg/L) *(11).* A solid phase microextraction (SPME) was applied to extract urea and SUHs. Chlorsulfuron, fluometuron, isoproturon, linuron, methabromuron and monuron were determined from environmental waters (5 sites) by GC with nitrogen-phosphorus detection (NPD). The indirect determination was used that the thermal decomposition products were phenylureas and triazineand identified online. The LOD was 0.04 ppb (μg/L) for linuron and 0.1 ppb (μg/L) for flumeturon and monuron. However, a unequivocal identification in environmental samples was done by GC/MS with SIM mode. *(10)*

1.3.2 Sulfonylurea analysis by Capillary Electrophoresis (CE)

Micellar electrokinetic capillary electrophoresis (MEKC) was used to study the degradation of SUHs in water. A triazinic heterocycle and O-benzene substitution structure, chlorsulfuron, metsulfuron, triasulfuron, ethametsulfuron and tribenuron decomposed to five or six possible products. The LOD was 25 ppm (mg/L). *(12)* MEKC also analyzed sulfonylureas in soil at ppb level by SPE enrichment. This method achieved low LOD; less than 0.01 ppm with 95.4±16.1% of recovery. *(13)* In 1995, CE reported crop determination. Five sulfonylurea herbicides from grain were extracted with acetone and partitioned with hexane. MEKC has a low detection limit at 0.02 ppm except for rimsulfuron and tribenuron methyl at 0.35 ppm (mg/L). *(14)* However, the major drawback of CE is low produce ability. The low injection volume required in CE may not yield the required sensitivity for certain applications.

1.3.3 Sulfonylurea analysis by Enzyme Linked Immuno-Sorbent Assay (ELISA)

The residue analysis of herbicides is presented at low-level. Therefore, the method must generally provide sensitivity, specificity and speed of analysis. The ELISA technique is being well used for detection of agricultural and pesticide residues. The principle is that antibodies recognize both analytes (pesticides) and pesticide enzyme-conjugates. Maria, Edward W. Christian and Stephen used enzyme immunoassay to determine chlorsulfuron from soil in 1985. The ELISA technique monitored down to

nanogram of chlorsulfuron. Chlorsulfuron in soil extracts can be detected at low concentration as 0.4 ppb in soil. *(15)* In 1999, Knopp et al. studied metsulfuron

methyl by ELISA in different water types. LOD with pure water samples was 40 ppb. However, the interference of matrices affected the ELISA linearity. *(16)* Triasulfuron residue quantities in soil with automated immunoassay were reported by Schlaeppi et al. in 1994. The resulting automated immunoassay had LOD at 0.02 ppb (μg/L) in aqueous media and 0.05 ppb (μg/kg) in soil. *(17)*

The advantages of this technique are speed, low cost and high specificity. The assay can detect other herbicides that are of similar chemical structure. Therefore, ELISA is not useful to do the multi-residue analysis.

1.3.4 Sulfonylurea analysis by High performance Liquid Chromatography (HPLC)

The use of liquid chromatography (LC) for application is increasing. LC is very effective in separating non-volatile and thermally labile compounds. The evaluation of SUHs by LC with many detectors was studied.

a) Photoconductivity Detector

Edward W. Zahnow analyzed chlorsulfuron, rimsulfuron *(18)* and sulfometuron methyl *(19)* in soil and water by using LC with a sensitive detector. The photoconductivity detector was used because it is sensitive to S, N, P and halogen atom. In the clean up step, tandem (C18, silica) SPE was used to eliminate unwanted components. Recoveries of chlorsulfuron, rimsulfuron and sulfometuron methyl at 0.2, 2.0 ppb (μg/L) were 80±16%. LOD was 0.2 ppb (μg/L) in the soil sample. *(18)* The isolation and clean up procedure for water used a C2 cartridge. This method presented recovery at 98-103% in working concentration 0.2 2.0 and 20.0 ppb (μg/L) in soil. In water, the recovery presented at 76.93% at the same concentration. LOD was 0.2 ppb (μg/L) in both samples. Moreover, Edward W. Zahnow has continued his work to determine sulfonylurea in three species of fish and four species of green plants. *(20)* James L. Prince and Richard A. Guinivan have reported the determination

of chlorimurom ethyl in straw, green plants, and oat grain in 1988. There were three clean up methods. Method 1 used double Bound Elute Si column. The second method

used silica Lichroprep. The last method used silica (turnips) or C18 (potatoes) Sep pack. *(21)* Photoconductivity detectors demonstrated undesirably long equilibration times and are no longer commercially available.

b) Ultraviolet (UV) Detector

Guido C. Galletti and a co-worker decided to study four sulfonylureas: chlorsulfuron, metsulfuron, chlorimuron and thifensulfuron by HPLC-UV in one injection. These were compared to the separation column between C6 and C18 column. It came out with C18 column application. SPE presented good recovery in both the soil and water sample and was more rapid than the classical extraction method *(22)*. In 1998, Font et al. selected reverse-phase HPLC (RPLC) with UV detection to determine five sulfonylureas in soil. The efficient clean up methods viz., SPE (MASE), and coupled column RPLC were investigated. A mild condition of microwave assisted solvent extraction (MASE) was used in fresh spike and aged soil samples. They have low levels of LOD between 20 to 1000 ppb (μg/kg). However, the MASE conditions are very complicated to produce and this preparative method is not used in many laboratories. *(23)* In 1998, Charles R. Powley and Peatricia A. de Bernard investigated a screening method for nine SUHs in environmental samples like soil and water. Soil samples were extracted by 0.1M ammonium carbonate/acetone and water samples were preconditioned with C18 SPE cartridge. Azimsulfuron, chrorimuron-ethyl, chlorsulfuron, ethametsulfuron-methyl, flupyrsulfuron-methyl, metsulfuron-methyl, sulfometiron-methyl, thifensulfuron-methyl and tribennuron-methyl presented LOQ at 1 and 0.1 ppb (μg/L) in soil and water, respectively. Therefore, LC/MS or LC/MS/MS would require confirming analytes in complex samples. *(24)* Trace analysis of acidic herbicides in water samples was conducted by coupled column LC with UV detection in 1999. The employing UV detection at low wavelength found a broad hump caused by humic substance. Co-extraction of humic and fulvic substances presented a severe baseline deviation. Analytical restricted access media (RAM) columns with semi–permeable-surface (SPS), internal surface reverse phase (ISRP) and Hisep

(SUPLECO) were studied. The ISRP/C18 column configuration proved to be most efficient for water sample. A medium DOC content, C18/SPS, appeared suitable for field studies. *(25)* The simultaneous determination of phenyl and sulfonylurea herbicides in water by SPE used liquid chromatography with UV diode array or mass spectrometry detection in 2002. It compared a pre-concentration tool by three-difference types of SPE. Oasis HLB and C18 presented similar behavior to acidic and neutral herbicides. Li Chrolut EN showed the deficient recovery to polar analytes (sulfonylurea). Acetonitrile extraction reported permitted recovery (70 to 90%) for all compounds. The chromatogram by DAD detection did not have adequate separation of chlorotuluron and flumeturon in a reasonable time. Thus, LC/MS was solving this situation. *(26)* The previous extraction methods required two approaches to do sample preparation and caused a high solvent consumption and a long operation time. The column switching (CS) system was the other competitive method. Min Zhou et al. introduced CS in 1996 to determine bensulfuron methyl in rice and crayfish. A sample was extracted by ethylene chloride and then cleaned up by an SPE cartridge. The first column was phenyl column and the second column was Rx C8 column. The total analytical time was 60 minutes per sample. The recovery presented at 90±8% over the concentration range 0.008 to 1 ppm. *(27)* The Ministy of Health, Labour and Welfare (MHLW) in Japan has already specified the tolerance level for sulfonylurea pesticide residues. Thus, determination of azimsulfuron, flazasulfuron and halosulfuron-methyl in grains, seeds, vegetable and fruits by HPLC-UV was reported in 2002. The clean up was performed using Sep-Pack Alumina N and Bound elute SAX cartridges. The recoveries of three analytes in fourteen agricultural products fortified at 0.05 to 0.5 ppm (mg/kg) were 70 to 120% with 10% CV. The result indicated that when measuring pesticides in complex interference the use of UV detection showed complicated chromatograms. The detected peaks were confirmed by selected ion monitoring (SIM) of LC/MS to improve the quantitative analyses. *(28)* For this reason, the confirmation would require a second analytical method. To avoid two methods of analysis, the combination technique of HPLC with a variety of ionization modes of mass spectrometry (MS) was studied.

c) Mass Spectrometry Detector

The analysis of sulfonylurea residues and their degradation metabolites by thermospray ionization was presented in 1992. Nicosulfuron, rimriduron and metabolites were extracted by simple and non-specific sample clean up methods. The recovery was studied in radiolabel material and ordinary analytes. LC/MS has analyzed at low level 0.02 ppm (mg/L). However, there are some problems with the thermospray evaporation process. The backpressure from the capillary interface was reported due to clogging of the in-line filter or probe tip. *(29)*

During the last few years; electrospray ionization (ESI) has become the most popular interfacing technique. In 1996, a multiresidual method for the determination of chlorsulfuron, metsulfuron-methyl, thifensulfuron methyl and triasulfuron in soil was reported. Ammonium carbonate was used to extract these herbicides from soil and then cleaned with C18 SPE. Recoveries of all four analytes were in the range of 80.1 to 100.5%. This method can detect < 0.1 ppb (µg/kg). *(30)* SPE cartridge was studied in 1997 and 1998 compared to carbograph 4 and octadecyl-bonded silica C18. *(31, 32)* Carbograph 4 can clean interference compounds in natural water better than C18. Thifensulfuron methyl, metsulfuron methyl, triaulfuron chlorsulfuron, rimsulfuron, tribenuron methyl and bensulfuron methyl had low LOD. In drinking water this was 0.6 to 2 ppt (ng/L), in ground water it was 2 to 9 ppt (ng/L) and in river water it was 13 to10 ppt (ng/L). *(31)* Imidasolinone, SUHs and arylphenoxy propionic acid (APPAS) were studied in water by C18 which caused loss of compounds especially, acidic pesticides (APPAS and SUHs). This study showed that the LOD was in the range of 0.5 to 4.5 ppt (ng/L). *(32)* The difference in groups of eighteen acid herbicides (phenoxy acids, sulfonylurea and phenols) in ground water were investigated by liquid chromatography with pneumatically assisted electrospray Ionization MS and tandem MS. They are most suitable for negative ion mode LC/MS. The detection limit using MS with SIM was in the order of 1 ppb (µg/L). The MS/MS detection increased the level of confidence based on produced ions by CID (collision induced dissociation) and the detection sensitivity reduced three to four fold. *(33)* Sixteen SUHs, imidazolinone and sulfonamide herbicides were determined and confirmed in surface water by ESI/MS. Surface water was extracted by RP-102

cartridges and then cleaned up by strong anion exchanger (SAX) stacked on top of an alumina cartridge. The quantitative analysis was demonstrated in a range of 0.1 to 1.0 ppb (μg/L), with average recovery between 70-114 % and RSD < 13%. The LOQ was 0.1 ppb for all analytes and six water types were studied. *(34)* Twenty-six base/neutral pesticides and thirteen acid pesticides were simultaneously investigated in drinking water, ground water and river water. (Antonio Di Carcia, 2000) The trace analysis used SPE with GCB cartridge. Results reported satisfactory recovery (80%). The important study indicated that LC eluent could perform best at pH 3.5 for acidic and non acidic analytes. Both positive and negative modes can be chosen. At this time, the pH of LC eluent and satisfactory separation could be performed by positive mode and raising LC eluent to pH 3.9. LOD for drinking water 0.05 and 1.5 ppt (ng/L), ground water and river water can be estimated by factor 2 and 4, respectively. *(35)* There was occurrence of SUHs, sulfonamide (SA) and imidazolinone (IMI) and other herbicides in different reservoirs of Midwestern United State in 1998. LC/MS was the determination technique with ESI and was operated in positive ion mode. The SAX cartridge removed the dissolved organic carbon (DOC) from the sample and the second one was RP-102 (styrene-divinyl benzene polymer) that retained SUHs, SA and IMI. Samples were also analyzed for 47 pesticides. *(36, 37)* In 2004, the water analysis continued to determine SUHs and urea herbicides. The LC/ESI/MS was selected for determination and quantization. There were three types of SPE in this method PS2 with polystyrene polymer resin, C18 ODS bonded silica resin and Oasis HLB. Pure water, tap water and river water were studied. The recoveries by 3 cartridges were carried out with good sensitivity (70 to 120%) and good precision (RSD 0.2 to 5.7%). *(38)*

d) Mass Spectrometry/Mass Spectrometry Detector (LC/MS/MS)

Determination of SUHs residues in soil samples with LC/MS/MS was studied by Liny Y. T. Li et al. Eight sulfonylurea herbicides and deuterium-labeled nicosulfuron were investigated. The selected reaction monitoring presented high sensitivity and specificity for SUHs in a complex matrix. The combination of HPLC retention time and unique product ions from the precursor provided the necessary information. The LOD was 0.05 ppb (μg/L) with a standard calibration curve of 0.05 to 10 ppb (μg/L). *(39)* Metsulfuron methyl was also analyzed by LC/MS/MS in soil samples in 1999. The SPE used extract analytes from soil and eliminated matrix. The C-14 labeled metsulfuron methyl indicated that microbial degradation affected field dissipation of sulfonamide part but triazine amine did not have the mineralization rate. *(40)* Large volume injection was used in determination of pesticides in vegetables and combined with LC/ESI/MS. The direct injection of a 900 μL sample extract was applied to Zorbax SB C18 column and determined by ESI tandem mass spectrometry. Optimum sensitivity was obtained with 10 mM ammonium formate-methanol gradient using selected reaction monitoring (SRM). LOD was 0.5 to 2.0 ppb (μg/kg) for potatoes and carrots in a good linearity range (2 to 100 ppb (μg/kg)). *(41)*

1.3.5 Sulfonylurea analysis by other techniques

Horseradish peroxides were used as the screen electrode to determine chlorsulfuron. A membrane with bienzyme immunoassay was attached to the electrode. Pure chlorsulfuron-glucose oxidase completely attached to binding sites of the membrane immobilized anti chlorsulfuron antibodies. The quantitative detection range was 0.01-1 ppb (ng/mL). *(42)*

A flow injection analysis with micellar-enhanced photochemical induced fluorescence detection was measured four SUHs in water. Chlorsulfuron, methylsulfuron methyl, 3-rimsulfuron and sulfometuron methyl had LOD between 0.1 to 1 ppb (μg/L). The micellar was photolysis when herbicides were located inside the micell core. *(43)*

Zue et al applied molecular imprinting SPE to determine SUHs in water in 2002. Metsulfuron methyl was a template molecule. The specific polymers were quantitating nicosulfuron, thifensulfuron methyl and chlorsulfuron in natural water and soil with HPLC/UV. LOD presented from 5 to 12 ppb (μg/kg) in soil. *(44)*

The SPE column can provide a rapid clean up of pesticide residues in complex sample matrices. SPE was studied to ascertain its efficiency in removing matrix from fresh fruit and vegetables. The graphite carbon black (GCB) removed only pigments. Aminopropyl (NH_2) and primary secondary amine (PSA) achieved clean up of extracts, especially fatty acids in samples. GC/ECD, GC/FPD and GC/MS were detectors. This paper compared the two methods from FDA method and Canadian PMA method. The result showed that a NH_2 and PSA column provided the most effective clean up and matrix, as well as a combination of C18+GCB+SAX. *(45)*

1.3.6 Sample Preparation of Sulfonylurea Herbicide

Sample preparation of sulfonylurea herbicides was extracted with water soluble organic solvents such as acetonitrile, methanol, acetone etc. Afterward, removal of co-extract compounds used SPE cartridge. Bonded phase of silica backbone produced reversed-phase application such as octadecyl (C18) octyl (C8) or ethyl (C2). C18 has been the universal extraction sorbents. These sorbents have hydrophobic properties that can be retained on alkyl chain of sorbent whereas polar analytes are eluted. *(14, 18, 19, 24, 26, 31, 32, 36, 37, 38, 45, 58, 59, 60, 61)*

The most common polar sorbents used for normal phase SPE is silica $(SiO_2)_x$, alumina (Al_2O_3) and magnesium silicate ($MgSiO_3$ or Florisil). Hydrophilic compounds are retained on polar sorbent while acidic herbicides are eluted. *(18, 19, 21, 24, 25, 28, 58, 60)*

Boned silica sorbents with cyanopropyl or aminopropyl can clean up and isolate sulfonylurea herbicides. Polar substances are retained on these sorbents. NH_2 cartridge was applied to prepared sulfonylurea herbicides found in environmental and food samples to eliminated hydrophilic matrix. *(45)* Ion exchange sorbents contain

ionic functional groups for example, quaternary amines, sulfonic acid or ionizable functional group like primary/secondary amines, or carboxylic acid. Strong anion exchange resin was used in the determination of sulfonylurea herbicides. *(28, 34, 36, 37, 60, 61)* The basic functional group (quaternary amine) is charged and attracts strongly with ionic compounds.

Graphitized carbon sorbent is successful in extraction of very polar analytes which were retained on sorbents. Moreover, graphitized carbon sorbents act as mixed mode sorbents (reverse phase and anion exchange). The sorbent surface consists of carbon-oxygen complexes that act as anion exchange site and van der Waals interaction do not have microbore pore. Colour and pigment compounds in fruits, vegetables and grains were removed by a graphitized carbon sorbent. *(45, 63, 64)* Multiresidue method for acidic, basic and neutral herbicides in the variety of water samples were studied by graphitized carbon sorbent. *(35)*

Mixed mode sorbents are also used in the study of sulfonylurea herbicides. This sorbent has multiple retentive sites on an individual particle. A different retention mechanism occurred. Food *(45, 58)* and environmental samples *(38)* contained a variety of matrices property (polar, non polar compounds etc.). HLB and PSA are mixed mode sorbents and are also used for isolating sulfonylurea herbicides. *(62)*

A restricted access media column has wide-pores or large-pores at its surface from which large or macromolecules are excluded. Small analyte molecules enter are retained in the pore. Therefore, this cartridge is suitable for cleaning up humic and fluvic substances in environmental samples. *(25)*

Another SPE for preparation of sulfonylurea herbicides in water and soil is molecular imprinted polymeric sorbents (MIPs). *(44)* MIPs are synthesizing antibodies which are very selective for analytes. A target analyte presents a molecular template when the polymer is formed. This advantage of MIPs is difficult to completely desorb analyte.

1.4 Purpose of Study

From the review, many researchers have paid attention to environmental analysis. Low LOD was reported for analysis of SUHs in soil and water samples. HPLC with many detectors like DAD, UV, and photoconductivity were reported. In terms of sample preparation, there were many sample preparation methods that refer to different matrix and are suitable to analysis technique. SPE was isolating herbicides from the matrix. Many sorbents were reported therefore the study of proper SPE cartridge was interesting. According to MHLW, the Japanese government has set up regulations for bensulfuron-methyl at 0.1 mg/kg, chlosulfuron and metsulfuron-methyl at 0.05 mg/kg. Moreover, the main exporting agricultural product of Thailand is rice. Therefore, there should be a safety evaluation of SUHs residue in local market where the consumers get their products. Thus, the study of SUHs residue in food is very interesting. This thesis is specific to a study on Thai jasmine rice, which is very popular in worldwide markets and also in domestic markets. We intended to develop a sample preparation for determination of SUHs residues in jasmine rice. The multi-residual of chlorsulfuron (Chlor), Triflusulfuron-methyl (Tri), Metsulfuron-methyl (Met), Cinosulfuron (Cino), Bensulfuron (Ben), Pyrazosulfuron-ethyl (Pyra) and Sulfometuron (Sul) in jasmine rice is studied by the HPLC/MS.

CHAPTER II

THEORY

2.1 High Performance Liquid Chromatography (HPLC)

Liquid chromatography (LC) relies on the separation of two different phases or immiscible layers. The separation is caused by the different interaction of each component with two phases. HPLC is used to describe LC in which the mobile phase is mechanically pumped through a column. HPLC is the most widely used of all analytical separation techniques and the basic components are shown in figure 2.1. The instrument consists of: (1) a mobile phase reservoir, (2) pumping system, (3) sample introduction system, (4) column, and (5) detector.

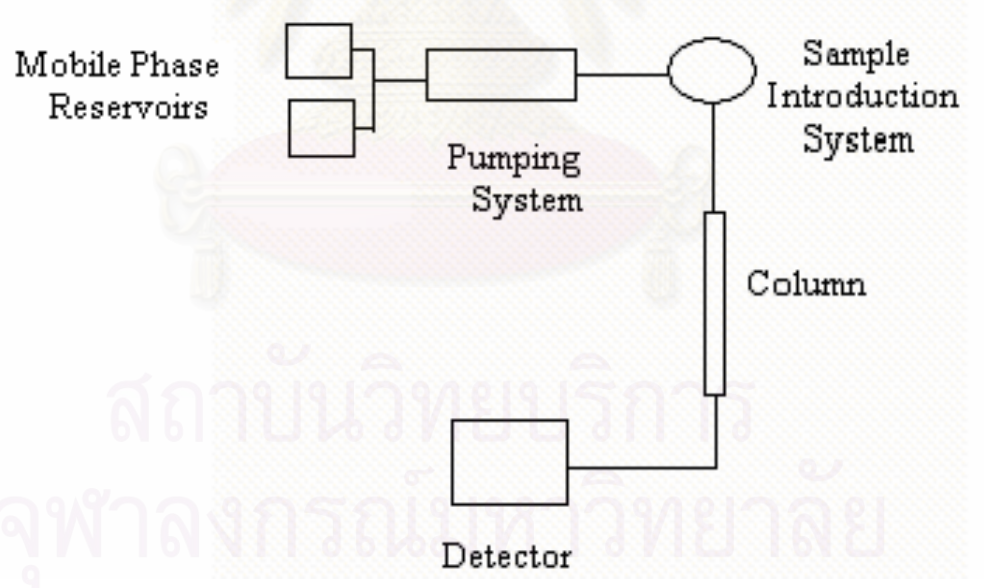


Figure 2.1 An apparatus of HPLC

2.1.1 Mobile Phase Reservoir

The reservoir is made with glass or stainless steel and can store 200 to 1000 mL of a mobile phase prior to it being fed into the pump. The container must be inert. All mobile phases should be de-gassed because the dissolved gases in the mobile phase can cause a separation by forming bubbles in the pump check valve and the detector. *(46)*

2.1.2 Pumping System

A wide variety of pump designs for the HPLC system have been developed, the most common being a reciprocating piston pump, invented in the 1980s. The pump head consists of check ball valves and a piston assembly. The small chamber pumps solvent with a motor driven piston. Two ball valves control the flow in and out of the chamber. The reciprocating pump produces a pulse flow which interferes with the detector and causes a noise on the baseline of the chromatogram. The minimization of pump pulsation is achieved by a two head design with a non circular cam to drive the piston 180^{o} out of phase.

2.1.3 Sample Introduction System

A sample loop is the most common way to introduce a sample to LC. This device is more precise than a syringe injection. The function of this device is to introduce the sample into solvent steam prior to the column. The injector should minimize dispersion and band broadening. Moreover, the injection system should not disturb the baseline especially in flow sensitive detectors like refractive index (RI) or conductivity.

2.1.4 Column (Stationary phase)

The most common packing for liquid chromatography is a silica based particle coated with a thin organic film chemically or physically bonded to the surface. Other packing material includes alumina particles, porous polymer particles and ion exchange resins. Bonded silica coated with C-18 is used most often in the reversed phase column.

2.1.5 Detector

Liquid chromatography detector is available in two basic types: the bulk property detector and the solute property detector. The bulk property detector responds to the universal property of mobile phase with or without an eluted solute. *(47, 48)* The solute property detector responds to some property of solute which is not exhibited by the mobile phase (MP) such as ultraviolet (UV) absorbance, fluorescence, or diffusion current.

2.2 Mass Spectrometer

The first mass spectrometer was invented by JJ. Thomson in 1912. Mass Spectrometer (MS) is an analytical spectroscopic tool primarily concerned with the separation of molecular (and atomic) species according to their mass. MS can be used for the establishment of the molecular weight and structure of compounds, or the identification and determination of the components. Gas, solid and liquid samples can be introduced through special inlet devices, into the ionization source of the instrument. Inside the ionization source, the sample molecules are ionised to gas phase ions. These ions are extracted into the analyzer region of the mass spectrometer where they are separated according to their mass to charge ratio (m/z). The analyzer is operated under a high vacuum, helping ions to travel safely to the detector with a sufficient yield. The separated ions are detected and the signal is sent to a data system where the m/z ratios and their relative abundance are presented in the spectrum. A mass spectrometer can be divided into four fundamental parts, namely: sample introduction, ionization source, analyzer, and detector, as shown in figure 2.2.

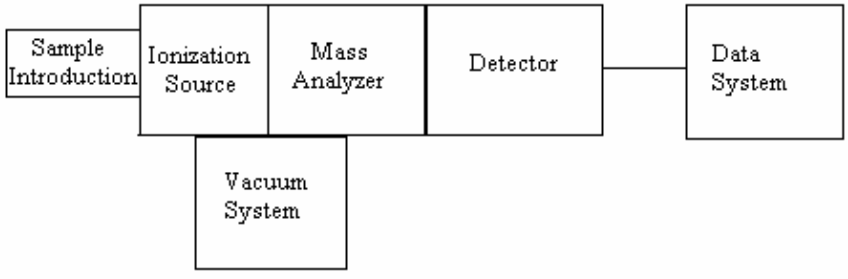


Figure 2.2 Mass Spectrometer block diagram

2.2.1 Sample Introduction

The purpose of a sample introduction is to introduce a very small amount of a sample into an ionization source depending on the ionization method being used, as well as the type and complexity of the sample. Direct insertion probe is a common sample introduction, in which a sample (solid or liquid) is placed on a probe and inserted, usually through a vacuum lock into the ionization region of the mass spectrometer. Then the sample is ionized and vaporized by thermal desorption. Likewise, a direct liquid introduction is chemical ionization (CI) or electron impact ionization (EI) method on liquid in MS.

2.2.2 Ionization Source

The ionization method depends on the sample type, specific information requirements and the mass spectrometer availability. Molecular compounds are converted into gas phase ions either before or during charging or the ionization process, which takes place in the ionization source. Ionization methods include electron impact ionization (EI), chemical ionization (CI), thermospray ionization (TSP), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), fast atom bombardment (FAB), field desorption/field ionization (FD/FI) and matrix assisted laser desorption ionization (MALDI).

a) Electron Impact Ionization (EI)

Electron impact ionization is the common and original ionization source which was developed by Dempster in 1918. Sample molecules in the vapor state are bombarded by fast moving electrons which produce 70 eV by passing the current through a filament.

$$M + e^{-} (70\ eV) \text{-----} > M^{+\cdot} + 2e^{-}$$

The electron beam gives excess energy. EI mass spectra contain fragment ion peaks and much smaller molecular ion peaks. Molecular can also lose an electron. The sample can be introduced to the EI source via a gas chromatography device or directly

via a solids probe device. Consequently, the EI ionization method is suitable for non-thermally labile compounds. *(46)* The common EI apparatus is presented in figure 2.3.

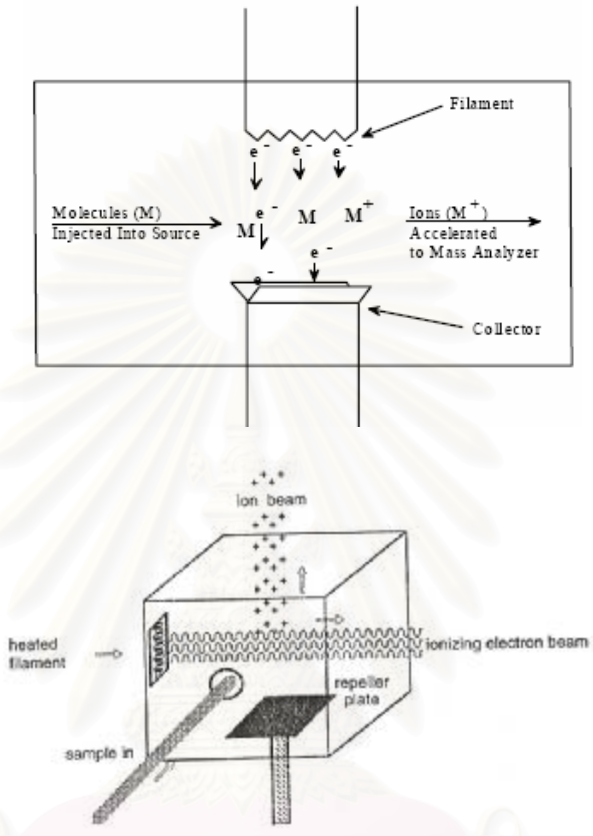


Figure 2.3 Electron impact ionization apparatus *(54)*

b) Chemical Ionization (CI)

Chemical ionization (CI) is an especially useful technique in determining molecular ions and confirming their mass-to-charge ratios. CI uses the same ion source device as electron impact; however, CI uses a tight ion source and a reagent gas. The reagent gas (methane, isobutene, and ammonia) is attacked by an electron beam and generates ionized reagent gas. Then, the reagent gas ions interact with sample molecules to form the sample ion. This phenomenon is called ion-molecule reactions. This is similar to EI source but requires less energy, because the sample molecule does not directly interactd with electron beams. Therefore, the product ions are more stable and not many fragments are present. Positive ions and negative ions can be formed in the CI process depending on the setup of the instrument. Some typical reactions in CI source are shown:

$$RH^+ + S \quad ---> \quad SH^+ + R$$

(R; CH_4 = reagent gas, S = sample, e = electron, . = radical electron, H = hydrogen)

A) Generation of reagent gas ions by electron beams:

$$CH_4 + e \quad ---> \quad CH_4^+. + 2e$$

B) Reaction of reagent gas ions to form adducts:

$$CH_4^+. + CH_4 \; ---> \; CH_3^+ + CH_5^+$$

OR $$CH_4^+. \quad ---> \; CH_3^+ + H.$$

$$CH_3^+ + CH \quad ---> \; C_2H_5^+ + H_2$$

C) Reaction of reagent gas ions with analyte molecules:

$$CH_5^+ + M \quad ---> \; CH_4 + MH^+$$

$$C_2H_5^+ + M \quad ---> \; C_2H_4 + MH^+$$

$$CH_3^+ + M \quad ---> \; CH_4 + (M\text{-}H)^+$$

Table 2.1 Advantages and disadvantages of ionization technique

Ionization Technique	Advantages	Disadvantages
EI	• Fragmentation pattern can identify unknown compounds. • Structural information from the fingerprint pattern.	• Limited decomposition by thermal desorption prior to the vaporization. • Too much fragmentation, affecting no observable molecular ion.
CI	• Soft ionization • Less fragmentation, mostly molecular ions	• Less fragmentation, fragment pattern not informative or reproducible enough for library search. • Sample must be thermally volatile with stable compounds. • Results depend on reagent gas type, reagent gas pressure, or reaction time, and nature of the sample. • Low mass range less than 1,000 Da.

2.2.3 Mass Analyzer

The mass analyzer is designed to separate and resolve the ions from the ionization source prior to their mass-to-charge (m/z) ratios. There are many mass analyzers currently available, the better known being the magnetic sector, quadrupole, time-of-flight (TOF) analyzer and fourier transform and quadrupole ion traps.

a) Magnetic Sector

The first mass spectrometer developed by J.J. Thomson used magnetic sector as the mass analyzer. Magnetic sector (Figure 2.4) separates ions in a magnetic field according to the momentum and charge of the ion. Ions are accelerated from the source region into the magnetic sector by a 1 to 10 kV electric field.

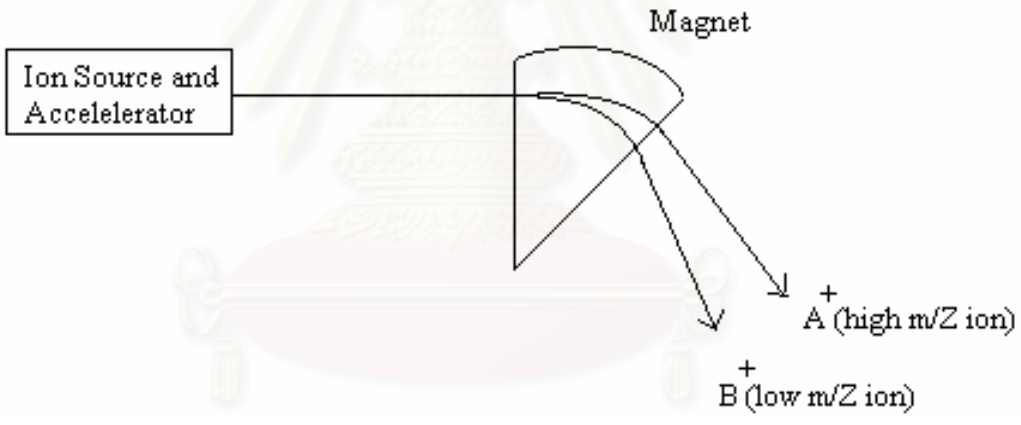


Figure 2.4 Magnetic sector device

Since the ions are charged, they move through the magnetic sector. This separates ions according to their momentum; so magnetic sectors are often called momentum analyzers. Analysis of equal mass and charge can follow the same path through the fixed magnetic field because the momentum (for a given mass the velocity (ν) or kinetic energy ($m\nu^2/2$) is constant. Double-focusing magnetic sector uses a magnetic and an electrostatic sector to focus and accelerate ions. The combination of two analyzers improves resolution and accuracy of the mass spectrometer and mass ranger. However, double-focusing magnetic sector has some tiny drawback such as reduction of sensitivity due to use of a narrow slit and decreased voltage.

b) Quadrupole

A quadrupole is the most common mass analyzer introduced by Paul, Steinwedel, Raether, Reihard and Von Zahn between 1953-1958. Quadruple mass analyzer is composed of four hyperbolic rods arranged in a square array. Opposite rods are connected electrically, one pair being attached to the positive side of an available DC source and the other to the negative terminal. The radio frequency (AC potential) is applied to each pair of rods. The operation of this analyzer is usually treated in terms of a stability diagram that is related to an application of DC potential, RF potential and frequency. The right size ions succeed in passing through the mass filter and can be measured.

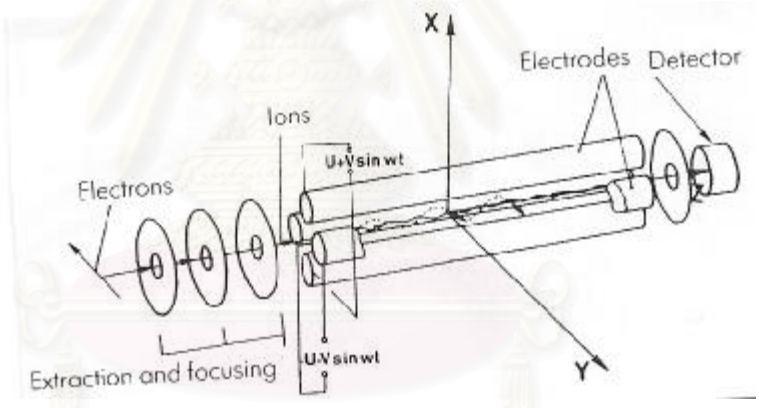


Figure 2.5 Four hyperbolic rods of a quadrupole *(56)*

The potential are applied by,

$$\phi = (\nu_{dc} + \nu_{rf} \cos(\omega.t)\ x^2 - y^2/r_o)$$

Where, ϕ = potential

ν_{dc} = DC potential

ν_{rf} = RF potential

ω = RF frequency

x,y = ion position

r_o = distance between rods

When $\nu_{dc} > \nu_{rf}$, the low mass ions are lost. Only heavier ions pass through rods (high mass filter) and project on the x-direction. At the same time in y-direction, the low mass ions are only passing through (low pass filter). The combination of both yields is a stability diagram, as seen on figure 2.4. Area in the plot where x and y are smaller than r_o (ions stay in the rods), present a stable path through the mass spectrometer.

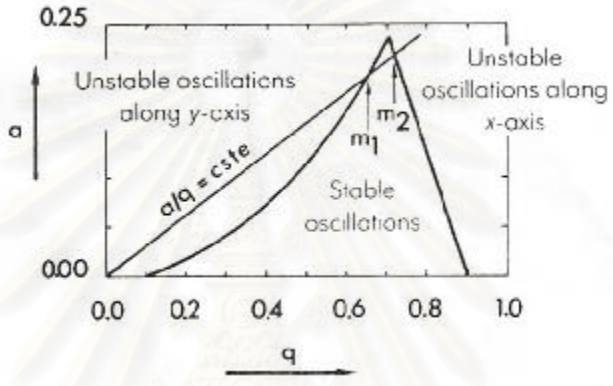


Figure 2.6 Stability diagram of quadrupole mass analyzer *(56)*

Table 2.2 Advantages and disadvantages of mass analyzer

Analyzer	Advantages	Disadvantages
Magnetic sector	• High resolution and mass accuracy. • Tandem MS are possible.	• Expensive instrument. • Scan speed limited by hysteresis and heating of the magnets. • Limited sensitivity, especially at high resolution.
Quadrupole	• Tolerant of high pressure. • Low potentials allow relatively high pressure and simple vacuum system. • Simple scanning method. • Low cost	• Low resolution systems, typically 1 Da. • Limited Mass range. (approximately *m/z* 4000)

2.2.4 Detector

The mass spectrometry detector monitors the ion current, amplifies it and the signal is then transmitted to the data system where it is the signal recored in the form of mass spectra. The type of detector is supplied to suit the type of analyzer. The mass spectrum is plotted between the intensities and the m/z value to present the information such as the molecular weight of each component and the relative abundance of the various components in the sample.

a) Faraday Cup

A faraday cup is a metal cup placed in the path of the ion beam. It is attached to an electrometer, which measures the ion-beam current. The basic principle involves charging a metal plate to create an electron flow resulting in a current production. A cup shape (Figure 2.7) of this detector minimizes loss of secondary electrons that would alter the current measurement. A deep cup with an electron repelled plate reduces the secondary electron loss.

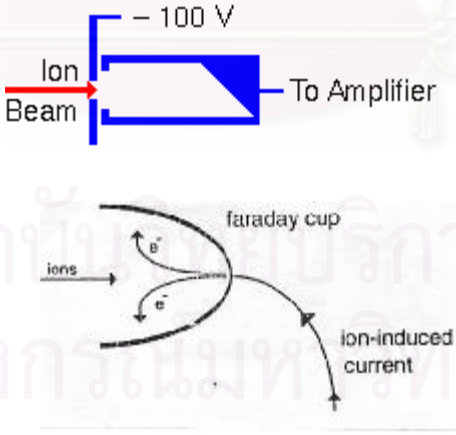


Figure 2.7 The principle component of faraday cup *(54)*

b) Electron Multiplier Tube

Electron multiplier is the most common type of detector. This detector consists of a layer of metal oxides (lead and tin oxide) on an anode glass tube (channeltron), or coat on a channel with a glass construction (channel plate). The principle of this detector is the amplification of electrons by producing secondary electrons when the ions from the analyzer hit a cascade of accelerated electrodes (dynode). These electrons are forced by a proper electric field to collide with the wall and these electrons act like ions, causing electrons to be emitted. A series of biased dynodes eject secondary electrons into the vacuum space. These secondary electrons travel down the channel and repeatedly collide with the next dynode to produce more secondary electrons. This process will continue until the resulting cloud of electrons exits the channel and are collected by the anode. Typical amplification or ion gain of an electron multiplier is 10^6 with a lifetime of 1 to 2 years due to surface contamination from incident ions or from a poor vacuum.

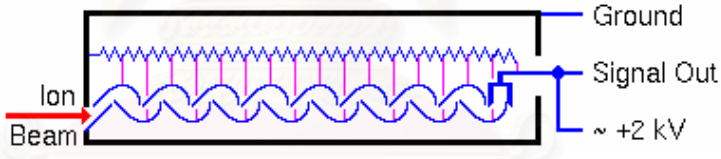


Figure 2.8 The principle component of electron multiplier tube and dynode

The electron multiplier can also be made from continuous dynode material such as a channeltron, which is horn-shaped, as shown in figure 2.9. This new design can improve the signal sensitivity.

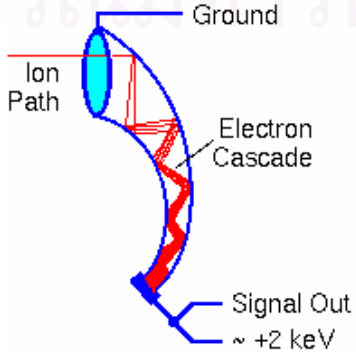


Figure 2.9 The continuous dynode (channeltron)

Most electron multipliers operate with sufficiently high gain to produce a detectable pulse for each ion arrival. Pulse counting is the most sensitive ion detection method. In order to detect both positive and negative ions, the electron multiplier case stands at ground potential and the output ends at high positive potential. The electron multiplier measures ions at arrival rates around 10^6 counts per second due to detector recovery time (dead time) of electronic devices. Pulse measurements consist of counting ions for a fixed period of time and the result takes the form of a counting number.

c) Photomultiplier Tube

A photomultiplier tube is a sensitive photocell used to convert a light signal of a hundred photons into a current pulse. A photomultiplier tube consists of a photo-emissive cathode (photocathode) followed by focusing electrodes, an electron multiplier and an electron collector (anode) in a vacuum tube. When light enters the photocathode, the photosensitive layer converts incident photons into low-energy electrons. The focusing electrode voltages towards the electron multiplier then direct these photoelectrons to an anode the output cotaines the multiplied electrons. After amplification, a typical scintillation pulse will give rise to 10^7-10^{10} electrons, sufficient to generate a charge signal that can be collected. The delay time of the original light pulse is about 20-50 ns. Most tubes will produce an electron pulse with a time width of few nanoseconds after delay time.

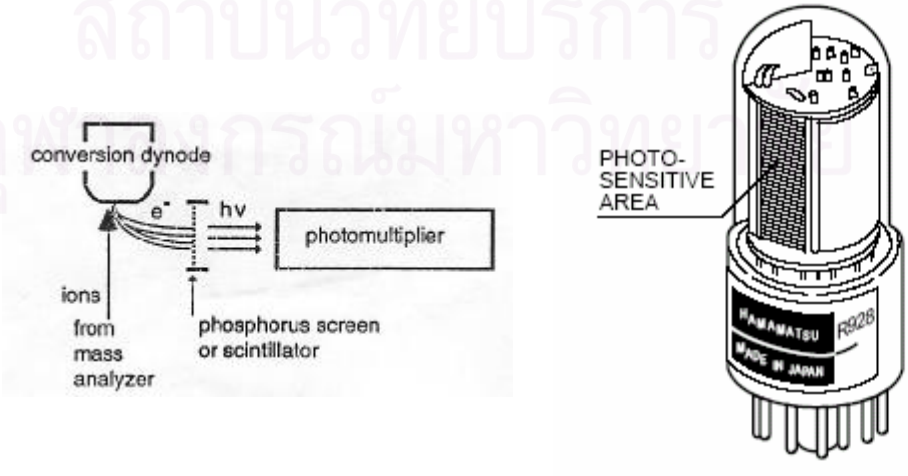


Figure 2.10 Photomultiplier tube *(54)*

d) High Energy Dynode Detector (HED)

HED are now commonly used to enhance the sensitivity of ion detectors by increasing the impact energy of input ions. High voltage (10 kV) is applied to the conversion dynode prior to the electron multiplier. This electrostatic field accelerates the ions before interacting with dynode surfaces. HED increases ion energy and signal intensity, which relates to greater sensitivity.

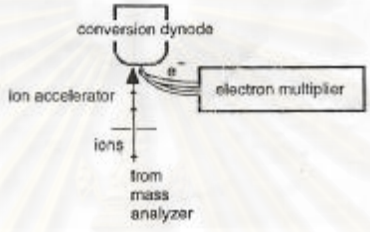


Figure 2.11 High-Energy Dynode Detector *(54)*

2.3 LC/MS Interfaces

For many years, high performance liquid chromatography (HPLC) with predominantly ultraviolet (UV), fluorescence or electrochemical detection has been employed. There are some limitations of the conventional HPLC detector. For example, UV detection is not very sensitive and suffers from low specificity. Fluorescence and electrochemical detection are limited to compounds with fluorescent or electro-active groups; to do otherwise requires derivatives. The excellent sensitivity and high selectivity of MS detection offers a powerful approach to do quantitative and qualitative analysis. The analyte is identified and detected not only by a molecular ion but also a typical fragment ion. The combination of the separation power like HPLC with the detection power like MS is called "*hyphenated technique*" or LC-MS in this case.

The fundamental problem in coupling LC with MS is the large volume formation and the vacuum requirement. Several interfaces have been developed for solving this problem. However, the combination of two powerful analytical techniques is not easy;

there are three major difficulties: the incompatibility of flow rate (from conventional LC column; 1mL/min into high vacuum MS), the incompatibility of solvent consumption by using a non-volatile mobile phase; salt and the ionization of non-volatile and/or thermal labile analytes.

Coupling methods are being developed to overcome these difficulties. This includes, a particle beam (PB), a moving belt/wire, a continuous flow fast atom bombardment (cf-FAB), a direct liquid introduction (DLI), a thermospray (TSP), an electrospray ionization (ESI) and an atmospheric pressure chemical ionization (APCI). *(49)* The LC-MS diagram is shown in figure 2.12.

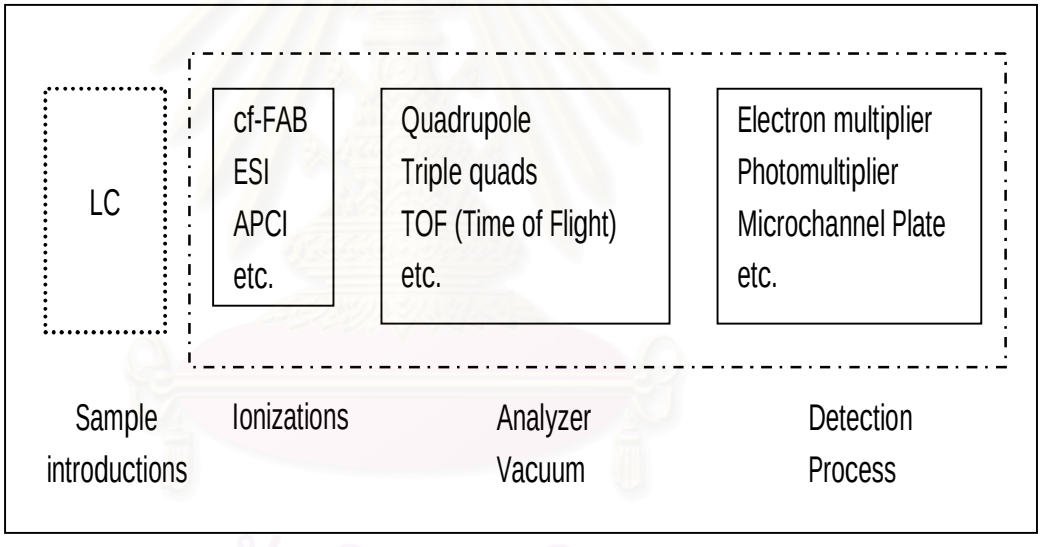


Figure 2.12 The ionization process diagram of LC/MS

2.3.1 Moving belt/wire Interface

Moving belt interface consists of an endless continuous moving kapton ribbon on which mobile phase of the LC is deposited and evaporated, as shown in figure 2.13. The mobile phase is removed via gentle heating and evaporation under vacuum chambers. According to cycling evaporation and ion transport from atmospheric pressure to MS, the two differently pumped vacuum locks require reduced pressure. Desorption of analyte at the tip of the moving belt interface provides analyte in a gaseous state to ionized by CI or EI. Nowadays, the moving belt interface devices are complex and have been replaced with the particle beam interface.

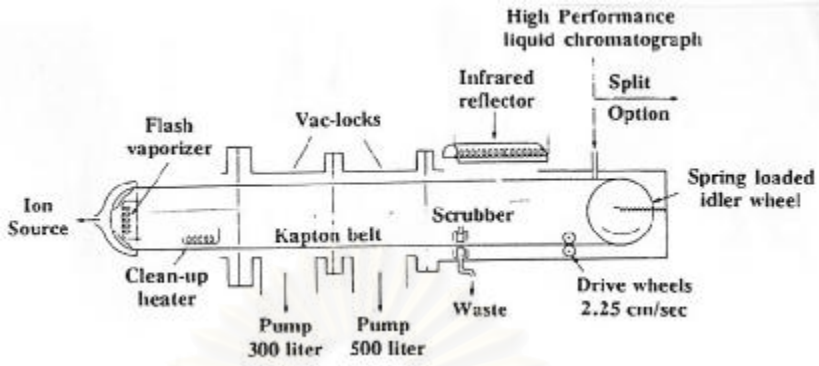


Figure 2.13 The component of moving belt/wire interface *(57)*

2.3.2 Direct Liquid Introduction

The capillary infusion is often used, because it efficiently introduces small quantities of sample into a mass spectrometer without destroying the vacuum. This device consists of a 5µm diameter pinhole between LC probe and mass spectrometer. The eluates from LC column transport through this probe, and reach the pinhole. Then, a vacuum in MS draws a proportion into the desolvation chamber. Mobile phase is not completely removed by this interface, thus the ionization process is restricted to chemical ionization. However, this technique can remove solvents by using large pump systems and assist the evaporation by using a heated desolvation charge. Then, the gas phase analyte is introduced directly into the source region through a needle valve.

2.3.3 Continuous Flow Fast Atom Bombardment (cf-FAB)

A continuous flow or dynamic fast atom bombardment interface (Figure 2.14) is a modification of the fast atom bombardment (FAB) technique that allows continuous on-line refreshing of the liquid on the FAB target. A small liquid stream is mixed with an appropriate FAB matrix solvent such as glycerol, thioglycerol, or nitrobenzyl alcohol. The FAB matrix is added to the LC effluent and transported through a narrow-bore fused-silica capillary. The special attention must be given to the addition of the matrix to LC eluents. Using pre-column addition might interfere with the separation; post column the choice of liquid junction or coaxial set-up has different effects on the chromatographic performance. It could flow through a capillary by a

stainless-steel frit or a gold-plated FAB target. These ions are generated by a bombardment of a liquid film of the sample by fast atoms (Ar or Xe, keV energy). Then, ions are sputtered out of the solution and sampled in the MS, leading to the ease of implementation of cf-FAB, especially at the magnetic sector instrument.

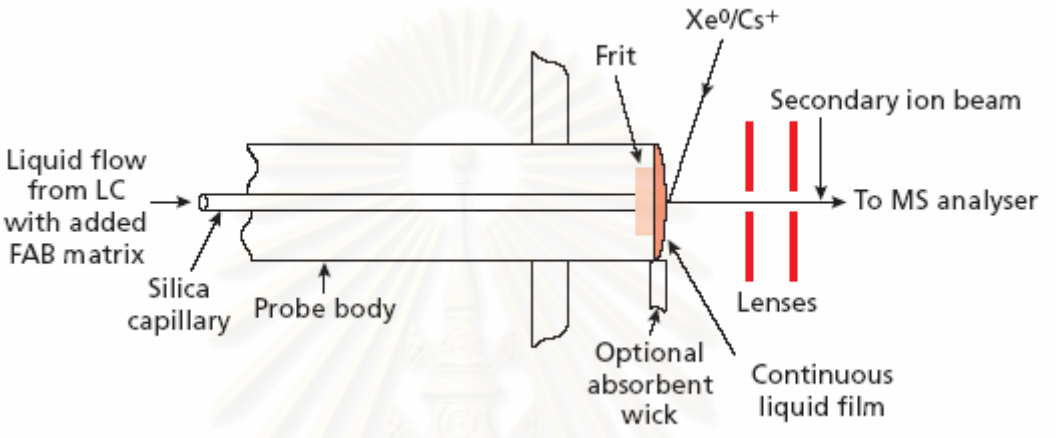


Figure 2.14 The principle component of cf-FAB probe *(50)*

2.3.4 Particle-beam Interface

Willoughby and Browner introduced the particle beam interface in 1984. This interface is also known as a mono-disperse aerosol generation interface (MAGIC). The apparatus of this interface is shown in figure 2.15. LC-eluent flows through a capillary neubulizer at a flow rate of 0.1-0.5 mL/min using a gas flow as a neubulizer gas. The closed desolvation chamber demands a total input higher than the vapor from LC. These droplets are evaporated in the desolvation chamber to solid particles, and then transported into MS. The heated transfer of the helium gas, heated capacities of the solvent, desolvation chamber temperature and the size of the droplets affect the evaporation of droplets. Therefore, the improvement of the performance of the particle beam interface is a cross-flow neubulization and a narrow droplet size distribution design. It also achieves a particle beam in the two-stage momentum separator.

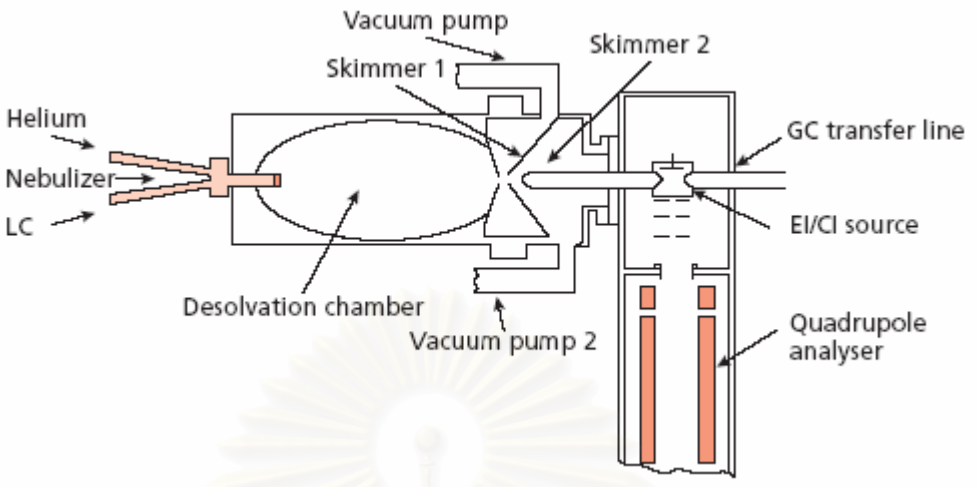


Figure 2.15 The principle component of particle beam or MAGIC interface *(50)*

2.3.5 Thermospray (TSP)

Blakely and Vestal developed a thermospray interface in 1983. Thermospray interface (Figure 2.16) permits a direct introduction of the high flow rate effluence from a column. Liquid is evaporated by a heated stainless steel capillary tube to form an aerosol jet. Thermospray produces ions from an aqueous solution that had been sprayed directly into the mass spectrometer. Thus, thermospray is not only an interface but also an ionization source. A solution containing salt and analyte is pumped into a heated steel capillary to produce a fine droplet spray, containing ions, solvent and sample molecules. This spray is an imbalance of charges originating from charged solutes present in the solution. The solvents evaporate from the droplet decreasing its size. The charge repulsion force overcomes the cohesive forces of the droplet in the “charged-residue” model. Coulomb explosions result in droplets containing a single solute molecule that accumulates charge as the remaining solvent is evaporated. Another view of the process is the “ion evaporation" model, in which the analyte ion is ejected from the droplet to alleviate the high electrical potential produced as the solvent evaporates, while most of the neutrals are removed by a vacuum pump. To conclude, the ionization takes place by solvent-mediated CI reactions and ion evaporation processes. The reagent gas for solvent-mediated CI can be generated either in a conventional way using energetic electrons from a filament or discharge electrode, or in a process called thermospray ionization, where the volatile

buffer dissolved in the eluent is involved. The ion source is equipped with a mechanical pump to evaluate the excess solvent vapour. The rapid heating and protective effects of the solvent allow the analysis of non-volatile samples without pyrolysis. The analyte ions enter the MS through a sampling cone. Thermospray interface provides an easy-to–use LC-MS interface and becomes the widely used LC-MS interface. One limitation of themospray ionization is the restriction to the aqueous mobile phase and some requirements of adding buffers to facilitate ion formation.

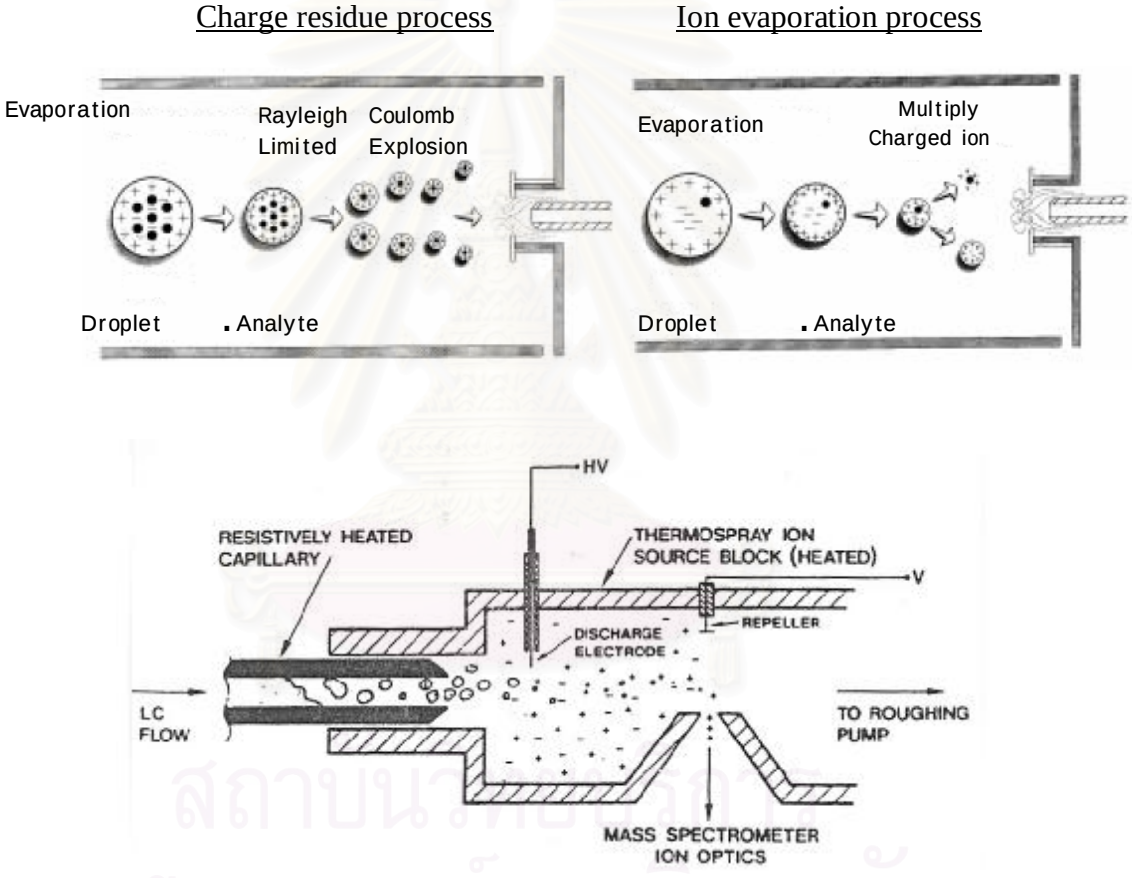


Figure 2.16 The principle component of thermospray apparatus *(55)*

2.3.6 Atmospheric Pressure Ionization (API)

The atmospheric pressure ionization (API) technique was developed in the 1990s. The advantages of API are, it is easy to handle with liquid from LC, suitable for the analysis of non-volatile, polar and thermal unstable compounds typically analysed by LC and API systems are very durable. An API technique combines the elimination and ionization step at atmospheric pressure (760 mmHg) and is considered a soft ionization source. Electrospray and atmospheric pressure chemical ionization are both API techniques.

a) Electrospray Ionization (ESI)

In the early 1970s, Dole, Mack, Hines, Mobley Ferguson and Alice attempted to use ESI as a MS interface. Electrospray is an ionization technique that uses electricity to form the droplets. The sample capillary tip sprays at atmospheric pressure and is floated at high potential. The metal capillary is surrounded by a nitrogen flow which also applies voltage. The potential difference is about 3 kV between the two electrodes. Ions of the same polarity migrate toward the liquid surface which is drawn out of the capillary, forming a taylor cone. These fine mists of droplets are an electrostatic spray of multiple charged droplets containing the ionized sample. The spraying process is assisted by nitrogen gas (neubulizer gas) flow. The solvent evaporation is reducing the droplet size and the charge density is increasing or the droplet is shrinking by solvent evaporation. Then, the droplet becomes a very small charged droplet and exposed because the surface of the liquid reaches the point where columbic forces overcome the surface tension of the liquid. The droplet repeats this process until analyte ions evaporate from the droplet. In order to reduce detrimental effects of deposit on the electrodes and skimmers, a gas curtain is applied effectively blowing the neutral and large particles from the MS entrance. These charged droplets produce analyte ions through the same ion evaporation process discussed above in the TSP section. ESI differs from TSP in the fact that it uses a high potential to impose a charge in place of the buffers used in TSP. This high potential has the unique advantage of being able to generate multiple charged ions. The effective mass range (less than 200,000 Da) can be extended due to multipled charged effect. Solubility of the analyte sample is essential for successful ESI analysis. ESI is preferred for

compounds that are ionic, very polar or thermal labile. The information consists of structural pattern and molecular weight. According to HPLC connection, flow rate affects both size and size distribution of the droplets formed during the electrospray process. To solve this situation, the HPLC column with reduced i.d. is now available. The use of a micro bore column is not yet routine and requires a much more rigorous control of parameters. Thus, the alternative is to split the flow from the conventional column. Solution is directly transported to the electrospray by a connection with pneumatic assisted neubulization and/or a heated source inlet. A slitting system can be used, because electrospray is a concentration-sensitive device; signal intensity is proportional to the concentration of analyte in mobile phase rather than the amount of analyte present.

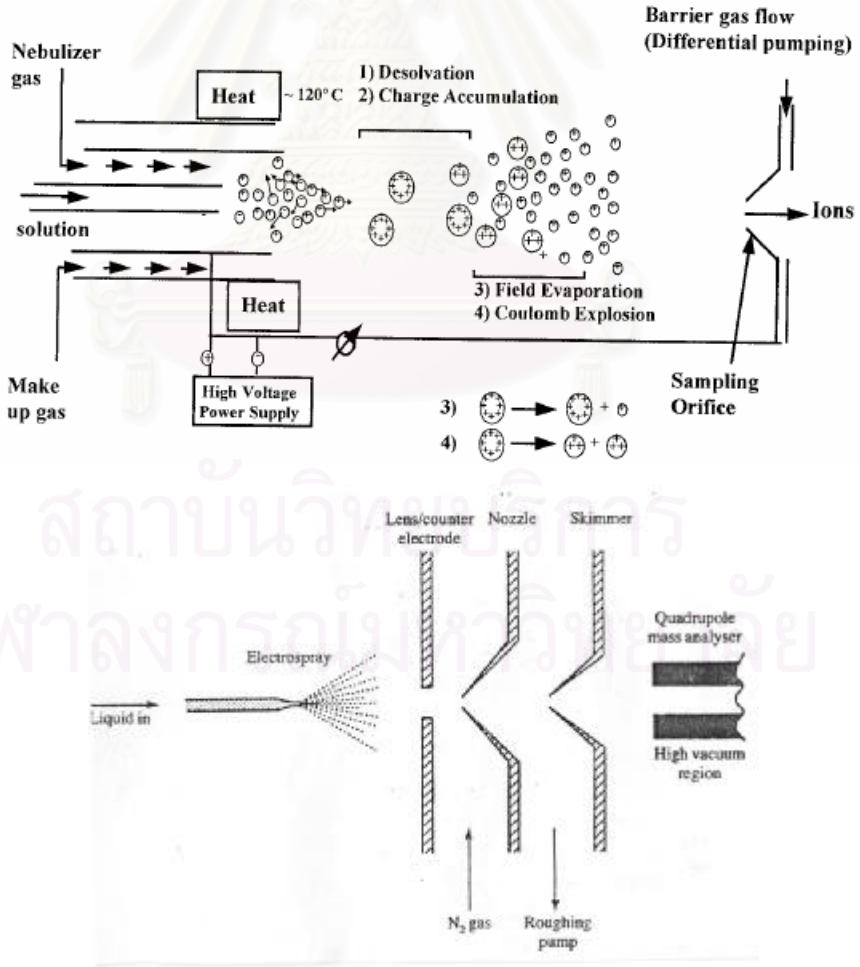


Figure 2.17 Ion formation and electrospray apparatus *(55)*

b) Atmospheric Pressure Chemical Ionization (APCI)

The APCI interface is another technique which transports and ionizes samples at the atmospheric pressure region; a corona discharge to mass spectrometer. The liquid flow from LC is sprayed and rapidly evaporated by a coaxial nitrogen stream and the neubulizer is heated to a high temperature (300-500^{o}C). Then the ions are transferred into the mass spectrometer. Although high temperature may degrade the analytes, the high flow rate and coaxial nitrogen flow prevent break down of the molecule. The discharge can ionize not only analyte molecules but also solvent molecules. The solvent ions can react with the analytes in the gas phase. Chemical ionization of sample molecules is very efficient at atmospheric pressure due to the high collision frequency. The moderating influence of the solvent clusters on the reagent ions, and the high gas pressure reduces fragmentation during ionization and results in primarily molecular ions. The reagent species in the positive ion mode may be considered to be protonated solvent ions, and in negative ion mode O_2^-, its hydrate and cluster. The ionization is very mild and leads to a molecular species with little or no fragmentation. Proton transfer (protonation $[MH]^+$) occurs in the positive mode and either electron transfer (proton loss, $[M-H]^-$) or electron capture in the negative mode. The ionization efficiency is better compared with CI, because it occurs at high temperature and the collision frequency is high compared with the process in a standard CI source. This technique is used as an LC/MS interface, because it can accommodate very high liquid flow rates (1mL/min). The destruction of corona discharge can occur, because the electrons are produced in the corona discharge.

Table 2.3 Advantages and disadvantages of LC/MS interface

Interface Technique	Advantages	Disadvantages
Moving belt/ wire interface	• Wide range of HPLC condition.	• Belt is prone to break during operation. • Interference from chemical background by belt material. • Not suitable for thermally labile and highly volatile compounds
Direct liquid introduction	• No heat (thermally labile). • Positive and negative ion CI spectra	• Intended for analyzing in volatile compounds. • Pin hold clogging. High background from mobile phase impurity.
cf-FAB	• Thermal labile material. • Lower material consumption than static FAB.	• Permanent chemical background from matrix material. • Pre-column addition curve chromatographic problem. • Inability for stable condition between analyte and matrix, irreproducibility of mobile phase gradient, because of low flow rate.
Particle beam	• Structural information. • Can study thermal labile and in volatile compounds.	• Sensitivity dependent on individual analyte and experimental condition. • Increase of water in mobile phase reduces the particle beam performance.

Interface Technique	Advantages	Disadvantages
Thermospray	• Handles high flow rate (up to 2 mL/min). • Able to study ionic and polar compounds. • Direct liquid flow into MS.	• Limited aqueous mobile phase (volatile buffer). • Limited to thermally labile compounds. • Adduct formation which may confuse the assignment of molecular weight.
ESI	• Soft ionization and multiple charge (high mass range). • Easy to connect with micro bore liquid chromatography. • Good sensitivity (concentration sensitive device).	• Low tolerance for mixture, purity of the sample is important. • Need polar sample and no application to non polar compounds.
APCI	• Medium to low polarity molecule of sample. • High flow operation (up to 2 mL/min). • Can be operated with normal phase HPLC.	• Corona discharge deteriorates (electron production). • Need volatile compounds and thermal stability compounds. • Not suitable for charged analyte. Mass spectra can contain adduct ions and cause complicated interpretation.

2.4 Sample preparation

Sample preparation is an essential part of HPLC analysis, and is intended to provide a reproducible and homogeneous solution that is suitable for injection onto the column. The aim of sample preparation is to make a sample aliquot that (1) is relatively free of interferences, (2) is not harmful to the column, and (3) is compatible with the intended HPLC method; that is, the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution. *(51)* For a complicated sample, sample preparation involves a clean-up procedure, making the sample simpler for further analysis. Moreover, sample preparation brings the analyte to a suitable concentration level for detection and typically includes enrichment. Several options are available for sample clean-up depending upon the analytes of interest, matrix and contaminants.

2.4.1 Liquid-Liquid Extraction (LLE)

Liquid-Liquid extraction (LLE) is useful for separating analytes from interferences by partitioning the sample between two immiscible liquids or phases. One phase in LLE is often aqueous and the others are organic solvents. More hydrophilic compounds prefer the polar aqueous phase, whereas more hydrophobic compounds will be found mainly in the organic solvent. Analytes extracted into the organic phase are easily recovered by evaporation of the solvent, while analytes extracted into the aqueous phase can often be injected directly onto a reversed phase HPLC column. The LLE organic solvent is chosen for the following characteristics: low solubility in water (< 10%), volatility for easy removal and concentration after extraction and high purity to minimize sample contamination. Some usual problems are associated with LLEs, including; emulsion formation which causes analytes to attach to particles and also mutual solubility of the two phases. Otherwise, LLE has some disadvantages like many organic consumptions, time consumptions, etc. *(51)*

2.4.2 Solid Phase Extraction (SPE)

Solid Phase Extraction (SPE) is considered as a replacement for LLE. SPE uses an absorbing medium to separate analytes according to their differing equilibrium affinities. SPE isolates analytes from a gas, fluid or liquid flowing sample stream by transfering and retaining a solid phase. The solid sorbent is packed into a small cartridge. SPE benefits are shorter processing time, lower solvent consumption and a simpler processing procedure. SPE is used for 3 main purposes in sample preparation; removal of interferences and column killers, concentration or trace of the analyte, and sample storage and transportation. In addition, it is easy it automate. Most commercially available bonded phase is a siloxane type, containing a Si-O-Si-C bond. The bonded phase contains the functional groups that are used for the types of separation. The separation mechanisms of SPE can be categorized into four types: normal phase, reverse phase, ion exchange, and mixed mode. *(52)*

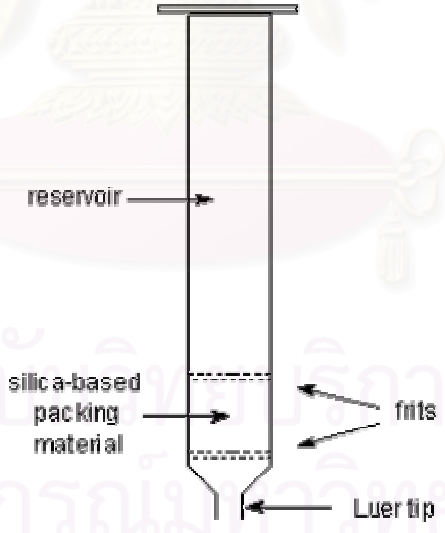


Figure 2.18 A solid-phase extraction column *(52)*

a) Mode of Solid Phase Extraction

- **Normal phase**

Normal phase SPE refers to the sorption of an analyte by a polar surface. It is a standard type of separation. The mechanism is polar interaction such as hydrogen bonding, dipole-dipole interaction, π-π interaction and induced dipole-dipole interaction. Polar-functionalized bonded silica (LC-CN, LC-NH_2, and LC–diol), and polar adsorption media (LC-Si, LC-Florosil, ENVI-Florisil, and LC-Alumina) are typically used in normal phase conditions. For example, silica base is extremely hydrophilic. This material adsorbs polar compounds from nonpolar matrix and elutes compounds with a more polar organic solvent than the original sample matrix.

- **Reverse phase**

Separation of a polar or moderate polar matrix normally uses a non-polar stationary phase. The interested analytes are usually moderate to non-polar. The hydrophobic interactions are non-polar and non-polar interactions, Van-Der Waals or dispersion forces. The secondary interaction between silica-based and analytes present. The endcapping is useful to reduce these interactions. However, secondary interaction may be useful in the extraction of highly polar compounds or matrices. Reversed-phase sorbents are packed with more hydrophobic material. The aqueous sample is commonly analyzed by reversed phase SPE. The reversed phase sorbents are nonpolar fuctionalized such as C-18, C-8, C-2, cyclohexyl and phenyl functional groups and bonded to the silica or polymeric sorbent.

- **Ion exchange**

Ion exchange can be used for compounds that are charged in a solution. The hydrophobic ion exchange is capable of exchanging both a cations or anions with free cations or anions in the solution. Ion exchange sorbent contains both weak and strong cation and anion functional groups. Strong cation-exchange sorbent consists of interaction sites like sulfonic acid groups and weak cation-exchange sites like carboxylic acidic groups. Strong anion–exchange sorbents would be quaternary amine, primary, and secondary. Tertiary amines refer to weak ion exchange. The

secondary nonpolar interaction with nonpolar portions can be provided. A decrease in the balance of pH, ionic strength, and organic content may be necessary for elution of interested analyte from these sorbents. The strong sites are always shown as an exchange site at any pH. Weak sites present are only at pH levels greater or less than the pKa. It has found many applications, for example, it is used for natural products, protein, cellulose, and trace enrichment.

- **Mixed-mode**

The deliberate use of two different function groups on the same sorbent is called "mixed-mode SPE". This sorbents are useful for complex samples that differ in polarity and ionization. Mixed-mode sorbent contains co-bonded ion exchange and alkyl group cartridge. Two different functional groups eliminate the complex sample matrix. For instance, a phase sorbent approach for the extraction of ionizable drugs from a biological matrix. The initial hydrophobic interaction is a function of the chain length, with shorter chains (C-4) being retained less than longer chains (C-18). An example of the mixed-mode is shown in figure 2.19, with reversed phase (hydrocarbon) and cation-exchange site of the sorbent (amino functional group).

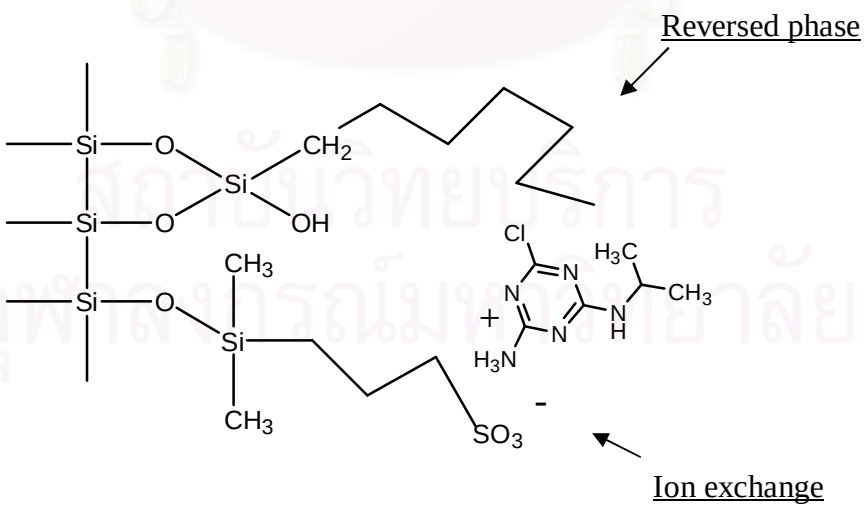


Figure 2.19 Mixed-mode SPE

Table 2.5 SPE mode and sorbent types

	Normal Phase	Reversed Phase	Ion Exchange
Sorbent polarity	High	Low	High
Typical solvent polarity	Low to medium	High to medium	High
Typical sample loading solvent	Hexane, Toluene, CH_2Cl_2, Buffers	H_2O, Buffers	H_2O, Buffers
Sample elution component order	Least polar component first	Most polar component first	Weakly ionized component first
Solvent charge require to elute	Increase solvent polarity	Decrease solvent polarity	Increase ionic strange or pH

b) Process of Solid Phase Extraction

There are four common steps of SPE process, containing a cartridge, loading a sample, washing and eluting analytes. *(53)*

In the conditioning step, the packing is passed by a few volumes of solvent, typically acetonitrile (ACN) or methanol (MeOH). In this step, any impurities that may be collected while the cartridge was exposed to the environment are removed and also the sorbent is solvated.

In the next step, samples are loaded into the cartridge. Many sample cases are in solid form, therefore, sampling needs to be homogenized and dissolved in an appropriate solvent before loading. Sample sizes must be scaled to suit the capacity of the cartridge. Also, the flow rate of a sample through the cartridge should be controlled. Moreover, the cartridge should not be allowed to dry out. Some matrix that has similar properties as analytes may retain the sorbent.

In the washing step, an appropriate solvent is passed through the cartridge. Weaker interferences are retained and washed out from the cartridge. Optimally, the washing step is discontinued, before the analyte begins to leave the cartridge.

In the last step, the analyte is eluted with a small portion of appropriate solvent. As a result, final analyte fraction volume is reduced. In any event, evaporation to dryness is often required.

CHAPTER III

EXPERIMENTAL

3.1 Instrument and Apparatus

3.1.1 High performance liquid chromatography (HPLC): A module 1100 ™ series consists of automatic vacuum degasser, pump, autosampler and column thermostat compartment, Agilent Technologies, Pola Alto, U.S.A.

3.1.2 Mass Spectrometry Detector (MSD): A module 1100 ™ with atmospheric pressure electrospray ionization (API-ES), Agilent Technologies, Pola Alto, U.S.A.

3.1.3 HPLC column: Zorbax SB C18 Narrow Bore 2.1 x 150 mm I.D., 3.5 μm, Agilent Technologies, Pola Alto, U.S.A.

3.1.4 A guard cartridge holder, Agilent Technologies, Pola Alto, U.S.A with C-8 high performance guard column, Agilent Technologies, Pola Alto, U.S.A.

3.1.4 LC/MS Grade Nitrogen Generators: Models 75-72-K727, Perker Hannifin Corporation, Haverhill, M.A.

3.1.5 Air Pump for N_2 generator, Model PAC-10, GAST, Michigan, U.S.A.

3.1.6 Liquid Nitrogen PCC, S size, 180 L, 350 psi, TIG (Thai Industrial Gag Limited), Bangplee, Samutplakarn, Thailand.

3.1.7 Milli-Q, Ultrapure Water Systems with Millipak® 40 Filter Unit 0.22 μm, model Millipore ZMQS5V00Y, Millipore, Billerica, M.A., U.S.A.

3.1.8 A Water Vacuum Pump, model DOA-P504-BN, with pressure regulator, GAST, Michigan, USA.

3.1.9 A rotary evaporator consists of BUCHI heating bath B-490, RüCHI rotavapor R-200, SIBATA and Circulating Aspirator WI-20, Buchi, Flawil, Switzerland.

3.1.10 A Glass Filter Set (300 ml Funnel, 1 L Flask, Glass base and tube cap, and 47 mm Spring Clamp) for HPLC mobile phase filtration, PALL, Germany, Laboratory)

3.1.11 Vortex mixer, Model G-5605, scientific Industries, Bohemia, New York, U.S.A.

3.1.12 Round bottle flask 50, 100 and 250 mL.

3.1.13 Volumetric flask 5.00, 10.00, 250.00 and 500.00 mL.

3.1.14 Beaker 10, 50, 150, 250 and 600 mL.

3.1.15 Separatory funnel, 250 mL.

3.1.16 Graduated cylinders10.0, 50.0 and 100.0 mL.

3.1.17 Volumetric pipettes 10.00 and 20.00 mL

3.1.18 HPLC amber vials 2 ml with PTFE caps, Agilent Technologies, Pola Alto, U.S.A.

3.1.19 Micro-pipettes 10-100 µL, 100-1000 µL and tips, Eppendrof, Hamburg, Germany.

3.1.20 Filter membrane 47 mm, 0.45 µm, type Teflon and nylon, Agilent Technologies, Pola Alto, U.S.A.

3.1.21 Syringe filter, Nylon 13 mm, 0.45 µm, Agilent Technologies, Pola Alto, U.S.A.

3.1.22 Laboratory blender consists of blender timer base only 2 speed, stainless container 1 L, stainless dry container 500 mL, WARING Commercial.

3.1.23 The Baker SPE-24G consists of 24 part vacuum manifold (glass vacuum basin, cover with lure fitting and gasket, individual flow control, stopcocks, stainless steel needles), sample collection rack, shelves, and vacuum gauge/controller, J.T. Baker Chemical Company, Denver, Holland.

3.1.24 Syringe adapter for 1.5 mL, 4.0 mL and 8.0 mL SPE, Alltech.

3.1.25 Glass syringe 2.00, 10.00 mL, Comet, Tokyo, Japan.

3.1.26 Solid Phase Extraction

- Supelclean ENVI-CARB 6 mL tubes, Supleco.
- Bakerbond C18 spe, Octadecyl C18, J.T. Baker Chemical Company, Denver, Holland.
- Bound elute C18, 500 mL, 3 mL, Varian.
- Sep-Pak® VAC NH_2, 500 mg, 6 cc, Waters.
- Bound elute PSA, 500 mL, 3 mL, Varian.
- OASIS HLB®, 500 mg, 300 ml, Waters Corporation, Milford, MASS.
- Sep-Pak® Sillica 500 mg, 6 cc, Waters Corporation, Milford, MASS.
- Alltech Alumina–N, 300 mL, 3 mL, Alltech.
- SPE PSA-SAX, 500 mg, 3 mL, Varian.
- SPE SAX, 500 mg, 3 mL, Varian.

All glassware was washed with detergent, rinsed with double distilled water and methanol before use.

3.2 Chemicals

3.2.1 Standard compounds

Cinosulfuron (Cino), Sulfometuron (Sul), Triflunsulfuron (Tri), were purchased from Dr. Ehrenstorfer (Augsberg, German) with 97.7%, 98.0% and 97.0% purity respectively. 100 ppm of each Pyrazosulfuron ethyl (Pyra), Bensulfuron (Ben), Chlorsulfuron (Chlor) and Metsulfuron (Met) supported by the Overseas Merchandise Inspection Co., Ltd. (OMIC).

3.2.2 Organic solvents

Acetonitrile (Ultra Resi-Analyzed) methanol and acetonitrile (ACS grade) were purchased from J.T. Baker Chemical Company, Denver, Holland. Acetone and ethyl acetate (J.T. Baker Chemical Company, Denver, Holland) were analytical grade. Dichloromethane and Hexane were analytical grade supplied by E. Merck, Darmstadt, Germany.

3.2.3 Reagents

Hydrochloric acid, acetic acid, di-sodium hydrogen phosphate dehydrate, potassium di-hydrogen phosphate, 85% ortho-phosphoric acid, and sodium chloride were purchased from E. Merck, Darmstadt, Germany. Ether anhydrous, ammonium acetate crystal, anhydrous sodium sulfate were ACS grade reagent from J.T.Baker Chemical Company, Denver, Holland. 28.0-30.0 % Ammonium hydroxide (Actual Analysis) supplied from J.T. Baker Chemical Company, Denver, Holland. Anhydrous sodium sulfate supplied from Kanto Chemical Co. Inc. 2-8 Nihonbashi Honcho 3-chome, Chuo-Ku, Tokyo, Japan. Ammonium hydrogen carbonate was purchased from MAY & BAKER Ltd. Dangenham, England. Di-potassium hydrogen phosphate, ammonium carbonate, oxalic acid dihydrate and trifluoroacetic acid were supplied from Fluka Chemica, Switzerland. 98-100% Formic acid was analytical reagent grade from Fisher Scientific UK.

3.3 Preparation of Standard Solution

3.3.1 Stock of Standard Solution

A 1000 ppm solution of each standard of Cinosulfuron (Cino), Sulfometuron (Sul), and Triflunsulfuron methyl (Tri), was prepared by dissolving 0.100 g in 100.00 mL volumetric flask with acetonitrile. These stock standard solutions were kept in amber-glass containers with teflon screw caps. Pyrazosulfuron (Pyra), Bensulfuron (Ben), Chlorsulfuron (Chlor) and Metsulfuron (Met) were already in solution with concentration of 100.00 ppm and were also stored in amber-containers with teflon screw caps.

A 100 ppm single standard was prepared by pipetting 0.500 mL of stick solution into a 5.00 mL volumetric flask and making up to the scale with acetonotrile. All single solutions were stored in amber bottle glass.

A single standard solution of 10.00 ppm (μg/mL) was prepared by pipetting 0.500 mL of 100 ppm (μg/mL) standard solution and diluting to 5.00 mL with acetonitrile in a 5.00 mL volumetric flask and stored in an amber container.

Each standard solution of 5.00 ppm (μg/mL) was prepared by pipetting 0.0500 mL of 100 ppm (μg/mL) standard solution and diluting to 1.00 mL with acetonitrile in 2.00 mL amber vial.

A 1.00 ppm (μg/mL) of single standard solution was prepared by pipetting 0.500 mL of 10 ppm (μg/mL) standard solution in a 5.00 mL volumetric flask and diluting with acetonitrile. The single standard solution was stored in an amber glass bottle.

3.3.2 Stock of Mixture Solution

A 100 ppm (μg/mL) of mix standard mixture solution was prepared by pipetting 0.500 mL of each 1000 ppm (μg/mL) single standard into a 5.00 mL volumetric flask and filled with acetonitrile.

A mixture of 10.0 ppm (μg/mL) standard solution was prepared by pipetting 0.500 mL of 100 ppm (μg/mL) mix standard into a 5.00 mL volumetric flask and diluting with acetonitrile. All mixture concentrations were contained in amber-glass bottles.

A 1.00 ppm (μg/mL) mixture of sulfonylurea herbicides was prepared by diluting 200 mL of 10.00 ppm mix standard solution with 2.00 mL of acetonitrile. The standard mixtures were stored in amber-glass bottles.

3.4 LC/MS Method Development

All analyses were performed using Agilent, HPLC module 1100^{TM} coupled with MSD module 1100^{TM} SL series with quadrupole mass analyzer. To optimize the MS conditions, the mobile phase composition, capillary voltage and fragmentor were evaluated under the flow injection analysis (FIA) in full scan mode.

3.4.1 Mass Spectrometric parameters

a) Mobile Phase Type

The six different buffer types were tested; ammonium acetate, trifluoroacetic acid (TFA), oxalic acid, acetic acid, formic acid and ammonium formate. The concentrations of different buffer types in that study are summarized in table 3.1. During this test, the 5.00 ppm single standard solution was injected and mobile phase was composed of 50% constituent in mobile phase (MP) A and 50% MP B. The MS condition was investigated under same static conditions that are presented in table 3.2.

Table 3.1 Mobile phase types and concentration

No.	MP A	MP B
1	20mM Ammonium acetate	ACN
2	0.1 % TFA	ACN
3	0.01 % TFA	ACN
4	5mM Oxalic acid	ACN
5	10mM Oxalic acid	ACN
6	0.1 % Acetic acid	ACN
7	0.01 % Acetic acid	ACN
8	0.1 % Formic acid	ACN
9	0.01 % Formic acid	ACN
10	5mM Ammonium formate	ACN
11	10mM Ammonium formate	ACN

Table 3.2 FIA static parameters

Parameters	
Injection volume	10 µL
Time between injection	1.0 minute
Injection loop flush time	0.5 minute
Ionization source	ESI/positive
Drying gas flow	10 mL/min
Neubulizer pressure	35 psig
Drying gas temperature	350° C
Scan mass range	200-600 *m/z*

b) Capillary Voltage and Fragmentor

In order to find MS condition, each herbicide was directly injected into the mass spectrometer and capillary voltage was varied at 3000, 3300, 3500 and 3700 V for the positive mode. 10 µL of each 5.00 ppm (µg/mL) sulfonylurea herbicide standard solution was injected. Acetic acid, ammonium acetate, ammonium formate, formic acid and oxalic acid were selected from the mobile phase study. The MS condition was applied as shown in table 3.1. The fragmentor was also studied at the same time by varying the voltage at 40, 70, 100, 120, 130 and 190 V.

3.4.2 High Performance Liquid Chromatographic Condition

The gradient program of appropriated mobile phases from MS optimized condition (2 mM oxalic acid and acetonitrile) was developed by varying percentage of mobile phase type. A flow rate of 0.25 mL/min with an oven temperature left 35 °C and right 40 °C was applied. A mixture of mix standard solution at 50.0 ppb (µg/mL) was injected at 5 µL. The gradient program was adjusted until it reached a baseline resolution for all herbicides. The operated condition followed table 3.3.

Table 3.3 The HPLC operating conditions for the separation of sulfonylurea herbicides

HPLC Parameter	Condition
Analytical column	Narrow Bore 2.1 x 150 mm,3.5 micron , Zorbax SB-C18
Mobile phase	2 mM oxalic acid : Acetonitrile
Injection volume	5 µL
Ionization source	ESI/positive
Drying gas flow	10 mL/min
Neubulizer pressure	35 psig
Drying gas temperature	350° C
Scan mass range	200-600 *m/z*

3.4.3 Selectivity Evaluation of LC/MS Condition

The selectivity of LC/MS can be divided into two parts. Retention time represented the chromatographic selectivity and diagnostic ions (target ion and qualified ion) represented the mass spectrometric selectivity. The SIM windows which is the specifics operating time to detect specific analytes. In this work, SIM window was selected by elution tine of analyte. A Mixture of mix standard solution at 50.0 ppb (µg/mL) was injected at 5 µL under control parameters in table 3.3.

3.5 Extraction Method

The study of extraction methods was spiking each sulfonylurea herbicide at two difference concentrations at 10.00 and 50.00 ppb. Seven extraction methods were studied which are summarized in appendix C. These methods are summarized in the following section.

Method I was applied from the New Analytical Method and Technique for food & Agricultural Analysis by Agilent. This vegetable was partitioned with 100 ml acetonitrile: 0.1 M NH_4HCO_3 (20:80). The matrix was removed by passing the extraction solution through ENVI-CARB column (0.5 g) and finally eluted with MeOH:CH_2Cl_2 (10:90)+0.1 M formic acid.

Method II was adjusted from the Hand Book of Residue Analytical Method for Agrochemicals 2003. First, the sample was extracted by liquid-liquid extraction and then passed through ENVI-CARB column and then eluted 20 mL of 0.1 N formic acid in methanol-dichloromethane (1:9v/v). After that, SAX and HLB cartridges were also used cleaning up and isolating the analytes.

Method III was "Simultaneous Determination of Azimsulfuron, Flazasulfuron and Halosulfuron-methyl in Grains, Seeds, Vegetables and Fruits by HPLC" *(28)* This method was carried out by liquid-liquid extraction. Samples were extracted with water and acetone and afterward were evaporated to dryness and then the residue was dissolved with 10% NaCl solution. The solution was adjusted to pH 3-4 with 1M HCl and extracted with ethyl acetate and the organic layer was collected and extracted with 2% di-potassium hydrogen phosphate and aqueous layer was partitioned with ethyl acetate. An organic layer was dehydrated with anhydrous Na_2So_4. Cleaning up and pre-concentration were using 2 SPE cartridges; Sep-Pak Plus Alumina N and Bound Elute SAX cartridge.

Method IV was adjusted from the screening analysis of 27 pesticides in high water content fruits and vegetables. *(58)* Supercritical fluid extraction was used to extract pesticides from the samples and the analytes were trapped by Extrelut NT Bond Elute C18 and then clean up by Sep–Pak Florisil and Bound Elute PSA.

Method V referred to Document No. AMR-132-83 by E.I. du Pont de Nemours and Company, Determination of bensulfuron methyl in rice grain was introduced in 1983. Analytes were isolated from polished rice by methylene chloride extraction. Interference was separated by acetonitrile–hexane partitioning. In addition, C18 Bond Elute collected bensulfuron methyl and elutedfrom column by acetonitrile.

Method VI This method was adjusted from three papers with the same sample preparation process. First, determination of sulfonylurea herbicides in water by Capillary Electrophoresis and by Liquid Chromatography/Mass Spectrometry *(59)* was concerned. A water sample was initially extracted by passing it through a RP-102 cartridge. The eluate was evaporated to dryness. A SAX cartridge was stacked on top of an Alumina cartridge to isolate analytes. A multiresidue method for determination of sulfonylurea herbicides in water by Liquid Chromatography with confirmation by Capillary Electrophoresis *(60)* was the second interesting process. The method is similar, but the cleaning up step by SPE used only a silica cartridge. The last extraction process was “Comparison of Capillary Electrophoresis and Liquid Chromatography for Determination of Sulfonylurea Herbicides in Soil” *(61)* Sample preparation used partitioning of analytes and phosphate buffer at pH 7. The extraction solution was adjusted to pH 3-3.5 and passed through a C18 cartridge. A silica cartridge was also used to prepare herbicides.

Method VII Screening method for nine sulfonylurea herbicides in soil and water by Liquid Chromatography with Ultraviolet Detection. *(24)* This method extracted soil and water sample with partitioning of 0.1 M ammonium carbonate/acetone. Interference was removed by a C18 cartridge and analytes were eluted with 0.1% glacial acetic acid in ethyl acetate. The solution was evaporated to dryness and residue

was dissolved in ethyl acetate. The extraction solution was passed through a silica cartridge. Interferences were isolated and extracted by 2-cartridges.

3.6 The Comparison of Solid Phase Extraction (SPE) cartridge

Extraction method II was used and two spiking levels were studied by:

3.6.1 Spike mix standard solution to 10.0 ppb by pipette into 50.00 g of rice sample.

3.6.2 The spike rice sample from 3.6.1 was extracted by extraction method II in appendix C, isolated, and cleaned up by C18+NH_2 cartridge.

3.6.3 The extract was injected into LC/MS under the optimization parameter in table 4.3 and 4.5.

3.6.4 The study of mixed mode sorbent followed sections 3.6.1 to 3.6.3 but with PSA and HLB cartridge.

3.7 Acidic pH effect to Extraction

Sulfonylurea herbicides have pKa during 3.5-5.8. Therefore, the study of the effect of pH on extraction was investigateded. Two spiking levels were applied. The pH effect was studied by:

3.7.1 Spike mix standard solution to 10.0 ppb by pipette into 50.00 g of rice sample.

3.7.2 The spike rice sample from 3.6.1 was extracted by extraction method II in appendix C.

3.7.3 Net spiking level was 50.00 ppb following a similar procedure as the lower level as mentioned in 3.6.2.

3.7.4 The extract was injected into LC/MS under the optimization parameter in tables 4.3 and 4.5.

3.8 Method Validation for C18+NH_2 Cartridge

The purpose of method validation is to study the method performance parameter and demonstrate a particular method for quantitative measurement of analytes in matrix (rice). There were many parameters to study such as selectivity, MQL and MQL, linearity and range, precision, accuracy and robustness. *(62, 63, 64 and 66)*

3.8.1 Selectivity

The selectivity of LC/MS can be divided into retention time represented by the chromatographic selectivity and diagnostic ions (target ion and qualified ion), represented by the mass spectrometric selectivity.

a) Preparation of the dilution solvent from the sample by extracting rice following extraction method II in appendix C, using tandem SPE to clean up and extract rice sample.

b) Mixture of matrix standard at spiking level 10.0 ppb (µg/mL) was prepared by using the matrix blank in section a).

c) Standard mixed sulfonylurea herbicides in matrix were injected into HPLC under LC/MS optimized conditions in table 3.4 and SIM windows in table 3.5.

3.8.2 Method Detection Limit (MDL) and Method Quantitation Limit (MQL) of C18+NH_2 Cartridge

MDL and MQL were studied under the parameters in table 3.4 and 3.5.

Table 3.4 The HPLC chromatographic optimization conditions

HPLC Parameter	Condition		
Analytical column	Narrow Bore 2.1 x 150 mm, 3.5 micron , Zorbax SB-C18		
Mobile phase	2 mM oxalic acid : Acetonitrile		
Flow rate	0.25 mL/min		
Injection Volume	5 μL		
Gradient program	Time	% A	% B
	0	67	33
	5	67	33
	12	0	100
Column temperature	Left 35 ^{o}C, Right 40 ^{o}C		

Table 3.5 Time schedule multiple-ion SIM conditions for monitoring of seven sulfonylurea herbicides

Group	Compound	SIM window (min)	Quasi-molecular ion $(M+H)^+$	Qualification ion
1.	Cinosulfuron	2.0-11.0	414.15	415.00
	Metsulfuron l		382.20	383.10
	Sulfometuron		365.10	366.12
	Chlorsulfuron		358.05	360.00
2.	Bensulfuron	11.0-17.0	411.15	412.20
	Pyrazosulfuron ethyl		415.20	416.10
	Triflusulfuron l		493.05	494.10

a) Spiked samples were prepared by spiking the standard solution into 50.0 g of rice sample and extracted following appendix C (extraction method II by tandem cartridge).

b) A blank sample was prepared with the same extraction method as section a} but without adding the standard solution.

c) The spiked sample from a) was injected into LC/MS system under the optimum conditions in table 3.4 and 3.5.

d) The MDL of each compound was determined from injection of a spiked sample, as the concentration of each compound gave a signal to noise ratio of 3.

e) The MQL of each compound was investigated using the same procedure as MDL, but the signal to noise ratio was 10.

3.8.3 Standard Calibration Curve

The standard calibration curve was studied by:

a) Mixed standard solutions run with the concentrations of 1.00, 5.00, 10.0, 20.0, 40.0, 60.0, 80.0, 100, 200 and 300 ppb, and injected into LC/MS under optimal conditions (table 3.4 and 3.5).

b) The calibration curves presented intercepts, slopes and correlation coefficients (R^2).

3.8.4 Linearity

Linearity was studied by:

a) Mixed sulfonylurea herbicides prepared at 1.00, 5.00, 10.0, 20.0, 40.0, 60.0, 80.0, 100, 200, 300, 400 and 500 ppb and injected into LC/MS under optimal conditions (table 3.4 and 3.5).

b) Linearity was determined by plotting peak area against standard concentration.

3.8.5 Matrix Calibration Curve

The matrix calibrations curve was studied by:

a) Preparation of dilution solvent from a sample by extracting rice following extraction method II in appendix C, using tandem SPE to clean up and extract the rice sample.

b) Matrix calibration curves were prepared in two ranges. Metsulfuron, sulfometuron and chlorsulfuron were prepared at the following concentration of 5.00, 10.0, 20.0, 40.0, 60.0, 80.0, and 100 ppb. Cinosulfuron, bensulfuron, pyrazosulfuron ethyl, and triflusulfuron were prepared at 1.00, 5.00, 10.0, 20.0, 40.0, 60.0 and 80.0. The blank solution from a) was used as a dilution solvent.

c) Standard mixed sulfonylurea herbicides in matrix were injected to HPLC under LC/MS optimized conditions in table 3.4 and 3.5.

d) The calibration curves were determined by plotting peak area against concentration of anlytes.

3.8.6 Matrix Effect

The matrix effect was studied by comparison between standard mixtures (acetonitrilel used as a dilution solvent) and matrix standard mixtures (rice extract used as a dilution solvent. A paired *t*-test at 95 % confidential level was used to study the standard calibration curve and matrix-based calibration curve.

3.8.7 Method Precision for C18+NH_2 Cartridge

Precision is subdivided into: within-day precision, which assesses precision during a single analytical run; and between-day precision, which measures precision between different analytical runs or at different times. In this thesis, precision was measured using a minimum of 2 concentrations (MQL and 5-MQL) and 6 determinations pre concentration. The spiking level is shown in table 3.6.

Table 3.6 Spiking level at method quantitation limit and 5-fold method quantitation limit of each sulfonylurea herbicides for the study of method precision in rice matrix (C18+NH_2 cartridge)

No.	Compound	Spiking level	
		Method Quantitation Limit (ppb)	5-Method Quantitation Limit (ppb)
1	Cinosulfuron	1.30	6.50
2	Metsulfuron	6.00	30.0
3	Sulfometuron	7.15	35.75
4	Chlorsulfuron	6.06	30.3
5	Bensulfuron	0.91	4.55
6	Pyrazosulfuron ethyl	0.97	4.85
7	Triflusulfuron	2.40	12.0

a) The within-day precision was measured by:

a-I) Spike sample standard solution at MQL and extracted using extraction method II in Appendix C (tandem cartridge).

a-II) Matrix blank was prepared by the same extraction method as a-I without adding the standard solution.

a-III) Injection of extract from a-I) and a-II) was operated under LC/MS optimized conditions as table 3.4 and 3.5.

a-IV) The recovery of each sulfonylurea herbicide and percent relative standard deviation (%RSD) were calculated.

a-V) For higher concentration levels, at the concentration of five times MQL was spiked into 50.00 g of rice sample.

a-VI) The extraction was similar to that in sections a-II) to a-IV).

b) The between-day precision was performed: using the same analytical procedure as within-day precision but this procedure was applied repeatedly on two different days.

3.8.8 Method Accuracy of C18+NH_2 Cartridge

Method accuracy is considered to be the closeness of the determination value to the true value. In this research, recovery is indicative of accuracy by spiking standard solution into a rice sample at three spiking levels: MDL, MQL and 5-MQL (table 3.7). After that, spiked rice was extracted under extraction procedure II in appendix C. The accuracy was obtained by measuring the mean recovery of each compound.

Table 3.7 Spiking level at method detection limit (MDL), method quantitation limit (MQL) and 5-method quantitation limit (5-MQL) of each sulfonylurea herbicide in rice matrix (C18+NH_2 cartridge)

No.	Compound	Spiking level (ppb)		
		MDL	MQL	5-MQL
1	Cinosulfuron	0.80	1.30	6.50
2	Metsulfuron	3.00	6.00	30.0
3	Sulfometuron	4.00	7.15	35.75
4	Chlorsulfuron	5.00	6.06	30.3
5	Bensulfuron	0.64	0.91	4.55
6	Pyrazosulfuron ethyl	0.71	0.97	4.85
7	Triflusulfuron	1.90	2.40	12.0

3.8.9 Method Robustness of C18+NH_2 Cartridge

There are many parameters that affect analysis of sulfonylurea herbicides in rice. Thus, the screening design was applied because it is able to study many factors with few runs. Plackett-Burman experimental design studies on N-1 variables and it needs only N experiments to evaluate the variables. In this work, this experimental design was applied to study seven parameters that affect the extraction of sulfonylurea herbicides in rice. These are extraction time, waiting time, volume of solvent elution, evaporation temperature, solvent grade, light explosion and amount of NaCl. The variations of each parameter are based on two levels that can occur in the experiment. The procedure for the study of method robustness was

a) Preparing standard solution at 5-MQL into 50.00 g of rice sample.

b) Each spiked sample was extracted by extraction method II in appendix C. The extraction parameters followed the experimental design at the two levels of each parameter presented in tables 3.8 and 3.9.

c) Different value of each parameter was calculated from

$$D_A = \frac{(s+t+u+v)}{4} - \frac{(w+x+y+z)}{4}$$

Table 3.8 Plackett- Burman experimental designs; capital letter is a normal method value and small letter is an alternative value

Parameters	Experiment No.							
	1	2	3	4	5	6	7	8
A or a Extraction time	A	A	A	A	a	a	a	a
B or b Waiting time	B	B	b	b	B	B	b	b
C or c Volume of elution	C	c	C	c	C	c	C	c
D or d Evaporation temperature	D	D	d	d	d	d	D	D
E or e Solvent grade	E	e	E	e	e	E	e	E
F or f Light explosion	F	f	f	F	F	f	f	F
G or g Amount of NaCl	G	g	g	G	g	G	G	g
Observe result	***s***	***t***	***u***	***v***	***w***	***x***	***y***	***z***

Table 3.9 Seven experimental parameters for Plackett- Burman experimental designs by normal parameters and alternative parameters

Conditions	Normal	Alternative
Extraction time	30 min	20 min
Waiting Time	5 min	10 min
Volume of elution	10.00 mL	15.00 mL
Evaporation Temperature	33°C	38°C
Solvent Grade	HPLC	ACS
Light Explosion	No	Yes
Amount of NaCl	5.00 g	10.00 g

3.9 Method Validation for PSA Cartridge

Partial method validation was carried out so that critical parameters could be studied. Validation of important parameters for an analytical method is usually taken to optimize a methods performance. Performance characteristics of analytical methods are MDL and MQL, precision, and accuracy for PSA method validation.

3.9.1 Selectivity

The selectivity of LC/MS can be divided into retention time representing the chromatographic selectivity and diagnostic ions (target ion and qualified ion) representing the mass spectrometric selectivity. Sulfonylurea herbicides were extracted by PSA. Thus, selectivity was rechecked by using the same parameters in table 3.4 and 3.5

3.9.2 Method Detection Limit and Method Quantitation Limits for PSA Cartridge

Method Detection Limit (MDL) and Method Quantitation Limits (MQL) were studied by: the same procedure as described in section 3.8.2. The clean up and extraction cartridge was PSA SPE cartridge. The MDL of each compound was determined from injection of spiked sample as concentration of each compound gave a signal to noise ratio of 3. MQL of each compound was investigated using the same procedure as MDL, but the signal to noise ratio was10.

3.9.3 Method Precision for PSA Cartridge

Method precision describes the closeness of the individual measure of an analyte when the process is applied repeatedly. Within-day precision was studied by determining % RSD of a single analytical run. In this thesis, precision was measured using three concentrations (MDL, MQL and 5–MQL) and 6 determination preconcentration. Three spiking levels are shown in table 3.10. Within-day precision study was performed by the same analytical method as in section 3.8.7, but cleans up was by PSA cartridge. The recovery and % RSD results were calculated.

Table 3.10 Spiking level at method detection limit (MDL), method quantitation limit and 5-fold method quantitation limit of each sulfonylurea herbicides for the study of method precision in rice matrix (PSA cartridge)

No.	Compound	Spiking level		
		Method Detection Limit (ppb)	Method Quantitation Limit (ppb)	5-Method Quantitation Limit (ppb)
1	Cinosulfuron	1.95	4.00	20.0
2	Metsulfuron	6.24	8.00	40.0
3	Sulfometuron	7.15	9.50	47.5
4	Chlorsulfuron	5.85	8.50	42.5
5	Bensulfuron	0.91	2.00	10.0
6	Pyrazosulfuron ethyl	0.91	1.50	7.50
7	Triflusulfuron	1.30	4.30	21.5

3.9.4 Method Accuracy of PSA Cartridge

Method accuracy is considered to be the closeness of the determination value to the true value. There are many ways to determine method accuracy like using certified reference material. In this research, recovery indicative of accuracy by spiking standard solution into a rice sample at three spiking levels; MDL, MQL and 5-MQL (table 3.10). Recovery studies are an essential component of the validation. Spiked rice was extracted under extraction procedure II from appendix C following the same procedure as section 3.8.8.

CHAPTER IV

RESULT AND DISCUSSION

4.1 LC/MS Method Development

4.1.1 Optimization of Mass Spectrometric Parameters

To optimize the mass spectrometric (MS) conditions, parameters that influence the ionization efficiency which are mobile phase composition, capillary voltage and fragmentor voltage were evaluated under electrospray ionization. Mass spectrometer sets up at a scan mode over an appropriate mass range (200-600 *m/z*).

a) Mobile Phase Type

Mobile phase is important for LC/MS analysis and mostly consists of buffer which can improve ionization efficiency and control the degree of ionization of analytes. The important properties of buffers using in LC/MS are easily protonate and volatile. The common buffers are ammonium acetate, trifluoroacetic acid, oxalic acid, acetic acid, formic acid and ammonium formate. The effect of different mobile phase types was studied. The MS condition was investigated under same stipulated conditions. The optimal condition was 50 % a acetonitrile – 50 % buffer as the mobile phase at a flow rate 0.5 mL/min. Peak height represented the sensitivity which are pseudo-molecular ion of each herbicide was used to study a suitability of mobile phase type. The result of triflusulfuron which is representing all SUHs is shown in figure 4.1. All results are shown in appendix B (figure B1-B7).

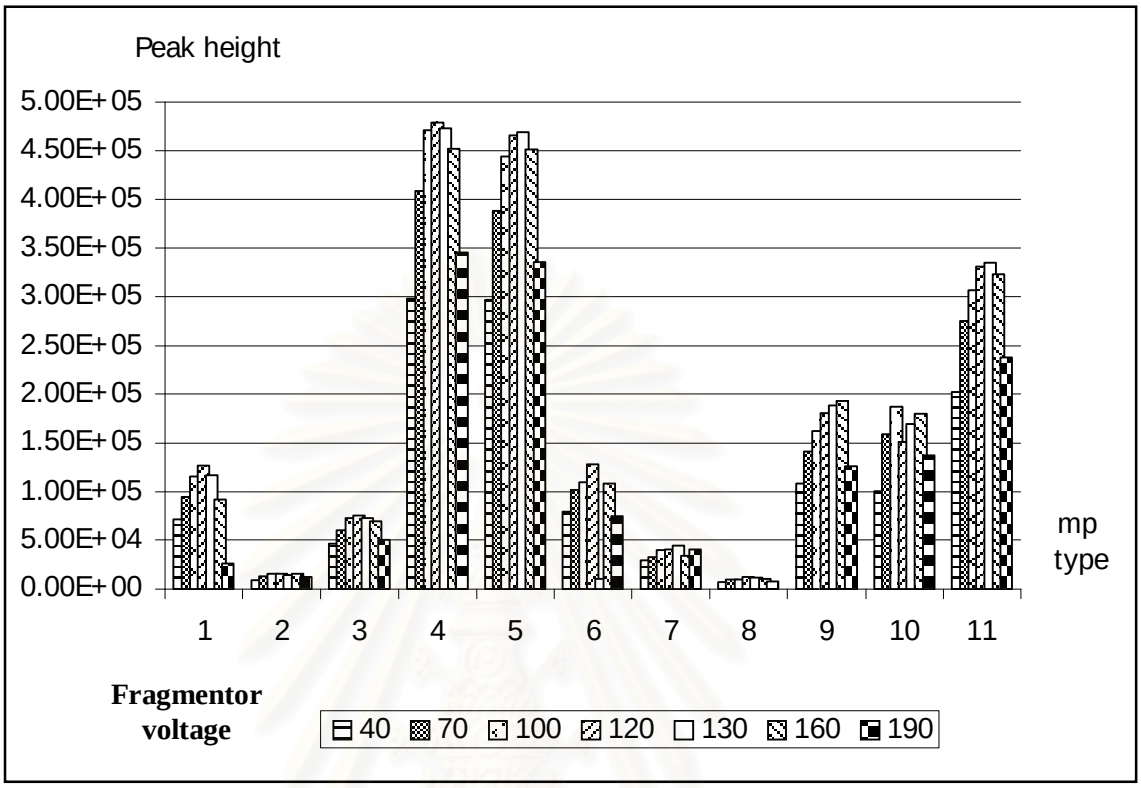


Figure 4.1 The comparison of triflusulfuron peak height at 5.00 ppm using various buffers in mobile phase. Mobile phase buffer: 1 =20mM Ammonium acetate, 2=0.1 % TFA, 3= 0.01 % TFA, 4 = 5mM Oxalic acid, 5 = 10mM Oxalic acid, 6 = 0.1% Acetic acid, 7 = 0.01 % Acetic acid, 8 = 0.1 % Formic acid, 9 = 0.01 % Formic acid, 10 = 5mM Ammonium formate and 11 = 5mM Ammonium Formate

According to figure 4.1, ammonium acetate (MP No. 1), TFA (MP No. 2 and 3), acetic acid (MP No. 6 and 7) and 0.1% formic acid (MP No. 8) showed very low peak height. Therefore, these buffers are not appropriate for analyzing SUHs. 0.01% formic acid (MP No. 9) and ammonium formate (MP No. 10 and 11) were present a moderate peak height and lastly, oxalic acid (MP No. 4 and 5) showed very high peak height. Therefore, oxalic acid was chosen to be the mobile phase. Generally, LC/MS mobile phase was operated under small amount of buffer. Therefore, the concentration of oxalic acid was next studied by varying from 1, 2, 5 and 10 mM. The results of mobile phase study are present in appendix B (figure B8-B14).

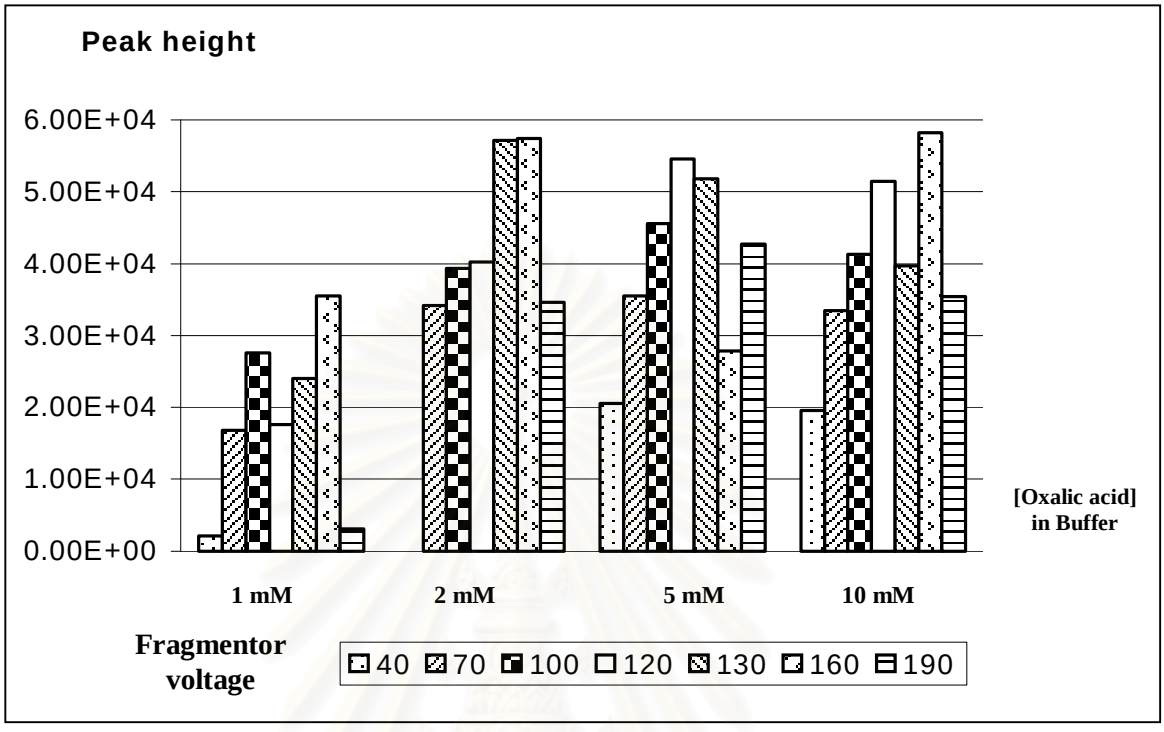


Figure 4.2 The comparison of triflusulfuron peak height at 5.00 ppm using various oxalic acid buffers concentrations in mobile phase

From figure 4.2, the response of peak height to sulfonylurea herbicides can be separated to 2 groups. Sulfometuron, chlorsulfuron, pyrazosulfuron ethyl and triflusulfuron reported the highest peak height by 2mM oxalic acid (MP No. 2). 10mM oxalic acid (MP No. 4) reported the highest peak height for metsulfuron, bensulfuron and cinosiulfuron. However, 2mM oxalic acid (MP No. 2) showed optimum peak height and achieved the best signal response for most of sulfonylurea herbicides. Moreover, the high buffer concentration can cause the appearance of salt residue and which then deposit on the needle of the ionization source. Salt causes deteriorating effects on the mass spectrometer especially to the detection efficiency. Therefore, 2 mM oxalic acid was chosen as a mobile phase buffer.

b) Capillary Voltage

Capillary voltage is the repelling voltage to induce the target ion into MS. The capillary voltage must be optimized because it will affect the signal sensitivity of the mass spectrometer. Sulfonylurea herbicides are weak acidic compounds therefore these herbicides are easy to protonate. For this first reason, capillary voltage in positive mode is suitable to detect the protronated sulfonylurea herbicides. The capillary voltage was at 3000, 3300, 3500 and 3700 V. Then, the extraction ions should be the most suited to find out the capillary voltage.

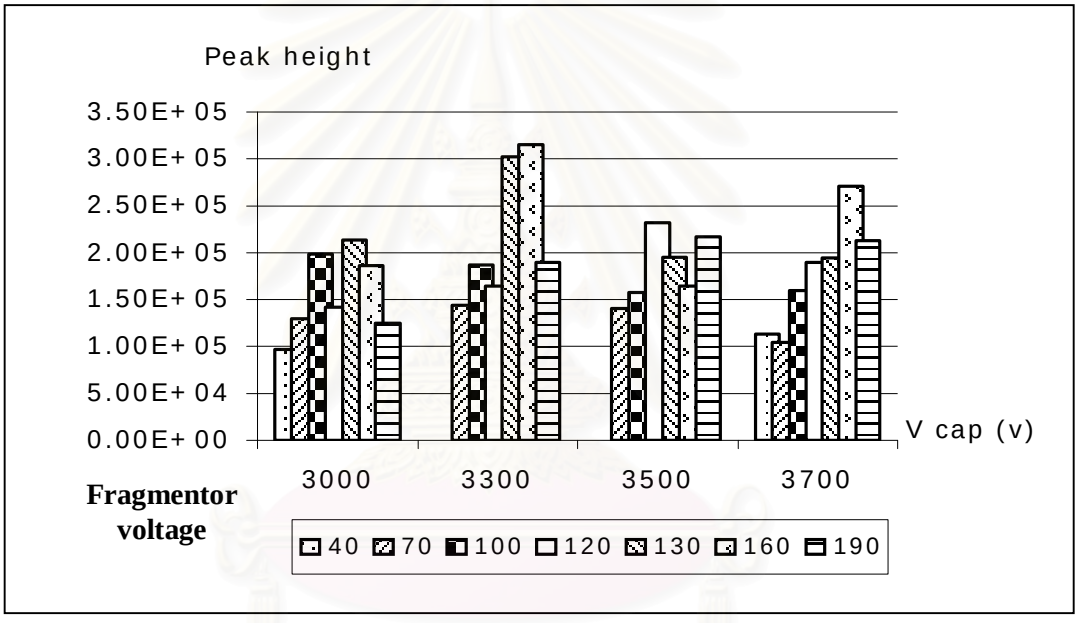


Figure 4.3 The comparison of triflusulfuron peak height at 5.00 ppm using various capillary voltage.

The capillary voltage was study at high field to low field. The result of triflusulfuron summarized in appendix B (figure B15-B21). Capillary voltage did not show obvious trends to improve signal sensitivity. However, at 3700 V, there were many adducted ions and the pseudo-molecular ion presented peak height was lower than that at 3000 V. Therefore, the MS was operated in the positive ion mode by applying a capillary voltage at 3000 V. Because, all target ions presented satisfactory sensitivity. 2 mM oxalic acid and positive ion mode at 3000 V was selected to be an optimization condition for further study.

c) Fragmentor Voltage

Fragmentor is one of powerful analytical tools for identifying structure of analytes. The fragmentor can affect the transmission and fragmentation of sample ion. Sulfonylurea herbicides have individual appropriate fragmentor voltage to rise to their highest sensitivity. The protonated pseudo-molecular ion (target ion) was selected because it present the based peak for each SUHs. In quantitative analysis by MS other ion was selected to confirm analyte. Therefore, protonation ion observed at two mass units above the molecular mass $(M+2)^+$ was used as qualified ion to confirm and monitor herbicides. Another aspect of compounds containing chlorine atom is their unique isotope patterns. Therefore, it can use the isotopic abundance to elucidate chlorsulfuron. According to that, the MS spectra is obtained not only from pseudo-molecular ion $(M+H)^+$, but also from the corresponding M+2 (^{37} Cl) isotope peak. M+ ^{37} Cl presented the static abundance ratio with M+ ^{35} Cl which is 1:3 which can be used as confirming parameter as well. Table 4.1 presents the optimum voltage for each SUHs with their target ion and qualified ion.

Table 4.1 The optimum voltage for determination of sulfonylurea herbicides

No.	Compound	Ion (Relative abundance)		Fragmentor voltage (V)
		Quasi-molecular ion $(M+H)^+$	Qualification ion	
1.	Cinosulfuron	414.00 (100)	415.05 (13.5) 415.95 (6.8)	120
2.	Metsulfuron	381.90 (100)	383.10 (13.0) 384.00 (4.0)	120
3.	Sulfometuron	365.10 (100)	366.15 (12.7) 367.05 (8.7)	100
4.	Chlorsulfuron	357.90 (100)	358.95 (18.7) 360.00 (32.3)	120
5.	Bensulfuron	411.00 (100)	412.05 (17.0) 413.10 (5.5)	70
6.	Pyrazosulfuron ethyl	415.05 (100)	415.95 (13.9) 417.00 (6.5)	100
7.	Triflusulfuron	492.90 (100)	493.95 (19.6) 495.00 (7.8)	130

According to table 4.1, the higher voltage produces more fragmentation ions. On the other hand, low potential presented in a different way. Mass spectra of chlorsulfuron represented all SUHs. Using high voltage (figure 4.4A) at 190 V generated many fragment ions and molecular ion presented low intensity because the applied voltage was too high. Figure 4.4B illustrated the using low fragmentor voltage (70 V). Lower fragment ion was present. Thus, the protonated pseudo-molecular ion $(M+H)^+$ was predominated ion at 120 V (figure 4.4 C). It is important to select the compromised conditions and target ions for each compound. However, the instrumental ability necessary considers. It causes only one voltage was selected. Therefore, fragmentor voltage was selected at 120 V because it compromised between seven SUHs and instrumental requirement. The optimum condition of monitoring ions and fragmentor voltages listed in table 4.2 and mass spectra of each sulfonylurea herbicides are shown in appendix B (figure B22-B28).

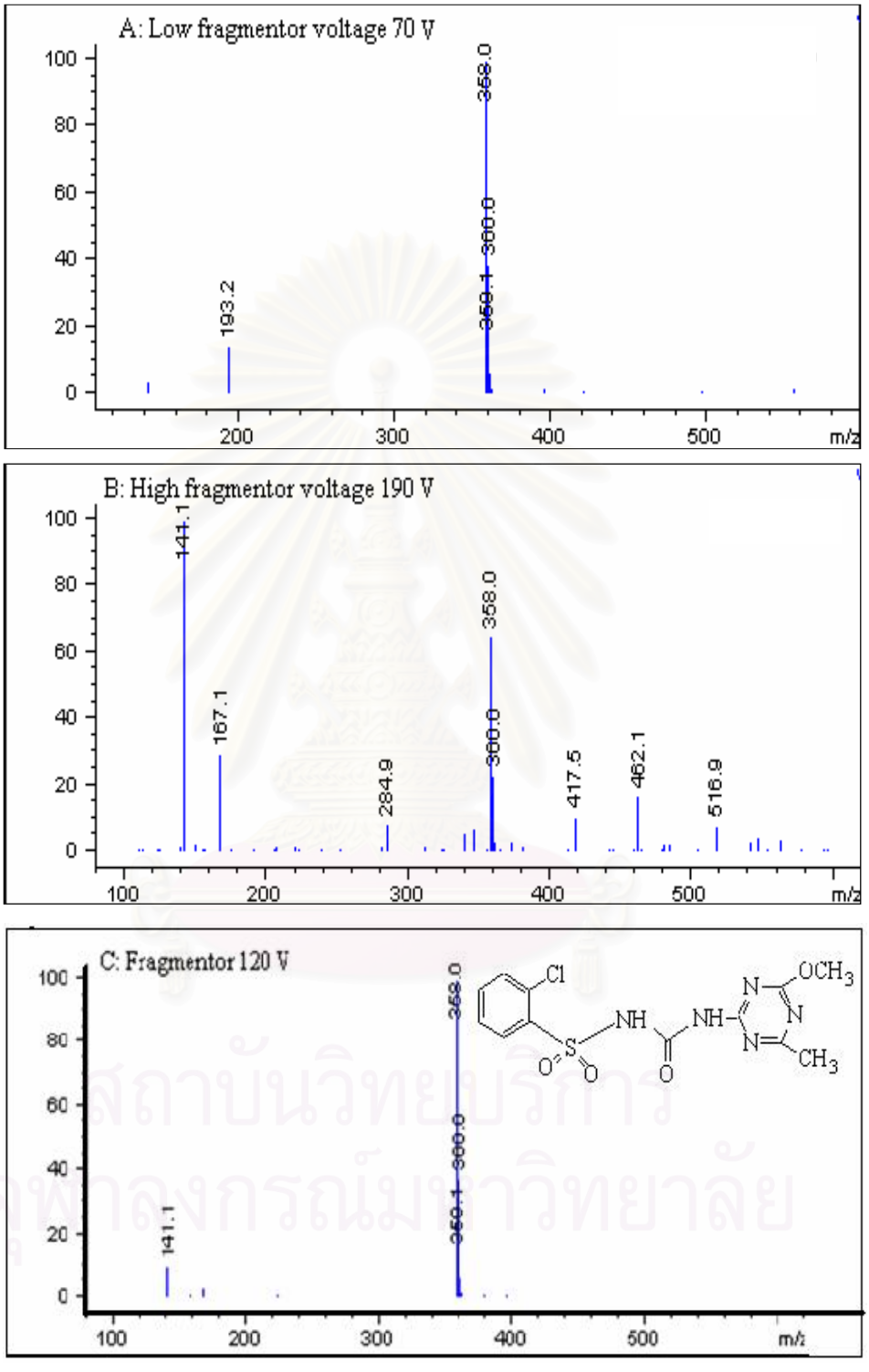


Figure 4.4 The mass spectra of 5.00 ppm chlorsulfuron at A) low fragmentor voltage, B) high fragmentation voltage and C) selected fragmentor voltage

Table 4.2 Mass spectrometric conditions for determination of sulfonylurea herbicides

No.	Compound	Ion (Relative abundance)		Fragmentor voltage (V)
		Quasi-molecular ion $(M+H)^+$	Qualification ion	
1.	Cinosulfuron	414.15 (100)	415.00 (16.8) 416.10 (6.8)	120
2.	Metsulfuron	382.20 (100)	383.10 (19.4) 384.15 (6.2)	120
3.	Sulfometuron	365.10 (100)	366.12 (17.3) 367.2 (6.5)	120
4.	Chlorsulfuron	358.05 (100)	359.1 (15.1) 360.00 (35.3)	120
5.	Bensulfuron	411.15 (100)	412.20 (18.3) 413.10 (7.8)	120
6.	Pyrazosulfuron ethyl	415.20 (100)	416.10 (18.7) 417.15 (8.1)	120
7.	Triflusulfuron	493.05 (100)	494.10 (22.8) 495.15 (8.2)	120

4.2 High Performance Liquid Chromatographic Condition

HPLC method was developed and compromised with the scanning mode (200-600 *m/z*). The appropriated mobile phase from MS optimized condition (2mM oxalic acid) was applied. The gradient elution was developed to reach baseline resolution. After testing many conditions for the separation of seven herbicides, the preferable gradient program was described in table 4.3.

Table 4.3 The HPLC chromatographic optimization conditions

HPLC Parameter	Condition		
Analytical column	Narrow Bore 2.1 x 150 mm, 3.5 micron , Zorbax SB-C18		
Mobile phase	2 mM oxalic acid : Acetonitrile		
Flow rate	0.25 mL/min		
Injection Volume	5 μL		
Gradient program	Time	% A	% B
	0	67	33
	5	67	33
	12	0	100
Column temperature	Left 35 ^{o}C, Right 40 ^{o}C		
Detector	Mass spectrometer		

The chromatogram under condition in table 4.3 in total scan mode was present in figure 4.5 and a SIM mode in figure 4.6. Co-elution of cinosulfuron and metsulfuron methyl occurred at retention time of 6.369 min. However, these two compounds have different molecular weight. They can be distinguished easily in SIM mode by selected at their quasi-molecular ion and qualification ion.

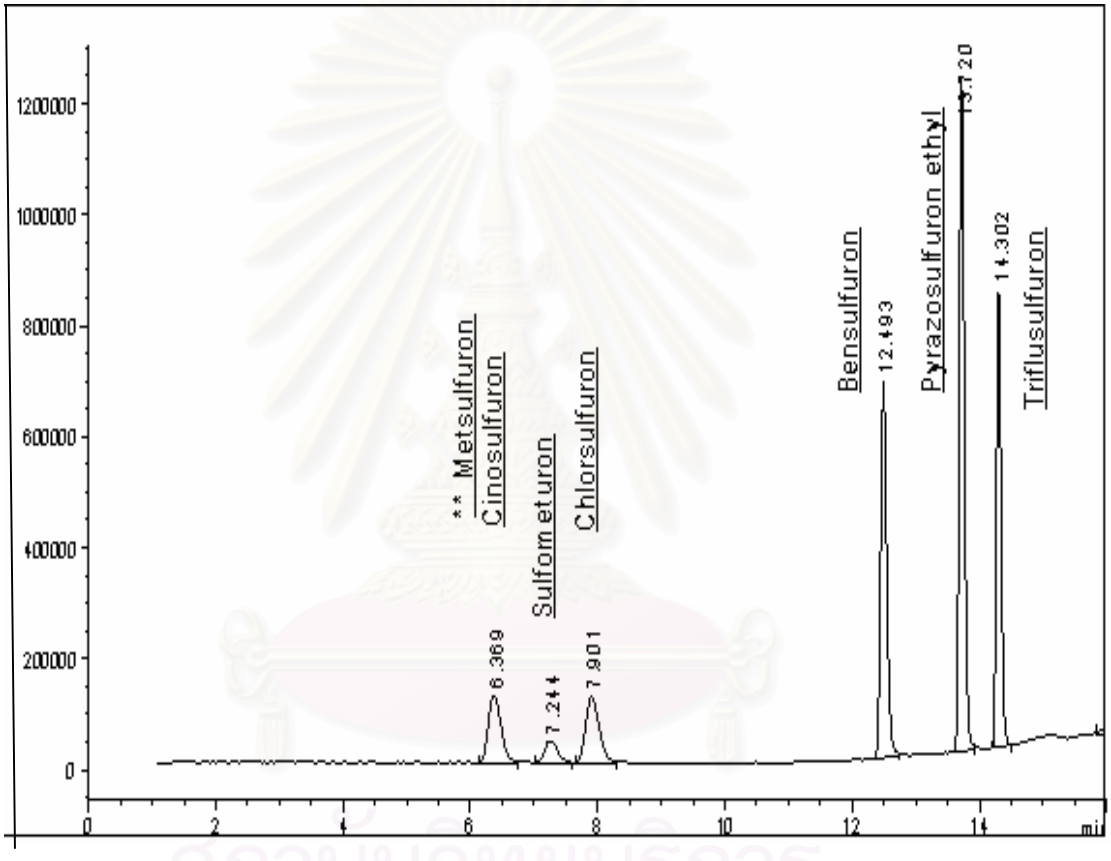


Figure 4.5 Chromatogram of Sulfonylurea herbicides mixture at 50.0 ppb

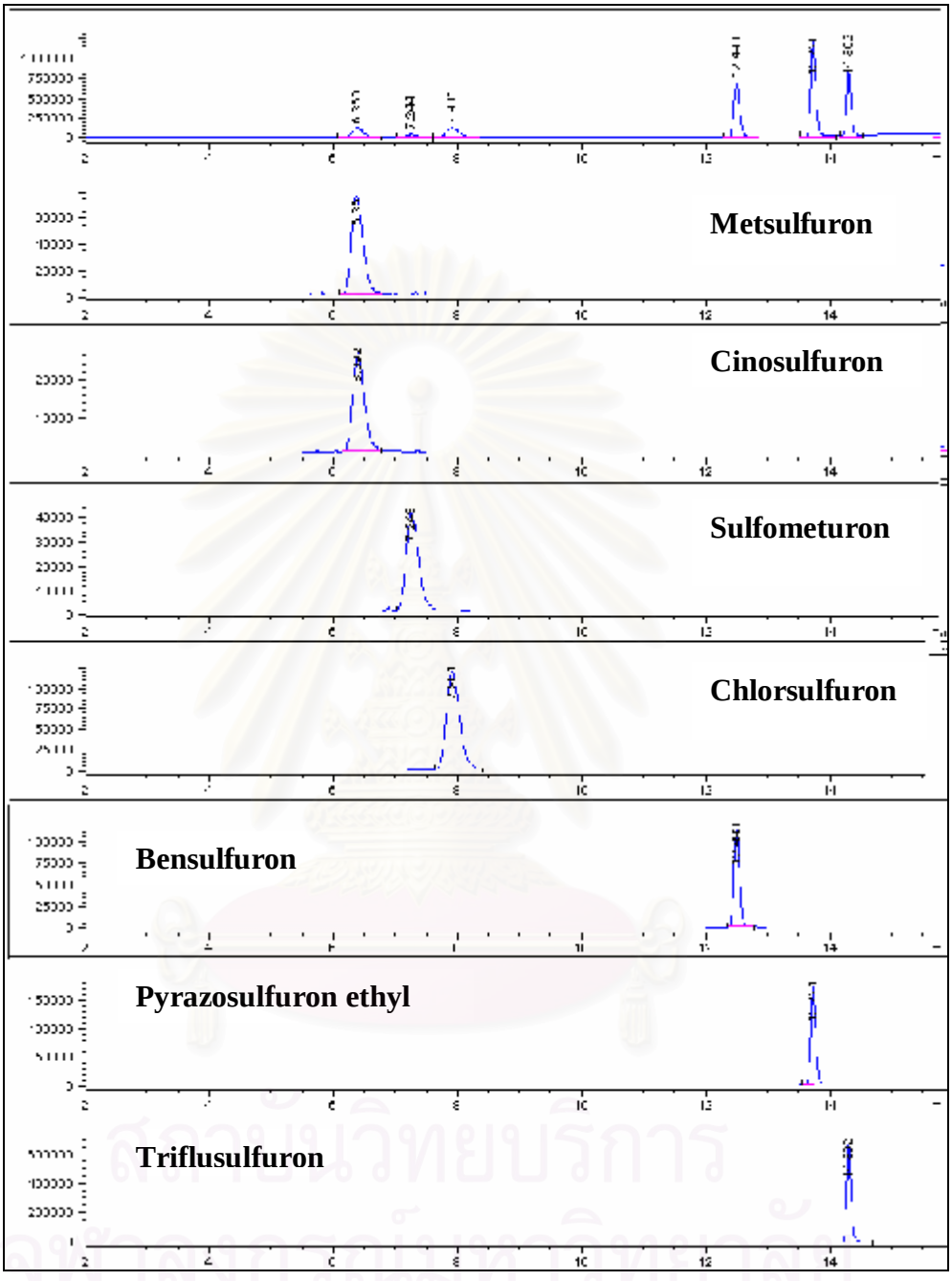


Figure 4.6 Extract ion Chromatogram of sulfonylurea herbicides mixture at 50.0 ppb (ng/mL)

4.3 LC/MS Selectivity

The selectivity of HPLC method was peak retention time and MS method was confirmed by target ion and qualified ion with the static abundance. Table 4.4 summarized the selectivity data of the developed method in acetonitrile solution.

Table 4.4 Retention time and characteristics ions of seven sulfonylurea herbicides

No.	Compound	t_R (min)	Quasi-molecular ion $(M+H)^+$	Qualification ion
1.	Cinosulfuron	6.392	414.15	415.00
2.	Metsulfuron	6.356	382.20	383.10
3.	Sulfometuron	7.243	365.10	366.12
4.	Chlorsulfuron	7.901	358.05	360.00
5.	Bensulfuron	12.493	411.15	412.20
6.	Pyrazosulfuron ethyl	13.721	415.20	416.10
7.	Triflusulfuron	14.302	493.05	494.10

The selected ion monitoring (SIM) experiment is very sensitive because the mass spectrometer can dwell for a longer time over a certain mass range. Therefore, SIM is more specific than scan mode and SIM window increases the sensitivity. The narrower mass range presented a more specific signal. Accordingly, the detection windows of substances were divided into two time schedules. Mass spectrometer opened at 2.00 to 11.00 minutes for the first window and investigated metsulfuron methyl, cinosulfuron, sulfometuron and chlorsulfuron by theirs select ions. The other

period was from 11.00 until 17.00 minutes when the rest of SUHs group was studied. Table 4.5 shows the detection window for monitoring sulfonylurea herbicides.

Table 4.5 Time schedule multiple-ion SIM conditions for monitoring of seven sulfonylurea herbicides

Group	Compound	SIM window (min)	Quasi-molecular ion $(M+H)^+$	Qualification ion
1.	Cinosulfuron	2.0-11.0	414.15	415.00
	Metsulfuron		382.20	383.10
	Sulfometuron		365.10	366.12
	Chlorsulfuron		358.05	360.00
2.	Bensulfuron	11.0-17.0	411.15	412.20
	Pyrazosulfuron ethyl		415.20	416.10
	Triflusulfuron l		493.05	494.10

According to the previous study, the optimized conditions and all further condition lists are in tables 4.3 and 4.5 at a fragmentor voltage 120 V.

4.4 Sample Preparation

Rice samples have two major components, polar such as carbohydrates and sugars and non polar such as macromolecules and pigment. Sample preparations were then required to isolate SUHs from a very complex rice matrix. The selected extraction methods (I-VII) concerned the most suited appropriate SPE were examined. Because their MRLs set up at a very low level (0.05 ppm for metsulfuron and chlorsulfuron by a ministry of health and labour welfare of Japan), therefore the comparison of peak

areas between spiked sample and spike blank was used. There are two spiking levels; medium and high concentration at 10.0 ppb and 50.0 ppb respectively. Recoveries of each sulfonylurea herbicides were calculated and the detail of extraction method is listed in appendix C.

4.4.1 Method I

Rice was extracted by ACN and 0.1 M NH_4HCO_3 and the solution was further cleaned up by ENVI-CARB cartridge. The recovery is presented in table 4.6. Bensulfuron presented the highest recovery from 74.54% to 69.60% at 10.0 and 50.0 ppb, respectively. But overall recovery was low because ENVI-CARB cartridge is effective in removing coloring or pigment substances from the sample but rice contains mostly starch and sugar.

Table 4.6 Percent recovery for seven sulfonylurea herbicides in rice under extraction method I (n=2)

No.	Compound	Spiking level	
		10.0 ppb	50.0 ppb
1	Cinosulfuron	14.03±1.02	26.53±10.98
2	Metsulfuron	17.47±2.17	21.92±6.91
3	Sulfometuron	3.49±2.97	11.84±5.05
4	Chlorsulfuron	21.24±3.80	21.61±6.33
5	Bensulfuron	74.54±15.19	69.60±12.03
6	Pyrazosulfuron ethyl	18.48±11.13	44.65±7.68
7	Triflusulfuron	52.43±12.00	50.23±1.20

4.4.2 Method II

Acetonitrile and water were used for extraction sulfonylurea herbicide in rice. Tandem SPE (C18+NH_2) was used to cleanup and preconcentrates the rice sample. The recovery range was from 85.00-131.10 % for C18+NH_2 cartridge. A high recovery of SUHs may result from a sufficient clean up of tandem SPE. Starch and a high molecular weight compounds in rice was trapped on the C18. The polar components in rice such as amino acid, sugar were cleaned by NH_2.

Table 4.7 Percent recovery for seven sulfonylurea herbicides in rice under extraction method II (n=2)

No.	Compound	Spiking level	
		10 ppb	50 ppb
1	Cinosulfuron	110.22±12.26	92.16±10.03
2	Metsulfuron	110.85±6.46	79.42±5.30
3	Sulfometuron	105.16±10.88	85.00±3.55
4	Chlorsulfuron	104.93±8.65	103.34±7.22
5	Bensulfuron	114.64±64.57	115.52±7.15
6	Pyrazosulfuron ethyl	120.31±12.83	119.72±5.03
7	Triflusulfuron	131.10±3.64	118.89±3.41

4.4.3 Method III

Method III has many extraction presented in the procedure. Herbicides were immerged and extracted with of water and re-extracted by acetone and ethyl acetate. The solution was transferred to 2% potassium hydrogenphosphate solution. The herbicides were extracted again into ethyl acetate and cleaned up using Alumina N and SAX cartridge. Result of this extraction method showed loss of sulfonylurea herbicides. SUHs were extracted by water and it may hydrolyze SUHs. Therefore; extraction with water using a long immersion time was not suitable. The other parameter which caused loss of analytes was emulsion was appearing in liquid-liquid extraction. Consequently, the analytes can not be separated completely in the immiscible phase.

Table 4.8 Percent recovery for seven sulfonylurea herbicides in rice under extraction method III (n=2)

No.	Compound	Spiking level	
		10 ppb	50 ppb
1	Cinosulfuron	ND	ND
2	Metsulfuron	ND	ND
3	Sulfometuron	ND	ND
4	Chlorsulfuron	ND	ND
5	Bensulfuron	ND	ND
6	Pyrazosulfuron ethyl	ND	ND
7	Triflusulfuron	ND	ND

4.4.4 Method IV

The sample was extracted by acetonitrile and dried by evaporation. Residue was re-dissolved in 2 mL of n-hexane. The extract passed through tandem SPE of Bound Elute PSA over Sep-Pak®Florisil cartridge. According to table 4.9, sulfometuron showed recoveries at 50.50% and 54.25% at 10.00 and 50.00 ppb whereas recoveries of bensulfuron ranged from 30.83 and 25.89% at the same spiking levels. This method was applied from multiresidue analysis in fresh fruits and vegetables. Therefore, this method may not suitable for dry sample like rice and present low recovery.

Table 4.9 Percent recovery for seven sulfonylurea herbicides in rice under extraction method IV (n=2)

No.	Compound	Spiking level	
		10 ppb	50 ppb
1	Cinosulfuron	ND	ND
2	Metsulfuron	ND	ND
3	Sulfometuron	50.50±38.64	54.25±14.4
4	Chlorsulfuron	ND	ND
5	Bensulfuron	30.86±18.90	25.89±6.30
6	Pyrazosulfuron ethyl	3.31±3.82	0.52±0.32
7	Triflusulfuron	2.38±1.30	0.81±0.16

4.4.5 Method V

Extraction of sulfonylurea herbicides by Dupont used C18 to clean up and remove rice matrix. Dichloromethane extracted the analytes from the sample and interference was separated by acetonitrile–hexane partitioning. The clean up step was to pass the extract to the C18 cartridge. The result is illustrated in table 4.10. Recovery of seven sulfonylurea herbicides was presented from 25.10 to 129.31% for two fortified levels. The results can be categorized into 2 groups, Cinosulfuron, bensulfuron, pyrazosulfuron and triflusulfuron showed the high recoveries (more than 80%). Metsulforon and chlorsulfuron have the low recovery at 25.10 to 49.23%. According to the original chromatogram of DuPont, this method showed interference peak in blank chromatogram. It refers to inadequate clean up steps and interference peaks can affect the quantitative analysis of herbicides. Moreover, dichloromethane is a carcinogen and this method is used to partition analytes.

Table 4.10 Percent recovery for seven sulfonylurea herbicides in rice under extraction method V (n=2)

No.	Compound	Spiking level	
		10 ppb	50 ppb
1	Cinosulfuron	82.90±1.78	75.46±3.45
2	Metsulfuron	28.79±0.97	36.58±117
3	Sulfometuron	41.77±0.94	49.23±1.29
4	Chlorsulfuron	25.10±0.78	31.95±1.06
5	Bensulfuron	93.78±0.86	80.21±3.55
6	Pyrazosulfuron ethyl	71.77±0.86	90.69±3.87
7	Triflusulfuron	129.31±1.26	86.12±2.46

4.4.6 Method VI

This method was taken from three papers using the same sample preparation process. Water samples were extracted with an acidic solution, such as 1% acetic acid solution whereas soil samples were extracted with 0.07 M phosphate buffer. Two different SPEs (polar and non-polar) were used to clean up. The pH in the solution was adjusted to 3.0-3.5 before passing through PSA. From table 4.13, recoveries were below 40.0%. Triflusulfuron has the highest recovery at 36.02% at 10 ppb whereas metsulfuron gave the lowest recovery at 0.43% at 50.00 ppb. Polar cartridge (Alumina or Silica) was applied to clean up and isolate herbicides as same as C18 cartridge. *(61)* However, SAX cartridges can be used alone to extract herbicides in water samples. *(59)* Because rice samples have difference matrix from water and soil samples therefore, a different SPE is needed. PSA is the primary secondary amines which consist of two amine groups which are suitable to retain polar compounds. Ammonium carbonate/acetone was used to extract analytes from a complicated matrix.

Table 4.11 Percent recovery for seven sulfonylurea herbicides in rice under extraction method VI

No.	Compound	Spiking level	
		10 ppb	50 ppb
1	Cinosulfuron	22.89	3.17
2	Metsulfuron	16.16	0.43
3	Sulfometuron	2.94	29.00
4	Chlorsulfuron	8.22	1.05
5	Bensulfuron	17.82	23.87
6	Pyrazosulfuron ethyl	20.40	4.90
7	Triflusulfuron	36.02	2.95

4.4.7 Method VII

The screening method used for the determination of nine sulfonylurea herbicides present in environmental samples by HPLC-UV was reported. This extraction method extracted rice with ammonium carbonate 3 times and cleaned up by two SPEs. Table 4.2, the recovery of chlorsulfuron (table 4.12) was placed at the highest level at 67.36% (50.0 ppb). The recoveries ranged from 25.00 to 67.36% and the lowest recovery was cinosulfuron at a low spiking level (10.0 ppb). C18 was firstly adsorbed the macromolecule of rice sample. The eluate was extracted and cleaned up by silica cartridge. Silica is a polar sorbent which can interact with polar molecules like sugar. Therefore, the analytes were finally separated from the matrix and injected into LC/MS system.

Table 4.12 Percent recovery for seven sulfonylurea herbicides in rice under extraction method VII

No.	Compound	Spiking level	
		10 ppb	50 ppb
1	Cinosulfuron	25.00	42.33
2	Metsulfuron	26.60	47.62
3	Sulfometuron	31.88	44.56
4	Chlorsulfuron	35.32	67.36
5	Bensulfuron	54.76	60.40
6	Pyrazosulfuron ethyl	53.26	28.62
7	Triflusulfuron	44.71	53.29

According to extraction methods, there were consideration from eliminate rice matrix and isolate sulfonylurea herbicides. Rice sample has polar and non polar compounds. Therefore, the sample preparations need solid phase extraction to isolate analytes from very complex matrix. The results from the study of sample preparation showed that extraction method II was suitable and has potential to extract sulfonylurea herbicides residue in rice. Extraction method showed the recovery was range from 85.00-131.10 % for C18+NH_2 cartridge at two spike levels, 10 and 50 ppb. Most of recoveries obtained within acceptable range.

4.5 Comparison of difference of Solid Phase Extraction (SPE) Cartridge

Extraction method II presented the satisfy recovery. Tandem cartridge consisted of hydrophobic sorbent which retain the macromolecule of rice matrix. The second cartridge was ion exchange sorbent; NH_2. This column can utilize both hydrogen bonding and anion exchange. NH_2 functional group interact with polar molecule like sugar, protein which containing in rice sample. Therefore, mixed mode SPE is alternative for cleaning rice matrix. SPE contains polar and non-polar property in one cartridge is interesting. Recently, new sorbents are produced and released to the market. The SPE development shows easy, reproduce and effective clean up of new cartridge. This SPE has two different functional groups in single sorbent. Primary Secondary Amine (PSA) is mixed mode cartridge represents anion exchange cartridge which also contained reverse phase property. Oasis HLB (Hydrophilic Lipophilic Balance) cartridge is polymeric sorbent which combines both hydrophilic and hydrophobic property in same cartridge. Therefore, comparison of difference SPE type was interesting by using same sample preparation process. Clean up and isolation was using C18+NH_2, PSA and HLB and following extraction method II. The result was summarized in table 4.13.

Table 4.13 Percent recovery for seven sulfonylurea herbicides in rice at 10 ppb under extraction method II by different cartridge (n=2)

No.	Compound	C18+NH_2	PSA	HLB
1	Cinosulfuron	110.22±12.26	104.58±17.09	109.60±7.58
2	Metsulfuron	110.85±6.46	114.65±20.93	100.35±0.53
3	Sulfometuron	105.16±10.88	105.16±10.88	125.21±2.58
4	Chlorsulfuron	104.93±8.65	106.28±18.29	104.03±3.17
5	Bensulfuron	114.64±64.57	114.01±11.25	97.89±1.41
6	Pyrazosulfuron ethyl	120.31±12.83	121.82±16.83	103.85±4.33
7	Triflusulfuron	131.10±3.64	109.69±18.85	101.06±2.50

In this study, extracts of rice samples were obtained using extraction method II in appendix C. Different cartridges presented different recovery and extraction efficiency. According to table 4.13, the combination of reversed phase (C18) and anion exchange (NH_2) presented the recovery at 104.93 to 131.10 % (which is higher than 80.0%). The recovery of sulfonylurea herbicides was higher than 80.0% for mixed mode cartridges (PSA and HLB) with PSA clean up, the recovery was placed in satisfactory range of not less than 80 %. HLB also presented a good recovery from 97.89-125.21%. To summarize, the overall recoveries were in a satisfactory range. Both tandem and mixed mode cartridges can be applied to the quantitative analysis of sulfonylurea herbicides in rice.

4.6 Acidic pH effect on Extraction

Sulfonylurea herbicides have pKa between 3.5-5.8. At a low pH, analytes are presented in the neutral form (pH<pKa). Therefore, loading solution was present in only one form. pH control was setting up at 3.0-3.5 and comparing with non control pH. From section 4.5, tandem (C18+NH_2) and PSA showed the accepted recovery; 104.93 to 131.10 % and 105.16 to 121.82 %, respectively. PSA was chosen to a representative of mixed mode cartridge for a further studies because PSA has two retention mechanisms and is cheaper than the HLB cartridge. Therefore, tandem and PSA cartridge was used to isolate herbicide from the matrix and studied at difference pH values. The results are summarized in table 4.14.

Table 4.14 Percent recovery for seven sulfonylurea herbicides in rice at two fortifications level under extraction method II by control and no control pH

No.	Compound	Spiking level (ppb)	No control pH		Acidic pH	
			$C18+NH_2$	PSA	$C18+NH_2$	PSA
1	Cinosulfuron	10.0	110.22	104.58	100.40	11.65
		50.0	92.16	84.52	125.30	4.46
2	Metsulfuron	10.0	110.85	114.65	98.52	0.16
		50.0	79.42	79.42	120.68	7.72
3	Sulfometuron	10.0	105.16	105.16	99.67	60.36
		50.0	85.00	85.00	55.18	48.71
4	Chlorsulfuron	10.0	104.93	106.28	96.98	60.62
		50.0	103.34	75.58	68.66	36.21
5	Bensulfuron	10.0	114.64	114.01	96.30	67.74
		50.0	115.52	86.54	107.98	47.03
6	Pyrazosulfuron ethyl	10.0	120.31	121.82	54.25	7.76
		50.0	119.72	94.70	107.97	0.50
7	Trflusulfuron	10.0	131.10	109.69	128.38	8.00
		50.0	118.89	94.06	113.08	5.22

Table 4.14 reported the extraction method II with control pH of extraction solution before load through cartridge tandem cartridge presented the recoveries from 54.25 to 128.38 %. The highest recovery (128.38%) was received from triflusulfuron at 10.0 ppb whereas pyrazosulfuron ethyl gave recovery at 54.25 % at same spiking level. PSA was showed recovery from 0.16-67.74%. Values decreased from not adjust pH. C18+NH_2 presented recovery over than 80 % and PSA cartridge showed nearly recovery as tandem cartridge at higher than 79 %. It can explain from the property of PSA cartridge. PSA consists of two anion exchanger therefore; analytes placed in neutral form can trap on the active site. PSA can be most effective clean up sulfonylurea herbicides at higher pH than 3.5 (ionic form). Tandem SPE presented the good recoveries at two concentration level therefore pH did not effect to determination of sulfonylurea herbicides in rice matrix.

4.7 The Result of C18+NH_2 and PSA Cartridge to Clean Up Efficiency

According to extraction method development, tandem SPE and PSA presented the very good % recovery. Therefore, the matrix removing is one of parameter to compare the efficiency of tandem cartridge (C18+NH_2) or PSA cartridge.

4.7.1 Matrix removing by C18+NH_2 Cartridge

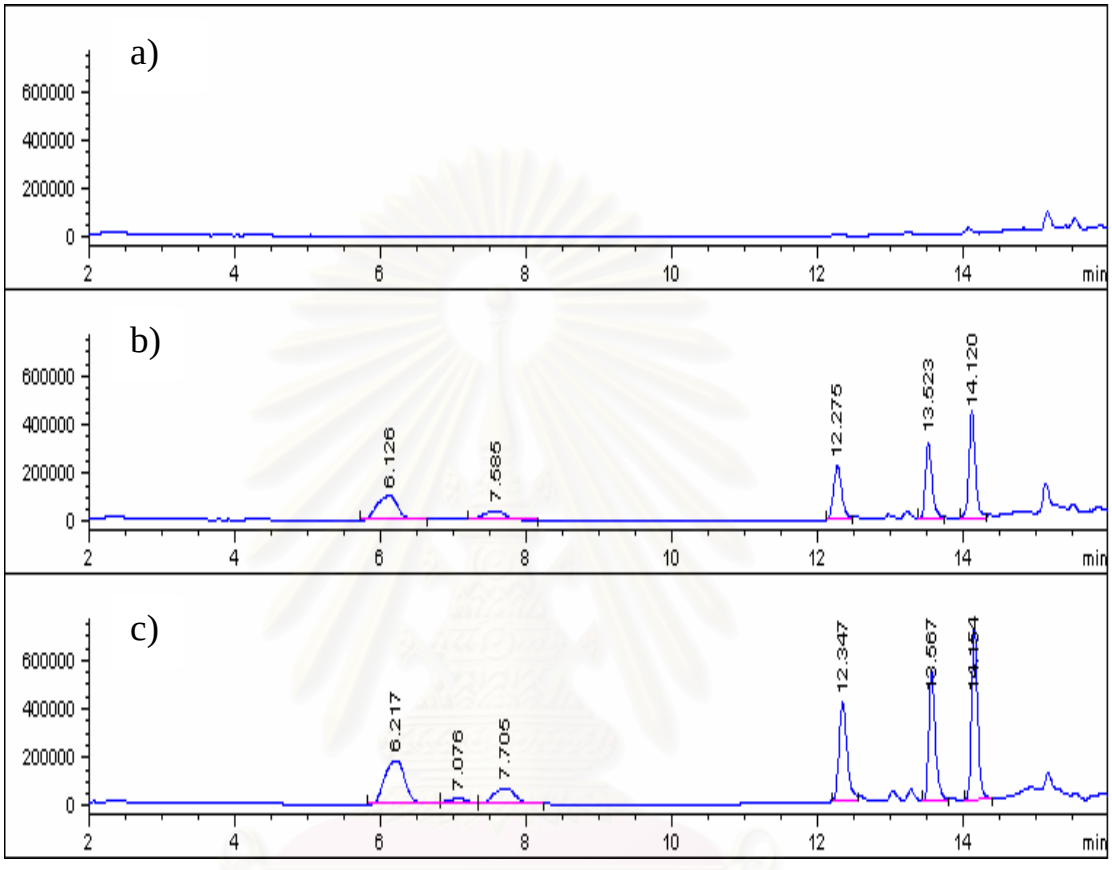


Figure 4.7 Chromatogram of tandem clean up cartridge (a) blank extract, (b) spiking at 10 ppb and (c) spiking at 50 ppb

According to the figure 4.7, tandem was effective in removing matrix from sample extracts. From the appearance only, tandem seemed to have the better effect to clean up the rice matrix. In this case, C18 is a reverse phase sorbent, which can trap starch and macromolecules (non polar molecules) in rice component. Sugar is retained in aminopropyl (NH_2) cartridge. Aminopropyl also extracted polar compounds because the active sizes are weak anion exchange. Therefore, the contamination of unwanted baseline of blank extract, and spiked blanks were very low.

4.7.2 Matrix removing by PSA cartridge

PSA sorbent is a new mixed mode sorbent produced by combining polar and non polar function. The property of this sorbent may same as tandem SPE. To achieve an efficient and rapid clean up, the study of PSA extraction was carried out.

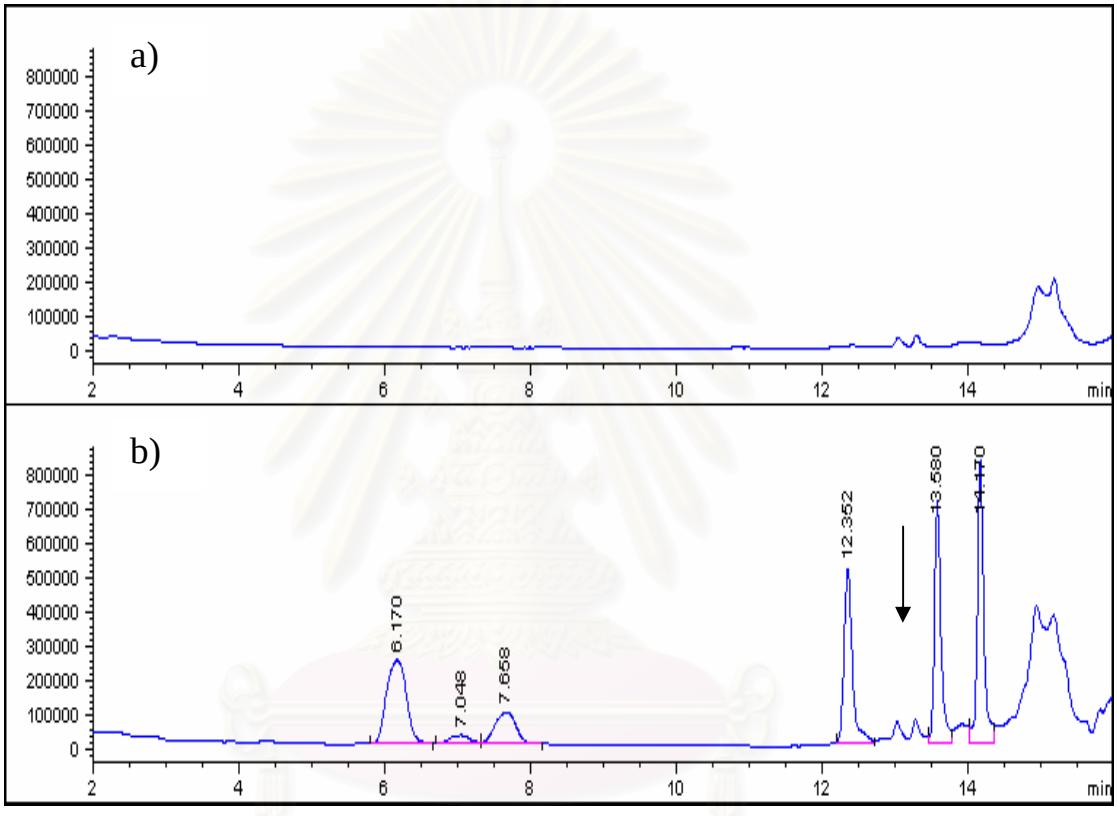


Figure 4.8 Chromatogram of PSA clean up cartridge
(a) Blank extract and (b) Spiking at 10 ppb

The chromatogram shows the removal of matrix from rice extract. However, the hump of signal was presented in PSA clean up chromatogram. The interference was present during 12.3-13.5 minutes.

4.7.3 Comparison of matrix removing by C18+NH_2 and PSA cartridge

The different cartridges of SPE (C18+NH_2 and PSA) were tested in order to indicate effective matrix removal. The results are presented in figure 4.9.

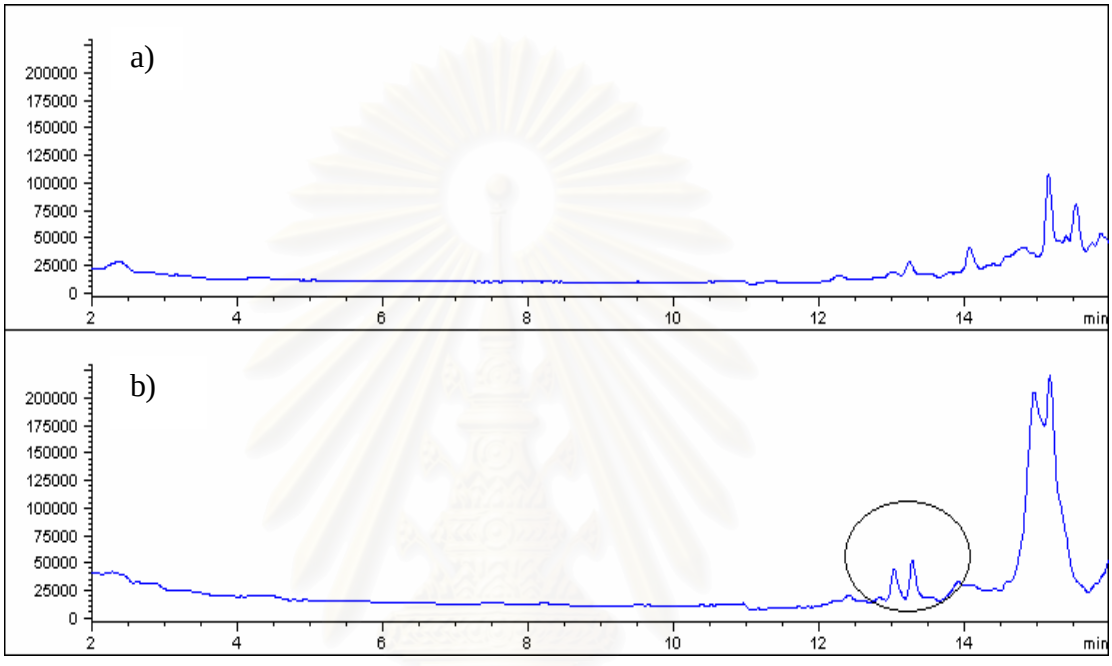


Figure 4.9 Chomatogram of tandem and PSA clean up cartridge
(a) blank extract by tandem cartridge
(b) blank extract by PSA cartridge

When tandem SPE was used for sample preparation, the baseline was very clean and less interference appeared. Mixed mode (PSA) SPE showed interference peaks (figure 4.9, (b)) which could affect the determination of sulfonylurea. Therefore, tandem SPE was more effective to remove rice interference and made it possible to detect pesticides residues at spiking level 10 and 50 ppb. However, the next extraction method in this study will be to compare sulfonylurea herbicides extracted by C18+NH_2 and PSA cartridge. Because, matrix was not present in retention time of analytes. The extraction method for sulfonylurea herbicides was following method II in appendix C.

4.8 Method Validation of C18+NH_2 cartridge

Method validation is an important requirement in the practice of chemical analysis. The purpose is to study the method performance parameter and demonstrat a particular method for quantitative measurement of analytes in the matrix (rice). The parameters for this validation include selectivity, linearity and range, MDL and MQL, precision, accuracy and robustness. *(62, 63, 64 and 66)*

4.8.1 Selectivity

This parameter refers to the reliability of the measurements in the presence of interferences, which is particularly important. The selectivity of analytes by LC/MS can be defined in two parameters. The selectivity of HPLC method was peak retention time and MS method was confirmed by target ion and qualified ion. Therefore, chromatogram of sulfonylurea herbicides are presented in figure 4.10 and t_R and characteristic ions were carried out and summarized in table 4.15.

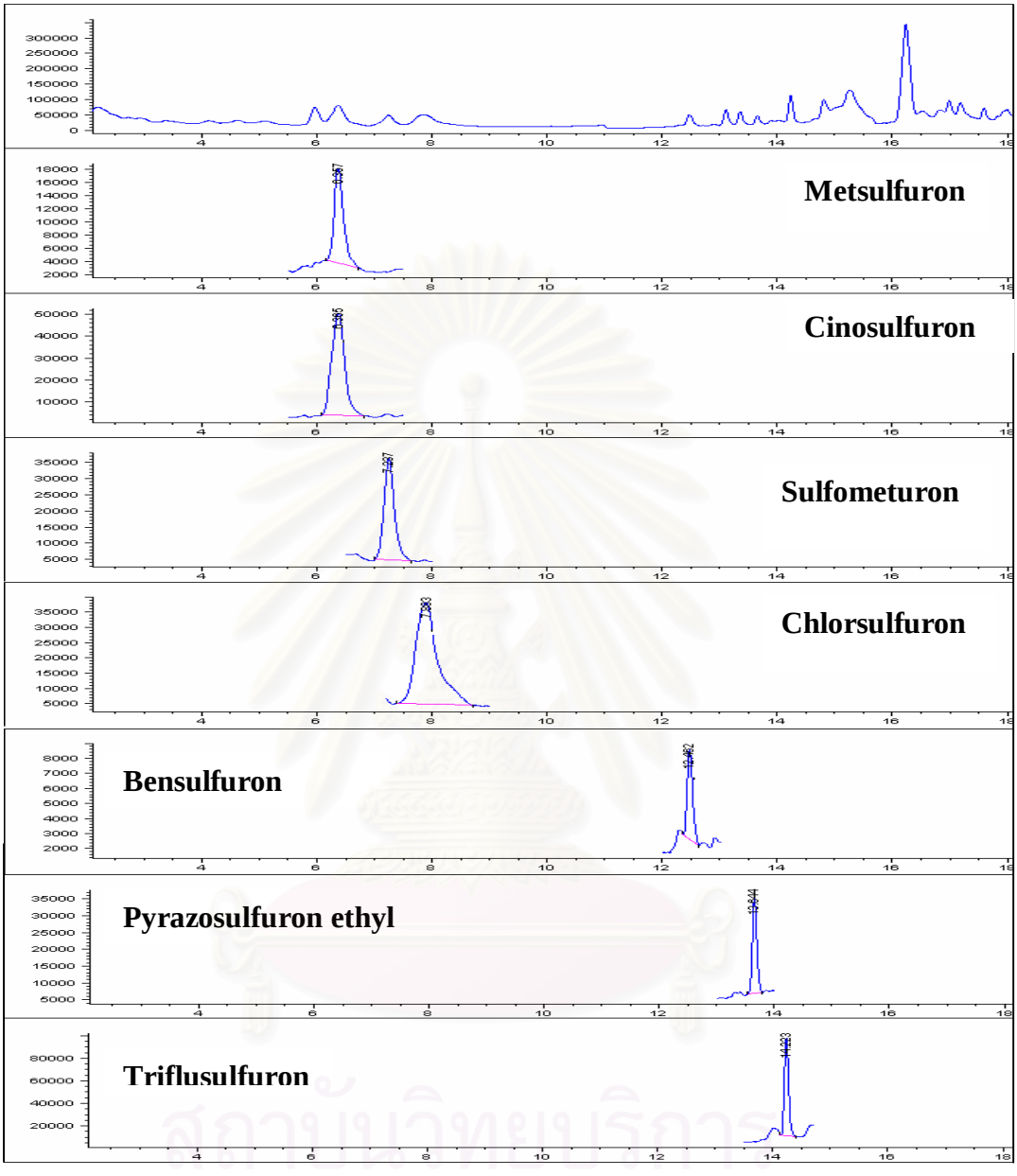


Figure 4.10 Extract ion Chromatogram of sulfonylurea herbicides mixture at MQL by C18+NH_2 cartridge

Seven compounds were not necessary to complete separation by LC/MS analysis. Co-elution of cinosulfuron and metsulfuron methyl occurred. Cinosulfuron and metsulfuron methyl have different molecular weights, therefore these two compounds can be distinguished easily. According to "Document N° SANCO/17476/2003; quality control procedures for pesticide residues analysis", diagnostic ion should have peaks of similar retention times and mass spectrum. Where increased sensitivity obtained by selected ion monitoring (SIM), the minimum requirement is for data from two ions of *m/z* over 200. *(65)* According to SANCO recommendation; the confirmation of results need at least two ions for monitoring and quantitation purposes. SIM has more sensitivity than the scan mode because the mass spectrometer can dwell for a longer time over a smaller mass range. The narrower mass range presented a more specific signal and improved the sensitivity. Accordingly, the substances were divided into two time schedules. Table 4.15 presents optimized conditions from column separation and time windows.

Table 4.15 Time schedule multiple-ion SIM conditions for monitoring of seven sulfonylurea herbicides

Group	Compound	SIM window (min)	Quasi-molecular ion $(M+H)^+$	Qualification ion
1.	Cinosulfuron	2.0-11.0	414.15	415.00
	Metsulfuron l		382.20	383.10
	Sulfometuron		365.10	366.12
	Chlorsulfuron		358.05	360.00
2.	Bensulfuron	11.0-17.0	411.15	412.20
	Pyrazosulfuron ethyl		415.20	416.10
	Triflusulfuron l		493.05	494.10

4.8.2 Method Detection Limit (MDL) and Method Quantitation Limit (MQL) for C18+NH_2 Cartridge

The method detection limit is the lowest concentration of analytes in a sample, which can be detected at a signal to noise ratio of 3. The method quantitation limit (MQL) is the lowest concentration of analyte in a sample that can be quantitatively determined with an acceptable level of precision. It is also defined by various concentrations to be the analytes concentration corresponding to present signal to noise of 10. MDL and MQL obtained by determining SUHs in rice matrix following extraction method II in appendix C. The results are summarized in table 4.16.

Table 4.16 Method detection limit and method quantitation limit of each sulfonylurea herbicides in rice matrix (C18+NH_2 cartridge)

No.	Compound	Method Detection Limit (ppb)	Method Quantitation Limit (ppb)
1	Cinosulfuron	0.80	1.30
2	Metsulfuron	3.00	6.00
3	Sulfometuron	4.00	7.15
4	Chlorsulfuron	5.00	6.06
5	Bensulfuron	0.64	0.91
6	Pyrazosulfuron ethyl	0.71	0.97
7	Triflusulfuron	1.90	2.40

4.8.3 Standard Calibration Curve

The standard calibration curves of sulfonylurea herbicides were investigated in a range from 1.00-300 ppb and analyzed by the LC/MS under the conditions listed in table 4.3 and 4.5. Regression coefficients (R^2) are summarized in table 4.17. The regression lines of the peak area and concentration are shown in appendix C.

Table 4.17 Linear least-squares regression coefficients of standard calibration curves of Sulfonylurea herbicides at a range 1.00- 300 ppb (10 points, duplicate analyses)

No.	Compound	Slope	y-Intercept	R^2
1	Cinosulfuron	36209	260182	0.9931
2	Metsulfuron	22169	151993	0.9923
3	Sulfometuron	8491.9	33950	0.9966
4	Chlorsulfuron	23367	152294	0.9939
5	Bensulfuron	35141	292576	0.9933
6	Pyrazosulfuron ethyl	46528	417406	0.9930
7	Triflusulfuron	47636	430268	0.9927

The ten-point calibration curves used a least-square regression analysis. Correlation coefficient (R^2) higher than 0.99 were determined for all compounds. Metsulfuron showed the lowest correlation coefficient (R^2) at 0.9923 whereas the highest correlation coefficient (R^2) was sulfometuron at 0.9966. The slope values are between 8491.9 and 47636.

4.8.4 Linear Range

The study of calibration curve is carried out at the analyte concentration of 1.000- 300 ppb. To determine the linear range of the analytical method, the concentration was extended to cover higher level than calibration from 1.00 to 500 ppb. The concentration and peak area was plotted and using linear least-square regression to predict best-fit curve of this range. The regression coefficient data is reported in table 4.18.

Table 4.18 Linear least-squares regression coefficients of SUHs at a range 1.000- 500.000 ppb (12 points, duplicate analyses)

No.	Compound	Slope	y-Intercept	R^2
1	Cinosulfuron	35276	310168	0.9976
2	Metsulfuron	20770	236606	0.9958
3	Sulfometuron	81461.1	53779	0.9983
4	Chlorsulfuron	21581	264041	0.9947
5	Bensulfuron	30949	527335	0.9896
6	Pyrazosulfuron ethyl	42735	638127	0.9948
7	Triflusulfuron	42229	743468	0.9913

The studies on linearity were performed using a standard solution. Correlation coefficient (R^2) was higher than 0.9000 for all compounds and ranged 0.9896 to 0.9983. The correlation coefficient suggests the developed method had excellent linearity over the concentration range of 1.00 – 500 ppb.

4.8.5 Matrix Calibration Curve

The matrix calibration curve was created using extracts from procedure II. The blank rice extract was spiked with sulfonylurea herbicides and the concentration range is shown in section 3.13. The relationship between the peak area and concentration was plotted and regression coefficient data is reported in table 4.19.

Table 4.19 Regression coefficients of Sulfonylurea herbicides at a range 1.00- 100 ppb (7 points, duplicate analyses)

No.	Compound	Slope	y-Intercept	R^2
1	Cinosulfuron**	8623.3	5642.5	0.9961
2	Metsulfuron*	5059.2	8033.4	0.9979
3	Sulfometuron*	2736.5	-1127.9	0.9944
4	Chlorsulfuron*	4866.7	58063	0.9949
5	Bensulfuron**	33464	184926	0.9994
6	Pyrazosulfuron ethyl**	45895	18140	0.9948
7	Triflusulfuron**	43503	36789	0.9958

* Matrix calibration range from 5.00-100 ppb

** Matrix calibration range from 1.00-80.00 ppb

From section 4.8.2, the seven sulfonylurea herbicides have different MQL therefore, the working range is also different. Because, a lower end of concentration range is values of individual MQL for each herbicide. The seven-point matrix calibration curves ranged from 5.00-100 ppb for metsulfuron, sulfometuron and chlorsulfuron and 1.00-80.0 ppb for cinosulfuron, bensulfuron, pyrazosulfuron ethyl and triflusulfuron. Working range existed a linear response range and presented a good

linearity due to R^2 reported from 0.9944 to 0.9994. The MRL (Maximum Residual Limit) by a ministry of health and labour welfare of Japan (MHLW) was defined of sulfonylurea herbicides in rice at 0.05-0.1 mg/kg. Therefore, it is not necessary to study this at a high concentration level. Sensitivity of each herbicide is different. According to the matrix calibration curve, triflusulfuron has the highest sensitivity whereas sulfometuron has the lowest sensitivity shown by the slope.

4.8.6 Matrix Effect

The matrix is one of the most important analytical measurements. When the analytical system is validated the matrix can be considered. A test for the matrix effect can be made by adding analytes into a standard solution compared with the matrix solution. Moreover, two calibration curves should cover the same working range of analysis. Matrix effect was studied by using paired *t*-test with mean of 95 % confidence limit. The *t*-values is given in table 4.20.

Table 4.20 *t*–calculated values of two tailed paired *t*-test at 95 % confidence level

No	Compound	Concentration (ppb)	Peak area		Pair *t*- test
			Standard solution	Standard in rice matrix	
1	Cinosulfuron	5.000	2.1464E+05	3.2868E+04	3.0201
		10.000	4.0605E+05	9.3977E+04	
		20.000	8.6013E+05	2.0532E+05	
		40.000	1.7168E+06	3.6440E+05	
		60.000	2.4975E+06	5.5178E+05	
		80.000	3.4651E+06	7.0484E+05	
2	Metsulfuron	5.000	1.3243E+05	3.3268E+04	2.9171
		10.000	2.4750E+05	7.0104E+04	
		20.000	4.1096E+05	1.1427E+04	
		40.000	1.0595E+06	2.1662E+04	
		60.000	1.5468E+06	3.1175E+05	
		80.000	2.1412E+06	4.1479E+05	

No	Compound	Concentration (ppb)	Peak area		Pair *t*- test
			Standard solution	Standard in rice matrix	
3	Sulfometuron	5.000	4.5678E+04	1.4975E+04	2.7807
		10.000	8.3312E+04	3.2180E+04	
		20.000	1.7324E+05	5.1841E+04	
		40.000	3.7213E+05	1.0346E+05	
		60.000	5.5375E+05	1.5397E+05	
		80.000	7.7006E+05	2.1424E+05	
4	Chlorsulfuron	5.000	1.3408E+05	7.1848E+04	2.6142
		10.000	2.5064E+05	1.0565E+05	
		20.000	4.5003E+05	1.6572E+05	
		40.000	1.1221E+06	2.3136E+05	
		60.000	1.5999E+06	3.5110E+05	
		80.000	2.2006E+06	4.3200E+05	
5	Bensulfuron	5.000	2.3491E+05	3.4574E+05	1.2403
		10.000	4.5869E+05	5.1234E+05	
		20.000	8.6879E+05	8.6636E+05	
		40.000	1.6458E+06	1.5627E+06	
		60.000	2.5672E+06	2.1030E+06	
		80.000	3.4402E+06	3.1540E+06	
6	Pyrazosulfuron ethyl	5.00	3.0631E+05	2.5565E+05	1.7251
		10.00	6.0362E+05	4.3920E+05	
		20.00	1.2125E+06	9.4309E+05	
		40.00	2.4082E+06	1.8312E+06	
		60.00	3.5356E+06	2.5407E+05	
		80.00	4.5212E+06	3.7390E+06	
7	Triflusulfuron	5.00	2.9435E+05	2.3864E+05	2.6445
		10.00	5.7567E+05	4.9126E+05	
		20.00	1.1543E+06	9.6683E+05	
		40.00	2.5066E+06	1.8212E+06	
		60.00	3.6184E+06	2.4457E+06	
		80.00	4.7884E+06	3.6205E+06	

Tests were carried out by the same concentration between standard and matrix calibration curve. From table 4.20, bensulfuron and pyrazosulfuron ethyl have lower *t*-calculated than the *t*–critical. Most of the *t*-calculated values were higher than the *t*-critical value; cinosulfuron and metsulfuron, sulfometuron and chlorsulfuron triflusulfuron which were 3.0201, 2.9171, 2.7807 2.6142 and 2.6445. Therefore; there was a significant difference between the standard calibration curve and the matrix calibration curve. The rice matrix shows significant difference between the standard calibration curve and the matrix calibration curve. This significance means the matrix affects to the analysis and therefore the matrix calibration curves were used for this study.

4.8.7 Method Precision for C18+NH_2 Cartridge

The precision of an analytical method refers to the scattering of results from multiple analyses and the closeness between independent test results under stipulated conditions. Because the matrix has significant differences, the precision was carried out by studying in the matrix. Two concentrations in range of expected concentration was studied. Therefore, method precision at MQL and 5-MQL in rice sample were studied.

a) Method precision at MQL level in rice matrix

The method precision at MQL was studied on 2 consecutive days and each concentration was repeated 6 times. The mean % recovery, the standard deviation and relative standard deviation were calculated and the results were summarized in table 4.21-4.23.

Table 4.21 Percent recovery and RSD of spiked rice matrix at MQL level (First day, n = 6, C18+NH_2 cartridge)

No.	Compounds	% Recovery						Mean	% RSD
		1	2	3	4	5	6		
1	Cinosulfuron	80.6	80.1	81.6	75.6	72.9	66.3	76.2±5.88	7.72
2	Metsulfuron	61.9	78.2	65.4	76.7	62.4	70.2	69.1±7.10	10.27
3	Sulfometuron	88.2	74.8	92.1	75.3	74.8	75.0	80.0±7.93	9.91
4	Chlorsulfuron	81.5	90.7	85.7	75.9	73.5	76.0	80.6±6.67	8.28
5	Bensulfuron	84.8	104.2	105.6	93.0	83.9	100.3	95.3±9.55	10.02
6	Pyrazosulfuron ethyl	87.1	101.1	109.8	99.4	97.5	92.0	97.8±7.82	7.99
7	Triflusulfuron	98.4	97.1	85.9	88.0	89.5	88.2	91.2±5.23	5.74

Table 4.22 Percent recovery and RSD of spike rice matrix at MQL level (Second day, n = 6, C18+NH_2 cartridge)

No.	Compounds	% Recovery						Mean	% RSD
		1	2	3	4	5	6		
1	Cinosulfuron	80.9	99.3	82.9	80.7	70.1	88.7	83.8±9.70	11.58
2	Metsulfuron	86.8	67.4	76.3	64.0	62.4	73.7	71.8±9.14	12.73
3	Sulfometuron	77.0	95.8	90.2	74.0	94.5	82.7	85.7±9.18	10.71
4	Chlorsulfuron	90.0	88.1	90.6	76.7	85.6	79.0	85.0±5.85	6.88
5	Bensulfuron	82.1	75.4	77.3	95.2	76.4	97.8	84.0±9.96	11.85
6	Pyrazosulfuron ethyl	81.5	83.7	80.2	82.7	85.7	90.8	84.1±3.78	4.50
7	Triflusulfuron	90.6	96.9	89.0	82.1	92.4	93.8	90.8±5.05	5.57

Table 4.23 Overall % recovery and % RSD of spiked rice matrix at MQL level (n=2, C18+NH_2 cartridge)

No.	Compounds	% Recovery		Mean	% RSD
		1	2		
1	Cinosulfuron	76.2	83.8	80.0±5.37	6.41
2	Metsulfuron	69.1	71.8	70.5±1.91	2.66
3	Sulfometuron	80.0	85.7	82.9±4.03	4.70
4	Chlorsulfuron	80.6	85.0	82.8±3.11	3.66
5	Bensulfuron	95.3	84.0	89.7±7.92	9.42
6	Pyrazosulfuron ethyl	97.8	84.1	91.0±9.69	11.52
7	Triflusulfuron	91.2	90.8	91.0±0.28	0.31

Table 4.24 One way ANOVA of spiked rice matrix at MQL level at 95 % confident level (n=6, C18+NH_2 cartridge)

No	Compounds	Analysis Day	% Recovery						*P-value*
			1	2	3	4	5	6	
1	Cinosulfuron	First	80.6	80.1	81.6	75.6	72.9	66.3	0.1327
		Second	80.9	99.3	82.9	80.7	70.1	88.7	
2	Metsulfuron	First	61.9	78.2	65.4	76.7	62.4	70.2	0.5895
		Second	86.8	67.4	76.3	64.0	62.4	73.7	
3	Sulfometuron	First	88.2	74.8	92.1	75.3	74.8	75.0	0.2793
		Second	77.0	95.8	90.2	74.0	94.5	82.7	
4	Chlorsulfuron	First	81.5	90.7	85.7	75.9	73.5	76.0	0.2474
		Second	90.0	88.1	90.6	76.7	85.6	79.0	
5	Bensulfuron	First	84.8	104.2	105.6	93.0	83.9	100.3	0.0734
		Second	82.1	75.4	77.3	95.2	76.4	97.8	
6	Pyrazosulfuron ethyl	First	87.1	101.1	109.8	99.4	97.5	92.0	0.0031
		Second	81.5	83.7	80.2	82.7	85.7	90.8	
7	Triflusulfuron	First	98.4	97.1	85.9	88.0	89.5	88.2	0.8999
		Second	90.6	96.9	89.0	82.1	92.4	93.8	

Precision is subdivided into repeatability and reproducibility. This work was studying repeatability by the performance method using the same laboratory and the same equipment. This thesis further studied the within-day precision or repeatability which assesses precision during a single analytical run (day). According to AOAC Peer-Verified method, Nov 1993 recommendation, the acceptable RSD can be calculated by "Horwitz equation" and is presented in table 4.25. *(65)*

Table 4.25 The acceptable RSD at MQL level by AOAC Peer-Verified methods, November 1993

No.	Compounds	Method Quantitation Limit (ppb)	% RSD*
1	Cinosulfuron	1.30	29.14
2	Metsulfuron	6.00	23.15
3	Sulfometuron	7.15	22.55
4	Chlorsulfuron	6.06	23.11
5	Bensulfuron	0.91	30.75
6	Pyrazosulfuron ethyl	0.97	30.45
7	Triflusulfuron	2.40	26.44

* Horwitz equation; $RSD < 2^{(1-0.5\log C)} \times 0.67$

a-I) Within-day Precision

Therefore, the closeness of the agreement was determined by the percent of RSD. Six replicate at MQL were presented the % RSD ranged from 5.75 to 12.86 on the first day and 5.59 to 15.42 on the second day. According to AOAC recommendation, the accepted value ranges from 22.55 to 30.75%. Therefore, this method shows the % of RSD is less than the recommended values on both studying days.

a-II) Between-day Precision

Between-day precision or repeatability is measuring precision using different times. This work was carried out over two consecutive days. The comparison between the % recovery of the first (n=6) and the second (n=6) day was determined by ANOVA. *P*-value at 95 % confidence limit of each compound was shown in table 4.20. Cinosulfuron, metsulfuron, sulfometuron, chlorsulfuron, bensulfuron and triflusulfuron presented a *P*-value greater than 0.05 at 95 % confidence. Therefore, these six sulfonylurea herbicides showed no significant difference at this concentration on the two consecutive days. One the other hand, pyrazosulfuron ethyl was presented *P*-value at 0.0031 at 95 % confidence. This value showed a significant difference determination of pyrazosulfuron ethyl on two different days. However, this method still fairly well reported.

b) Method Precision at 5-MQL level in rice matrix

Method precision at 5-MQL used the same procedure as MQL. The mean % recovery, the standard deviation and relative standard deviation were calculated and the results are summarized in table 4.26-4.27.

Table 4.26 Percent recovery and RSD of spike rice matrix at 5-MQL level (First day, n = 6, C18+NH_2 cartridge)

No.	Compounds	% Recovery						Mean	% RSD
		1	2	3	4	5	6		
1	Cinosulfuron	79.4	76.9	87.6	83.5	72.4	79.0	79.8±5.26	6.60
2	Metsulfuron	73.4	80.2	79.3	91.9	75.9	83.3	80.7±6.49	8.05
3	Sulfometuron	81.4	89.0	5.7	94.6	79.8	68.6	84.9±10.31	12.15
4	Chlorsulfuron	87.8	66.8	79.0	76.2	80.2	79.9	78.3±6.83	8.73
5	Bensulfuron	85.9	105.6	106.6	97.8	100.4	78.2	95.8±11.36	11.87
6	Pyrazosulfuron ethyl	88.0	100.7	86.4	89.9	101.9	97.1	94.0±6.75	7.18
7	Triflusulfuron	93.0	98.7	93.6	80.4	109.9	80.9	92.8±11.16	12.04

Table 4.27 Percent recovery and RSD of spike rice matrix at 5-MQL level (Second day, n = 6, C18+NH_2 cartridge)

No.	Compounds	% Recovery						Mean	% RSD
		1	2	3	4	5	6		
1	Cinosulfuron	80.0	84.5	87.1	78.2	93.6	71.5	82.5±7.67	9.30
2	Metsulfuron	85.6	82.2	76.8	81.9	83.6	80.7	81.8±2.97	3.63
3	Sulfometuron	81.5	90.4	79.5	87.7	74.5	83.1	82.8±5.72	6.90
4	Chlorsulfuron	77.1	84.1	72.4	72.5	82.9	70.3	76.6±5.84	7.62
5	Bensulfuron	85.6	100.4	80.3	74.8	97.8	76.6	85.9±10.89	12.68
6	Pyrazosulfuron ethyl	85.9	93.9	83.1	79.9	91.5	81.7	86.0±5.60	6.51
7	Triflusulfuron	104.0	90.8	102.7	98.4	80.5	88.3	94.1±9.16	9.74

Table 4.28 Overall Percent recovery and RSD of spiked rice matrix at 5-MQL level, (n=2, C18+NH_2 cartridge)

No	Compounds	% Recovery		Mean	% RSD
		1	2		
1	Cinosulfuron	79.8	82.5	81.2±1.91	2.31
2	Metsulfuron	80.7	81.8	81.3±0.78	0.95
3	Sulfometuron	84.9	82.8	83.9±1.48	1.79
4	Chlorsulfuron	78.3	76.6	77.5±1.20	1.57
5	Bensulfuron	95.8	85.9	90.9±7.00	8.15
6	Pyrazosulfuron ethyl	94.0	86.0	90.0±5.66	6.58
7	Triflusulfuron	92.8	94.1	93.5±0.92	0.98

Table 4.29 One way ANOVA of spiked rice matrix at 5-MQL level at 95 % confident level (n=6, C18+NH_2 cartridge)

No	Compounds	Analysis Day	% Recovery						*P-value*
			1	2	3	4	5	6	
1	Cinosulfuron	First	79.4	76.9	87.6	83.5	72.4	79.0	0.4959
		Second	80.0	84.5	87.1	78.2	93.6	71.5	
2	Metsulfuron	First	73.4	80.2	79.3	91.9	75.9	83.3	0.7055
		Second	85.6	82.2	76.8	81.9	83.6	80.7	
3	Sulfometuron	First	81.4	89.0	5.7	94.6	79.8	68.6	0.3617
		Second	81.5	90.4	79.5	87.7	74.5	83.1	
4	Chlorsulfuron	First	87.8	66.8	79.0	76.2	80.2	79.9	0.6405
		Second	77.1	84.1	72.4	72.5	82.9	70.3	
5	Bensulfuron	First	85.9	105.6	106.6	97.8	100.4	78.2	0.1569
		Second	85.6	100.4	80.3	74.8	97.8	76.6	
6	Pyrazosulfuron ethyl	First	88.0	100.7	86.4	89.9	101.9	97.1	0.0494
		Second	85.9	93.9	83.1	79.9	91.5	81.7	
7	Triflusulfuron	First	93.0	98.7	93.6	80.4	109.9	80.9	0.8214
		Second	104.0	90.8	102.7	98.4	80.5	88.3	

AOAC has introduced “Peer Verified Method program” for the validation method. *(65)* The acceptable RSD was calculated by Horwitz equation and shown in table 4.30.

Table 4.30 The acceptable RSD at 5-MQL level by AOAC Peer-Verified methods, November 1993

No.	Compounds	5-Method Quantitation Limit (ppb)	% RSD*
1	Cinosulfuron	6.50	22.86
2	Metsulfuron	30.00	18.17
3	Sulfometuron	35.75	17.69
4	Chlorsulfuron	30.30	18.14
5	Bensulfuron	4.55	24.13
6	Pyrazosulfuron ethyl	4.85	23.90
7	Triflusulfuron	12.00	20.85

* Horwitz equation; $RSD < 2^{(1-0.5\log C)} \times 0.67$

b-I) Within-day Precision

On the first day, SD ranged from 6.75 to 11.36 at 5-MQL level. The SD indicated good within-day precision. % of RSD was reported at 6.60-12.15%. On the second day, analysis presented SD at 2.97 to 10.89 and accepted RSD in range 6.90 to 12.68%. In terms of precision, acceptable precision could be based on the Horwitz equation. RSD should range between 17.69 to 24.15 % at 4.55 to 30.30 ppb. Therefore, this method presented the RSD value to be less than the AOAC recommended values.

b-II) Between-day Precision

Cinosulfuron, metsulfuron, sulfometuron, chlorsulfuron, bensulfuron and triflusulfuron presented *P*-value at 0.4959, 0.7055, 0.3617, 0.6405, 0.1569 and 0.8214 at 95 %confident for six replication times. *P*-value is greater than 0.05 at 95 % confident therefore, this is no significance different between two working days. Pyrazosulfuron ethyl only showed significance difference at 95 % confidence (*P*-value= 0.0494). However, this method was precise because six in seven herbicides showed non significant difference.

To conclude, the newly developed method was very precise by studying at MQL and 5-MQL level of individual herbicides. The precision of analytical method was study 2 terms; within-day and between-day precision in matrix based. % RSD represented the precision and fairly well precision was reported. *P*-value was shown the precise between two days. The result was indicated that higher level (5-MQL) is smaller deviation than lower level (MQL). It can be explained by matrix interference more disturb at MQL.

4.8.8 Method Accuracy for C18+NH_2 Cartridge

Accuracy expresses the closeness of a result to a true value (samples containing known amount of analyte). The true value can be determined from certified reference material (CRMs), reference materials, used of a reference method and used of spiking/recovery. Recovery was the determining method accuracy used this time. The most widely used recovery study, is performed by spiking analyte in a blank sample matrix. The analyte is added to a blank matrix at MDL, MQL and 5-MQL. The recovery at each level was determined by comparison to the known amount added and the results are showen in table 4.31-4.32.

Table 4.31 Percent recovery and RSD of spike rice matrix at MDL level (n = 6; C18+NH2 cartridge)

No.	Compounds	% Recovery						Mean	% RSD
		1	2	3	4	5	6		
1	Cinosulfuron	68.6	58.5	66.4	65.0	58.4	70.9	64.7±5.19	8.03
2	Metsulfuron	73.8	80.2	70.9	71.5	67.0	73.8	72.9±4.36	5.98
3	Sulfometuron	66.9	77.6	79.8	59.4	76.4	87.1	74.5± 9.89	13.27
4	Chlorsulfuron	58.4	59.0	51.8	62.6	62.8	67.3	60.3±5.25	8.71
5	Bensulfuron	89.1	90.8	87.2	91.5	70.9	102.0	88.6±10.07	11.37
6	Pyrazosulfuron ethyl	85.6	81.6	79.4	89.1	91.2	80.9	84.6±4.78	5.65
7	Triflusulfuron	87.8	90.1	92.3	95.0	91.3	92.4	91.5±2.42	2.65

Table 4.32 Summarize percent recovery for spiked rice matrix at method detection limit (MDL), method quantitation limit (MQL) and 5-method quantitation limit (5-MQL) level (C18+NH_2 cartridge)

No.	Compounds	% Recovery		
		MDL level	MQL level	5-MQL level
1	Cinosulfuron	64.7±5.19	80.0±5.37	81.2±1.91
2	Metsulfuron	72.9±4.36	70.5±1.91	81.3±0.78
3	Sulfometuron	74.5± 9.89	82.9±4.03	83.9±1.48
4	Chlorsulfuron	60.3±5.25	82.8±3.11	77.5±1.20
5	Bensulfuron	88.6±10.07	89.7±7.92	90.9±7.00
6	Pyrazosulfuron ethyl	84.6±4.78	91.0±9.69	90.0±5.66
7	Triflusulfuron	91.5±2.42	91.0±0.28	93.5±0.92

Method accuracy is represented by the closeness of the mean test results to the true concentration. The recovery of spike rice at MDL was reported between 60.3 to 91.5% from concentration range 0.64 to 5.00 ppb. % Recoveries at MQL were higher than lower level access from 70.5 to 91.0%. 5-MQL, recoveries reported from 77.5 to 93.5 %. Cinosulfuron and chlorsulfuron reported lower recovery at the MDL level at 64.7 and 60.3 %, respectively. AOAC has introduced the “Peer Verified Method program” *(65)* for the validation of method”. In terms of method accuracy, it is defined that the acceptable recovery at 1 ppb should range from 40-120 % and 10 ppb range between 60 and 115 %. Therefore, this method was accurate according to AOAC recommendations.

4.8.9 Method Robustness for C18+NH_2 Cartridge

Robustness test is studying on effect of varying parameters to the analysis. The effective way to determine method robustness is with a statistical experimental designed to evaluate many parameters simultaneously. A proper design can minimize the number of experiments needed while still providing effective information. In this work, Plackett-Burman design was applied to study seven parameters that affect the extraction of sulfonylurea herbicides in rice in eight runs. The extraction parameters are illustrated in table 3.1. This design is based on the screening of two levels for each parameter that is also shown in table 3.1. The recovery of each experiment was applied to calculate the different value (D). The different values were shown in table 4.33. Data analysis and corresponding statistic *t*-value for the effect of seven parameters on the extraction of sulfonylurea herbicides in rice was summarized in table 4.34.

Table 4.33 Comparisons of difference value for spike rice matrix at 5-MQL level by following Plackett- Burman experimental designs

No.	Compounds	Different						
		D_A	D_B	D_C	D_D	D_E	D_F	D_G
1	Cinosulfuron	-1.38	7.54	12.85	36.58	-20.96	-17.92	-4.48
2	Metsulfuron	-5.81	-0.55	15.04	16.28	-6.92	-2.30	-4.27
3	Sulfometuron	-3.28	0.62	15.70	28.41	-11.25	-11.62	-5.42
4	Chlorsulfuron	4.97	6.61	1.54	17.47	-10.55	-9.75	-8.17
5	Bensulfuron	8.09	0.40	-3.56	-1.04	-3.59	2.78	0.60
6	Pyrazosulfuron ethyl	4.07	11.88	5.67	33.01	-10.58	-12.63	-8.76
7	Triflusulfuron	1.25	5.85	-0.91	-2.07	9.25	3.32	-12.28

Table 4.34 *t*-value of spike rice matrix at 5-MQL level by following Plackett- Berman experimental designs (95% confidence limit)

No.	Compounds	*t*-value						
		A	B	C	D	E	F	G
1	Cinosulfuron	0.07	0.40	0.68	0.93	1.11	0.95	0.77
2	Metsulfuron	0.54	0.05	1.39	0.50	0.64	0.05	0.39
3	Sulfometuron	0.22	0.04	1.04	1.88	0.74	0.77	0.36
4	Chlorsulfuron	0.52	0.69	0.16	1.81	1.09	1.01	0.85
5	Bensulfuron	2.01	0.10	0.89	0.26	0.89	0.69	0.15
6	Pyrazosulfuron ethyl	0.26	0.77	0.37	2.16	0.69	0.82	0.57
7	Triflusulfuron	0.19	0.91	0.14	0.32	1.44	0.52	1.91

It is apparent from table 4.25 that the extraction time, waiting time, volume of solvent elution, evaporation temperature, solvent grade, light and amount of NaCl had *t*-value ranging from 0.07 to 2.01 at 95% confidence limit. The critical value for *t* (0.05, 7) is 2.36. Since *t*-experiment from table 4.24 is less than *t*-critical, variations on the parameters do not have any significant effect on this method performance.

4.9 Method validation of PSA cartridge

Partial method validation aims to check only significant parameters. According to section 4.5 (comparison of difference of solid phase extraction cartridge), tandem SPE and mixed mode SPE showed a good recovery over extraction method II. PSA cartridge *(62, 63, 64 and 66)* is another effective clean up and isolation sulfonylurea herbicides in rice. The selecting parameters provided on extraction process.

4.9.1 Selectivity

Selectivity refers to the reliability of measurements in the presence of interferences. The selectivity of analytes by LC/MS can be defined in two parameters. The selectivity of HPLC method was peak retention time and the MS method was the target ion and the qualified ion. Selectivity was already studied. Tandem SPE was used to clean up and isolate analytes in section 4.8.2. Therefore, the selectivity parameters in table 4.5 were also used and checked in this section. The results of PSA cartridge are presented in figure 4.11.

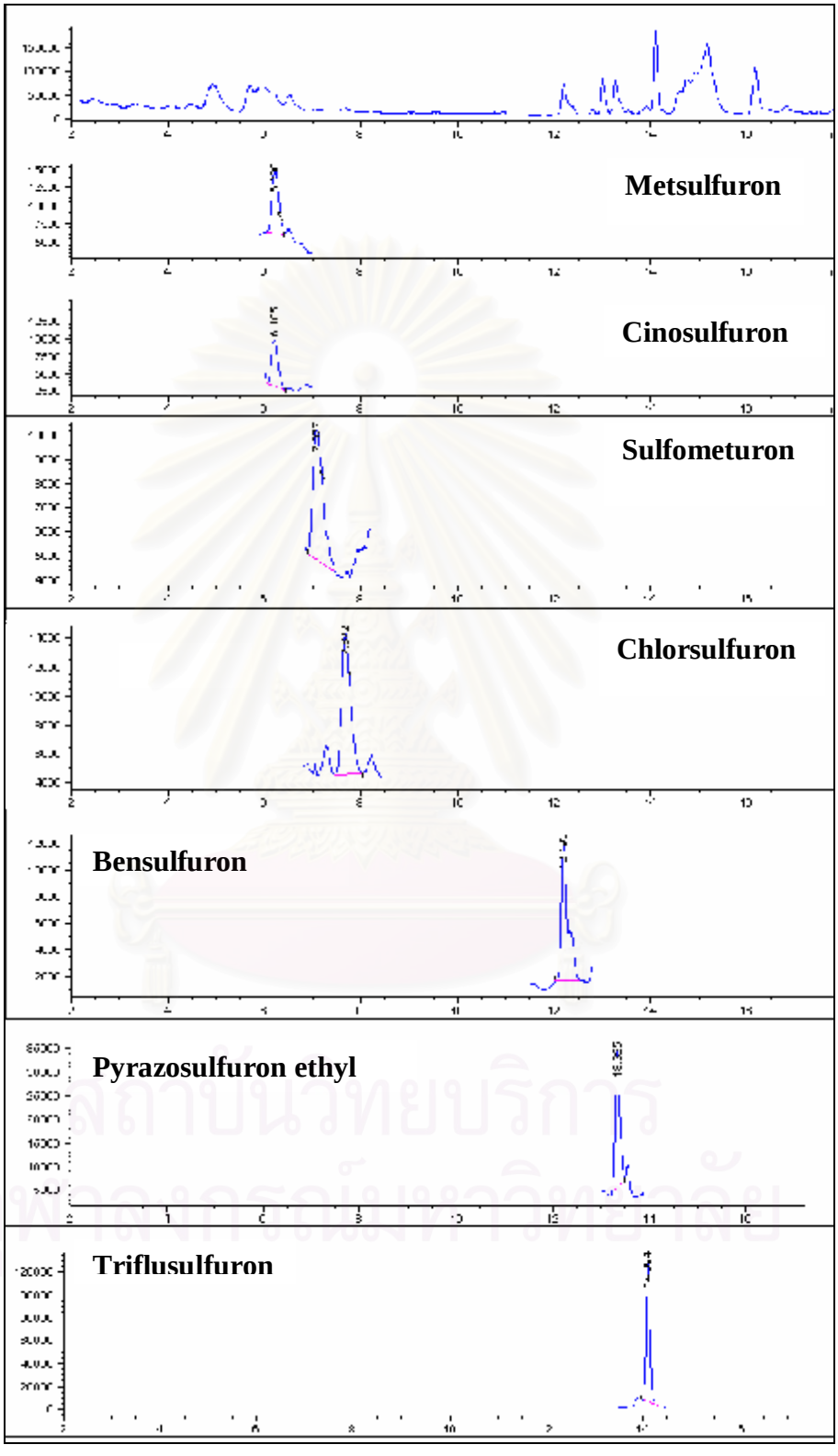


Figure 4.11 Extract ion Chromatogram of sulfonylurea herbicides mixture at MQL by PSA cartridge

4.9.2 Method Detection Limit (MDL) and Method Quantitation Limit (MQL) for Primary Secondary Amine (PSA)

Method Detection Limit (MDL) and method quantitation limit (MQL) are important parameters and these values are affected by the separation condition, separation method and instrumentation. The MDL refers to the amount of analytes in a sample, which are detected at a signal to noise ratio equal to 3. The method quantitation limit (MQL) is the lowest amount of analyte that can be quantitatively determined with suitable precision. MQL is detected at signal to noise equal to 10. The MDL and MQL values obtained by the extraction method II (appendix C) and cleaned up with mixed mode SPE (PSA cartridge). The results are summarized in table 4.35.

Table 4.35 Method detection limit (MDL) and method quantitation limit (MQL) of each sulfonylurea herbicide in rice matrix (PSA cartridge)

No.	Compound	Method Detection Limit (ppb)	Method Quantitation Limit (ppb)
1	Cinosulfuron	1.95	4.0
2	Metsulfuron	6.24	8.0
3	Sulfometuron	7.15	9.5
4	Chlorsulfuron	5.85	8.5
5	Bensulfuron	0.91	2.0
6	Pyrazosulfuron ethyl	0.91	1.5
7	Triflusulfuron	1.30	4.3

4.9.3 Method Precision for Primary Secondary Amine (PSA) Cartridge

Method precision is the closeness of agreement between independent test results obtained under stipulated conditions. For a single laboratory validation, a precision is operated under repeatable conditions during one day. Precision often varies with analyte concentration. In this work, three concentrations (MDL, MQL and 5-MQL) were indicated precision. The spiking level was presented in table 3.7.

a) Method precision at MDL level in rice matrix by PSA cartridge

The simultaneous of method precision at MDL was studied in a single run and repeated 6 times. The mean % recovery, the standard deviation and relative standard deviation were calculated and summarized in table 4.36.

Table 4.36 Percent recovery and RSD of spiked rice matrix at method detection limit (MDL) level (n=6, PSA cartridge)

No.	Compounds	% Recovery						Mean	% RSD
		1	2	3	4	5	6		
1	Cinosulfuron	70.9	68.8	67.0	70.6	59.3	58.9	65.9±5.48	8.32
2	Metsulfuron	67.0	72.6	72.0	63.7	72.0	64.4	68.66±4.14	6.02
3	Sulfometuron	51.2	58.9	72.3	77.0	65.5	71.2	66.01±9.57	14.50
4	Chlorsulfuron	62.5	73.7	61.4	63.9	68.0	69.9	66.56±4.79	7.19
5	Bensulfuron	96.1	97.7	89.9	80.5	75.0	74.9	85.71±10.28	12.00
6	Pyrazosulfuron ethyl	60.8	58.0	63.6	72.3	54.6	59.9	61.53±6.06	9.85
7	Triflusulfuron	73.4	81.9	74.7	83.9	72.1	78.6	77.45±4.79	6.18

Table 4.37 presented the AOAC "Peer Verified Method program" for the method validation. The Horwitz equation calculated an acceptable RSD.

Table 4.37 The acceptable RSD at MDL spiking level by AOAC Peer-Verified methods, November 1993

No.	Compounds	Method Detection Limit (ppb)	% RSD*
1	Cinosulfuron	1.95	27.47
2	Metsulfuron	6.24	23.01
3	Sulfometuron	7.15	22.55
4	Chlorsulfuron	5.85	23.17
5	Bensulfuron	0.91	30.75
6	Pyrazosulfuron ethyl	0.91	30.75
7	Triflusulfuron	1.30	29.14

* Horwitz equation; $RSD < 2^{(1-0.5\log C)} \times 0.67$

a-I) Within-day precision

Herbicides were obtained and repeatedly analysed in one day. The method showed a good within-day precision value. SD ranged from 4.14 to 10.28 at MDL level (0.91-7.15 ppb).The %RSD was reported at 6.02-14.50 %. The AOAC recommended % RSD should be based on the Horwitz equation. From table 3.4, an acceptable RSD could not be greater than 30.75 %.RSD should be range between 22.55 to 30.75 % at 0.91 to 7.15 ppb. Therefore, this method presented good method precision because the RSD value was less than the AOAC recommended values.

b) Method precision at MQL level in rice matrix by PSA cartridge

Method precision at MQL was studied by repeated 6 times and analysed in the same day. The mean % recovery, the standard deviation and relative standard deviation were calculated and summarized in table 4.38.

Table 4.38 Percent recovery and RSD of spiked rice matrix at method quantitation limit (MQL) level (n=6, PSA cartridge)

No.	Compounds	% Recovery						Mean	% RSD
		1	2	3	4	5	6		
1	Cinosulfuron	64.5	71.2	72.5	60.8	55.6	63.3	64.7±6.37	9.85
2	Metsulfuron	71.2	77.6	75.7	83.3	85.8	84.2	79.6±5.72	7.18
3	Sulfometuron	64.6	72.6	72.9	69.2	60.1	72.6	68.7±5.26	7.67
4	Chlorsulfuron	72.1	67.1	62.6	66.8	63.3	74.5	67.8±4.74	8.28
5	Bensulfuron	78.5	91.8	93.4	96.7	80.8	91.2	88.7±7.35	8.28
6	Pyrazosulfuron ethyl	58.9	56.6	60.9	58.9	65.8	73.1	62.4±6.10	9.79
7	Triflusulfuron	96.3	90.2	92.9	90.9	89.4	93.5	92.1±2.60	2.83

The acceptable RSD is recommended by AOAC “Peer Verified Method program” for the method validation. The Horwitz equation was calculated and presented in table 4.39.

Table 4.39 The acceptable RSD at MQL spiking level by AOAC Peer-Verified methods, November 1993

No.	Compounds	Method Quantitation Limit (ppb)	% RSD*
1	Cinosulfuron	4.0	24.61
2	Metsulfuron	8.0	22.16
3	Sulfometuron	9.5	21.61
4	Chlorsulfuron	8.5	21.96
5	Bensulfuron	2.0	27.31
6	Pyrazosulfuron ethyl	1.5	28.52
7	Triflusulfuron	4.3	24.34

* Horwitz equation; $RSD < 2^{(1-0.5\log C)} \times 0.67$

b-I) Within-day precision

The method presented good within-day precision value. AOAC recommended that the % RSD should range from 21.61 to 28.52 at spiking level 1.5 to 9.5 ppb. According to table 4.35, the %RSD was reported at 2.83- 9.79%. Therefore, this method shows that % RSD is less than the recommended values. SD ranged from 2.60 to7.35 at MQL level.

c) Method precision at 5-MQL level

Method precision at 5-MQL used same procedures as MDL and MQL The mean % recovery, the standard deviation and relative standard deviation were calculated and are summarized in table 4.40.

Table 4.40 Percent recovery and RSD of spiked rice matrix at 5-method quantitation limit (5-MQL) level (n=6, PSA cartridge)

No.	Compounds	% Recovery						Mean	% RSD
		1	2	3	4	5	6		
1	Cinosulfuron	78.2	76.6	73.0	88.2	78.7	70.2	77.5±6.17	7.97
2	Metsulfuron	79.4	76.8	75.1	80.9	85.1	81.2	79.6±3.55	4.55
3	Sulfometuron	73.2	80.2	75.2	81.4	74.6	98.4	75.5±4.76	6.30
4	Chlorsulfuron	72.5	75.8	67.5	65.3	72.5	72.7	71.1±3.84	5.40
5	Bensulfuron	84.5	90.2	92.7	84.5	96.4	100.7	93.2±5.54	5.95
6	Pyrazosulfuron ethyl	76.2	66.1	61.7	76.4	76.9	70.4	71.3±6.37	8.93
7	Triflusulfuron	95.4	97.7	109.8	95.5	94.8	92.3	97.6±6.23	6.38

Horwitz equation presented acceptable RSD by the AOAC for the method validation under “Peer Verified Method program”. The recommended values were calculated and are presented in table 4.41.

Table 4.41 The acceptable RSD at 5-MQL spiking level by AOAC Peer-Verified methods, November 1993

No.	Compounds	5-Method Quantitation Limit (ppb)	% RSD*
1	Cinosulfuron	20.0	19.31
2	Metsulfuron	40.0	17.40
3	Sulfometuron	47.5	16.96
4	Chlorsulfuron	42.5	17.24
5	Bensulfuron	10.0	21.11
6	Pyrazosulfuron ethyl	7.5	22.38
7	Triflusulfuron	21.5	19.77

* Horwitz equation; $RSD < 2^{(1-0.5\log C)} \times 0.67$

c-I) Within-day precision

SD ranged from 3.55 to 6.37 at 5-MQL level (7.5 to 47.5 ppb). The %RSD was reported from 4.55-8.93 %. In terms of precision, it is defined that the acceptable precision could be based on the Horwitz equation. The %RSD should range between 16.96 to 22.83% at 7.5 to 47.5 ppb. It is seen that this method presented the %RSD value to be less than the AOAC recommended value and also showed the method precision of the PSA cartridge.

In summary, the newly developed method with PSA cartridge is very precise. Individual herbicides were studied at MDL, MQL and 5-MQL level. The precision of the PSA column was studied by within-day precision in matrix. % RSD represented the precision and good precision followed the AOAC recommendation.

4.9.4 Method Accuracy for Primary Secondary Amine (PSA) cartridge

Method accuracy is the degree of how to observe results correspond to the true value of analytes in samples. There are many ways to determine the true value. Spike is one determination method. The appropriate range of analyte concentrations should be investigated because recovery may be concentration-dependent. Consequently, the accuracy of this method is based on studies at 3-concentration level, method detection limit (MDL), method quantitation limit (MQL) and 5-method quantitation limit (5-MQL), as shown in table 3.7. The recoveries of spiked sample at MDL, MQL and 5-MQL are presented in table 4.42.

Table 4.42 Overall percent recovery of spiked rice matrix at Method detection limit (MDL), method quantitation limit (MQL) and 5-method quantitation limit (5-MQL) of each sulfonylurea herbicide (n=6, PSA cartridge)

No.	Compounds	% Recovery		
		MDL level	MQL level	5-MQL level
1	Cinosulfuron	65.9±5.48	64.7±6.37	77.5±6.17
2	Metsulfuron	68.66±4.14	79.6±5.72	79.6±3.55
3	Sulfometuron	66.01±9.57	68.7±5.26	75.5±4.76
4	Chlorsulfuron	66.56±4.79	67.8±4.74	71.1±3.84
5	Bensulfuron	85.71±10.28	88.7±7.35	93.2±5.54
6	Pyrazosulfuron ethyl	61.53±6.06	62.4±6.10	71.3±6.37
7	Triflusulfuron	77.45±4.79	92.1±2.60	97.6±6.23

According to table, 4.36, recovery reported was between 61.53 to 85.71% for the MDL level that ranged from 0.91 to 7.15 ppb. The MQL spiking level was 1.5 to 9.5 ppb and the recovery ranged from 62.4 to 92.1 %. Accuracy of the new method developed was also test at a higher concentration range. 5-MQL reported a satisfactory recovery at 71.1 to 97.6 %. According to the AOAC recommendation, the proper recovery should place at 40 to 120 % for spiking level 1 ppb and 60 to115 % at 10 ppb. Therefore, the method developed by the PSA cartridge showed a closeness of recovery result (illustrate method accuracy) according to the AOAC recommendation.

4.10 Comparison of Method Detection Limit (MDL) and Method Quantitation Limit (MQL) of C18+NH_2 and PSA Cartridge

MDL and MQL represented the method property. It is important to know the lowest concentration of analytes that can be confidently detected by the method. Comparison of two values by different extraction process was investigated. Table 4.43 indicated MDL and MQL by C18+NH_2 and PSA cartridge.

Table 4.43 Comparison of method detection limit (MDL) and method quantitation limit (MQL) of sulfonylurea herbicide in rice matrix using two different cartridges

No.	Compound	MDL (ppb)		MQL (ppb)	
		C18+NH2	PSA	C18+NH2	PSA
1	Cinosulfuron	0.80	1.95	1.30	4.0
2	Metsulfuron	3.00	6.24	6.00	8.0
3	Sulfometuron	4.00	7.15	7.15	9.5
4	Chlorsulfuron	5.00	5.85	6.06	8.5
5	Bensulfuron	0.64	0.91	0.91	2.0
6	Pyrazosulfuron ethyl	0.71	0.91	0.97	1.5
7	Triflusulfuron	1.90	1.30	2.40	4.3

From table 4.43, MDL and MQL for both cartridges were very similar. However, C18+NH_2 cartridge has a lower MDL and MQL level for all sulfonylurea herbicides. The MDL level of metsulfuron and cinosulfuron by tandem SPE were about 2 times lower than the other clean up cartridge. Only triflusulfulron had the MDL at similar 1.90 and 1.30 ppb by tandem and PSA cartridge, respectively. The highest MQL was by sulfometuron of PSA clean up at 9.5 ppb, whereas, the MQL value of tandem SPE ranged from 0.91 to 6.06 ppb. However, the different of MDL and MQL between the two cartridges is not much. MQL of PSA placed at 1.5 to 9.5 ppb which present trace level of method development. Therefore, PSA can be an alternative cartridge for preparation of sulfonylurea herbicides in rice instead of C18+NH_2 cartridge.

CHAPTER V

CONCLUSION AND SUGGESTION FOR FUTHER STUDY

A new method development for simultaneous analysis of cinosulfuron, metsulfuron, sulfometuron, chlorsufuron, bensulfuron, pyrazosulfuron ethyl and triflusulfuron was developed. The analysis was carried out using High Performance Liquid Chromatography/Mass Spectrometry (LC/MS). The work covered the LC/MS parameter. For the MS parameter study, there were three parameters to optimize. Electrospray ionization was applied to optimize all parameters for full scan mode. 2 mM oxalic acid presented the highest sensitivity to most sulfonylurea herbicides. Capillary voltage was also studied, and the optimized voltage for this work was 3000 V positive ionization mode. Fragmentor was also important to observe analytes in mass spectrometer. Seven sulfonylurea herbicides have differt fragmentor to obtain their highest sensitivity. The result was summarized in table 5.1.

Electrospray ionization is a soft ionization technique. The quatitative confirmation selected at their pseudo-molecular ion $(M+H)^+$ and other diagnostic ions were monitoring at $(M+2)^+$ which is isotope ion. For example, chlorsulfuron methyl has monitored ion by selected at $(M+\text{isotope ion})^+$. Because Cl has isotope atom at ^{37}Cl, the abundance ratio between ^{37}Cl:^{35}Cl is (1:3).

Table 5.1 Quantitative ions for the analysis of seven sulfonylurea herbicides

No.	Compound	Ion (Relative abundance)	
		Quasi-molecular ion $(M+H)^+$	Qualification ion
1.	Cinosulfuron	414.15 (100)	415.00 (16.8)
2.	Metsulfuron	382.20 (100)	383.10 (19.4)
3.	Sulfometuron	365.10 (100)	366.12 (17.3)
4.	Chlorsulfuron	358.05 (100)	360.00 (35.3)
5.	Bensulfuron	411.15 (100)	412.20 (18.3)
6.	Pyrazosulfuron ethyl	415.20 (100)	416.10 (18.7)
7.	Triflusulfuron	493.05 (100)	494.10 (22.8)

A chromatographic separation condition was achievd on reversed-phase gradient elution with 2 mM oxalic acid and acetonitrile as a mobile phase. The gradient program was applied to separate seven sulfonylurea herbicides, as is presented in table 5.2.

Table 5.2 High Performance Liquid Chromatography Conditions

Time	% A (2 mM Oxalic acid)	% B (Acetonitrile)
0	67	33
5	67	33
12	0	100

The selectivity of LC/MS method was measured by retention time, and mass pattern; quantitative ions and abundance. Although co-elution of sulfonylurea herbicides was occurred in the study but the analysis of SUHs could be achieved. LC/MS can distinguish analytes by their mass spectrum pattern and retention time. Mass spectrum pattern is the characteristic profile to confirm analyte forms the different compounds especially from the interferences. The selected ion monitoring (SIM) used in analysis of Sulfonylurea herbicides with their molecular ion and qualify ions. SIM is more sensitive, because of it has longer dwelling time with small mass range. The chromatographic separation and mass spectrometric detection can optimize by dividing the SIM window into 2 time windows. The first window started at 2.0 to 11.0 minute for cinosulfuron, metsulfuron methyl, sulfometuron and chlorsulfuron. The second window was began at 11.0 to 17.0 minute for mornitering of bensulfuron methyl pyrazosulfuron ethyl and triflusulfuron methyl. The determination parameters of sulfonylurea herbicides are summarized in table 5.3.

Table 5.3 Time schedule of SIM program for the monitoring of seven sulfonylurea herbicides

Group	Compound	SIM window (min)	Quasi-molecular ion $(M+H)^+$	Qualification ion	Fragmentor (V)
1.	Cinosulfuron	2.0-11.0	414.15	415.00	120
	Metsulfuron		382.20	383.10	120
	Sulfometuron		365.10	366.12	120
	Chlorsulfuron		358.05	360.00	120
2.	Bensulfuron	11.0-17.0	411.15	412.20	120
	Pyrazosulfuron ethyl		415.20	416.10	120
	Triflusulfuron		493.05	494.10	120

Sulfonylurea herbicides have recently been used in the general paddy area. Thus, organic rice became a representative of rice matrix and was used for the screening extraction process. Sample preparation by screening was also studied from previous works. Screening of sample preparation was presented as one effective method. Method II was reported recovery ranged from 79.4 to 115.8% which following AOAC recommend.

The study of difference SPE cartridge was provided by tandem (C18+NH_2) and mixed mode cartridge (PSA and HLB). The result at same spiking level (10 ppb) showed the satisfactory recovery from both cartridge and follows the AOAC recommendation at 70-120 %.

The study of the pH effect was controlled at two values, control pH at 3.0-3.5 and non control pH compare between C18+NH_2 and PSA cartridge. The sample preparation by method II with C18+NH_2 satisfied recovery at control and non-control pH. On the other hand, PSA is effective clean up at non-control pH. Thus, C18+NH_2 and PSA were used to prepare of sulfonylurea herbicides in rice.

The comparison of matrix removal between C18+NH_2 (tandem) and PSA cartridge was investigated. Sample preparation of sulfonylurea herbicides by C18+ NH_2 compared to with PSA cartridge illustrated satisfactory matrix clean up. Thus, PSA is an alternative cartridge to isolate analytes instead of tandem cartridge.

Method validation is the establishment of the performance and limitations of a method. C18+NH_2 was validated on these parameters: selectivity, MDL and MQL, linearity and range, precision, accuracy and robustness. *(62, 63, 64 and 66)*

The confirmation of sulfonylurea herbicides was investigated under interferences present. Selectivity of a method is its application to both quantitation and qualitation analysis. The selectivity of LC/MS method was measured by retention time, target ion and qualified ion which is presented in table 4.15. Method detection limit (MDL) and method quantitation limit (MQL) for C18+NH_2 cartridge raged from 0.64 to 5.0 ppb

and 0.91 to 7.15 ppb, respectively. These values represented the method response of C18+NH_2 cartridge which presented at ppb level. The matrix effect must be assessed in validation. The study of the matrix effect indicated that the matrix in a sample has a significant effect on determination of sulfonylurea herbicides in rice. Consequently, a matrix calibration curve was used. This method showed good analytical characteristics, having good linear relationship at R^2 >0.9900. MDL was lower than MHLW recommended, as shown in table 5.4.

Table 5.4 Characteristics validation data consists of correlation coefficient (R^2) method detection limit (MDL) and method quantitation limit (MQL) of each compound in rice matrix

No.	Compound	R^2	Method Detection Limits (ppb)	Method Quantitation Limits (ppb)
1	Cinosulfuron	0.9961	0.80	1.30
2	Metsulfuron	0.9979	3.00	6.00
3	Sulfometuron	0.9944	4.00	7.15
4	Chlorsulfuron	0.9949	5.00	6.06
5	Bensulfuron	0.9994	0.64	0.91
6	Pyrazosulfuron ethy	0.9948	0.71	0.97
7	Triflusulfuron	0.9958	1.90	2.40

It is necessary to establish the signal produced at the measurement stage. Method precision and accuracy were studied at two concentration levels (MQL and 5-MQL) in the rice sample. Within-day and between-day precision were tested for method precision. The results showed excellent precision which follows AOAC recommended. Method accuracy was also investigated by studying percent recovery. The result presented acceptable recovery level, which is greater than 70 %, and following AOAC recommendations. *(65)*

Method robustness was studied by using a statistical model. Plackett-Burman experimental design demonstrated the extraction effect of extraction time, waiting time, volume of solvent elution, evaporation temperature, solvent grade, light explosion and amount of NaCl in only eight run. Statistical *t*-critical at 95 % confidence limit of all parameters is above *t*-experiment. Thus, the study of method precision indicated that the seven studying parameters have no effect on the extraction procedure for this work. This shows the robust of the new method.

Primary secondary amine (PSA) cartridge was also studied using the same process as tandem SPE. Partial method validation was carried out to study critical parameters. Financial constrains could dictate the method of validation, thus important analytical parameters were studied. *(62, 63, 64 and 66)* First, selectivity by PSA cartridge was re-checking. Retention time, target and qualified ion selected same as C18+NH_2 cartridge. Second, method detection limit and method quantitation limit was studied by spike sample method precision and accuracy was studied at three concentration levels: MDL, MQL and 5-MQL. In terms of precision, the acceptable precision could be based on the Horwitz equation. This method presented RSD values less than calculated by the Horwitz equation and following AOAC recommended values. Recoveries represented method accuracy, and percent recoveries ranged from 61.53 to 97.6 % for the three concentration levels. Last, method accuracy of PSA cartridge to clean up and isolation was under AOAC limits.

In comparison of MDL and MQL of two different cartridges, tandem cartridge has lower MDL for metsulfuron and cinosulfuron by about two times. Method quantitation limit for each of the analytes was determined to be 0.91 to 7.15 ppb in rice sample. MQL was defined as the lowest fortification level evaluated at acceptable average recoveries and precision. This quantitation limit reflects lower than regulation of ministry of health labour and welthfare (MHLW) of Japan limits (50 ppb). Thus, C18+NH_2 and PSA cartridge illustrated nearly MDL and MQL level two different cartridges can also be used to prepare sulfonylurea herbicides in a complex matrix (rice).

The new method development can be released for determination of sulfonylureas herbicides in rice LC/MS with good method accuracy, precision and robustness. Further work should be concentrated on determination of SUHs in rice samples from the markets. The study can be extended to residue of sulfonylurea herbicides in consumer rice which is exported. The result of sulfonulurea herbicides will present the contamination and consumption of herbicides in our rice field of Thailand. Moreover, other agricultural products like beans, wheat or corn are also the exports of Thailand. Therefore, the contamination level of sulfonulurea herbicides should be studied and controlled.

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APPENDICES

APPENDIX A

Table A Properties of Sulfonylurea Herbicide

Compound (MW)	CAS	pKa	Range of Use Rates (g ai/ha)	Activity
Chlorsulfuron (357.77) Formula : $C1_2H1_2ClN_5O_4S$	{*N*-[[(4,6-dimethoxy-1,3,5-triazin-2-yl)amino]carbonyl]-2-(2-methoxyethoxy) benzenesulfonamideis}	3.6	9-25 (cereals) 17-157 (veg mgt)	Triazinylsulfonylura Herbicides
Triflusulfuron ethyl (492.42) Formula : $C_{16}H_{17}F_3N_6O_6S$	{2-[[[[[4-(dimethylamino)-6-(2,2,2-trifluoroethoxy)-1,3,5-triazin-2-yl]amino] carbonyl]amino]sulfonyl]-3-methylbenzoic acid}	4.4	18-35 (sugar beat)	Triazinylsulfonylura Herbicides
Metsulfuron (381.36) Formula :$C_{13}H_{13}N_5O_6S$	{2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoic acid}	3.3	3-7.5 (cereals, rice) 14-168 (veg mgt)	Triazinylsulfonylura Herbicides
Cinosulfuron (413.40) Formula : $C_{15}H_{19}N_5O_7S$	{*N*-[[(4,6-dimethoxy-1,3,5-triazin-2-yl)amino]carbonyl]-2-(2-methoxyethoxy) benzenesulfonamide}			Triazinylsulfonylura Herbicides

Table A Properties of Sulfonylurea Herbicide (continue…)

Compound (MW)	CAS	pKa	Range of Use Rates (g ai/ha)	Activity
Bensulfuron methyl (410.40) Formula : $C_{15}H_{16}N_4O_7S$	{2-[[[[[(4,6-dimethoxy-2-pyrimidinyl)amino] carbonyl]amino] sulfonyl]methyl]benzoic acid}	5.2	20-70 (rice)	Pyrimidinylsulfonylurea herbicides
Pyrazosulfuron ethyl (414.39) Formula : $C_{12}H_{14}N_6O_7S$	{5-[[[[(4,6-dimethoxy-2-pyrimidinyl)amino] carbonyl]amino]sulfonyl]-1-methyl-1*H*-pyrazole-4-carboxylic acid}			Pyrimidinylsulfonylurea herbicides
Sulfometuron (364.37) Formula : $C_{14}H_{14}N_4O_5S$	{2-[[[[(4,6-dimethyl-2-pyrimidinyl) amino] carbonyl]amino] sulfonyl]benzoic acid}	5.2	26-420 (veg mgt)	Pyrimidinylsulfonylurea herbicides

Table A-2 Instrumental method set up

```
Method:D:\HPCHEM\1\METHODS\DUANGKAMOL.M of 2/20/2006 3:15:07
PM

                    1100 Quaternary Pump 1

Control
     Column Flow          :      0.250 ml/min
     Stoptime             :        20.00 min
     Posttime             :        10.00 min

Solvents
     Solvent A            :         0.0 % (H2O)
     Solvent B            :      Off
     Solvent C            :        67.0 % (MP-A)
     Solvent A            :        33.0 % (MP-B)

PressureLimits
     Minimum Pressure     :         0 bar
     Maximum Pressure     :      400 bar

Auxiliary
     Maximal Flow Ramp    :      100.00 ml/min^2
     Primary Channel      :      Auto
     Compressibility      :      100*10^-6/bar
     Minimal Stroke       :      Auto

Store Parameters
     Store Ratio A        :      Yes
     Store Ratio B        :      Yes
     Store Ratio C        :      Yes
     Store Ratio D        :      Yes
     Store Flow           :      Yes
     Store Pressure       :      Yes

                  Mass Spectrometer Detector

General Information
------- -----------

Use MSD                   :  Enabled
Ionization Mode           :  API-ES
Tune File                 :  atunes.tun
```

StopTime : 17.00
Time Filter : Enabled
Data Storage : Condensed
Peakwidth : 0.10 min
Scan Speed Override : Disabled

Signals

[Signal 1]

Polarity : Positive
Fragmentor Ramp : Not Applicable

Sim Parameters

Time (min)	Group Name	SIM Ion	Frag-mentor	Gain EMV	SIM Resol	Actual Dwell
2.00	Cpd.1-4	358.05	120	1.0	Low	37
		360.00	120			
		365.10	120			
		366.12	120			
		382.20	120			
		383.10	120			
		414.15	120			
		415.00	120			
11.0	Cpd.5-7	411.15	120	1.0	Low	289
		412.20	120			
		415.20	120			
		416.10	120			
		493.05	120			
		494.10	120			

[Signal 2]

Not Active

[Signal 3]

Not Active

[Signal 4]

Not Active

Spray Chamber

[MSZones]

Gas Temp	:	300 C	maximum 350 C
DryingGas	:	10.0 l/min	maximum 13.0 l/min
Neb Pres	:	35 psig	maximum 60 psig
VCap (Positive)	:	3000 V	
VCap (Negative)	:	0 V	

FIA Series

FIA Series in this Method : Disabled

Time Setting

Time between Injections	:	0.73 min
Injection Loop Flush Time	:	0.17 min

Agilent 1100 Autosampler 1

Injection

Injection Mode	:	Needle Wash
Injection volume	:	5.00 ml/min
Wash Vial	:	61
Optimization	:	none

Auxiliary

Drawspeed	:	100 ml/min
Ejectspeed	:	100 ml/min
Draw position	:	1.0 mm

Time

Stoptime	:	As Pump
Posttime	:	Off

Agilent 1100 Column Thermostat 1

```
Temperature setting
     Left temperature     :    35.0 °C
     Right temperature    :    40.0 °C
     Enable analysis      :    when Temp. is within setpoint
                               +/- 0.8 °C
     Store left temperature    :    Yes
     Store right temperature   :    No

Time
     Stoptime             :    As pump
     Posttime             :    Off

Column Switching Value    :    Column 1
```

APPENDIX B

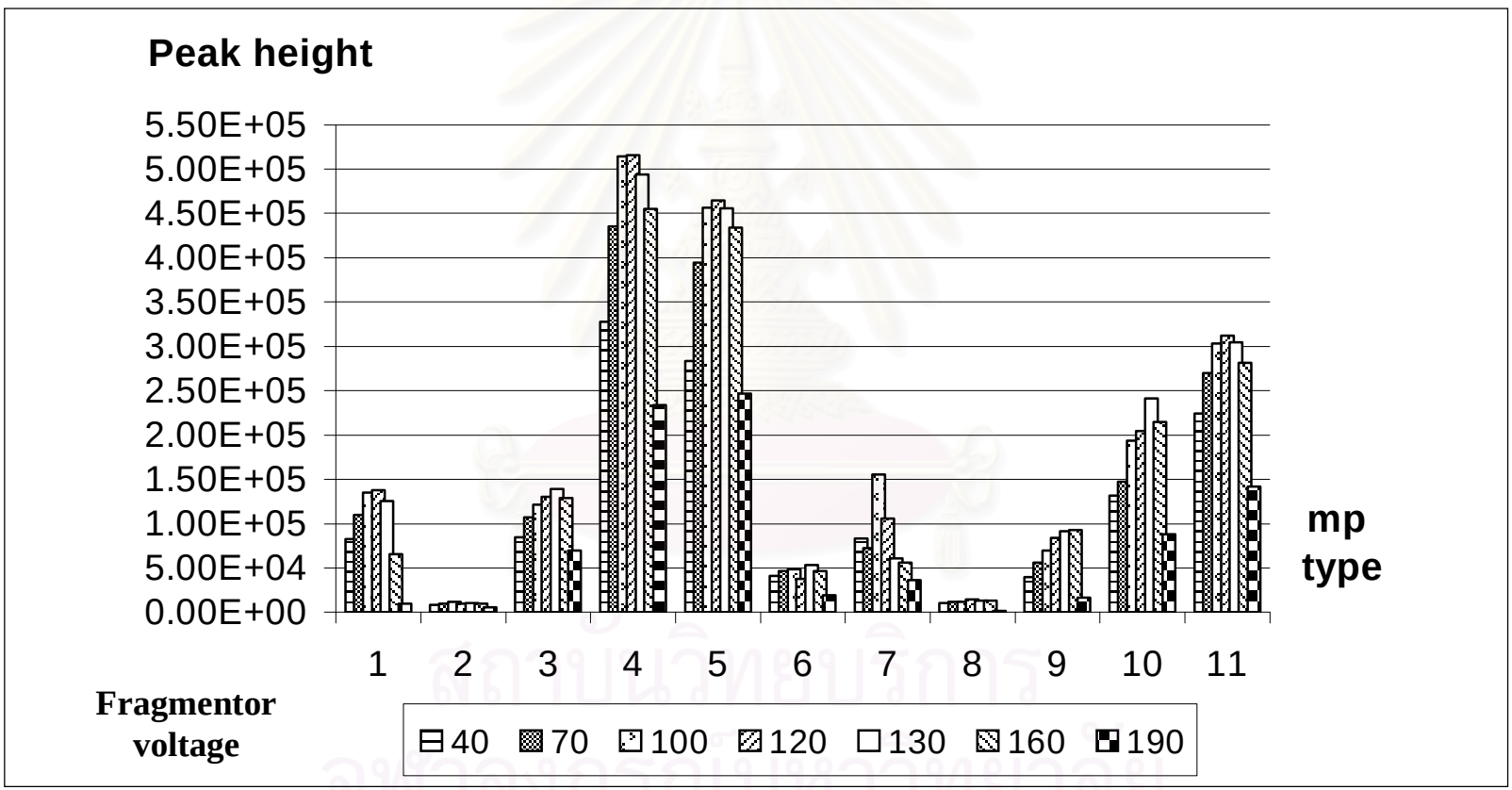

Figure B-1 The comparison of cinosulfuron peak height at 5.00 ppm using various buffers in mobile phase

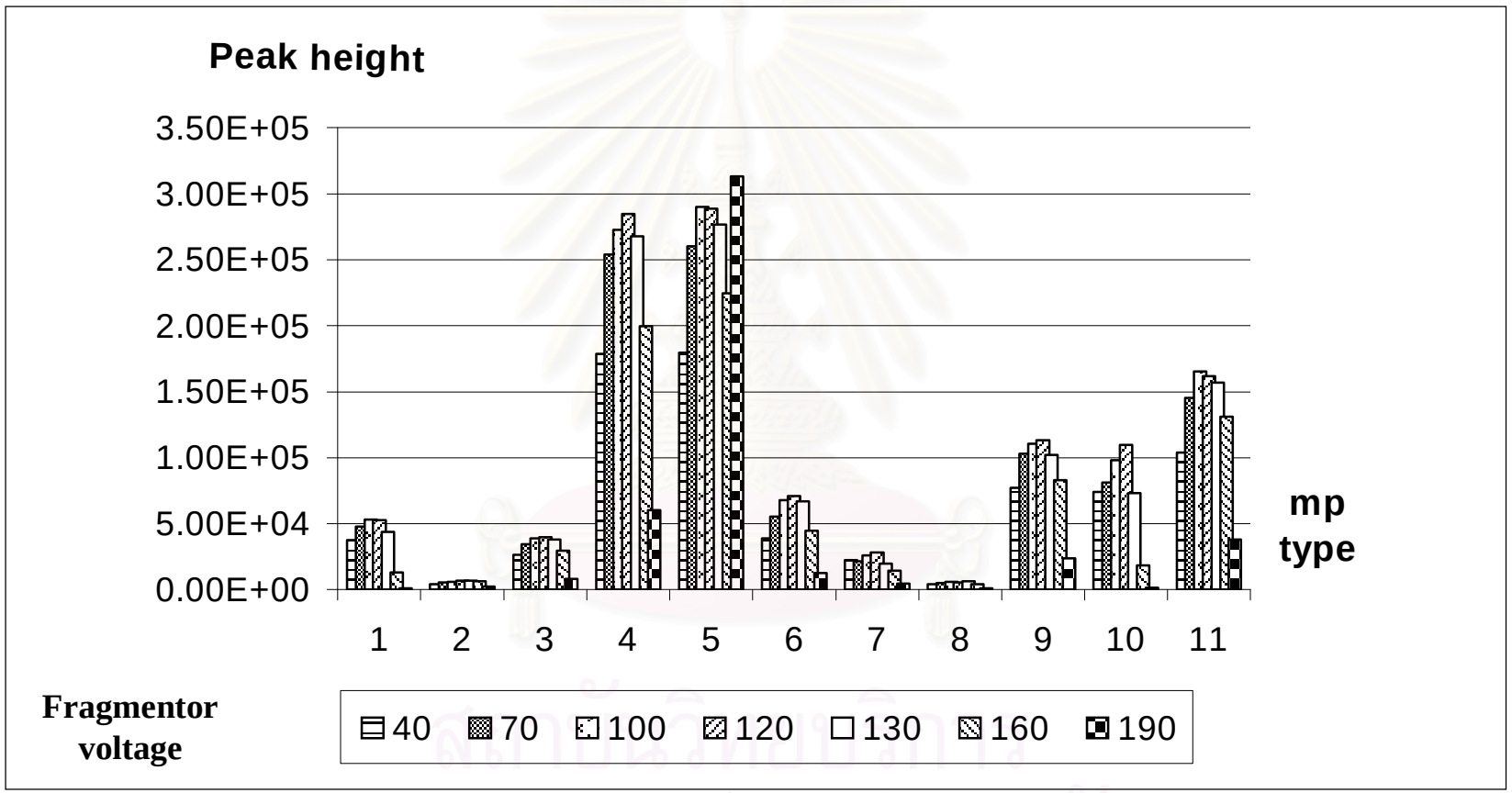

Figure B-2 The comparison of metsulfuron peak height at 5.00 ppm using various buffers in mobile phase

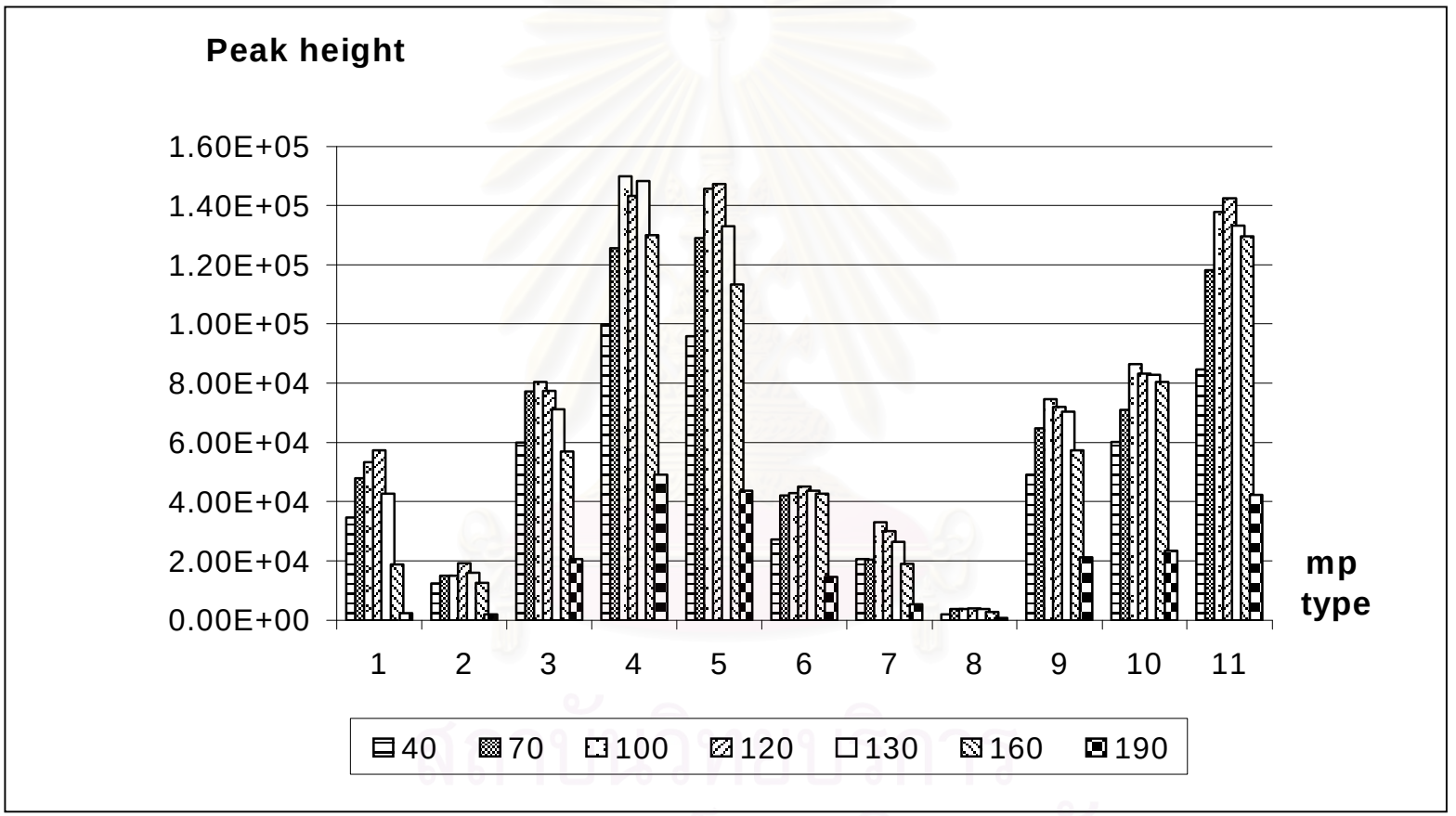

Figure B-3 The comparison of sulfometuron peak height at 5.00 ppm using various buffers in mobile phase

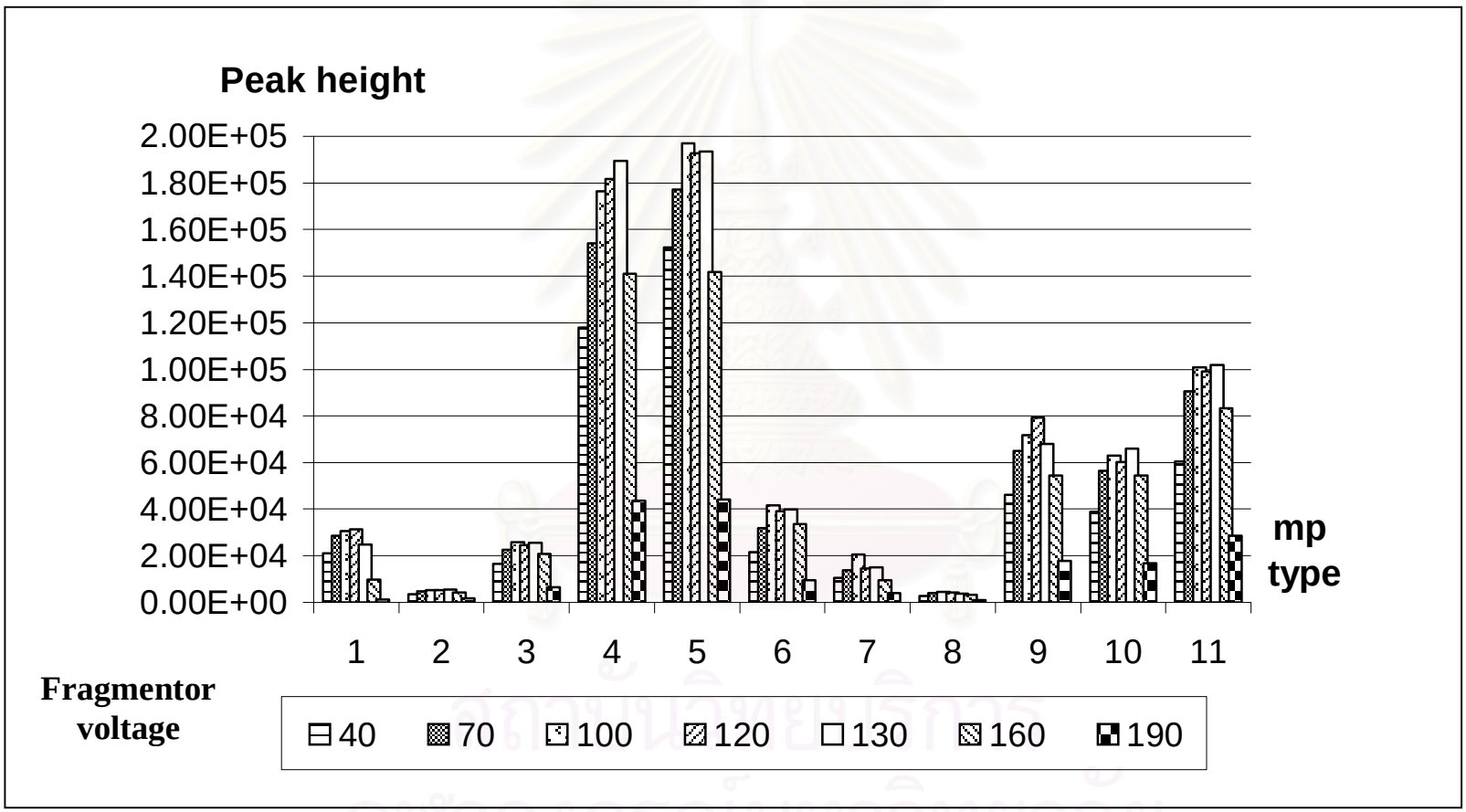

Figure B-4 The comparison of chlorsulfuron peak height at 5.00 ppm using various buffers in mobile phase

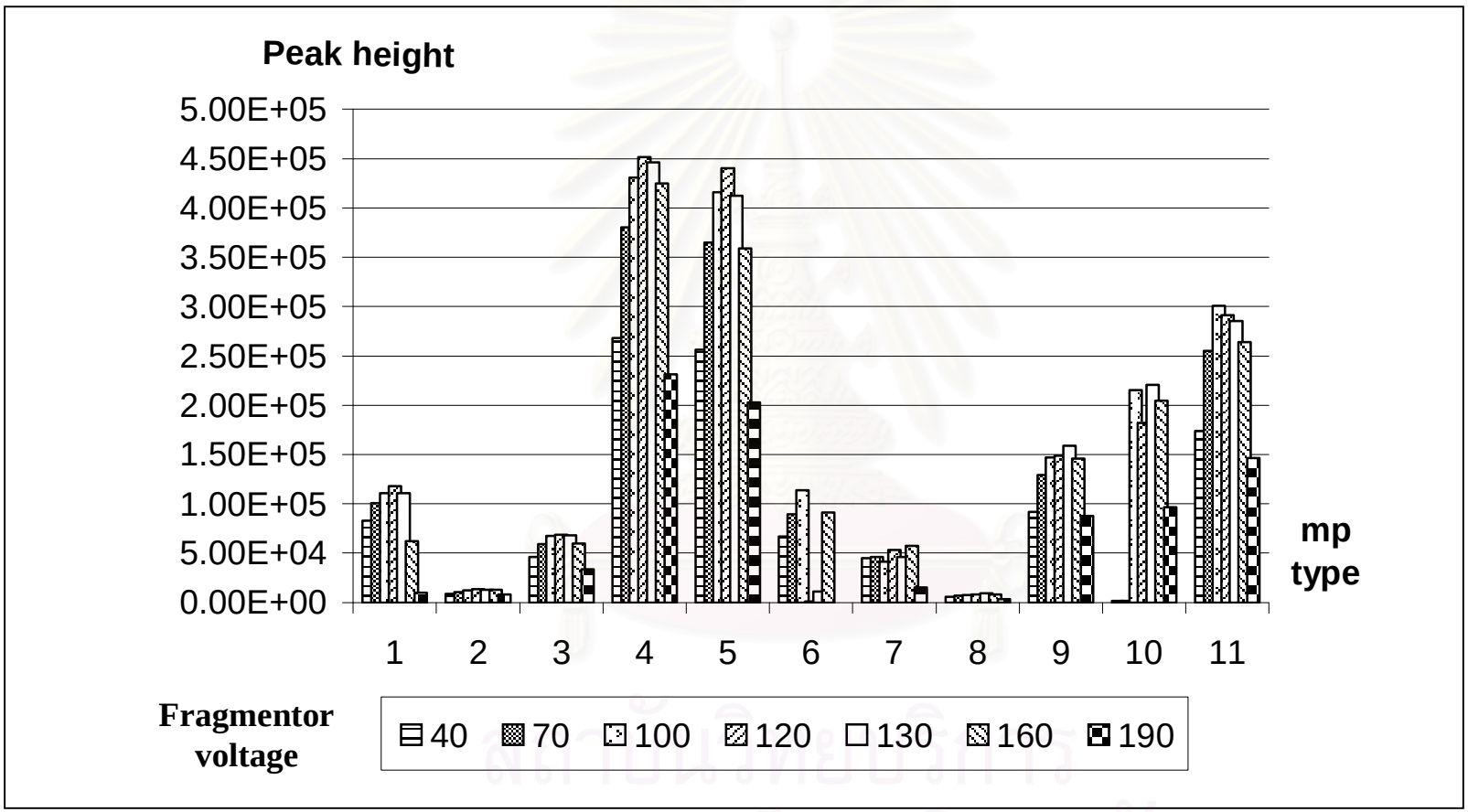

Figure B-5 The comparison of bensulfuron peak height at 5.00 ppm using various buffers in mobile phase

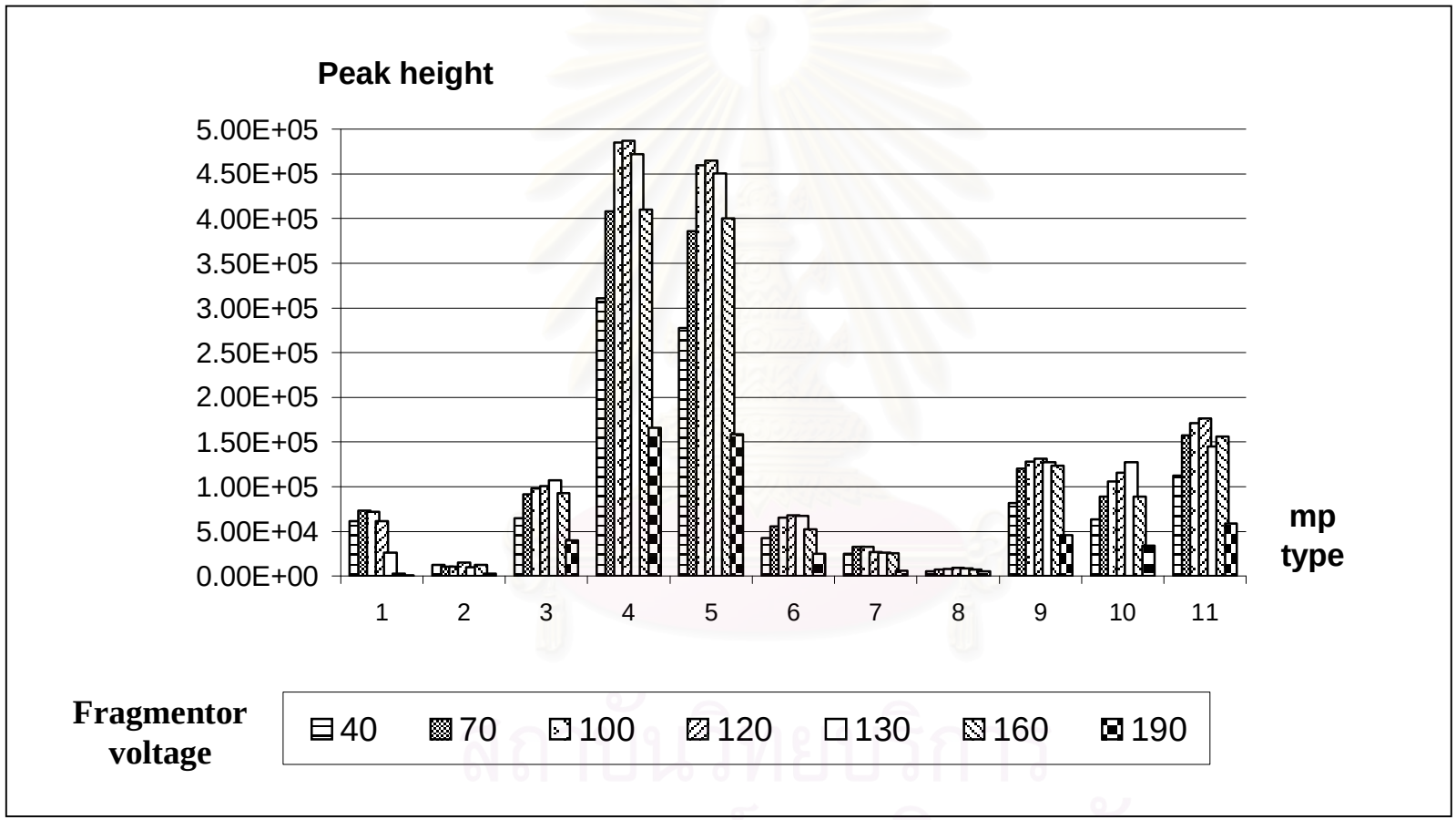

Figure B-6 The comparison of pyrazosulfuron peak height at 5.00 ppm using various buffers in mobile phase

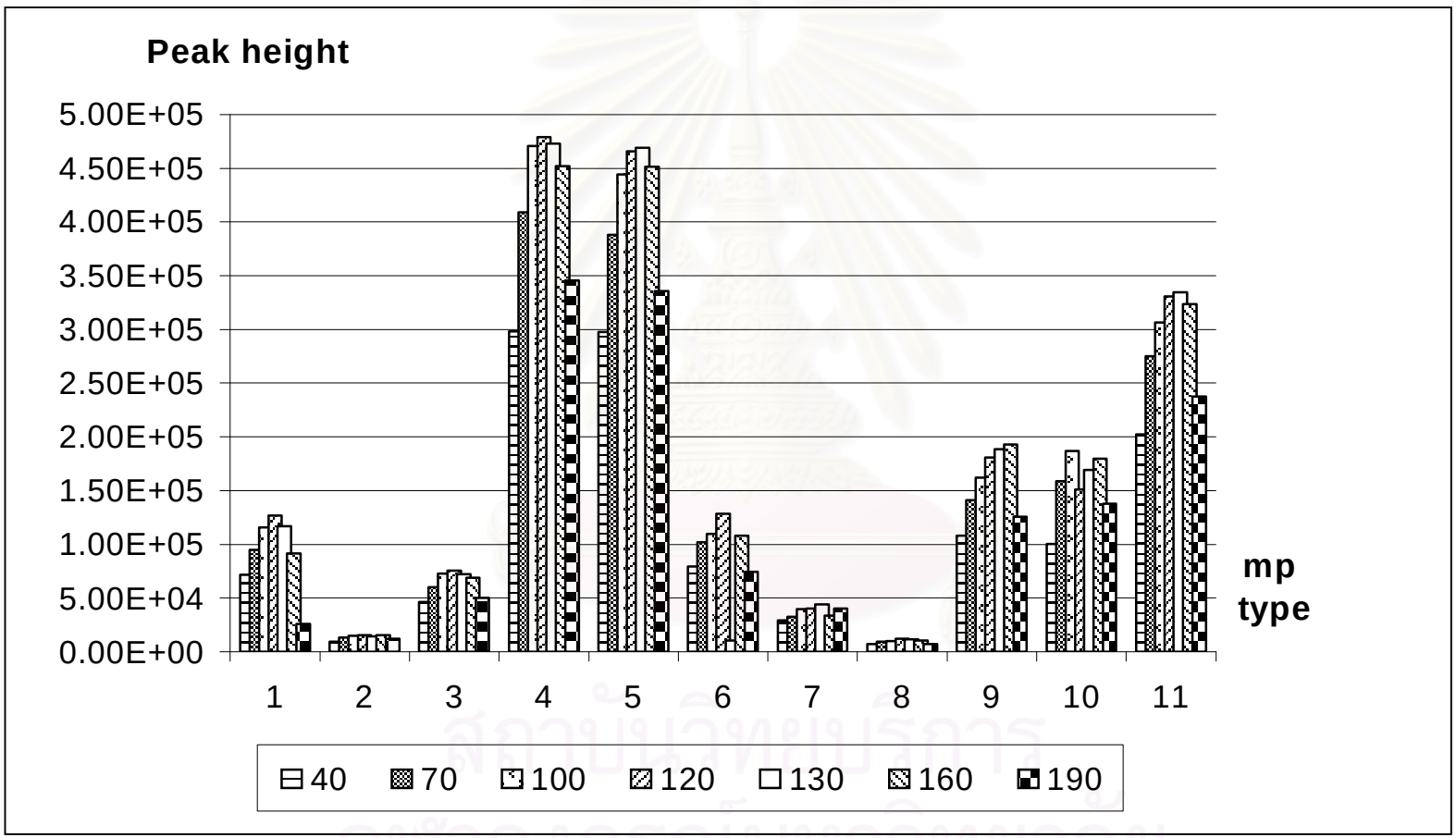

Figure B-7 The comparison of triflusulfuron peak height at 5.00 ppm using various buffers in mobile phase

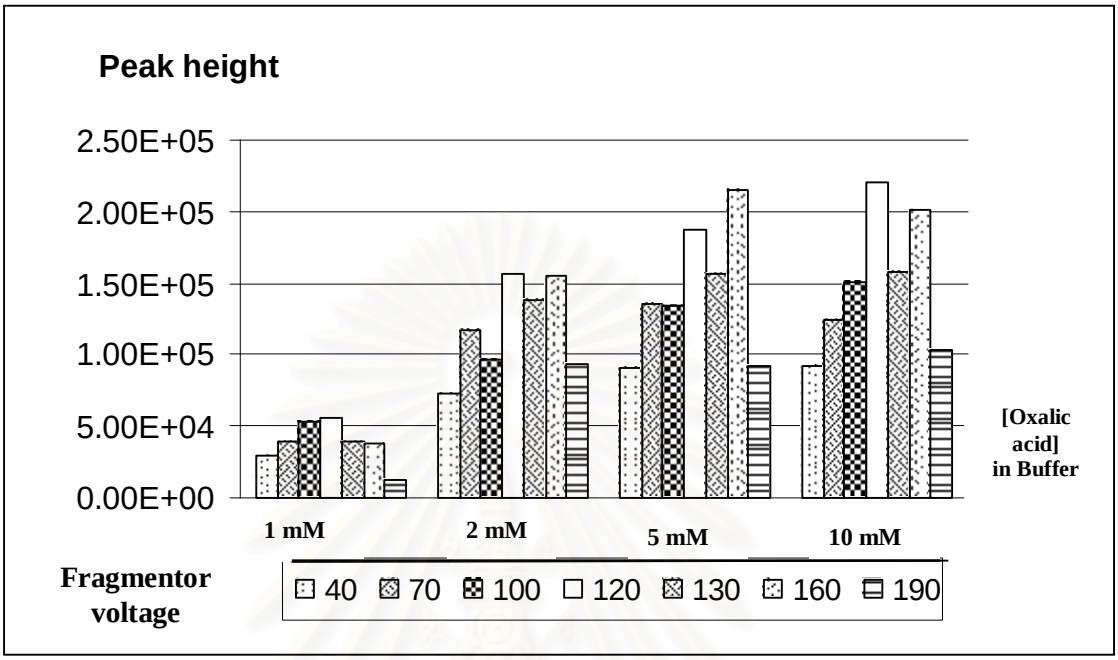

Figure B-8 The comparison of bensulfuron peak height at 5.00 ppm using various oxalic acid buffers concentrations in mobile phase

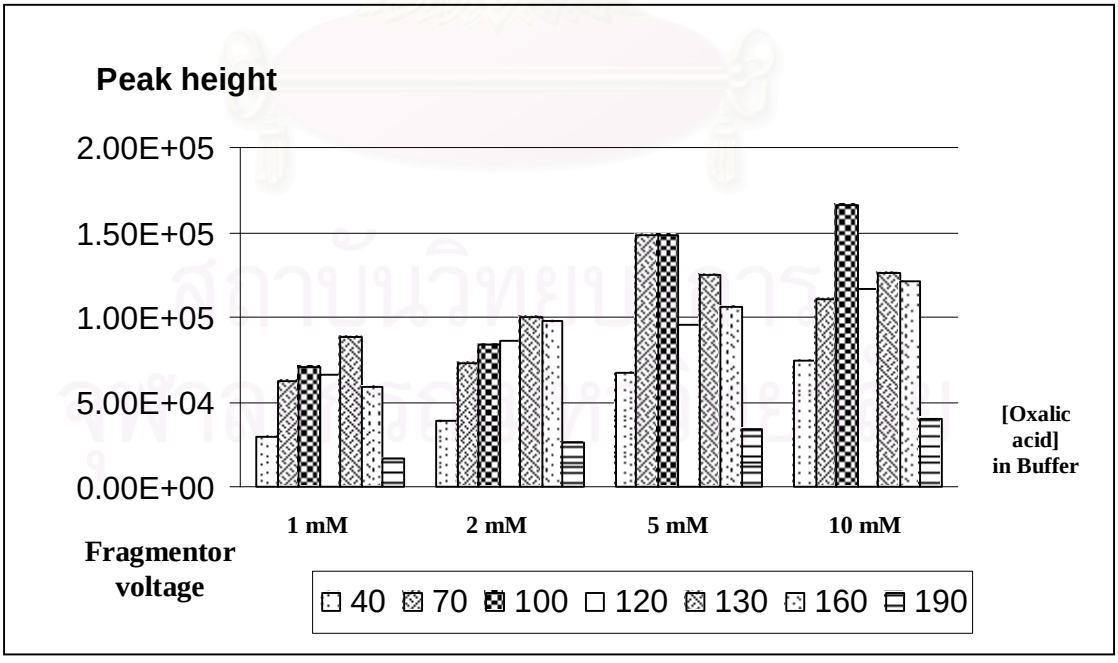

Figure B-9 The comparison of metsulfuron peak height at 5.00 ppm using various oxalic acid buffers concentrations in mobile phase

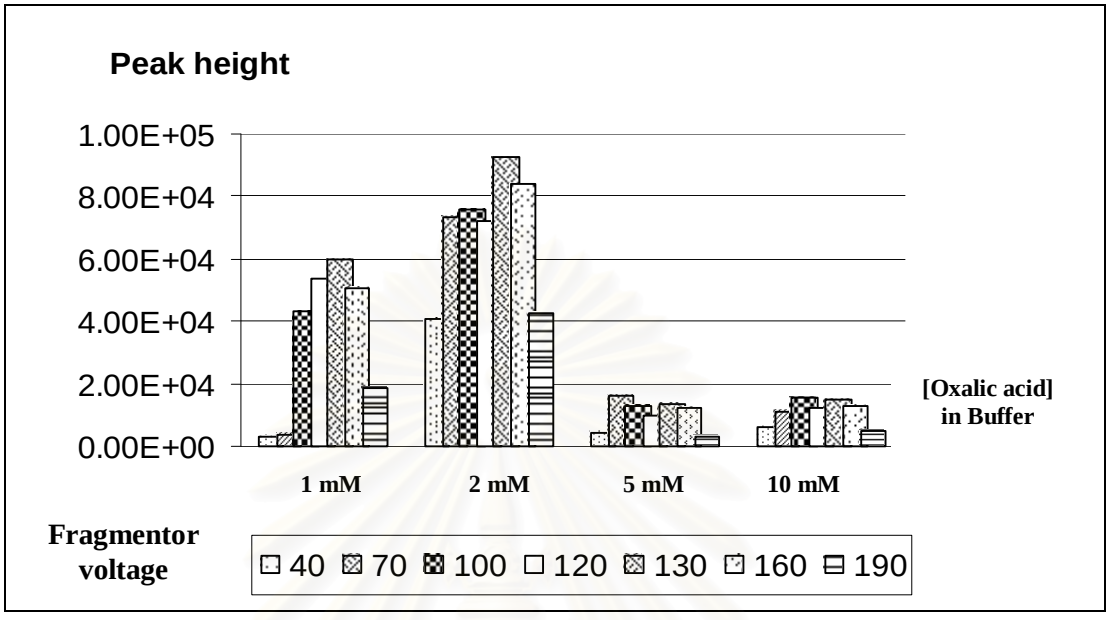

Figure B-10 The comparison of sulfometuron peak height at 5.00 ppm using various oxalic acid buffers concentrations in mobile phase

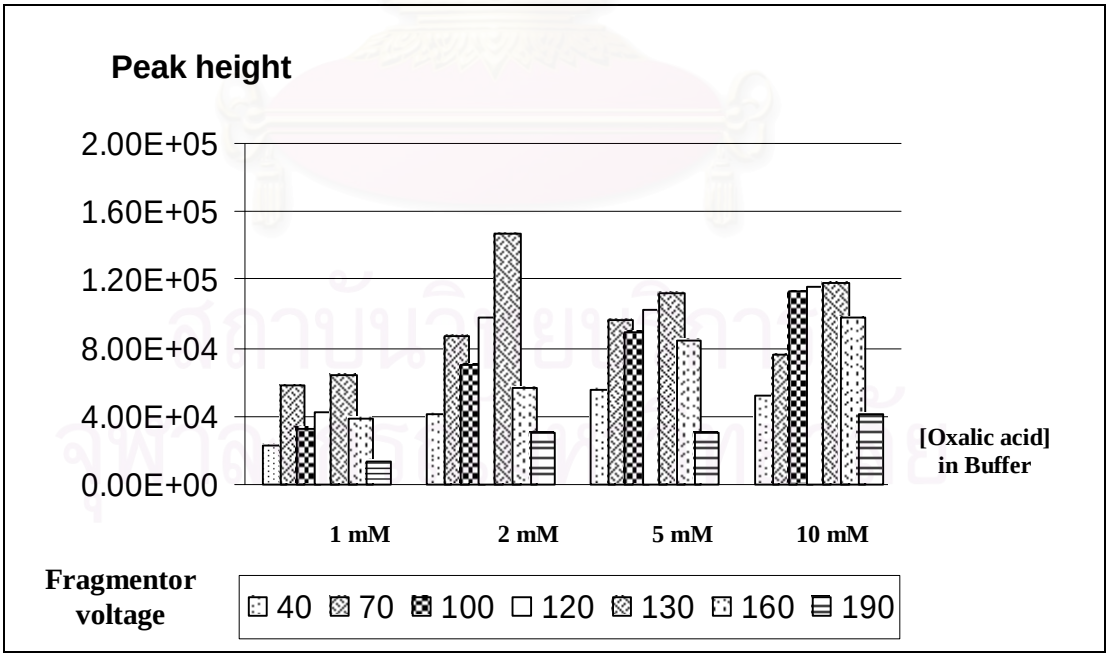

Figure B-11 The comparison of chlorsulfuron peak height at 5.00 ppm using various oxalic acid buffers concentrations in mobile phase

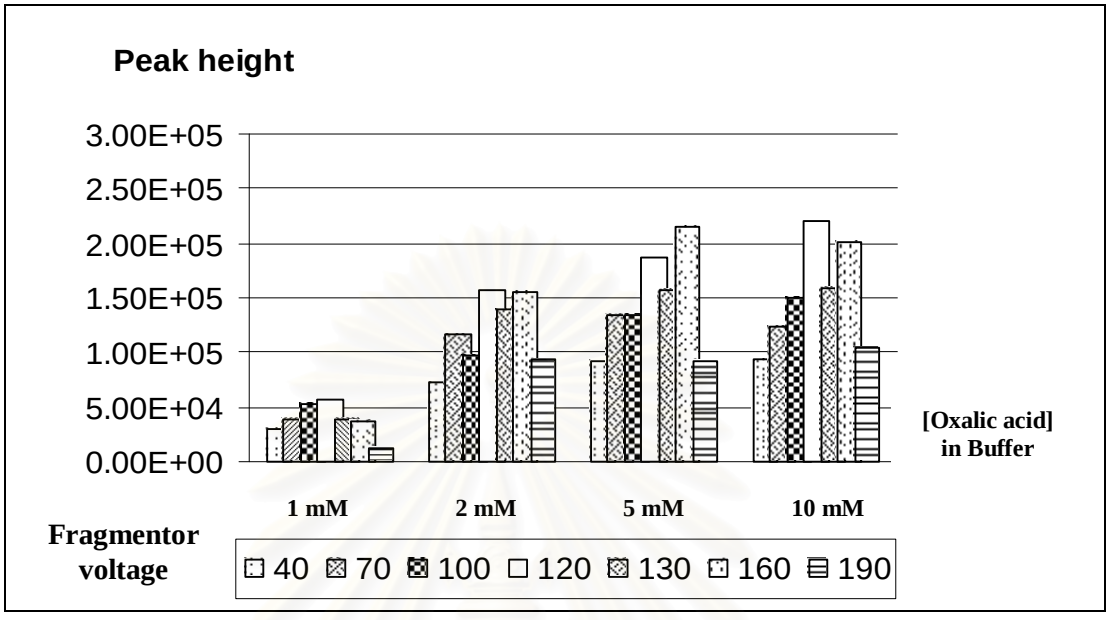

Figure B-12 Te comparison of bensulfuron peak height at 5.00 ppm using various oxalic acid buffers concentrations in mobile phase

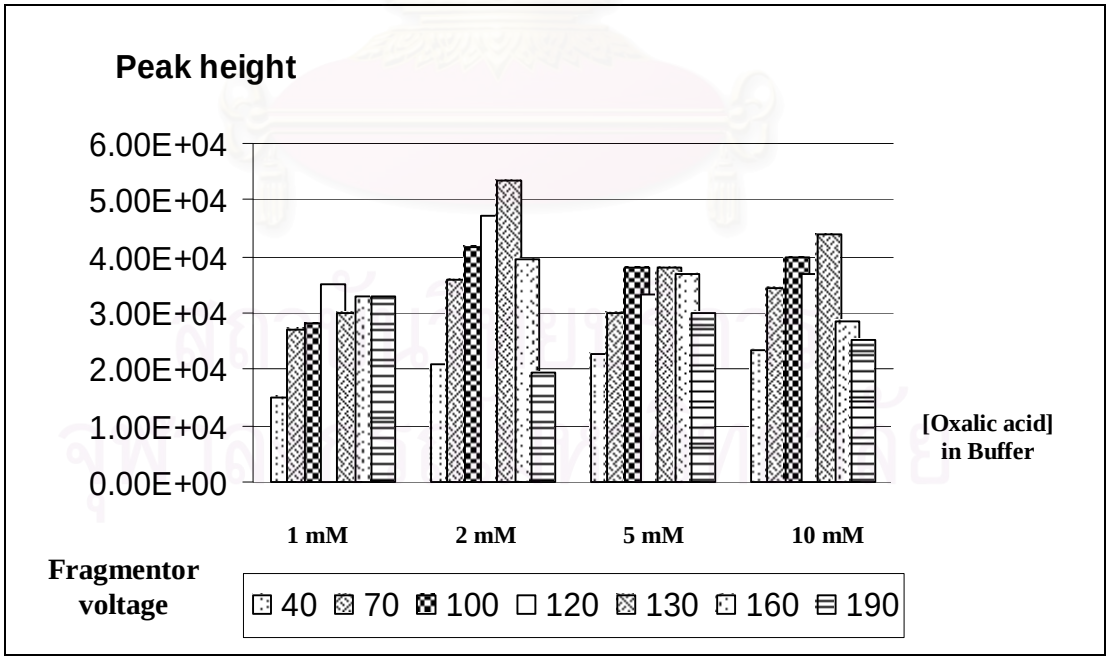

Figure B-13 The comparison of pyrazosulfuron ethyl peak height at 5.00 ppm using various oxalic acid buffers concentrations in mobile phase

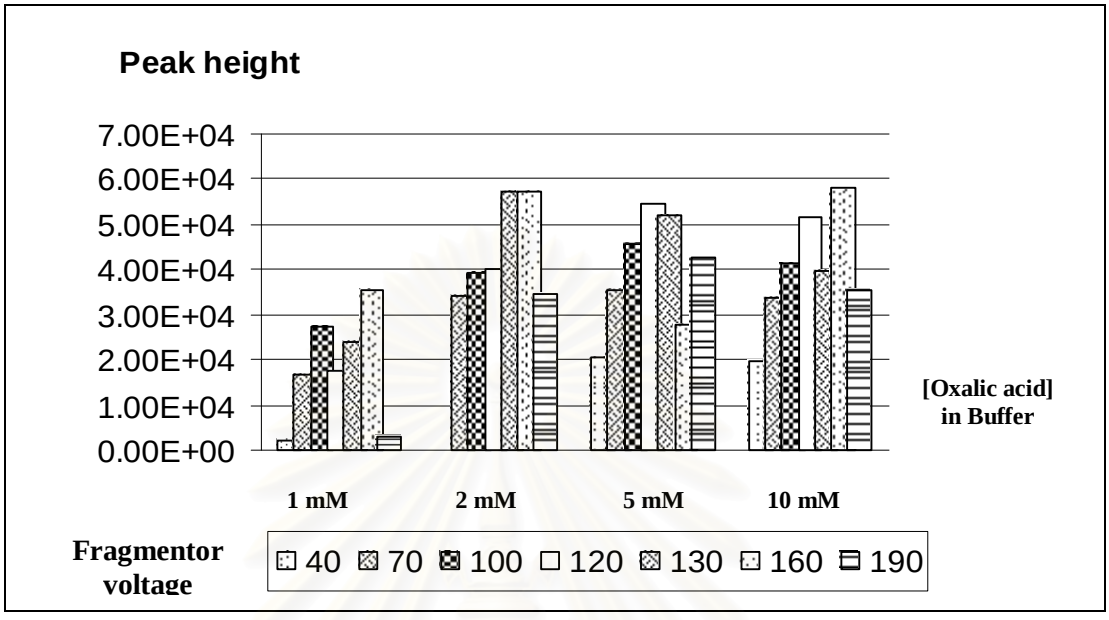

Figure B-14 The comparison of triflusulfuron peak height at 5.00 ppm using various oxalic acid buffers concentrations in mobile phase

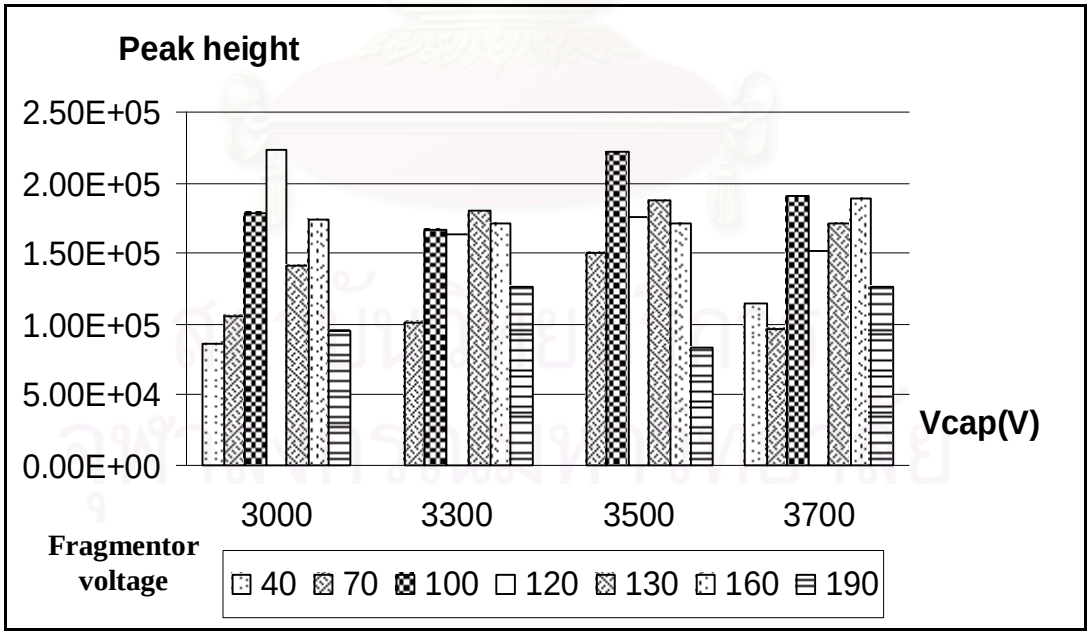

Figure B-15 The comparison of cinosulfuron peak height at 5.00 ppm using various capillary voltage in mobile phase (2 mM oxalic acid)

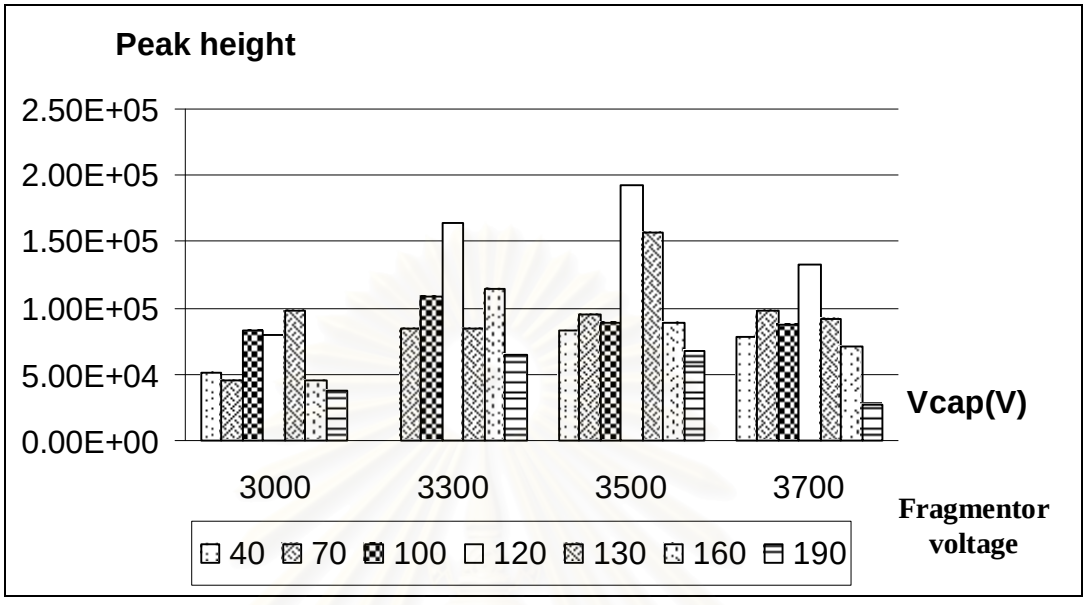

Figure B-16 The comparison of metsulfuron peak height at 5.00 ppm using various capillary voltage in mobile phase (2 mM oxalic acid)

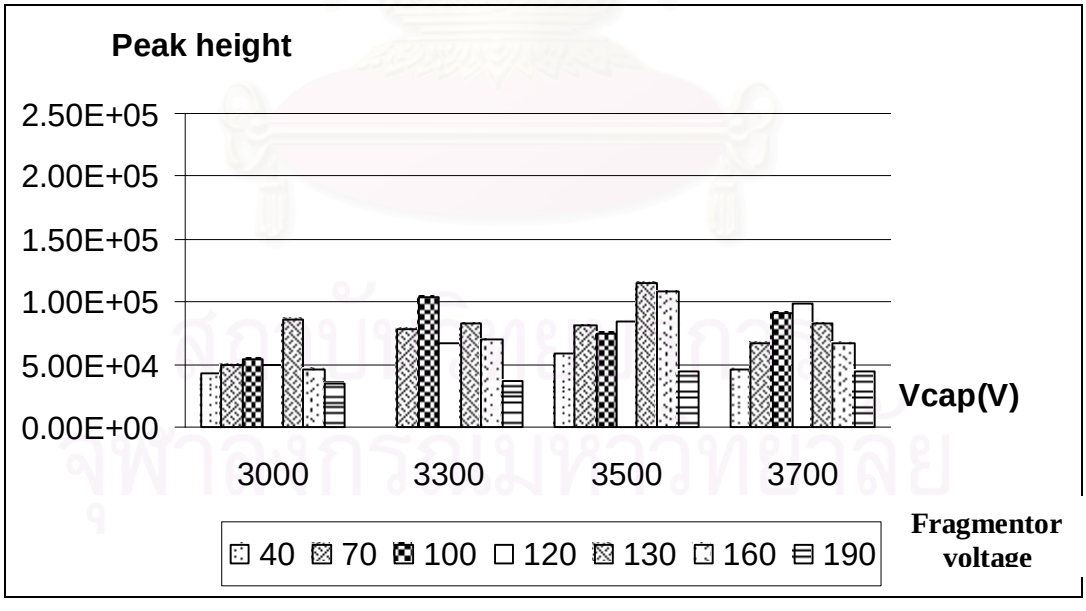

Figure B-17 The comparison of sulfometuron peak height at 5.00 ppm using various capillary voltage in mobile phase (2 mM oxalic acid)

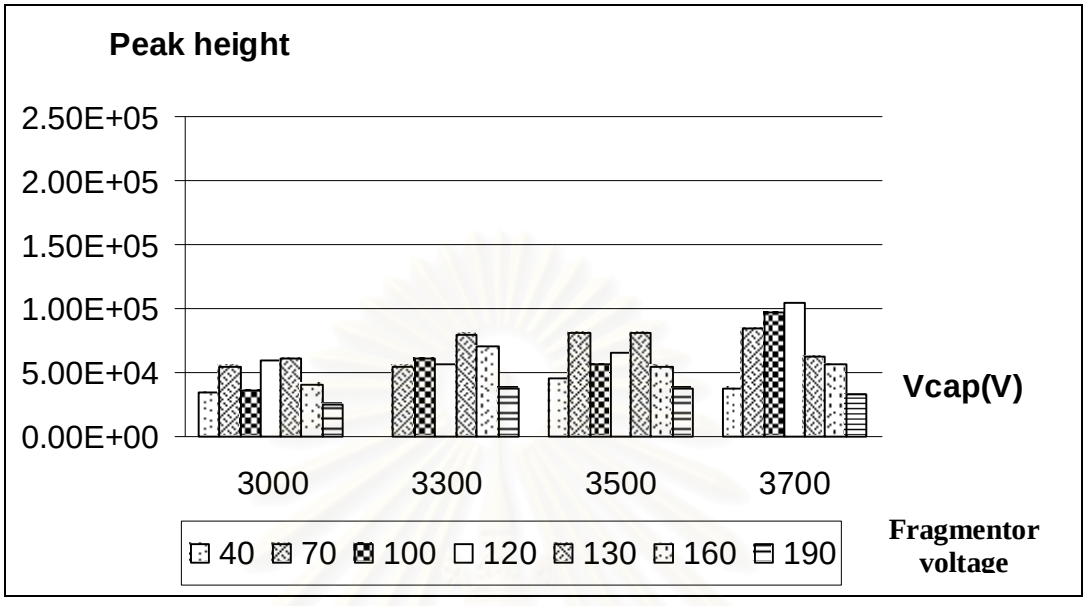

Figure B-18 The comparison of chlorsulfuron peak height at 5.00 ppm using various capillary voltage in mobile phase (2 mM oxalic acid)

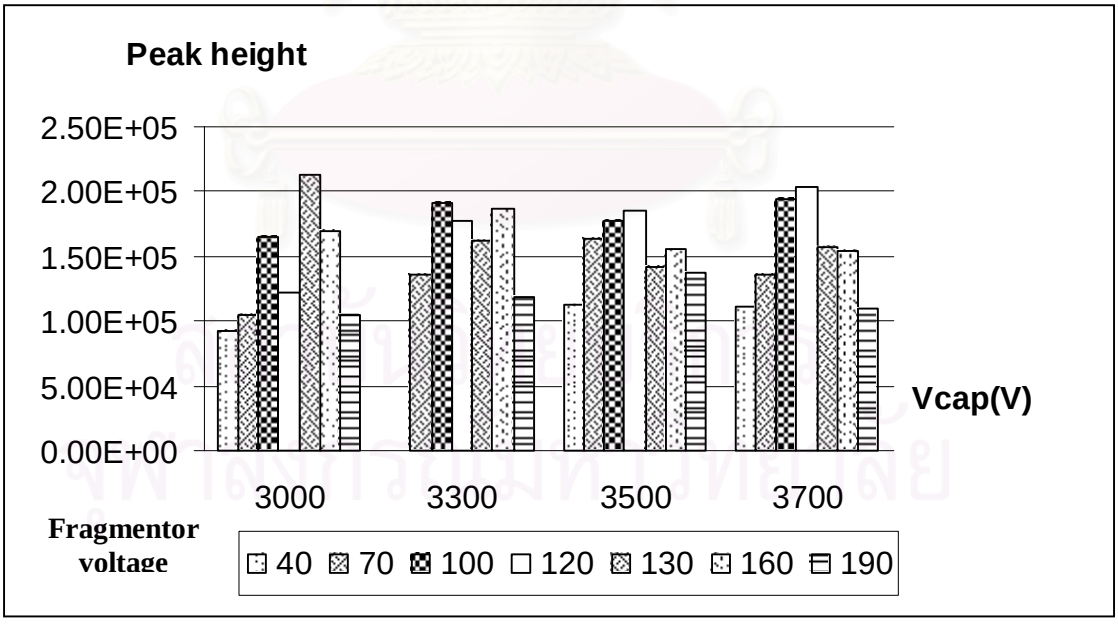

Figure B-19 The comparison of bensulfuron peak height at 5.00 ppm using various capillary voltage in mobile phase (2 mM oxalic acid)

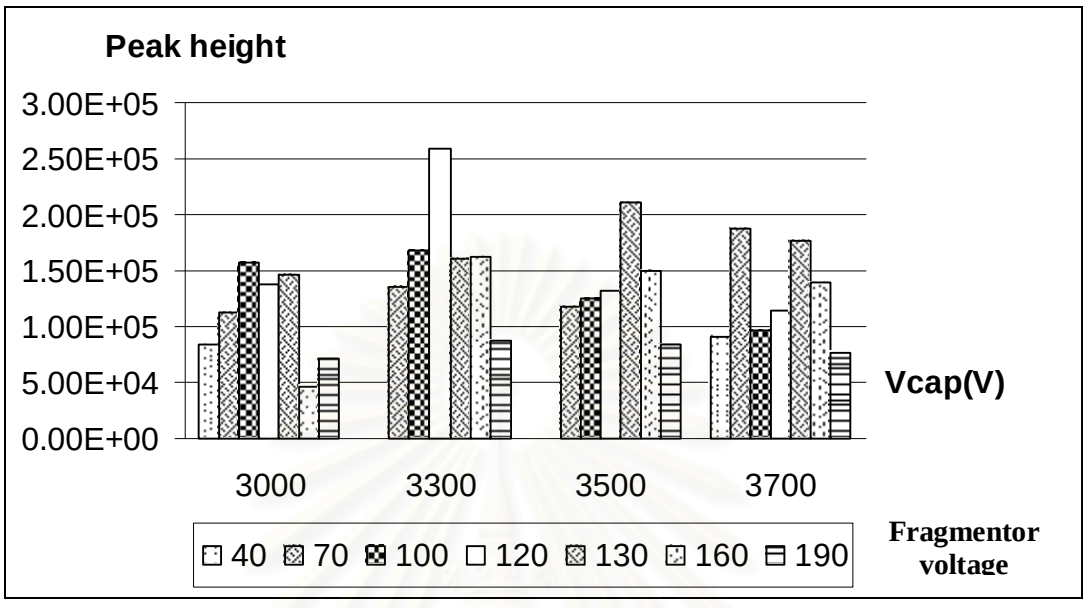

Figure B-20 The comparison of pyrazosulfuron ethyl peak height at 5.00 ppm using various capillary voltage in mobile phase (2 mM oxalic acid)

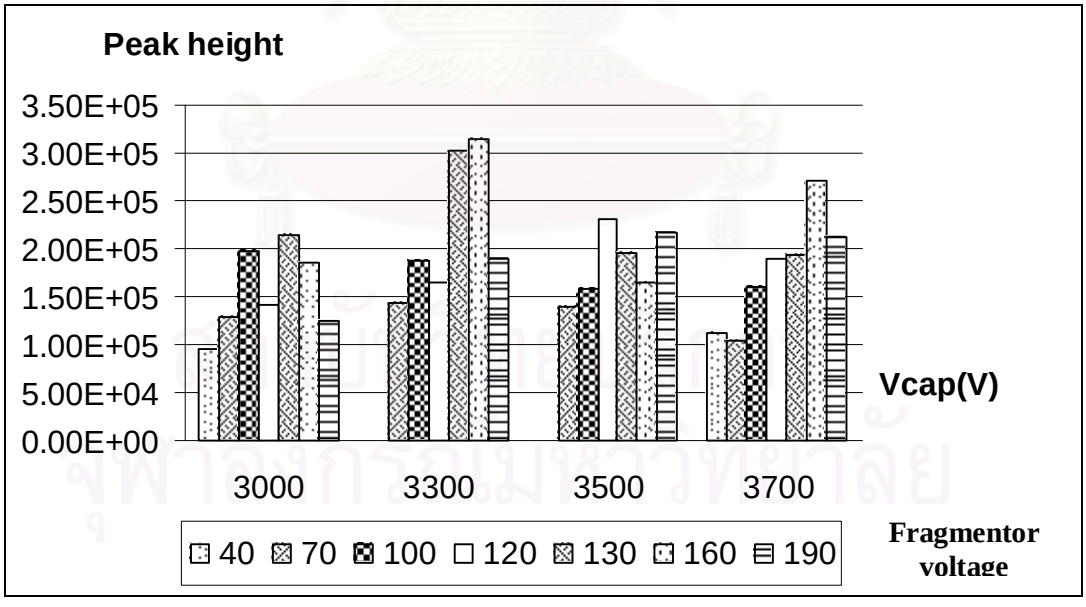

Figure B-21 The comparison of triflusulfuron peak height at 5.00 ppm using various capillary voltage in mobile phase (2 mM oxalic acid)

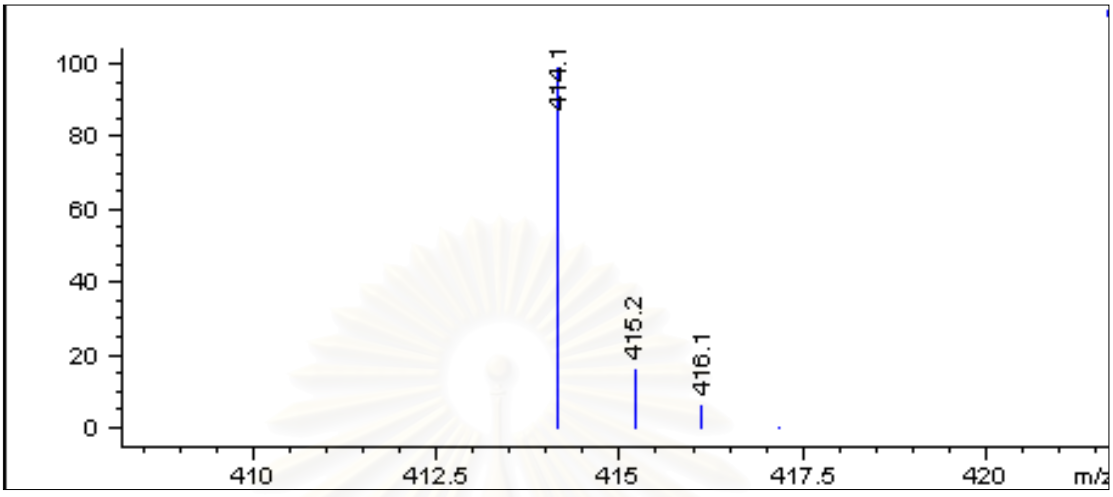

Figure B-22 Mass spectrum of standard cinosulfuron 5.ppm by FIA under condition follow table 4.2 and 4.3

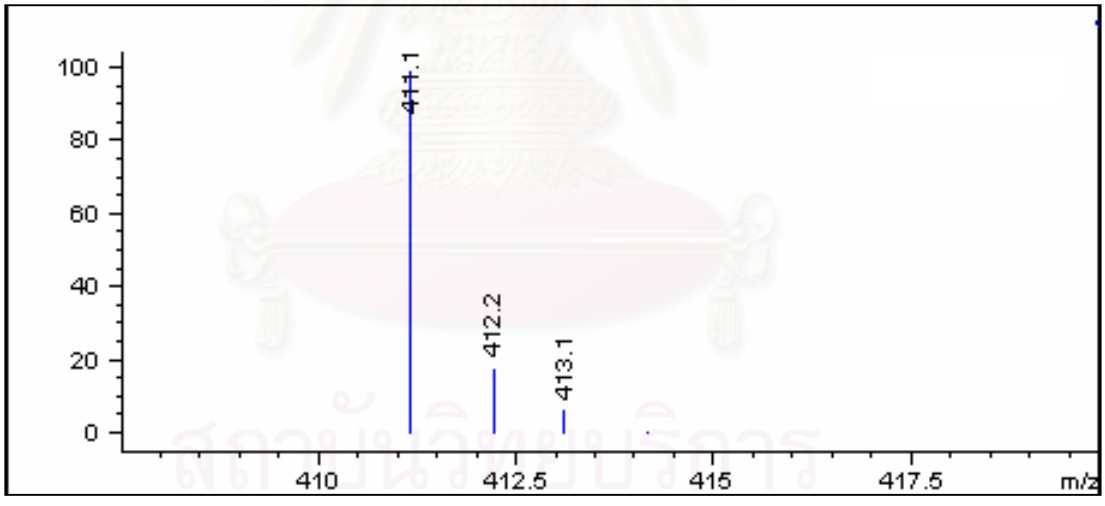

Figure B-23 Mass spectrum of standard metsulfuron 5.00 ppm by FIA under condition follow table 4.2 and 4.3

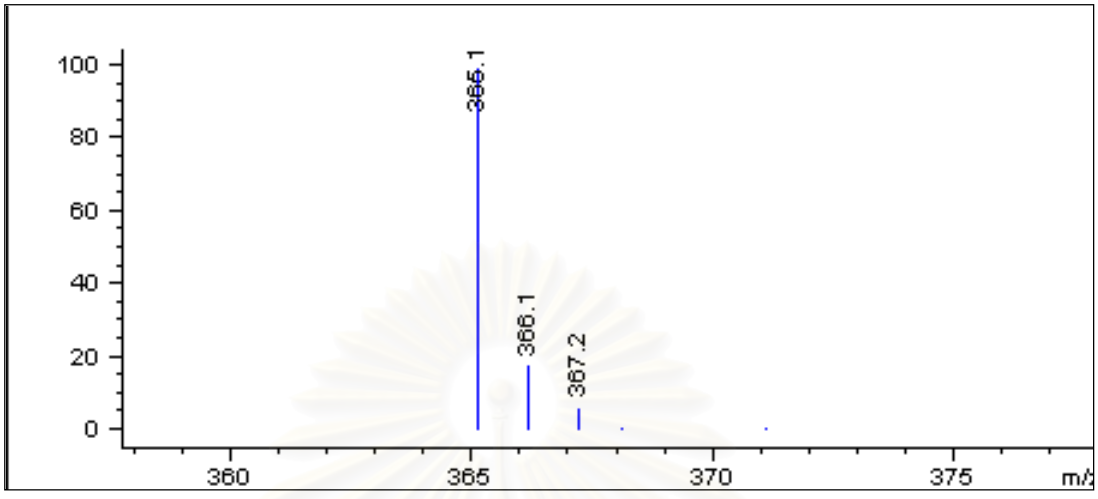

Figure B-24 Mass spectrum of standard sulfometuron 5.00 ppm by FIA under condition follow table 4.2 and 4.3

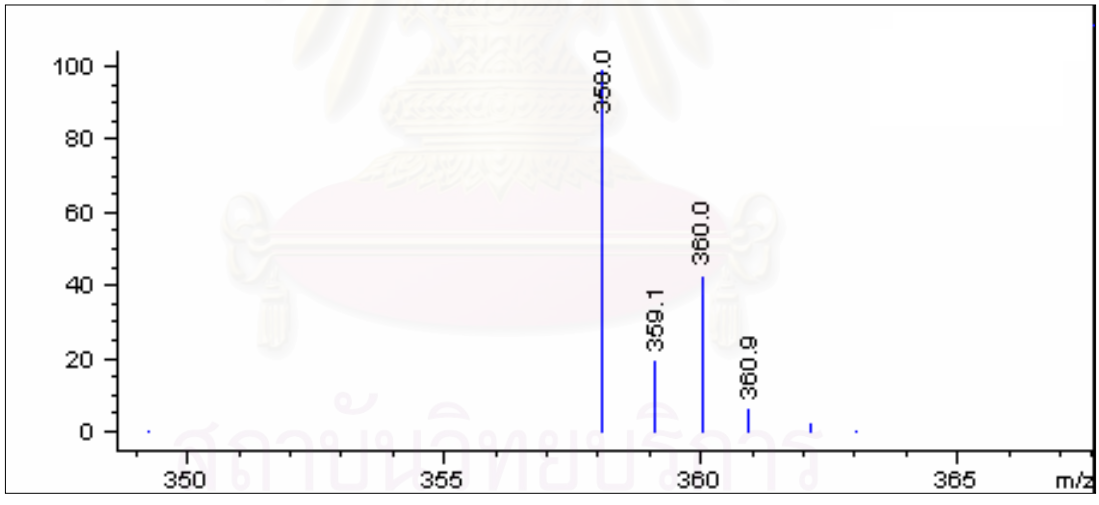

Figure B-25 Mass spectrum of standard chlorsulfuron 50 ppb by FIA under condition follow table 4.2 and 4.3.

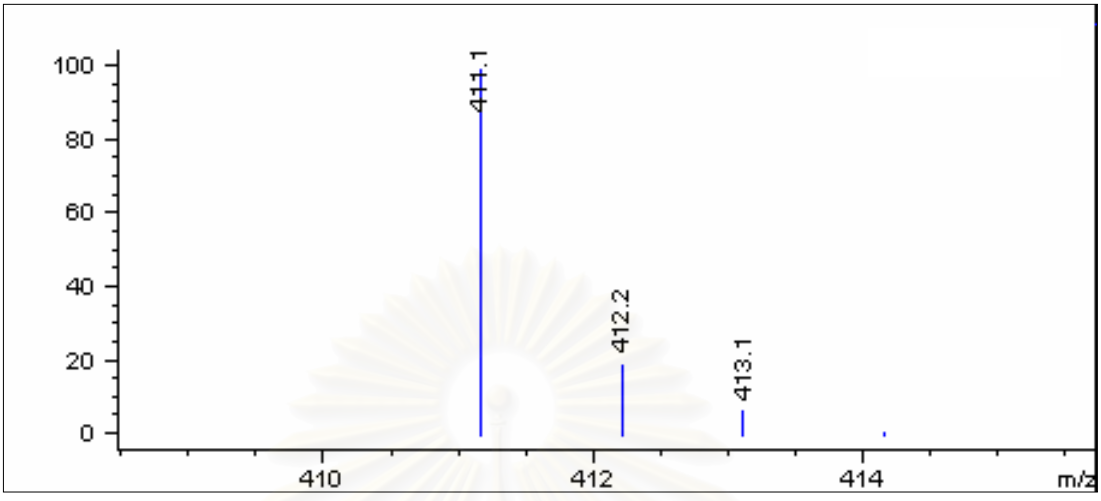

Figure B-26 Mass spectrum of standard bensulfuron 50 ppb by FIA under condition follow table 4.2 and 4.3

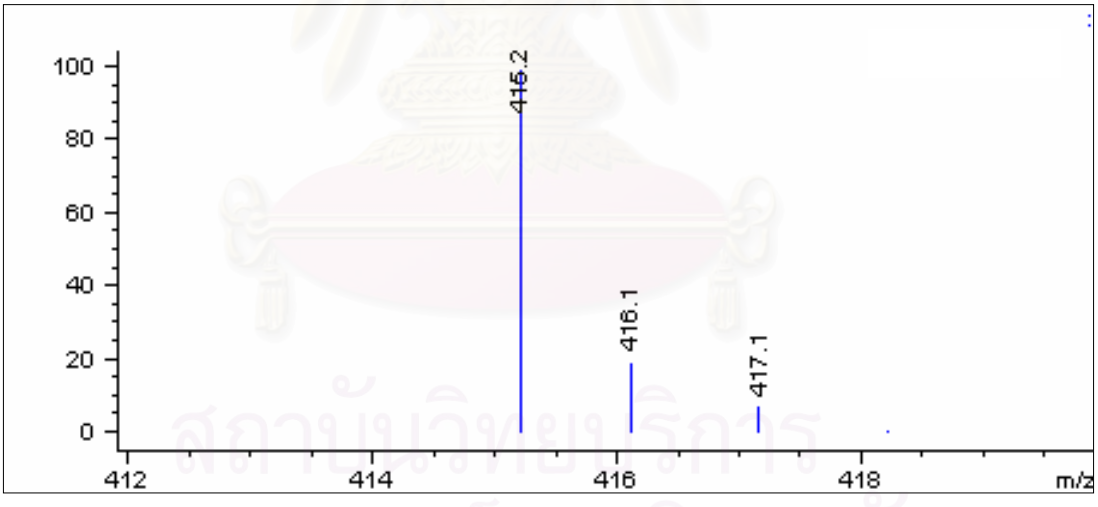

Figure B-27 Mass spectrum of standard pyrazosulfuron ethyl 50 ppb by FIA under condition follow table 4.2 and 4.3

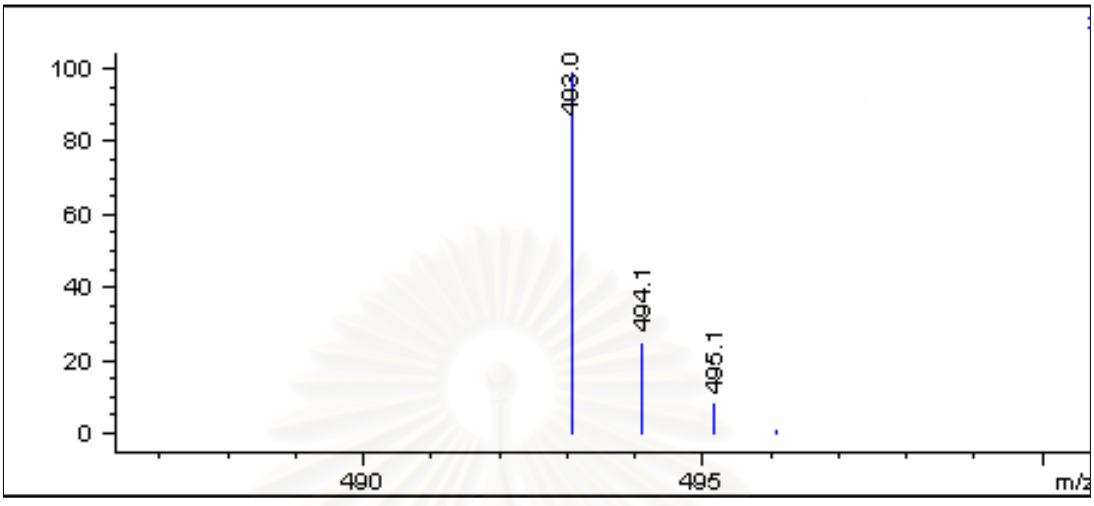

Figure B-28 Mass spectrum of standard triflusulfuron 50 ppb by FIA under condition follow table 4.2 and 4.3

APPENDIX C

Extraction Method I

1.1) Weighted 10.00 g of homogenized rice powder into conical flask.

1.2) Rice sample (from 1.1) was extracted by 100 mL of acetonitrile/0.1 M ammonium hydrogen carbonate.

1.3) The extract was homogenized for 5 minutes and centrifuged at 2000 rmp for 15 minute. (Spike mix standard in this step)

1.4) Clean up

1.4.1) SPE (ENVI-CARB) is pre-concentration by 5.00 mL methanol: dichloromethane (10:90) + 1 M formic acid.

1.4.2) Loaded 5.00 mL of sample extract (from 1.3) into ENVI-CARB cartridge.

1.4.3) Washed SPE with 10.00 mL water, followed by 5.00 mL methanol dichloromethane (10:90).

1.4.4) Eluted pesticide residues with methanol dichloromethane (10:90) + 1M formic acid and collected the extract in 25 mL round bottle flask.

1.5) The extract was concentrated with evaporation to dryness and reconstitutions to 1.00 mL by mobile phase.

1.6) The solution was filtered through a 0.45μm membrane and collected in 2.00 mL amber vial before LC/MS analysis.

Extraction Method II

2.1) A portion of 50.00 g of homogenized rice is extracted by 100.00 mL double distillation water:acetonitrile (30:100).

2.2) Solution was sonicated in Ultrasonic bath for 15 minute at room temperature.

2.3) In order to break emulsion, sodium chloride (10 g) was added to the extract.

2.4) Sodium chloride and the extract was homogenized by sonicate for 10 minutes. (Spike mix standard in this step)

2.5) The supernatant was filtered through filter paper No. 1 and the solution was transferred to a mixing cylinder.

2.6) Sodium sulfate (5 g) is added to dehydrate the extract and mixing the solution for 1 minute and leave it to stand of 5 minute.

2.7) Clean up

2.7.1) On top of NH_2 cartridge by C18 cartridge and precondition a tandem SPE before use with 15.00 mL acetonitrile.

2.7.2) Through the tandem by 20.00 mL of sample extract from 2.6

2.7.3) Afterward, the tandem is rinsed by 10.00 mL of ancetonitrile. The solution from 2.7.2 and 2.2.7.3 are collected in round bottle flask.

2.8) The elute solution is evaporated to dryness by rotary evaporator at 33 °C.

2.9) The residue was dissolved (vortex shaker) and made up to 1 mL by mobile phase and filtered with 0.45 μm membrane before LC/MS analysis.

Extraction Method III

3.1) Rice is weighing 10.00 g in 250 mL beaker with 20.00 ml of water and then homogenized for 5 minute.

3.2) The extract with 100.00 mL of acetone and homogenize for 3 minute, (Spike mix standard in this step) flitted through filter paper No 1 into a 300 mL of round bottle flask.

3.3) The extract is rinsed and filtered with 50.00 mL of acetone.

3.4) The solution was evaporated to dryness with a rotary evaporator.

3.5) Adding 100 mL of 10% sodium chloride to dissolve the residue and adjusted to pH 3-4 with 1 M hydrochloric acid.

3.6) The solution is transferred to separatory funnel and extracted with 50.00 mL × 2 of ethyl acetate. Collected the organic layer into separatory funnel.

3.7) Add 100.00 mL of n-hexane and 50 mL × 2 of 2 % di-potassium hydrogen phosphate, shaken for 5 minute and collect the aqueous layer in 250 mL beaker.

3.8) Adjust the solution to pH 3-4 by 6 M hydrochloric acid and transfer to separatory funnel.

3.9) Extract with 50 mL × 2 ethyl acetate and organic layer is dehydrated by 20 g of anhydrous sodium sulfate (shaken and stand for 30 min).

3.10) Filtered the extract from 3.9 through filter paper (rinse with 20.0 mL ethyl acetate) and then evaporated to dryness before clean up (A) step.

3.11) Clean up (1)

3.11.1) Precondition: A sep-Pak® Alumina N is conditioned by 10.0 mL of acetonitrile.

3.11.2) Dissolve the residue with 5.00 mL of acetonitrile and load through the column.

3.11.3) Rinse the cartridge with 10.0 mL of acetonitrile and elute with 15.0 mL of 20 % water in acetonitrile. Evaporate to dryness at 40 °C.

3.12) Dissolve with 30.0 mL ethyl acetated, and then transfer to 250 mL Erlenmeyer flask.

3.13) Dehydration with 20 g of anhydrous sodium sulfate (shaken and stand for 30 min). Filtered through filter paper (rinse with 20.0 mL ethyl acetate) and then evaporate (40 °C) to dryness before clean up (B) step.

3.14) Clean up (2)

3.14.1) Precondition a Bound Elute®SAX cartridge with 10.0 mL of 25 % n-hexane in acetone.

3.14.2) Dissolve the residue with 5.00 mL of 25 % n-hexane in acetone and load through the column.

3.14.3) Rinse the cartridge with 10.0 mL of 25 % n-hexane in acetone and elute with 15.0 mL of 10% methanol in acetone. Evaporate to dryness at 40 °C.

3.15) Dissolve the residue in 1ml of acetonitrile, shake by vortex shaker and filtration with 0.45 μm membrane before LC/MS analysis.

Extraction method IV

4.1) 5.00 g of homogenized rice were extracted with 40 mL of acetonitrile

4.2) Sonicate the mixture in Ultrasonic bath for 10 minute.(Spike mix standard in this step)

4.3) The eluate is filtered through filter paper and collected in 100 mL round bottle flask. Evaporate to dryness.

4.4) The residue is dissolved in 2 mL of n-hexane and applied through tandem SPE of Bound Elute PSA over Sep-Pak®Florisil.

4.5) Washing the tandem with

4.5.1) 20 mL of 15 % ether/ n-hexane

4.5.2) 20 mL of 15% acetone/ n-hexane

4.5.3) 20 ml of 50% acetone / n-heaxane.

4.6) Collect the extract from 4.5.1, 4.5.2 and 4.5.3 in round bottle flask

4.7) Evaporate to dryness by rotary evaporator.

4.8) Pass the solution through 0.45 membranes and collect in 2.00 mL amber vial before injection.

Extraction method V

5.1) Extract 25.00 g of rice sample three times with 100 mL of methylene chloride. In each extract, blender was set up high speed for 60 minute.

5.2) Transfer the extract to centrifuge tube and centrifuge for 10 minute at 2000 rpm.

5.3) Filter it through a filter paper and evaporate to dryness in a rotary evaporator at 35 °C

5.4) Dissolve the sample residue in 50.0 mL of acetonitrile

5.5) Transfer the solution to 250 mL separatory funnel and extract acetonitrile phase 3 times with 50 mL of n-hexane and shake the separatory funnel vigorously for each wash.

5.6) Discarding the hexane phase, evaporate the acetonitrile solution to dryness on a rotary evaporater at 35 °C.

5.7) Dissolve the residue by 50.0 mL of 0.15M ammonium hydroxide.

5.8) Clean up

5.8.1) Precondition the C-18 cartridge by 25 mL acetonitrile and 25 mL of 0.15M ammonium hydroxide.

5.8.2) Load the extract from 6.10 through the C-18 SPE and rinse the glassware with 10.0 mL of 0.15M ammonium hydroxide.

5.8.3) Elute with 10.0 mL of acetonitrile to round bottle flask.

5.9) Evaporate the extract to dryness with rotary evaporator.

5.10) Dissolve the residue by mobile phase to 1.00 mL before inject to LC/MS system.

Extraction method VI

6.1) Weighing 10.0 g of rice sample into 250 mL beaker.

6.2) Add 100 mL 80/20 of 0.1mM ammonium carbonate/ acetone in rice sample.

6.3) Sonicate the mixture of 20 minute and then centrifuged at 2000 rpm for 20 minute. (Spike mix standard in this step)

6.4) Supernatant is collected and extracted rice for a second time (repeat step 5.2 to 5.3) after break the platelet with spatula.

6.5) Collect the solution from step 5.3 and 5.4 and adjust to pH 3.0 to 3.5 by phosphoric acid.

6.6) Clean up

6.6.1) Precondition PSA cartridge by 10.0 mL of methanol and 10.0 mL of water.

6.6.2) Load 15.0 mL of acidic solution from step 5.5 through SPE.

6.6.3) Rinse PSA cartridge by 10.0 mL of 50 mM sodium acetate pH7/5 % methanol and followed by 10.0 mL of methanol.

6.6.4) Elute the analyte by 10.0 mL of 100 mM phosphoric acid/ acetonitrile.

6.7) Evaporate the solution to drytness at 35°C by rotary evaporator.

6.8) Dissolve the residue by mobile phase to 1.00 mL (vortex shaker) before pass through 0.45 μm membrane and inject to LC/MS system.

Extraction method VII

7.1) 5.00 g of homogenized rice is added into centrifuge bottle.

7.2) Add 100 mL of 80:20 (v/v) 0.1M ammonium carbonate/acetone and shake 2 minute by hand. (Spike mix standard in this step).

7.3) Place the bottle in and set up at 2000 rpm for 15 minute.

7.4) Supernatant passed through filter paper and collect into 250 mL beaker.

7.5) Spatula is used to break up the pellet and repeat step 7.2 to 7.3.

7.6) Combining the supernatant from step 7.4 and 7.5 and dried the solution by rotary evaporator at 35 °C.

7.7) Clean up (1)

- 7.7.1) Precondition of C-18 cartridge by 5.0 ml of methanol and then 10.0 ml of acetone.
- 7.7.2) The extract was adjusted to 3.0-3.5 by dilute phosphoric acid (1:10) and loaded through C-18 cartridge.
- 7.7.3) Rinse the cartridge with 5.0 ml double distillation water.
- 7.7.4) Elution the analyte with 10 mL of 0.1% (v/v) glacial acetic acid in ethyl acetate.

7.8) The eluate was evaporated to dryness under 35 °C water bath.

7.9) Reconstitution by 2 mL of ethyl acetated (vortex shaker) and added 8 mL of hexane (vortex shaker).

7.10) Clean up (2)

- 7.10.1) Precondition of Silica cartridge by 5.0 ml of ethyl acetate, followed by 5.0 mL of 80/20 (v/v) hexane/ethyl acetate.
- 7.10.2) Load the extract from step 7.9) through the SPE.
- 7.10.3) Elute with 0.1 % (v/v) glacial acetic acid in ethyl acetate.

7.11) The eluate was evaporated to dryness and reconstitute by 0.5 mL of methanol and followed by 1 mL of 30mM, pH 6.2 phophate buffer

7.12) Steam nitrogen and 35 °C water bath were used to reduce residue to 1 mL (vortex shaker).

7.13) Solution was passed through 0.45 μm membrane before LC/MS analysis.

APPENDIX D

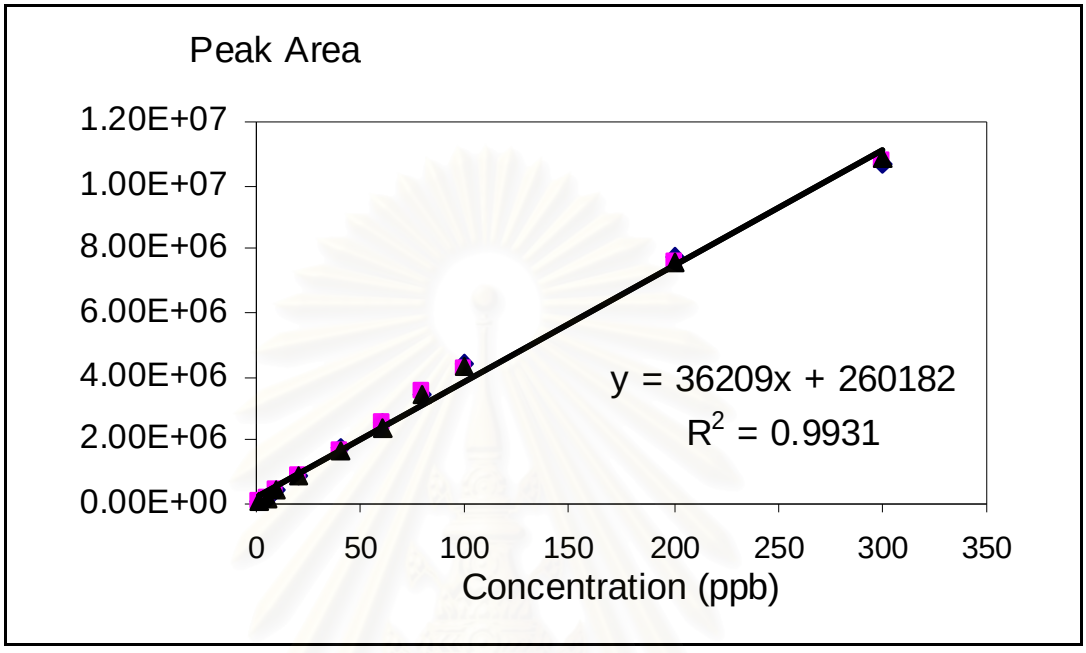

Figure D-1 Standard calibration curve of cinosulfuron by LC/MS condition in table 4.3 and 4.5.

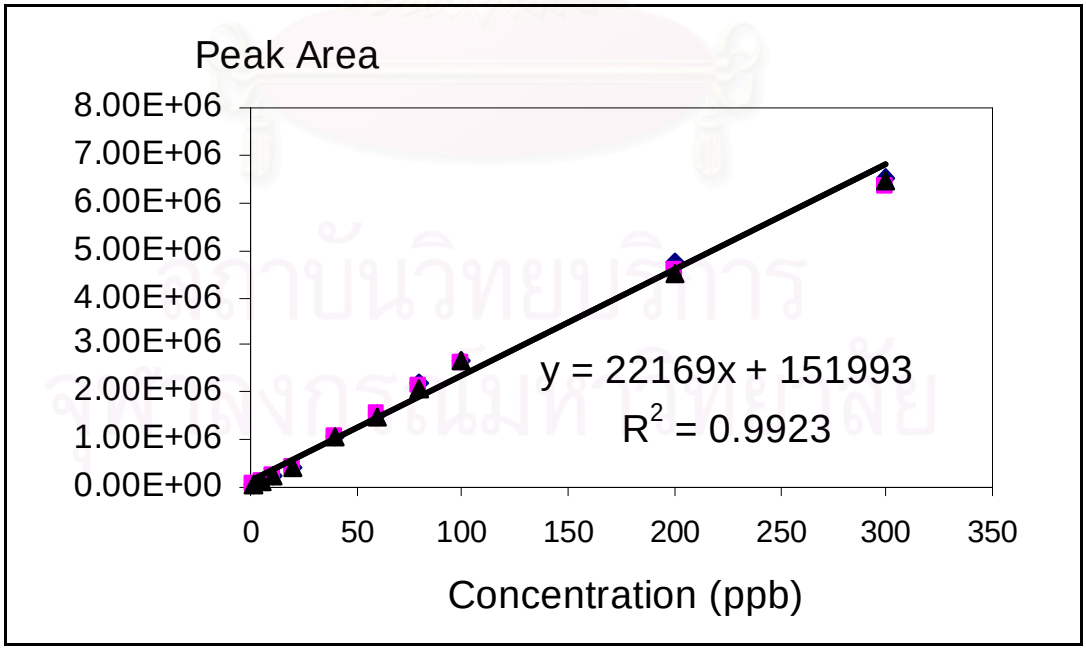

Figure D-2 Standard calibration curve of metsulfuron by LC/MS condition in table 4.3 and 4.5.

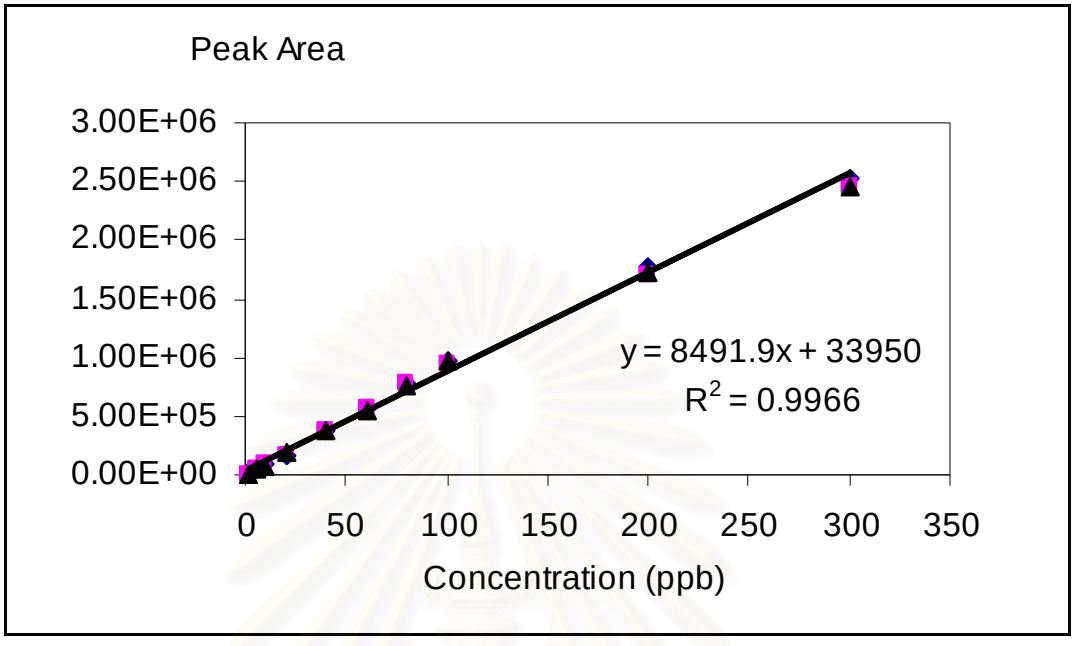

Figure D-3 Standard calibration curve of sulfometuron by LC/MS condition in table 4.3 and 4.5.

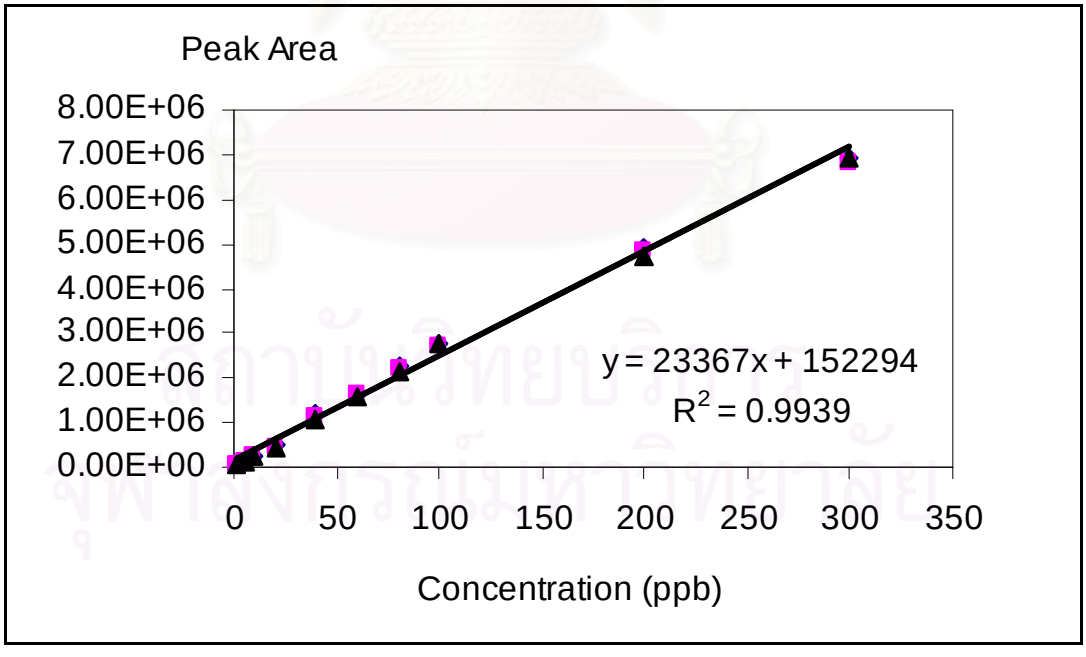

Figure D-4 Standard calibration curve of chlorsulfuron by LC/MS condition in table 4.3 and 4.5.

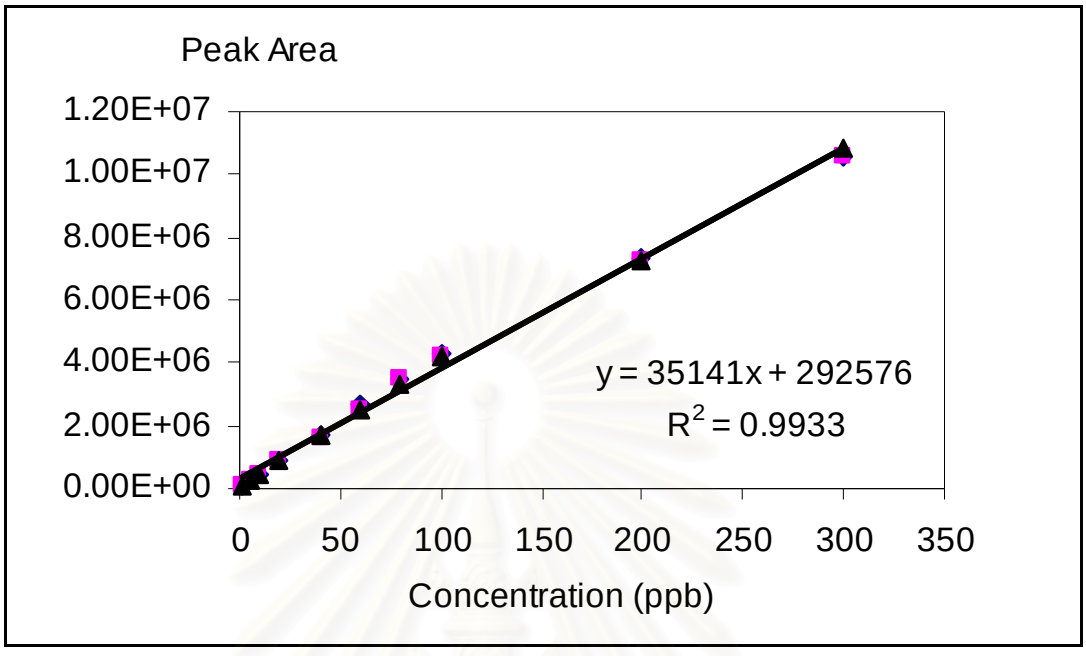

Figure D-5 Standard calibration curve of bensulfuron by LC/MS condition in table 4.3 and 4.5.

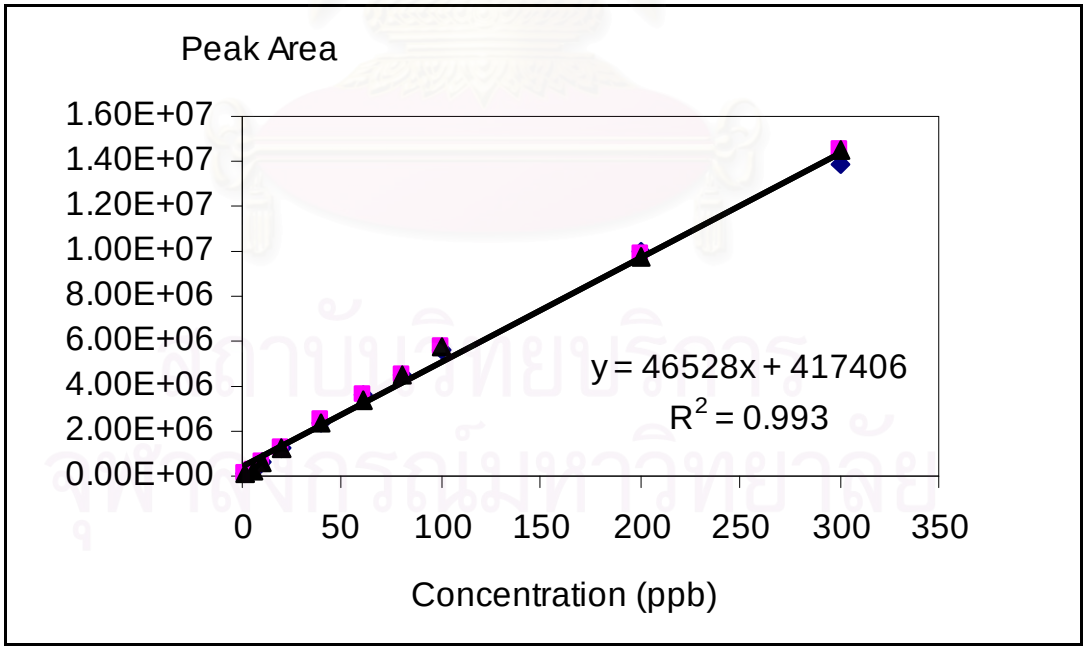

Figure D-6 Standard calibration curve of pyrazosulfuron ethyl by LC/MS condition in table 4.3 and 4.5.

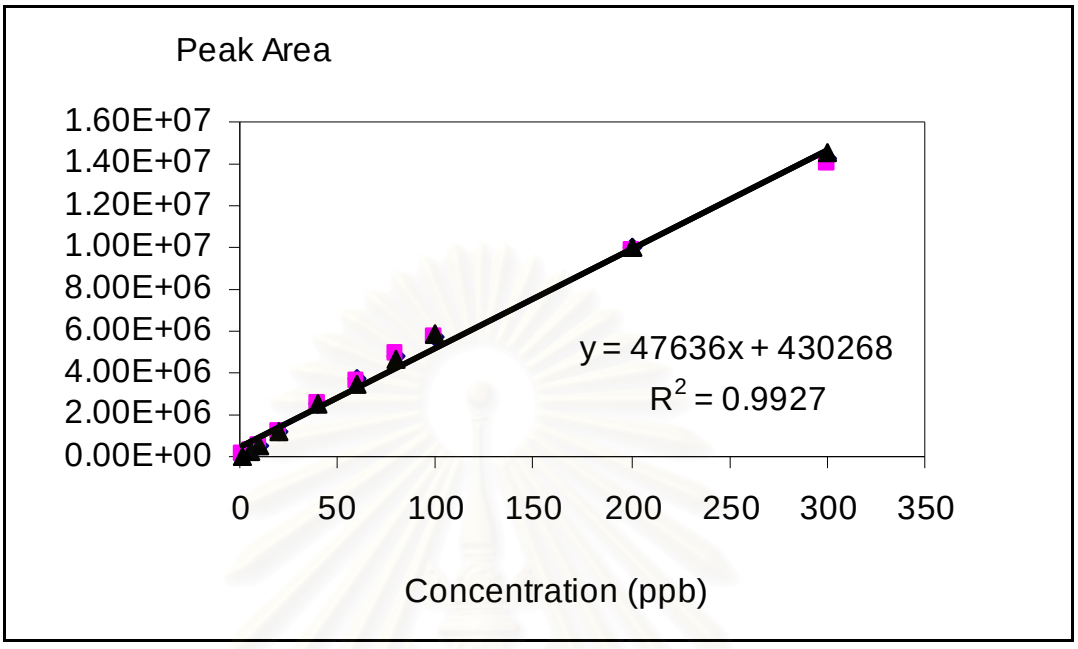

Figure D-7 Standard calibration curve of triflusulfuronby LC/MS condition in table 4.3 and 4.5.

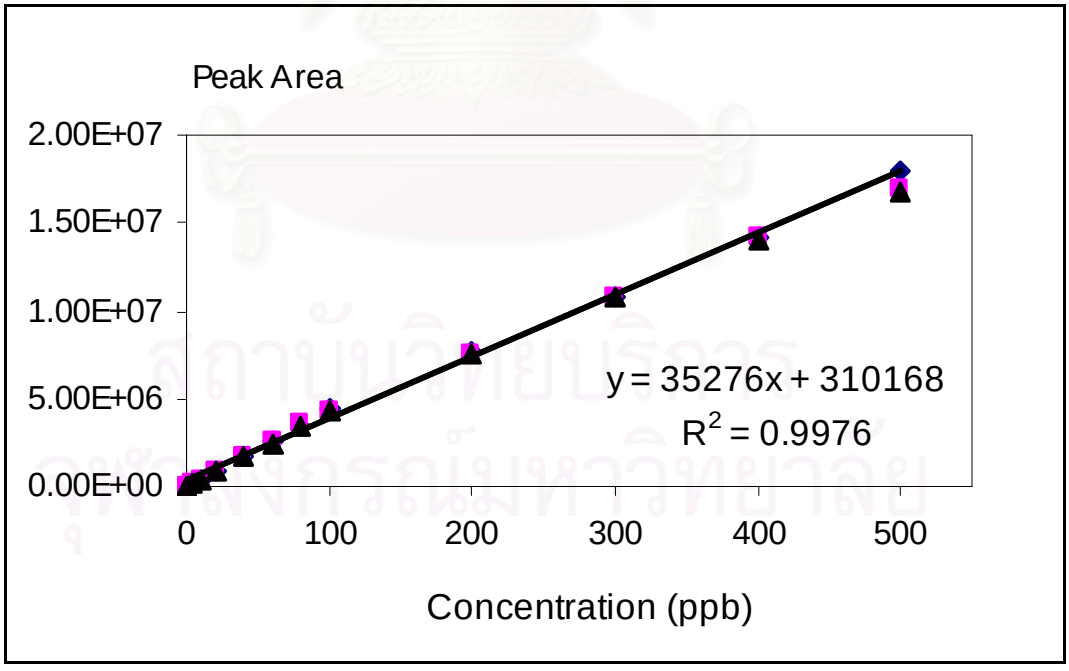

Figure D-8 The relationship between concentrations of cinosulfuron and peak area by LC/MS condition in table 4.3 and 4.5.

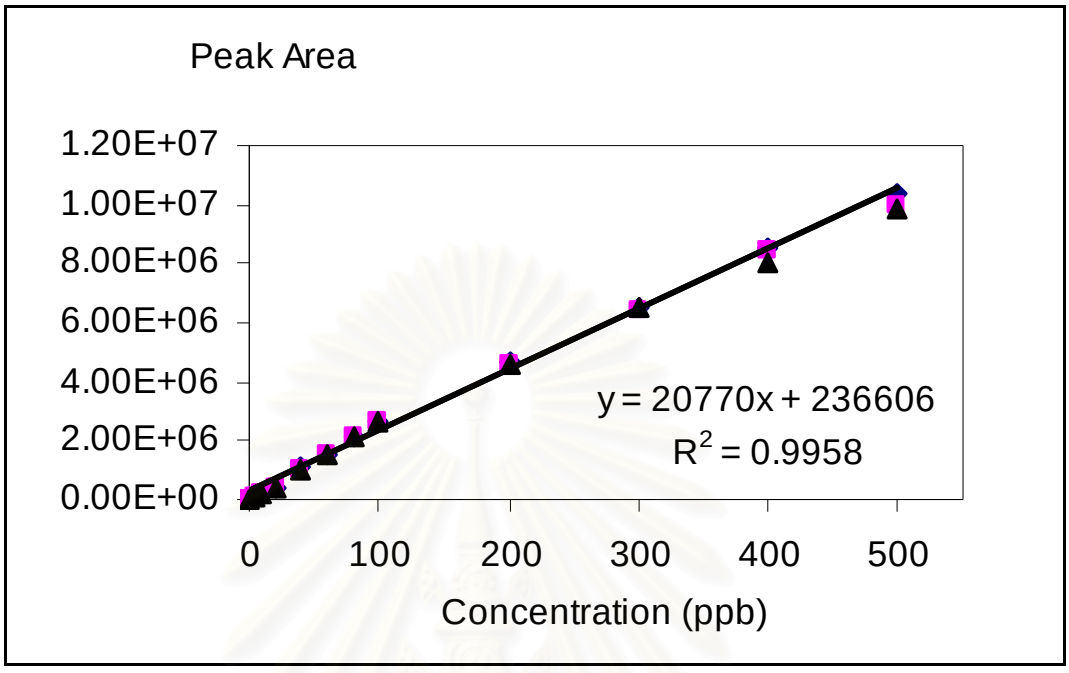

Figure D-9 The relationship between concentrations of methylsulfuron and peak area by LC/MS condition in table 4.3 and 4.5.

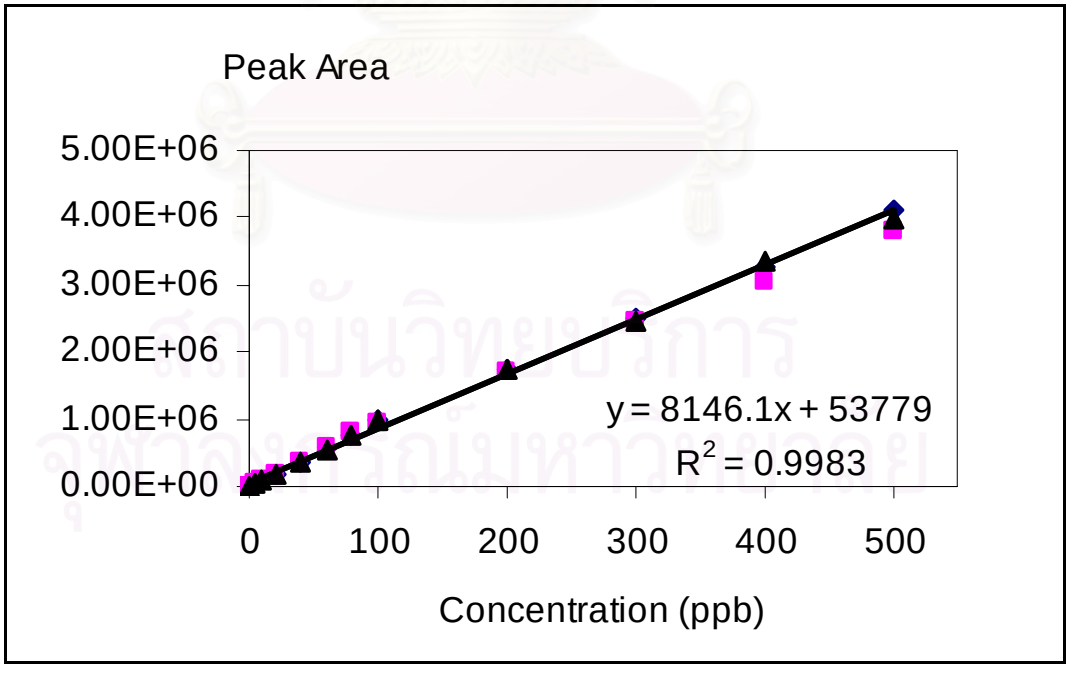

Figure D-10 The relationship between concentrations of sulfometuron and peak area by LC/MS condition in table 4.3 and 4.5.

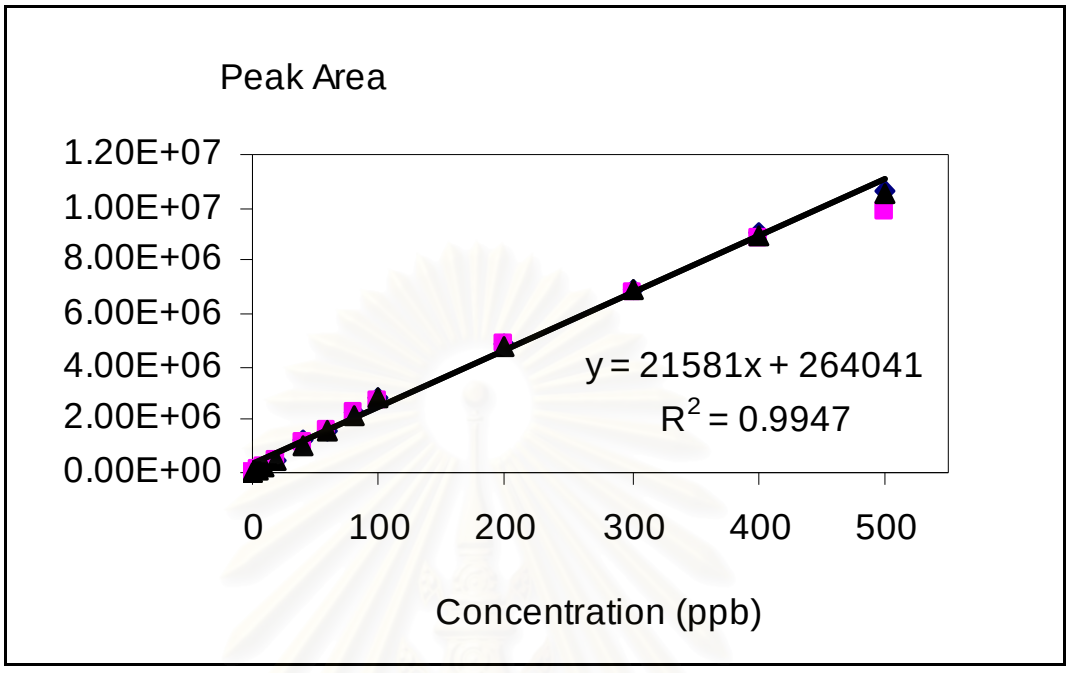

Figure D-11 The relationship between concentrations of chlorsulfuron and peak area by LC/MS condition in table 4.3 and 4.5.

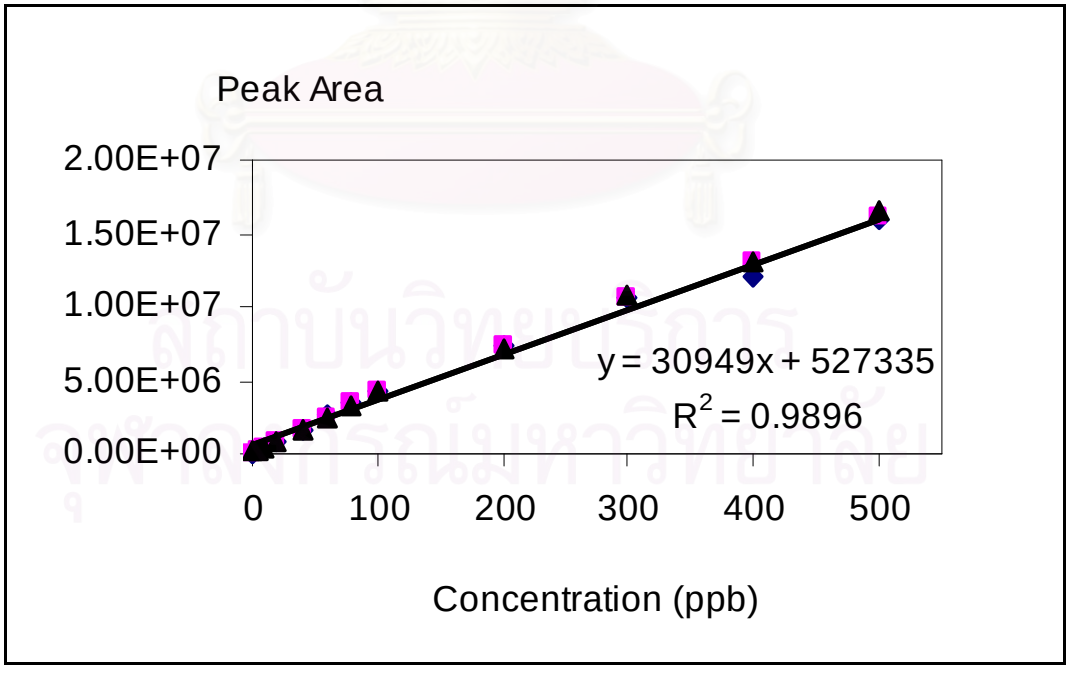

Figure D-12 The relationship between concentrations of bensulfuron and peak area by LC/MS condition in table 4.3 and 4.5.

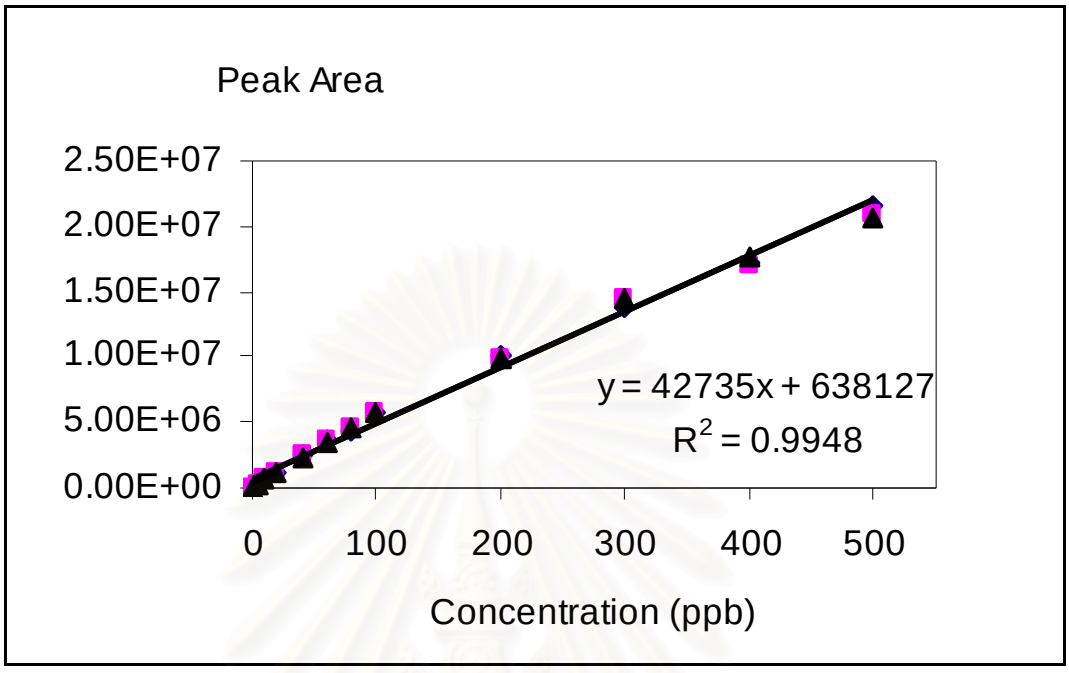

Figure D-13 The relationship between concentrations of pyrazosulfuron ethyl and peak area by LC/MS condition in table 4.3 and 4.5.

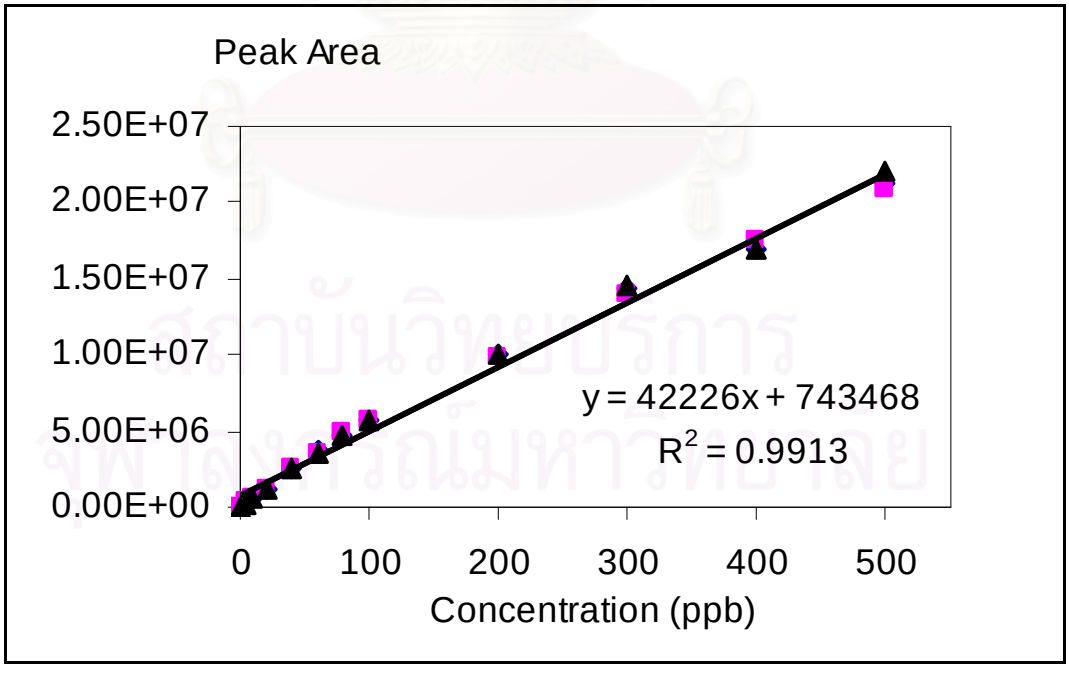

Figure D-4 The relationship between concentrations of triflusulfuron an peak area by LC/MS condition in table 4.3 and 4.5.

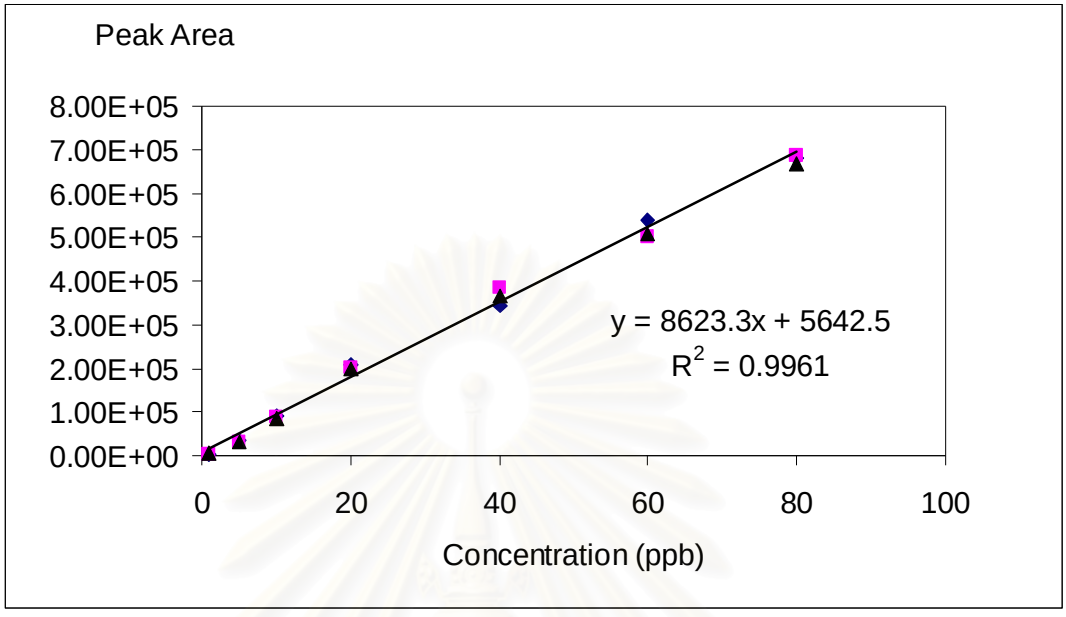

Figure D-15 Matrix calibration curve of cinosulfuron by LC/MS condition listed in table 4.3 and 4.5.

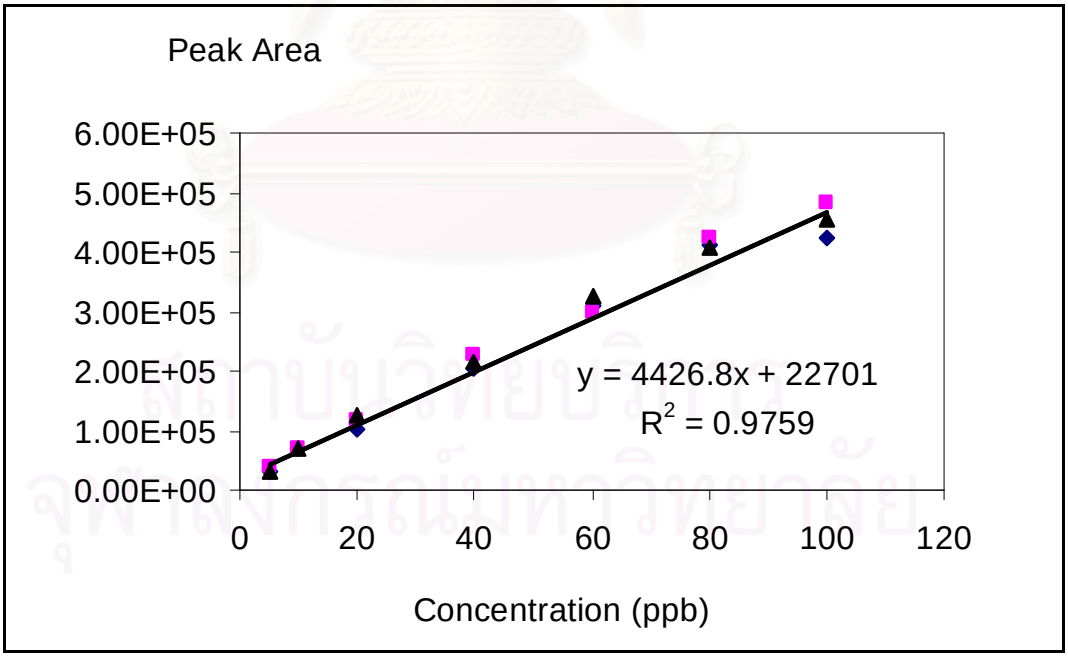

Figure D-16 Matrix calibration curve of metsulfuron by LC/MS condition listed in table 4.3 and 4.5.

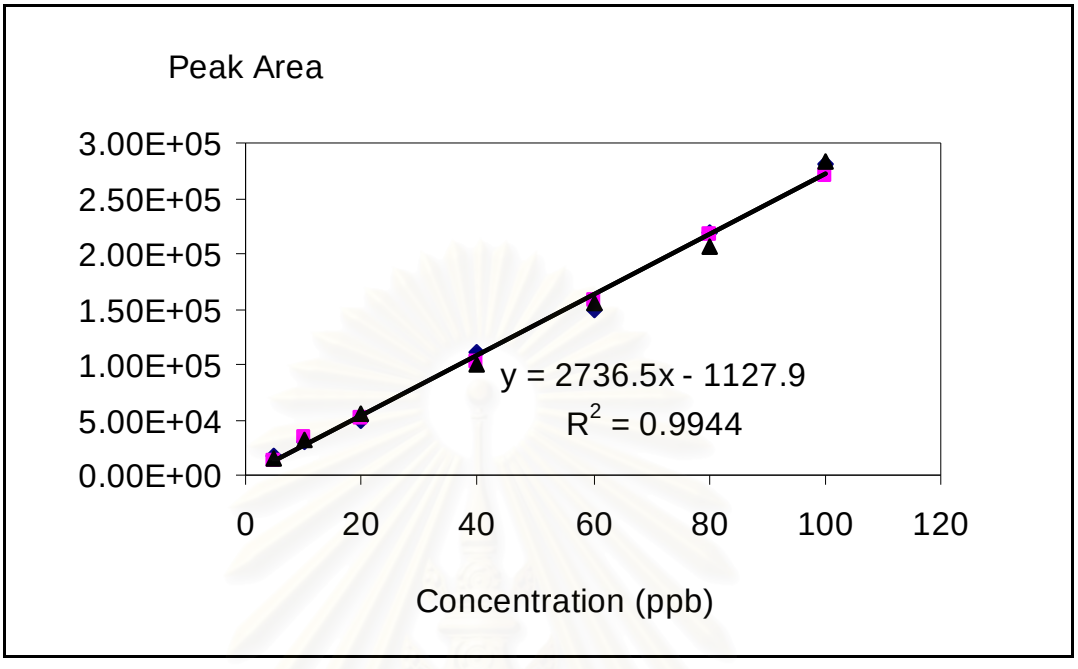

Figure D-17 Matrix calibration curve of sulfometuron by LC/MS condition listed in table 4.3 and 4.5.

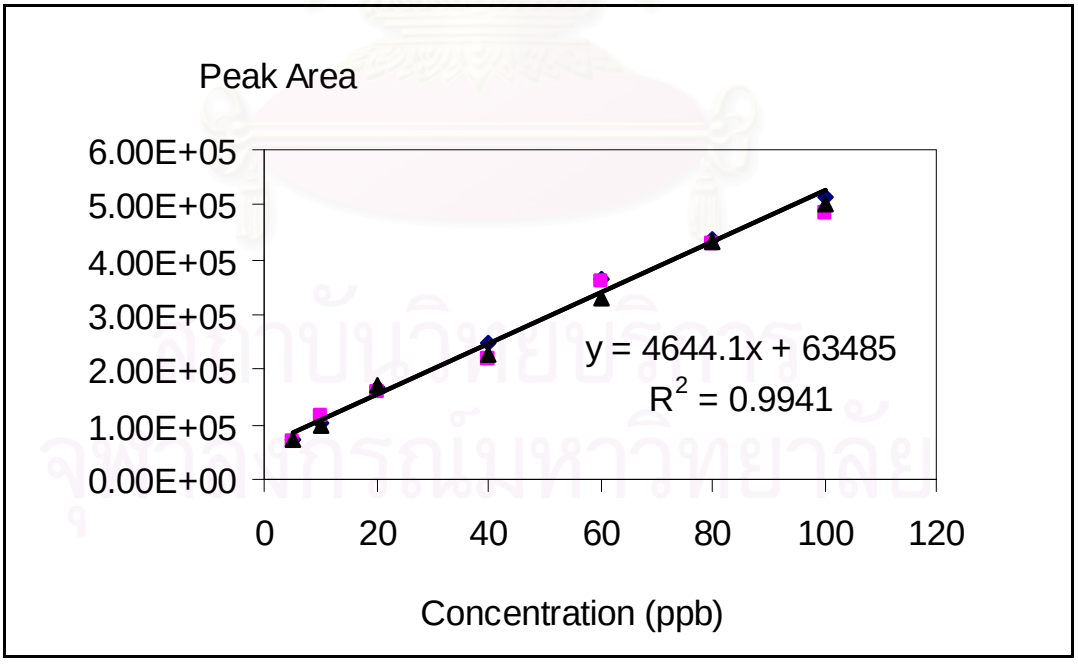

Figure D-18 Matrix calibration curve of chlorsulfuron by LC/MS condition listed in table 4.3 and 4.5.

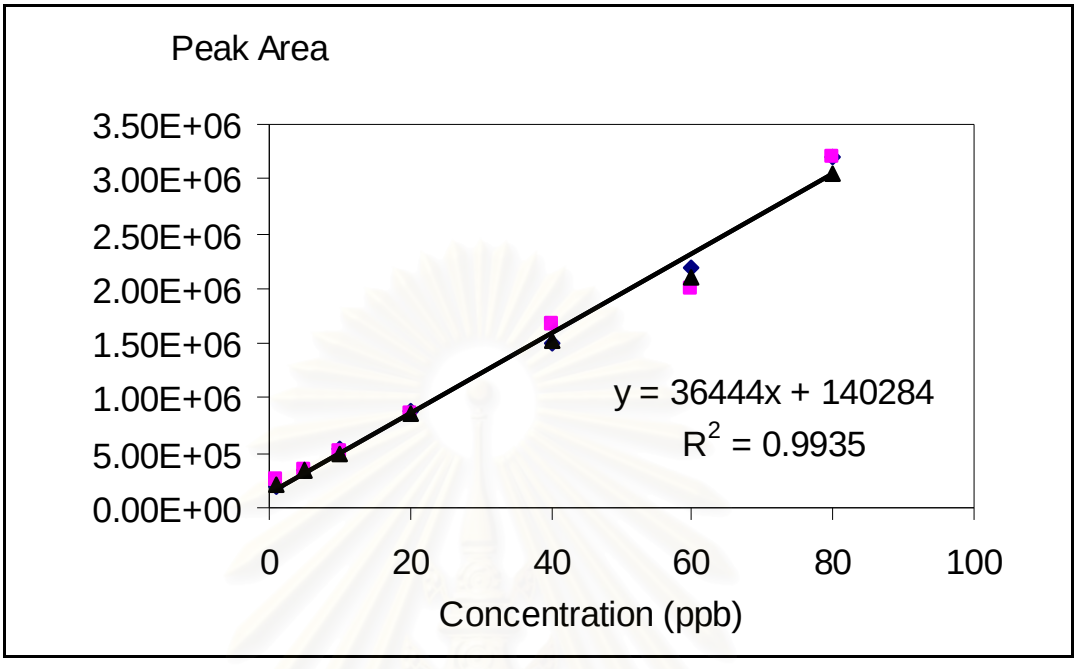

Figure D-19 Matrix calibration curve of bensulfuron by LC/MS condition listed in table 4.3 and 4.5.

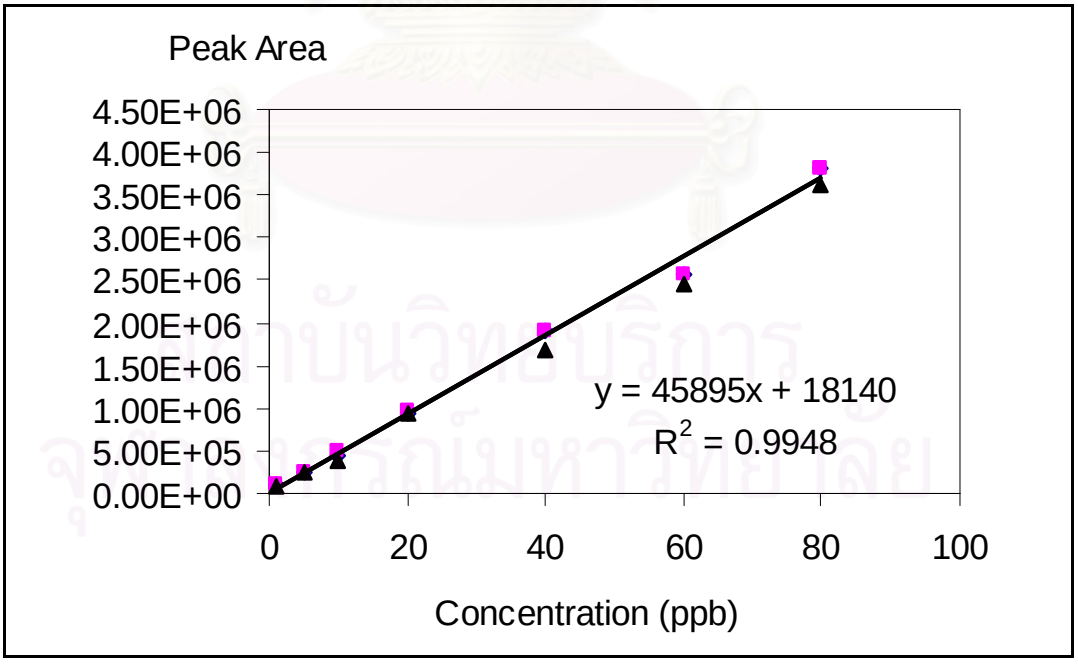

Figure D-20 Matrix calibration curve of pyrazosulfuron ethyl by LC/MS condition listed in table 4.3 and 4.5.

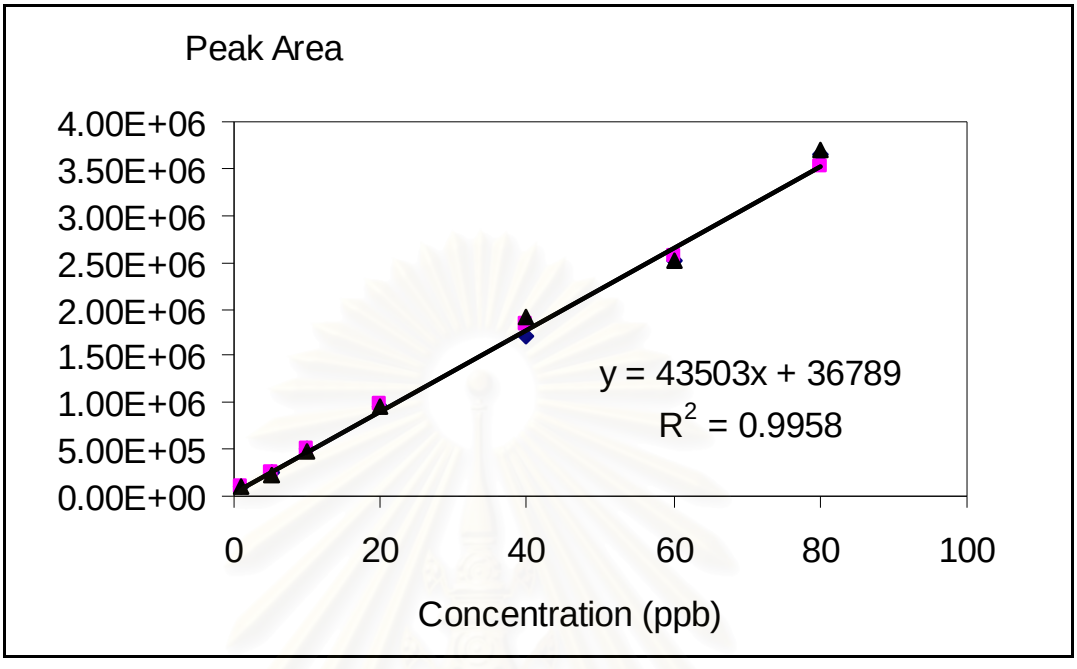

Figure D-21 Matrix calibration curve of triflusulfuron by LC/MS condition listed in table 4.3 and 4.5.

VITA

Miss Duangkamol Pinyo was born on October 22, 1978, in Surin, Thailand. She had graduated a Bachelor Degree in Chemical Science from Faculty of Science, Khon Kaen University in 2001. Afterward she had continued her academic education at Chemistry Department, Faculty of Science, Chulalongkorn University. Her work was concentrated on chromatography and separation science and will received her Master of Science Degree in July 2006.