DETERMINATION OF GLYPHOSATE, GLUFOSINATE AND THEIR METABOLITES BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Miss Walaiporn Chanchanasophon

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
Department of Chemistry
Faculty of Science
Chulalongkorn University
Academic Year 2012

การตรวจวัดไกลโฟเสต กลูโฟซิเนต และเมแทบอไลต์ด้วยลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมตรี

นางสาววลัยพร ชาญชนะโสภณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาเคมี ภาควิชาเคมี

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2555

Thesis Title	DETERMINATION OF GLYPHOSATE, GLUFOSINATE AND THEIR METABOLITES BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY
By	Miss Walaiporn Chanchanasophon
Field of Study	Chemistry
Thesis Advisor	Assistant Professor Natchanun Leepipatpiboon, Dr.rer.nat.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

.....Thesis Advisor
(Assistant Professor Natchanun Leepipatpiboon, Dr.rer.nat.)

.....Examiner
(Monpicha Srisa-Art, Ph.D.)

.....External Examiner
(Assistant Professor Panthira Ketkaew, Ph.D.)

วลัยพร ชาญชนะโสภณ : การตรวจวัดไกลโฟเสต กลูโฟซิเนต และเมแทบอไลต์ด้วยลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมตรี. (DETERMINATION OF GLYPHOSATE, GLUFOSINATE AND THEIR METABOLITES BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.ณัฐชนัญ ลีพิพัฒน์ไพบูลย์, 120 หน้า.

การพัฒนาเทคนิคลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมตรี สำหรับการตรวจวัดในวิธีการเดียวของไกลโฟเสต กลูโฟซิเนต และเมแทบอไลต์ (เอ็น-อะซีติล-กลูโฟซิเนต, กรดอะมิโนเมทิลฟอสฟอนิก และ กรด 3-(เมทิลฟอสฟินิโค) โพรพิโอนิก) ในเมทริกซ์ข้าว โดยศึกษาการเปรียบเทียบโหมดในการแยก เช่น ไฮโดรฟิลิกอินเทอเร็กชันลิควิดโครมาโทกราฟี, รีเวิร์สเฟสลิควิดโครมาโทกราฟี, ไอออนโครมาโทกราฟี และศึกษาตัวแปรต่างๆที่มีผลต่อโหมดการแยก ได้แก่ ชนิด ความเข้มข้นและค่าความเป็นกรดด่างของเฟสเคลื่อนที่ โหมดการแยกแบบไอออนโครมาโทกราฟีให้การแยกที่ดีกว่าไฮโดรฟิลิกอินเทอเร็กชันลิควิดโครมาโทกราฟี และรีเวิร์สเฟสลิควิดโครมาโทกราฟี ซึ่งมีความสามารถในการแยกที่ดีและรูปร่างพีกสมมาตร ช่วงความเป็นเส้นตรงมีค่า 0.05-2.8 ไมโครกรัมต่อกิโลกรัม ค่าสัมประสิทธิ์การตัดสินใจมากกว่า 0.89 ค่าเฉลี่ยร้อยละการคืนกลับและค่าความเบี่ยงเบนมาตรฐานสัมพัทธ์อยู่ในช่วง 87-110% และ 8-23% ตามลำดับ ที่ระดับความเข้มข้น MRL, 5-MRL และ 10-MRL วิธีการนี้มีวิธีการที่ง่าย รวดเร็ว ใช้สารเคมีน้อย และไม่ต้องทำการเตรียมอนุพันธ์

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555

##5372408023 : MAJOR CHEMISTRY
KEYWORDS : TANDEM MASS SPECTROMETRY / HYDROPHILIC INTERATION CHROMATOGRAPHY / REVERSED PHASE LIQUID CHROMATOGRAPHY / ION CHROMATOGRAPHY

WALAIPORN CHANCHANASOPHON : DETERMINATION OF GLYPHOSATE, GLUFOSINATE AND THEIR METABOLITES BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY. ADVISOR : ASST.PROF.NATCHANUN LEEPIPATPIBOON, Dr.rer.nat., 120 pp.

A liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been developed for the simultaneous determination of glyphosate, glufosinate, and their metabolites (aminomethylphosphonic acid (AMPA), 3-(methylphosphinico) propionic acid (3-MPPA), and *N*-acetyl-glufosinate) in rice. A comparative study of various chromatographic separation modes such as hydrophilic interaction liquid chromatography (HILIC), reversed phase liquid chromatography (RPLC), and ion chromatography (IC) were studied. Parameters affecting chromatographic separations such as type, concentration, and pH of mobile phase were studied and optimized separately. IC mode has a better separation than HILIC and RPLC mode, which were good resolutions and symmetric peak shapes. The linearity was achieved in the range of 0.05-2.8 mg/kg with acceptable values of correlation coefficient, $R^2 > 0.89$ for all compounds. The mean recovery and relative standard deviation (RSD) were in the range of 87-110% and 2-23% respectively, at MRL, 5-MRL and 10-MRL concentration level. This method was simple, fast, used less chemicals and had no derivatization step.

Department : Chemistry Student's Signature .....
Field of Study : Chemistry Advisor's Signature .....
Academic Year : 2012

ACKNOWLEDGEMENTS

First of all, I could never thanks enough for my thesis advisor, Assistant Professor Natchanun Leepipatpiboon, Dr.rer.nat., for her professional, intelligent guidance, attention, encouragement and critical proofreading. Thank you very much to the committee for their useful advices and comments.

I would like to thank the Higer Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (FW 0648I) for financial supports.

Furthermore, I am appreciated all members of the Chromatography and Separation Research Unit for their valuable suggestions. Many thanks to Miss Chayada Chiaochan, Miss Urairat Koesukwiwat and Miss Soparat Yudthavorasit for their helpfulness and suggestions as well as my friends for their friendship and encouragement.

Finally, I am very grateful to my beloved parents and sisters for their love, encouragement and their support in every step for my life. Thank also extend to everyone who has contributed to the success of thesis.

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LIST OF ABBREVIATIONS

%	percentage
°C	degree Celsius
µg/L	microgram per liter
µL	microliter
µm	micrometer
3-MPPA	3-(methylphosphinico) propionic acid
AMPA	aminomethyl phosphonic acid
AOAC	Association of official analytical chemists
APCI	atmospheric pressure chemical ionization
CAV	cell accelerator energy
CE	capillary electrophoresis
CEM	channeltron electron multiplier
CI	chemical ionization
CID	collision-induced dissociation
DC	direct current
EI	electron impact ionization
EPA	Environmental protection agency
ESI	electrospray ionization
EU	European union
FMOC-Cl	9-fluorenylmethyl chroformate
FT-ICR	fourier transform ion cyclotron resonance
g	gram
GC	gas chromatography
GFC	gel filtration chromatography
GPC	gel permeation chromatography
HILIC	hydrophilic interaction chromatography
HPLC	high performance liquid chromatography
hν	photons
IC	ion chromatography

kV	kilovolt
L/min	liter per minute
LC	liquid chromatography
LCL	lowest calibration level
LC-MS/MS	liquid chromatography-tandem mass spectrometry
M	molar
M.W.	molecular weights
m/z	mass per charge ratio
MDL	minimum detection levels
MeCN	acetonitrile
MeOH	methanol
mg	milligram
mg/kg	milligram per kilogram
mg/L	milligram per liter
min	minute
mL	milliter
mL/min	milliter per minute
mM	millimolar
mm	millimeter
MRL	maximum residue limit
MRM	multiple reactions monitoring
MS	mass spectrometry
ms	millisecond
MS/MS, MS^n	tandem mass spectrometry
MSPD	matrix solid-phase dispersion
NMR	nuclear magnetic resonance
NPLC	normal phase liquid chromatography
OPA	ortho-phthalaldehyde
PMT	photomultiplier tube
QIT	quadrupole ion trap
QQQ	triple quadrupole

R^2	correlation coefficient
RF-AC	radio frequency-alternating current
RI	refractive index
RPLC	reversed phase liquid chromatography
rpm	revolutions per minute
RSD	relative standard deviation
SLM	supported-liquid membrane
SLMTE	supported liquid membrane tip extraction
SPE	solid phase extraction
TOF	time of flight
t_R	retention time
UV	ultraviolet
V	voltage

CHAPTER I

INTRODUCTION

1.1 Problem Definitions

Herbicides are widely applied in agriculture for the control of weeds and also used to kill unwanted plants or inhibit their normal growth; farmers use herbicides to control weeds before planting their crops. The use of herbicides is important to the development of agriculture as it aids in the production of large volumes of agricultural products. Applications of herbicides allow farmers to timely and effectively control weeds with minimum cost and labor. Glyphosate and glufosinate are herbicides which are most widely used around the globe; including Thailand. In fact, the national import statistics indicated that glyphosate and glufosinate have topped the country's list of imported hazardous substances since 2002. Glyphosate was the number one agricultural substance imported in 2009 when a total of eighty-five thousand tons of herbicides was imported [1, 2]. These agricultural substances are sources of chemical residues which have an adverse affect on humans, animals and the environment. In the case of humans, exposure to chemical substances can be both direct and indirect. Farmers can suffer from direct exposure whilst consumers are exposed indirectly because the chemical substancees can accumulate in soil, water and agricultural products. The chemical will be able to get into the body through the skin, breathing and swallowing. The toxicity of the herbicides is both acute and chronic. Symptoms are dizziness, drowsiness, vomiting, cancer, paralysis or even death [3]. For these reasons, the European Union (EU) regulates herbicide residues in vegetables and fruits. The maximum residue limit (MRL) is set at 0.01 mg/kg depending on the particular herbicide and matrix type [4].

Glyphosate is mainly decomposed to aminomethyl phosphonic acid (AMPA), while *N*-acetyl-glufosinate and 3-(methylphosphinico) propionic acid (3-MPPA) are the degradation products of glufosinate. Glyphosate, glufosinate and their metabolites

are very difficult to analyze due to their extreme polarity. Their molecular structures contain both positive and negative ions (zwitterionic) and therefore, are extremely soluble in water. Other properties that contribute to their analytical difficulty are low volatility, and lack of chromophore or fluorophore. Chemical structures of these herbicides are illustrated in Figure 1.1.

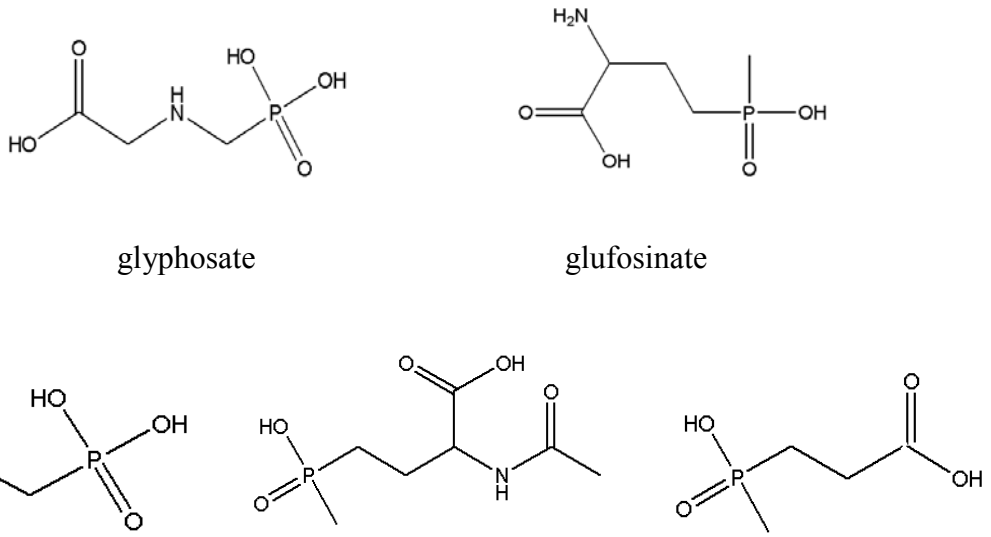


Figure 1.1 Chemical structures of glyphosate, glufosinate and their metabolites.

There are many analytical methods for determination of glyphosate, glufosinate and their metabolites such as liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE), UV-visible spectroscopy, fluorescence and nuclear magnetic resonance (NMR). These herbicides have a low volatility and lack of chromophore or fluorophore. Therefore the analysis techniques (GC, UV-visible, and fluorescence) require derivatization. Glyphosate, glufosinate and their metabolites are extremely soluble in water thus suitable for LC. LC is a technique that combines many detectors such as refractive index (RI), UV- visible, fluorescence, electrochemical, conductivity and mass spectrometry (MS). The refractive index detector is used in the isocratic program. A UV-visible and

fluorescence detectors are based on absorption hence derivatization is required before detection. Mass spectrometry (MS) measures the mass-to-charge ratio of a compound. MS is a powerful technique used for many applications as it has a very high sensitivity and selectivity. Sometimes these compounds also need derivatization in MS because they have less molecular weights and low selectivities. Derivatizing procedures often use toxic chemicals and are complicated, time consuming, expensive and sometimes needs a specific instrument.

Simultaneous determination of five compounds is very difficult to analyze. Commonly, an individual method for determination of these herbicides is required for each compound and metabolites are not usually determined. There are standard methods for determination of glyphosate, which are set by the U.S. Environmental Protection Agency (U.S.EPA) and the Association of Official Analytical Chemists (AOAC).

1.2 Regulations

Rice is the main food of the world's population including Asians and Thais. Rice planting is mostly found in countries in the Southeast Asia such as Vietnam, Myanmar, Laos, Cambodia, Malaysia, Indonesia, the Philippines and Thailand. Rice is an important crop in Thailand and is at the top of the export list of the country because Thailand is an agricultural country with rice being the main crop planted. At present rice is also in great demand both within the country and abroad. Before planting, famers use herbicides to control of weeds and glyphosate and glufosinate are the most popular herbicides used in the weed control. These agricultural substances are sources of chemical residues which have adverse affects on the rice. The EU therefore regulates herbicide residues in rice. The maximum residue limit (MRL) is set at 0.1 mg/kg (Table 1.1) [4].

Table 1.1 Herbicide residues in rice

Herbicides	MRL (mg/kg)
Glufosinate-ammmonium (sum of glufosinate, its salts, MPP and NAG expressed as glufosinate equivalents)	0.1
Glyphosate	0.1

1.3 Literature Reviews

Although glyphosate, glufosinate and their metabolites are very difficult to analyze, researchers have suggested a method of analysis. As these herbicides are toxic, many international organizations have proposed analytical methods for the determination of glyphosate, glufosinate and their metabolites.

In 1990, The U.S. Environmental Protection Agency (U.S.EPA) recommended a HPLC post-column derivatization with fluorescence detection for the analysis of glyphosate in drinking water (EPA method 547) [5]. The derivatizing reagents were ortho-phthalaldehyde (OPA) and 2-mercaptoethanol. An analysis column is Bio-Rad Aminex A-9 (cation exchange) and C18 packing is a guard column.

In 1996, the Association of Official Analytical Chemists (AOAC) recommended using an ion-exchange chromatography with UV-visible detection for determination of glyphosate in water (AOAC official method 996.12) [6]. An anion exchange column is used for separation.

In 2011, the European Union (EU) recommended the analysis of glyphosate, glufosinate, and their metabolites in vegetables and fruits using LC-MS/MS [7]. An analysis column is anion exchange. Sample preparation used is liquid extraction using water and acidified methanol for solvent extraction.

Other published works analyzed glyphosate, glufosinate, and their metabolites using many techniques.

In 2000, Royer, A. *et al.* [8] presented a method for determination of glufosinate and its metabolites (3-methylphosphonicopropionic acid and 2-methylphosphonicoacetic acid) in water using gas chromatography-tandem mass spectrometry. The method was clean-up by column chromatography on ion-exchange resin and derivatized for analysis. Derivatizing reagents were glacial acetic acid and a trimethylarthoacetate mixture. A fused-silica capillary column was used for separation. Mean recovery and relative standard deviation were in the range of 89-92% and 12-18%, respectively.

In 2001, Grey, L. *et al.* [9] studied the determination of glyphosate and AMPA in environmental water and vegetation matrices by LC/MS. 9-Fluorenylmethyl chroformate (FMOC-Cl) in borate buffer is a reagent for derivatization prior to LC analysis. A Zorbax SB-C18 column was used for the separation and gradient elution of 5 mM ammonium acetate and acetonitrile. This method has recoveries ranging from 58-117% depending on the matrix. Patsias, J. *et al.* [10] reported the use of on-line anion exchange solid phase extraction for the extaction of glyphosate and AMPA in water. The analytes were determinated using cation exchange liquid chromatography with post-column derivatization and fluorescence detection. Derivatizing reagents were *o*-phthalaldehyde (OPA) and *N*, *N*-dimethyl-2-mercaptoethylamine hydrochloride solution. An eluent was 5 mM KH_2PO_4 (potassium dihydrogenphosphate) in water and adjusted to pH 1.9 using orthophosphoric acid.

In 2004, Nedelkoska, T.V. *et al.* [11] presented separation of glyphosate and AMPA in water and plant materials using single and coupled polymeric amino columns, respectively, with fluorescence detection. The method used pre-column derivatization with FMOC-Cl and strong cation exchange-solid phase extraction for sample preparation. The minimum detection levels (MDL) obtained were 0.16 μg/L and 0.3 mg/kg for glyphosate in water and plant materials, respectively.

In 2005, Gracia de Llasera, M.P. *et al.* [12] described matrix solid-phase dispersion (MSPD) for extraction of glyphosate and AMPA in tomatoes using pre-column derivatization with FMOC-Cl. Silica based sorbent with amionopropyl (NH_2-

silica) and quaternary amine functional group (SAX-Cl anionic exchange silica) were used in the MSPD procedure. A technique used for the analysis was HPLC (high performance liquid chromatography) using a ResElut C18 column with fluorescence detection. Mean recoveries were 87% and 78% of 0.5 μg/g and 0.4 μg/g for glyphosate and AMPA, respectively. In the same year, Khrolenko, M.V. *et al.* [13] proposed the determination of glyphosate and AMPA in fruit juices by HPLC with UV detection. A supported-liquid membrane (SLM) and pre-column derivatization with *p*-toluenesulphonyl chloride (TsCl) were used for extraction and preconcentration.

In 2007, Coutinho, C.F.B. *et al.* [14] studied hydrophilic interaction chromatography (HILIC) with coulimetric detection (copper microelectrode) for determination of glyphosate in apple and grape juices. Sample preparation was centrifugation and filtration. The limit of detection was 0.1 mg/L. Linearity was in the range of 0.1-34 mg/L and the correlation coefficient was 0.9999. Recoveries presented at 92 and 90% for apple and grape juices, respectively.

In 2008, Piriyapittaya, M. *et al.* [15] validated supported liquid membrane micro-extraction (SLME) using a hollow fiber membrane for extraction of glyphosate and AMPA in water. A column used was a strong cation exchange column with a fluorescence detector and post column derivatization. *O*-phthalaldehyde (OPA) reagent and Thioflour® were reagents for derivatization. The detection limits were 0.22 μg/L for glyphosate and 3.4 μg/L for AMPA. Regarding other methods, Motojyuku, M. *et al.* [16] analyzed glyphosate, AMPA and glufosinate in human serum using gas chromatography-mass spectrometry. The method used solid phase extraction (SPE) and derivatization with *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) and 1% *tert*-butyldimethylchlorosilane (TBDMCS) for extraction. Recoveries were in the range of 88.2-103.7%.

In 2009, Li, X. *et al.* [17] used HILIC for separation of glyphosate and glufosinate in fruits and vegetables. Electrospray tandem ion-trap mass spectrometry (LC-MS/MS) and a diode array detector; DAD (LC-UV) were compared. The compounds were separated on a Venusil HILIC column using mobile phase of 0.1%

formic acid in water and acetonitrile. Recovery in the range of 63.6-107.3% and RSD < 15.3% were obtained.

In 2010, Dimitrakopoulos, I.K. *et al.* [18] reported a method for determination of glyphosate and AMPA in natural water and orange samples using ion chromatography with conductimetric detection. A dionex AS 18 anion exchange column separated the compounds which were then eluted by KOH. The linearity was 2.0-20 µg/L and the correlation coefficient was 0.9995. For another technique, See, H.H. *et al.* [19] validated capillary electrophoresis with conductivity detection for determination of glyphosate and AMPA in water. Supported liquid membrane tip extraction (SLMTE) was used for sample preparation. Extraction parameters such as pH of donor phase, type of membrane solvent, concentration of acceptor phase, agitation and extraction time were optimized. Recovery and relative standard deviation (RSD) were obtained in the range of 88-92% and 5.2-6.9%, respectively. The method showed linearity of 0.01-200 µg/L for glyphosate and 0.1-400 µg/L for AMPA, low limits of detection of 0.005 µg/L and 0.06 µg/L for glyphosate and AMPA, respectively.

In 2011, Qian, K. *et al.* [20] presented liquid chromatography-UV detection for analysis of glufosinate in maize. A sample preparation was used SPE and derivatization with 4-chloro-3,5-dinitrobenzotrifluoride (CNBF). Ion-pair reagents such as tetrabutyl ammonium bromide (TBAB), methyl trioctyl ammonium bromide (MTAB), dodecyl trimethyl ammonium bromide (DTAB), cetyltrimethyl ammonium bromide (CTAB) were optimized for separation. Glufosinate was separated on a Kromasil C18 column and mobile phase of acetonitrile and mixture solutions of 20 mM phosphoric acid pH 4 and 10 mM DTAB. Mean recovery and relative standard validation were in the ranges of 98.0-100.5% and 2.13-4.13%, respectively. Linearity was 0.1-20 mg/L and the correlation coefficient was 0.9998. In the same year, Yoshioka, N. *et al.* [21] developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) for determination of glyphosate, glufosinate, bialaphos, AMPA and 3-methylphosphinicopropinonic acid (MPPA) in human serum. Types of HILIC columns were compared for separation. The compounds were separated using an

Obelisc N column and the mobile phase was 0.1% formic acid in water and acetonitrile. Human serum was filtered via an ultrafiltration membrane to remove protein and washed with chloroform. The recovery was achieved in the range of 94-108% and RSD was within 5.9%.

1.4 Purpose of The Study

From the literature reviews there are many techniques for determination of glyphosate, glufosinate and their metabolites and the techniques chosen depend on sample preparation. The techniques for separation are gas chromatography, capillary electrophoresis and liquid chromatography. For liquid chromatography research, revered phase liquid chromatography, hydrophilic interaction chromatography and ion chromatography were studied for the separation. The most effective method for determination of these compounds is together with a pre- or post-column derivatization process due to their high polarity and lack of chromophores and fluorophore. However derivatizing procedures use toxic chemicals, and are complex, time consuming, and expensive. For post-column derivatization, a pump and a mixing valve must be added to the process. SPE is a process used for preconcentration and clean-up. SPE is a 4 step procedure (conditioning, loading, washing and eluting) thus it is difficult and time consuming. If a sample is solid, it must be extracted prior to the SPE process. Simultaneous determination of five compounds has rarely been studied. Furthermore, individual analysis methods must be used for determination of glyphosate and glufosinate.

The aim of this study was to develop method for simultaneous determination of glyphosate, glufosinate, and their metabolites (AMPA, *N*-acetyl glufosinate and 3-MPPA) using liquid chromatography–tandem mass spectrometry. A comparative study of various chromatographic separation modes such as hydrophilic interaction liquid chromatography (HILIC), reversed phase liquid chromatography (RPLC), and ion chromatography (IC) was studied. A sample preparation procedure for rice was optimized for simple, fast, less use of chemicals and no derivatization step. Tandem mass spectrometry provides high selectivity and sensitivity.

CHAPTER II

THEORY

2.1 Liquid Chromatography (LC) [22-25]

Liquid chromatography is a popular chromatographic technique for separation of a mixture of compounds based on interactions of compounds with stationary and mobile phases. The stationary phase is packed into a column which contains hydrophilic or hydrophobic materials. The mixture of compounds moves through the stationary phase and the mobile phase whereby the mobile phase carried the compounds through the column. Each compound will retain and be separated with a slow or fast speed depending on the interaction of the compounds with the stationary phase and the mobile phase. After that the compounds move through the detector and separation results will show the relationship of time (horizontal axis) and the signal measurement (vertical axis) which is called chromatogram. LC techniques are used to determine quantitatively and qualitatively for a variety of applications such as chemicals, pharmaceuticals, clinical, bioscience, environmental, food safety and consumer products. An LC system consists of a mobile phase reservoir, pumps, sample injection, a column and a detector for which a diagram is shown in Figure 2.1.

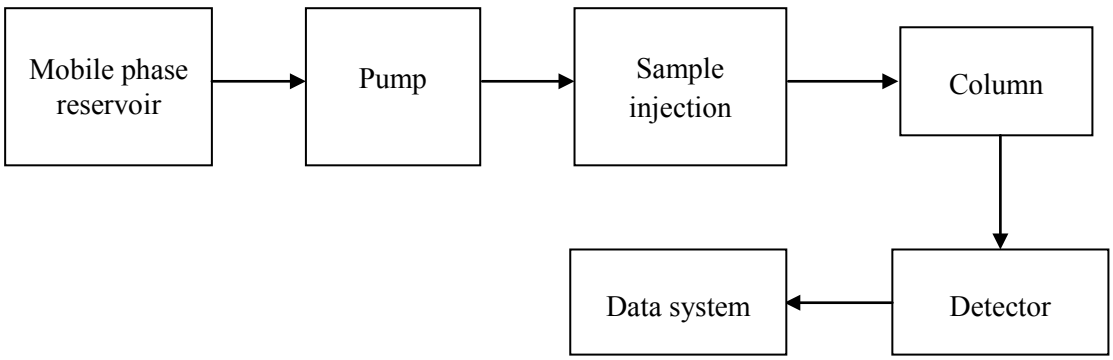


Figure 2.1 A schematic diagram of a typical LC instrument.

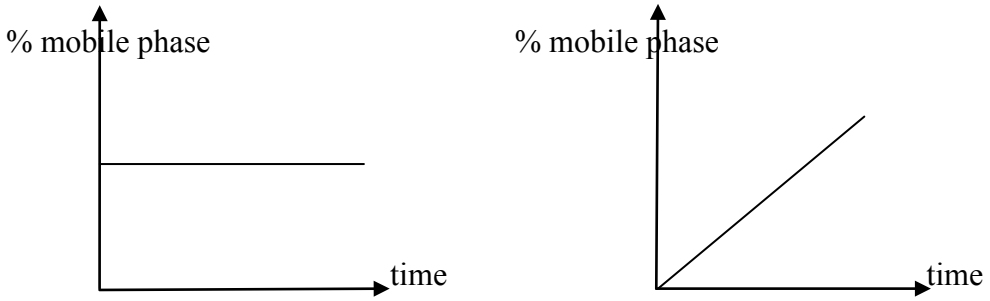
2.1.1 Part of the LC system

2.1.1.1 Mobile phase reservoir

The reservoir is made from glass and can contain 250 to 1000 mL. The mobile phase is stored in the reservoir before being pumped through the system. Selection of the mobile phase must consider the solubility, compatibility of the mobile phase and purity of solvent. For mobile phase preparation, a solvent is filtered through a micro-membrane to remove particles and impurities because small particles can cause blockages in the system. Degassing in the mobile phase removes dissolved gases because the degassed mobile phase will give a less noisy baseline. Therefore the mobile phase was filtered and degassed before being used.

A mobile phase is usually comprised of a mixture of two or more individual solvents or a modifier or an additive added in to the mobile phase. The mobile phase chosen affects the elution. For separation, there are two elution types which are

- Isocratic elution: solvent composition is in mobile phase is maintained at a constant mixing ratio during the experiment (Figure 2.2a).
- Gradient elution: the ratio of solvent in mobile phase is changed during analysis, which was suitable for separation of complex mixtures and compounds with different solubility properties (Figure 2.2b).



(a) isocratic elution (b) gradient elution

Figure 2.2 Elution types of the mobile phase.

2.1.1.2 Pump

The pump transports mobile phase from a reservoir through the system. Small particles (micro-particles) of stationary phase within a column cause resistance to the flow of the mobile phase. It is necessary to use a high-pressure pump to drive the mobile phase. Most commercial pumps are based on a reciprocating piston design. A driven motor pulls the piston back and forth in the head pump. The pump can provide a stable flow rate for the mobile phase during the experiment.

2.1.1.3 Sample injection

Samples which move through a column are very sensitive to the separation. Sample should go onto the column in a narrow band and not disturb the flow of the mobile phase. The injection used in the system is a micro-sample valve with a six-port rotary sampling injection valve, shown in Figure 2.3.

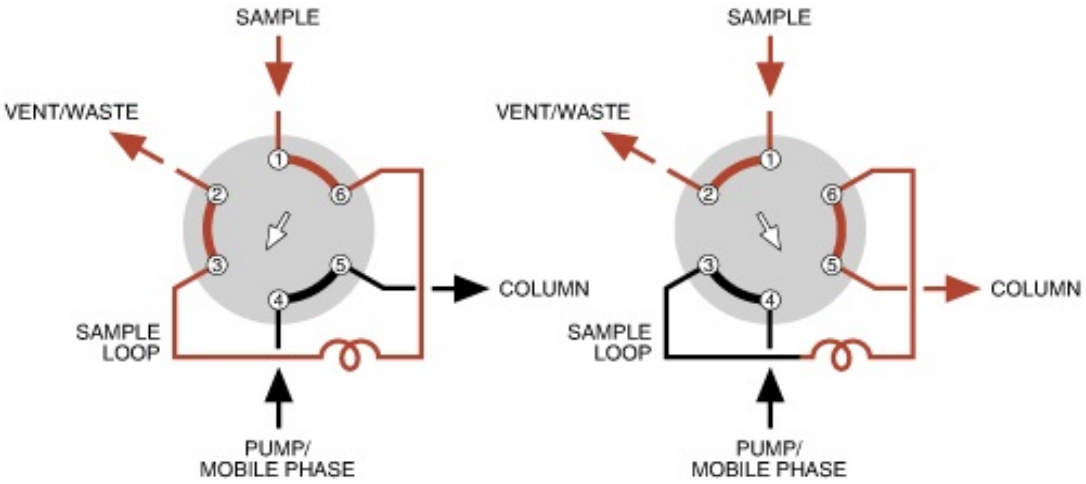


Figure 2.3 Six-port rotary sampling injection valve for sample injection system in LC. [26]

2.1.1.4 Column

A column is an important part in which separation occurs. A column used for LC analysis is made from polymer or stainless steel and has a length of 50-300 mm, an internal diameter of 2-4 mm and an extremely small particle diameter of 1.7-10 μm. The stationary phase is contained in the column in which the stationary phase identifies the separation mode.

2.1.1.5 Detector

A detector measures the signal of compounds from separation. The characteristics of a detector are sensitivity and signal response. Detector has no effect from temperature and flow rate of the mobile phase and is reliable. There are two basic types of detector; bulk property (general detectors) and solute property (selective detectors). The bulk property detector responds to the physical properties of the mobile phase and the solute such as a refractive index (RI) and conductivity detectors. The solute property detector responds to some properties of the solute which are not included with the mobile phase such as UV-visible, fluorescence and electrochemical detectors. Nowadays, mass spectrometry (MS) is popular whereby

the MS can determine quantitatively and qualitatively and has a high sensitivity. The choice of the detector for analysis depends on the characteristics and concentration of compounds in which each detector has particular advantages and disadvantages.

2.1.2 LC separation mode

2.1.2.1 Normal phase liquid chromatography (NPLC)

In normal phase liquid chromatography or adsorption chromatography, the stationary phase is more polar than the mobile phase. The stationary phase includes polar boned phase or bare silica such as cyano, amino and diol bonded phases. The mobile phase is an organic solvent such as hexane and ethyl acetate as this mode does not use water. Polar compounds slowly elute, resulting in retention times being increased. The mechanism of NPLC is based on polar adsorption or hydrogen-boning or dipole-dipole interaction. This mode separates the mixture of isomers and polarity compounds.

2.1.2.2 Reversed phase liquid chromatography (RPLC)

This separation mode is commonly used for analysis. The mobile phase is more polar than the stationary phase. The stationary phase is octadecylsilyl (C18) or octyl silyl (C8) hydrophobic and chemically bonded to the surface of a silica support particle. The mobile phase is water, buffer and an organic solvent such as methanol and acetonitrile. Nonpolar compounds slowly elutete when retention time is increased. The separation mechanism of RPLC is based on partition. RPLC is widely applied for neutral polar and nonpolar compounds with molecular weights below 2000 Daltons, weak acids and bases, proteins and peptides.

2.1.2.3 Hydrophilic interaction liquid chromatography (HILIC)

HILIC or aqueous normal phase chromatography is a combination of NPLC and RPLC modes. The stationary phase is a polar bond phase such as simple unbound silica, diol, amino, amide, cation, anion and zwitterionic

bonded phase. The mobile phase includes water, buffer with a small amount of water and an organic solvent such as acetonitrile and solvents miscible with water (methanol). Polar compound slowly elute when retention time is increased. The separation mechanism of HILIC is partition of analytes between the bulk mobile phase and the water-enriched layer which hydrates the hydrophilic stationary phase or electrostatic (ionic) interaction. Samples contain polar and hydrophilic compounds.

2.1.2.4 Ion chromatography (IC)

Analyte have ionic or polar molecules. Stationary phase of IC mode is a strong cation exchanger (SCX; sulfonates), a strong anion exchanger (SAX; quaternary ammonium), a weak cation exchanger (WCX; carboxymethyl) or a weak anion exchanger (WAX; diethylaminoethyl). Mobile phase is a buffer and counterion. The separation mechanism of IC is based on ion exchange or reversible binding of sample ions to charged support.

2.1.2.5 Size exclusion chromatography (SEC)

For size exclusion chromatography or gel permeation chromatography (GPC) or gel filtration chromatography (GFC), stationary phase is cross-linked gels with a defined pore size. Mobile phase of GPC is an aqueous solvent and of GFC is an organic solvent. The separation mechanism is based on molecular size. Main application is analysis of proteins, peptides, enzymes, lipids, nucleic acids, cosmetic products, rubber and printing products.

2.2 Mass Spectrometry (MS) [22,25, 27-28]

Mass spectrometry is a powerful technique for identification of unknown compounds. This technique separates molecules or ionized atoms by measuring their differences in mass to charge ratios (m/z). MS can be applied for quantitative and qualitative analysis or identification and characterization of compounds. Application is analysis of proteomics (proteins), pharmacokinetics, bioanalysis, forensic science, agrochemistry, food analysis, petrochemistry and cosmetics. The sample molecules

are ionized into gas phase ions at an ion source and then accelerated into a mass analyzer. The ions are measured at a detector and results are processed at the data system. The MS system is operated under a vacuum system with low pressure. The MS system consists of sample introduction, an ion source, a mass analyzer and a detector and a diagram is shown in Figure 2.4.

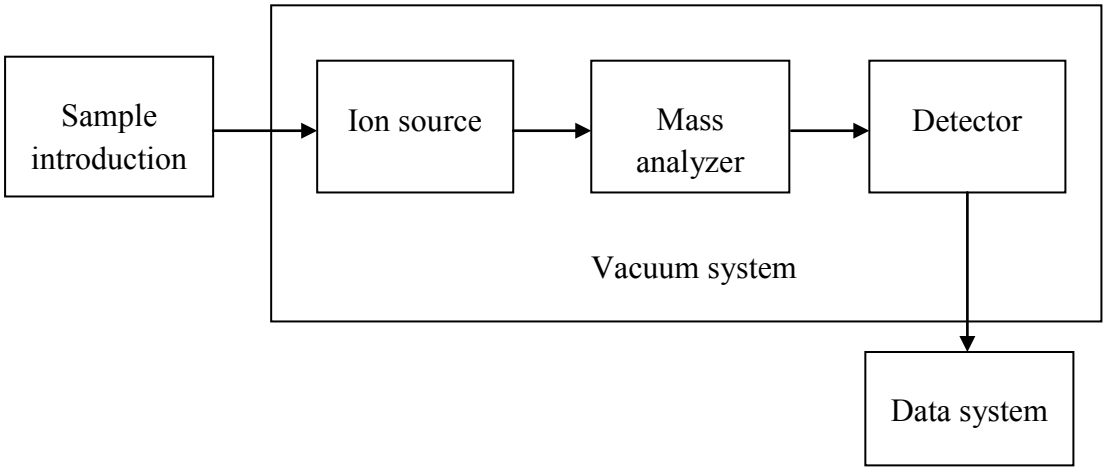


Figure 2.4 A schematic diagram of a typical MS instrument.

2.2.1 Sample introduction

Sample introduction is operated at atmospheric pressure. An inlet device loads samples into a chamber, depanding on the type of sample. The characteristics of samples are volatility and thermal stability. Gas samples are introduced directly into an ion source using a septum inlet or a GC inlet. Liquid and solid samples are heated to be volatile compounds.

2.2.2 Ion source

Samples are ionized at atmospheric or vacuum pressure. The samples are passed through an ion source to be gas phase ions. The ionization procedure depends on the type of samples such as organic (volatile and thermally stable organic molecules), non-volatile or thermally unstable organic compounds and macromolecules and inorganic compounds in which different samples have different ionization methods. Ionization methods include electron impact ionization (EI),

chemical ionization (CI), atmospheric pressure chemical ionization (APCI), fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI), thermospray ionization (TSI), electrospray ionization (ESI) and thermal ionization (TI). Ion sources which have been popular in a hyphenated technique (LC-MS) are APCI and ESI.

2.2.2.1 Atmospheric pressure chemical ionization (APCI)

APCI is chemical ionization at atmospheric pressure in which the sample is ambient air or mainly eluent from LC (flow rate of LC up to 2 mL/min). A sample is drawn (gas) or sprayed (liquid) through a heated probe with an inert nebulizing gas (N_2, He) into an ionization chamber, A high voltage potential from a corona discharge needle strips electrons out of solvent molecules (dominant species) turning them into reagent ions for CI reaction with analyte molecules. Ions are extracted through a small sample skimmer into MS for mass analysis. For samples from LC, the mobile phase easily becomes volatile and has a good pumping system. A schematic of an APCI source is shown in Figure 2.5.

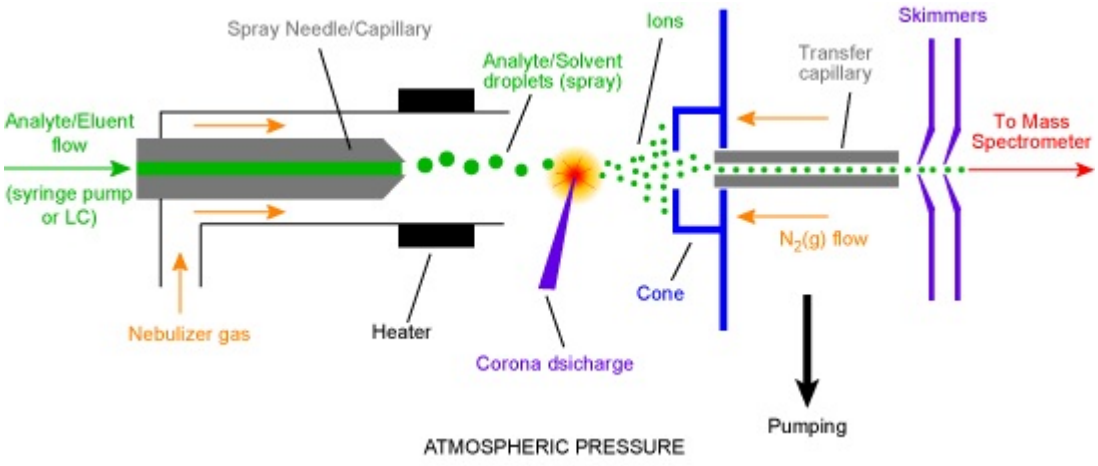


Figure 2.5 A schematic of the components of an APCI source [29].

2.2.2.2 Electrospray ionization (ESI)

ESI is a soft ionization technique whereby the samples with charge bearing potential include polar compounds, ionic molecules and compounds containing heteroatoms. The main process, ionization performed in atmospheric pressure and spraying is made via electrostatic interaction. The sample is dissolved in polar, volatile buffer and pumped through an electrospray capillary at a relatively low flow rate. Charged droplets are produced by a large electric field. Electron are stripped out of the sample molecule (positive mode) or by adding an electron to the sample molecule (negative mode) via an ES needle. Ionization mode is determined by polarity of the ES needle. Ions are preformed in the charged droplets in accordance with the characteristic of the dissolved analytes and chemistry of the sample solution. The system requires a different pumping system. A schematic of an ESI source is shown in Figure 2.6.

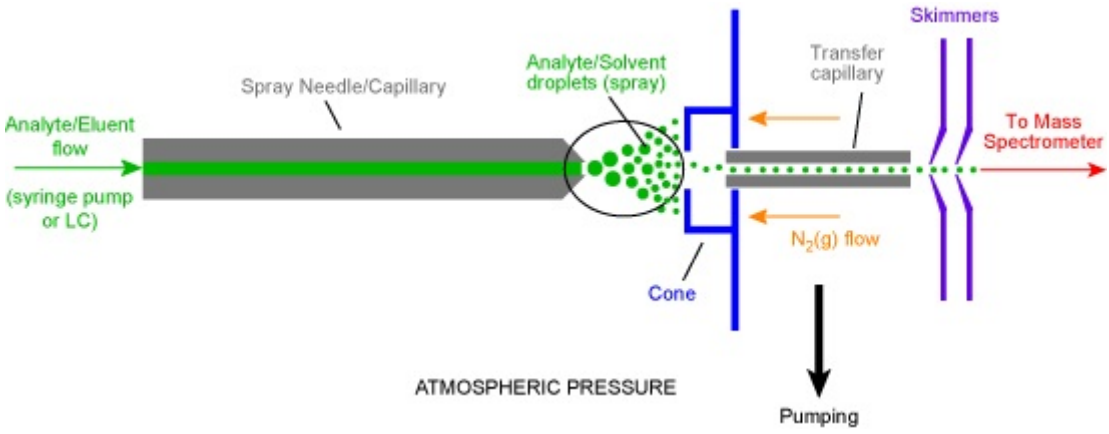


Figure 2.6 A schematic of the components of an ESI source [29].

2.2.3 Mass analyzer

Ionized atoms are separated or distinguished according to their differences in mass to charge ratios (m/z). The ions are formed in the ion source and then the ions are accelerated into the mass analyzer. Two types of mass analyzer are ion transmission and ion trapping systems. The ion transmission system includes

quadrupole, time of flight (TOF) and magnetic sector (double focusing). The ion trapping system includes quadrupole ion trap (QIT) and fourier transform ion cyclotron resonance (FT-ICR).

2.2.3.1 Quadrupole

This mass analyzer is most popular and widely used in various commercial systems. The detectable mass is limited in a range of 0-4,000 Daltons, so peaks above 4,000 are not well resolved with a low sensitivity that is not suitable for analysis of high mass ions such as macromolecules. Applications of quadrupole are analysis of organic molecules, inorganic elemental analysis and online detection for GC-MS and LC-MS. The configuration of quadrupole is four parallel rods with opposite pairs connected to DC (direct current) and RF-AC (radio frequency-alternating current) potentials of opposite polarity. Ions from the ion source enter the quadrupole (small accelerating voltage), experience the combined DC-RF field and move and transmit in complex trajectories (oscillation) through the rods. For giving DC and RF-AC amplitude, only ions of selected m/z (resonant ion) have stable oscillations and are transmitted, other non- resonant ions hit the rods and are destroyed (Figure 2.7).

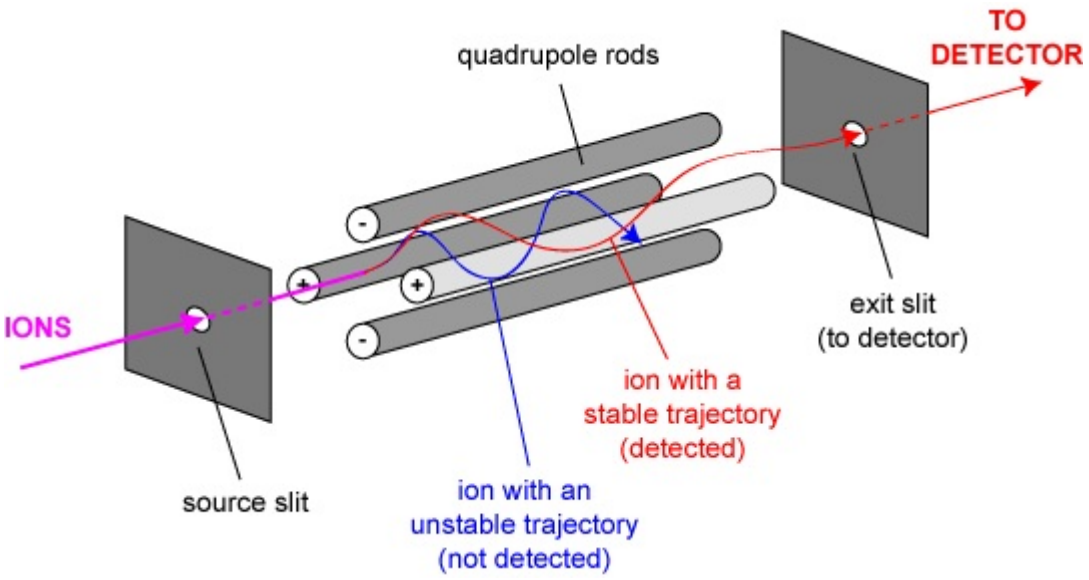


Figure 2.7 A schematic of a quadrupole mass analyzer [29].

2.2.3.2 Time of flight (TOF)

TOF is widely used in analysis of biomolecules and macromolecules with a very large m/z (up to > 500,000 Daltons). The operating principle is to accelerate ions under an electric field, followed by the free movement of ions in a field free flight tube. Ions of different masses (and different velocities) are separated by their travelling times in the flight tube.

2.2.3.3 Magnetic sector

A magnetic sector mass analyzer has two focusing instruments including single and double focusing instrument. With the single focusing instrument, ions of different m/z get separated under an applied magnetic field. The double focusing instrument is a high resolution mass spectrometer that combines magnetic and electrostatic fields (double focusing action). There are two types of double focusing instrument, which are a mass magnetic sector mass analyzer (mass focusing) and electrostatic analyzer (energy focusing) shown in Figure 2.8. The application of double focusing instrument is to determine elemental formula of questionable ions and limited detectable mass range (max ~10,000 Daltons at 10 kV accelerating voltage).

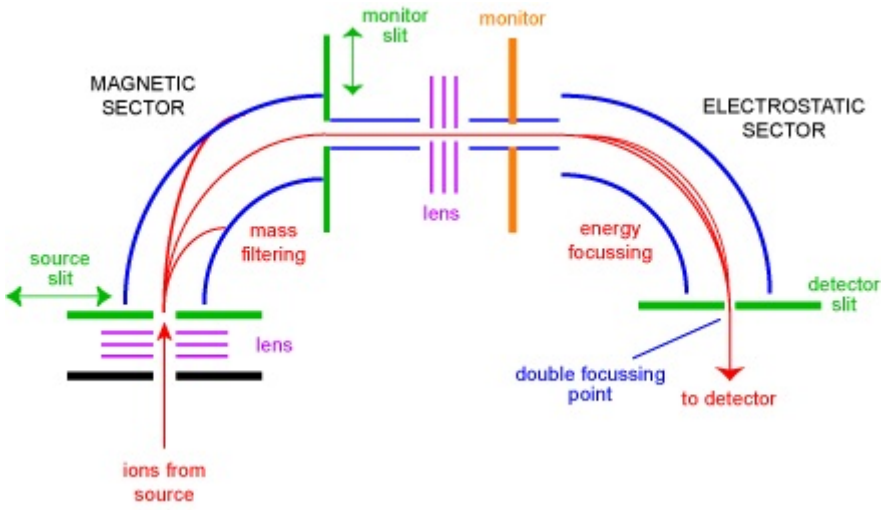


Figure 2.8 A schematic of a sector mass spectrometer [29].

2.2.3.4 Quadrupole ion trap (QIT)

An ion trapping device utilizes a three-dimensional quadrupole field to trap and analyze m/z of ions. An evacuated cavity comprises two end cap electrodes spaced by ring electrodes, connected to appropriate DC and RF potentials with ion injection and ion ejection orifices. Ions are either created inside the cell (by EI) or drawn in as a pulse from an external ion source. Ions are confined (stored) to the center of the QIT cell by application of an appropriate oscillating RF voltage on the ring electrodes. Ions are scanned (by ramping RF voltage) and ejected out to the detector selectively (Figure 2.9).

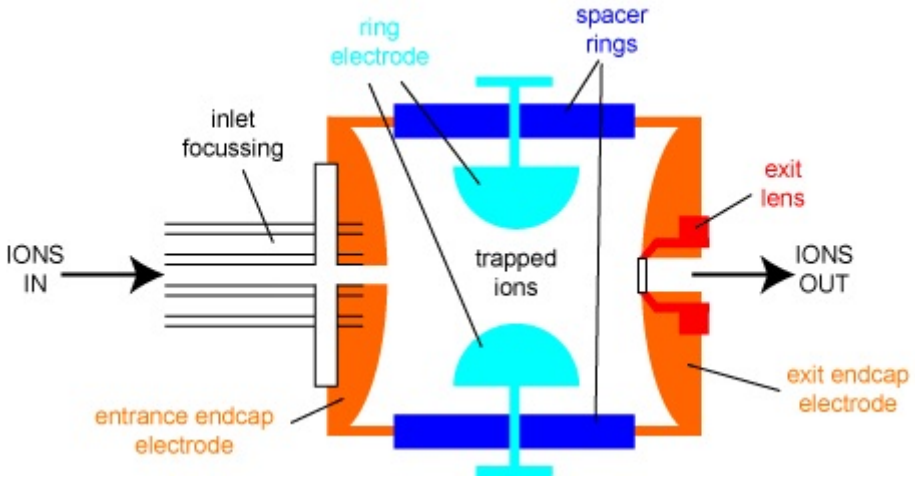


Figure 2.9 A schematic of a quadrupole ion trap mass analyzer [29].

2.2.3.5 Fourier transform ion cyclotron resonance (FT-ICR)

This trapping system is similar to the ion trap, but it uses different detection schemes. The operating principle is that ions are created inside the ICR cell or drawn in as a pulse from an external (continuous) ion source. Ions are trapped (stored) by an intense, constant magnetic field and voltage is applied in the ICR cell. The ions are then excited by an RF potential to circulate around inside the cell for detection (Figure 2.10).

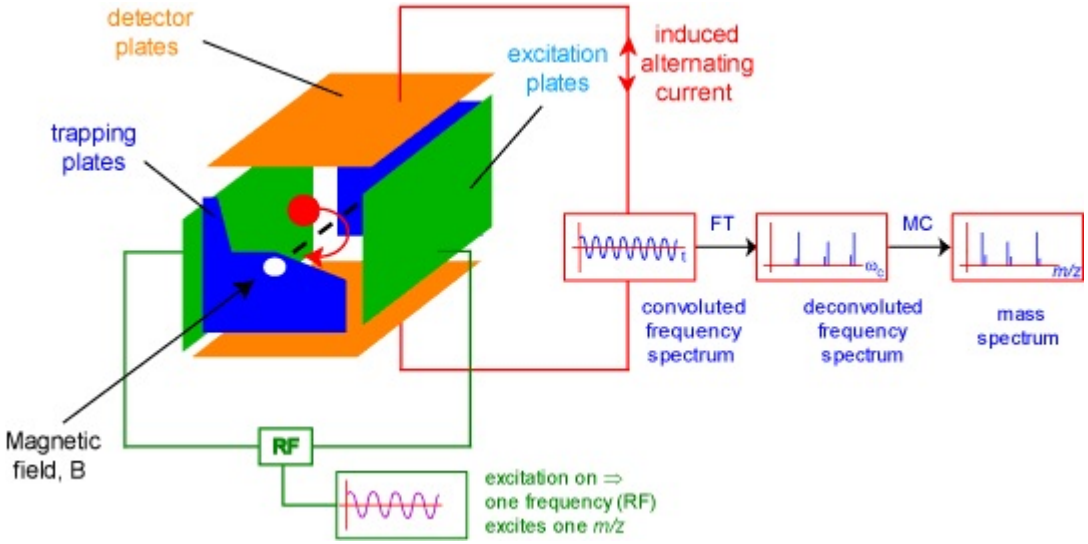


Figure 2.10 A schematic of FT-ICR [29].

2.2.4 Detector

A detector converts ions from the mass analyzer into electrical current or other forms of signal. Ion detectors are classified into no gain detectors vs. gain detectors or point detectors vs. array detectors. No gain detectors include a photographic detector and a faraday cup detector. Gain detectors include a daly detector (scintillator detector), channeltron electron multiplier (CEM) and discrete dynode electron multiplier.

2.2.4.1 Photographic detector

This detector is an array type detector and can produce simultaneous detection with high precision. The photographic detector has no amplification signal. Photographic film is mounted at the exit of the mass analyzer. Ions strike the plate coated with a sensitive material ($AgNO_3$) which creates permanent bands on the film. Recorded bands are optionally converted into current signals using a microdensitometer. The process has a slow processing time with a low resolution (limited space).

2.2.4.2 Faraday cup detector

This is a metal cup connected to an electrical measurement circuit. Ions impinge on the metal cup. The positive charges build up on the cup's surface and electrons are drawn from the measurement circuit to neutralize positive charges where the electrons (current) flow producing an electrical signal. Or the secondary electrons emitted upon ion strike whereby the electrons are captured by the cup wall and measured by the electrical measurement circuit (Figure 2.11). The detector is used as a multiple collector for simultaneous detection such as isotope ratio measurement with a magnetic sector MS.

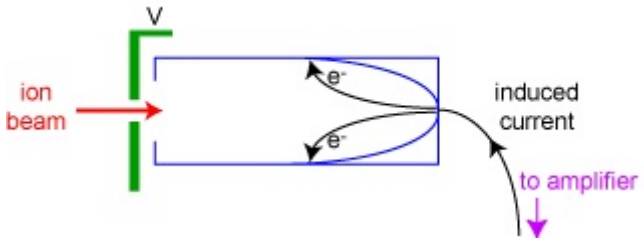


Figure 2.11 A schematic of a faraday cup [29].

2.2.4.3 Daly detector (scintillator detector)

The detector is an amplification signal which is operated at very low pressure ($<10^{-7}$ Torr). Ions from the mass analyzer hit the electrode which releases secondary electrons. Emitted electrons are accelerated onto the scintillator and emit photons (hν). The hν detecting is converted into an electrical signal and amplified by a conventional photomultiplier tube (PMT) in Figure 2.12.

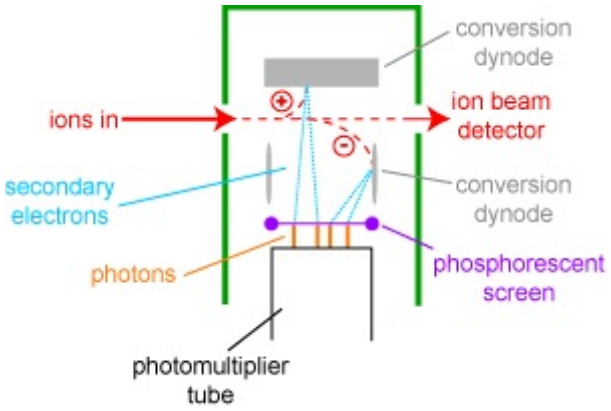


Figure 2.12 A schematic of a scintillator detector [29].

2.2.4.4 Channeltron electron multiplier (CEM)

The curved tube is made of glass or metal, with the inner interface of the tube coated with electron-emissive material (SiO_2, PbO_2) and biased at the entrance with a large potential of opposite charges to the analyzed ion. Ion entrance of detector, electrons are knocked out of the surface. The electrons accelerate to strike the next portion of the tube by electrostatic force that hits the wall and knocks off more electrons. Cascades of electrons are produced as the electron pack travels down towards the end of tube causing amplification of the electrical current (electrons). Pluses of the electron pack are counted by the electrical circuit and displayed as signal intensity in Figure 2.13. The detector has a limited lifetime due to the degraded surface.

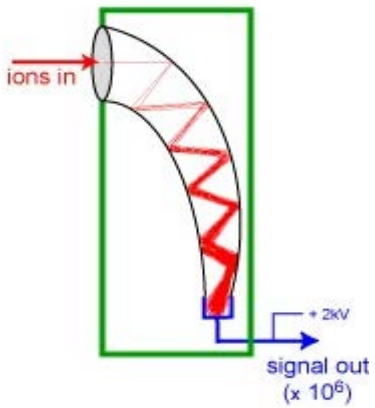


Figure 2.13 A schematic of a channeltron electron multiplier.

2.2.4.5 Discrete dynode electron multiplier

The operation is similar to a channeltron electron multiplier but this detector uses non-continuous electrodes (dynodes) for electron amplification. Cascading effect of the electron is the same as CEM but different in detecting. The detector has ability of measuring in both the analog mode for high ion density and digital mode for low ion density in Figure 2.14.

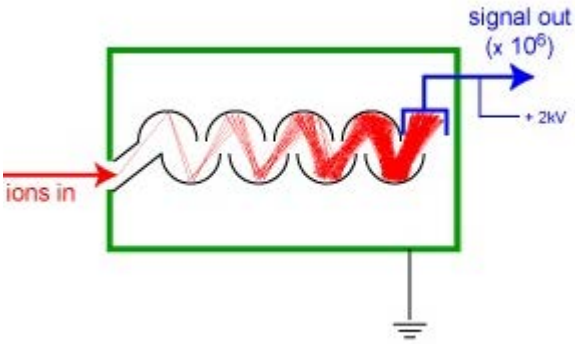


Figure 2.14 A schematic of a discrete dynode electron multiplier [29].

2.2.5 Vacuum system

The system is operated under vacuum system with low pressure (high vacuum). The two main pumps are a rotary vane pump (mechanical pump or rough pump) and a turbomolecular pump (turbo pump). The rotary vane pump is used mainly in the ion source to obtain immediate pressure (~10^{-4} up to 1 Torr) and a back up unit for the turbo pump. The turbomolecular pump is used to obtain high vacuum (<10^{-5} Torr) for mass analyzer and this pump has a high speed. MS system required a differential pumping system as multi pumps for pressure reduction.

2.2.6 Tandem mass spectrometry

Tandem mass spectrometry (MS-MS) is two or more MS systems coupled together for synchronized operation. The principle, molecular ions of all compounds

in mixture are made and separated in the 1st MS. Each molecular ion is fragmented or dissociated by collisional induced dissociation (CID). The fragment ions are detected or analyzed in the 2nd MS for identification or measurement of each component in the mixture in its scanning order. The CID uses of high energy, inert collision gas (He, N_2, Ar) to induce fragmentation of a selected parent ion (precursor ion).

Triple quadrupole (QQQ) is tandem mass spectrometry instrumentation whereby the ions get separated in space (tandem in space). The first quadrupole (Q1) is the selected parent ion. The second quadrupole (Q2), the process is called collisional induced dissociation (CID), is operated in the RF mode as an ion guiding system to transfer all ions and allow collision/fragmentation, no mass resolving action and multi devices such as hexapole or octapole are optionally used for better transportation efficiency. The third quadrupole (Q3) is to scan or measure fragmented ions in Figure 2.15. QQQ is most widely used hyphenated technology such as GC-MS/MS and LC-MS/MS.

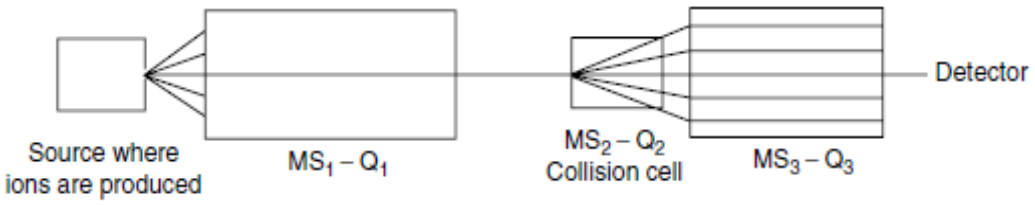


Figure 2.15 A schematic of a triple quadrupole mass spectrometer [25].

Multi-sector and hybrid tandem mass spectrometry are various combinations of multiple mass analyzer including Q-TOF, TOF-Q, TOF-TOF.

Quadrupole-time of flight, the ions get separated in space (tandem in space) as shown in Figure 2.16.

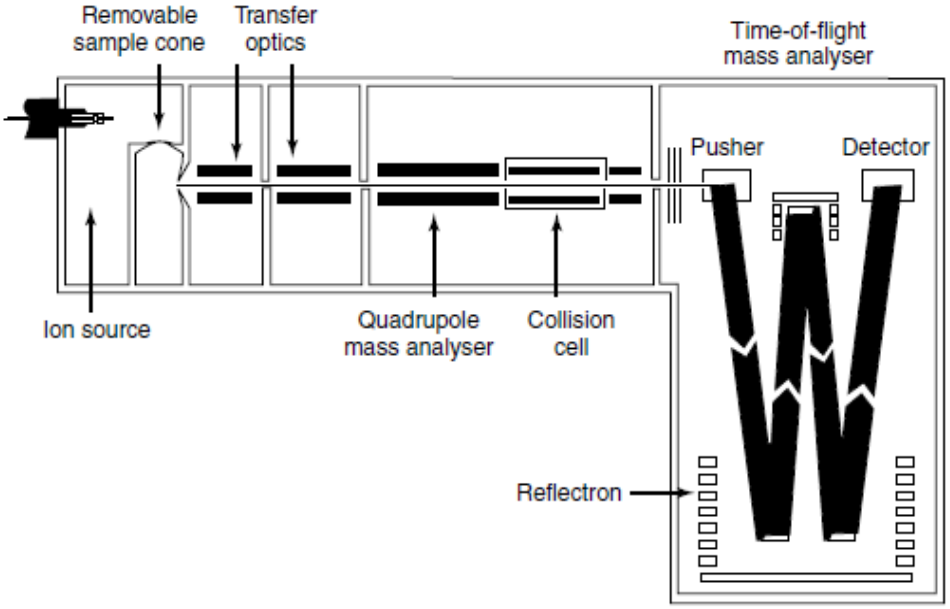


Figure 2.16 A schematic of a quadrupole-time of flight mass spectrometer [25].

Quadrupole ion trap is the ability to do tandem MS (MS^n) without additional equipment (standalone tandem MS). A quadrupole ion trap is tandem mass spectrometry instrumentation whereby the ions get separated in time (tandem in time). In step 1, the process creates parent ions by soft ionization and then in step 2, QIT traps only selected ions and rejects other ions. In step 3, collision gas is added into QIT cell and RF tickle is applied to end caps to induce axial motion of selected ions. In step 4, this tandem MS scan or measure fragmented ions for analysis of selected component. After that in the step 5, the process trap selected daughter ion for more tandem analysis (repeat step 2-4). QIT has a higher sensitivity than QQQ because all ions are trapped during the collision process.

2.3 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Liquid chromatography-tandem mass spectrometry is a hyphenated technique whereby the mixture is separated according to chemical characteristic by the

appropriate chromatography technique into pure compounds. Each individual component is detected or analyzed by mass spectrometry. The technique has very high sensitivity and selectivity for many applications.

CHAPTER III

EXPERIMENTAL

3.1 Instrument and Apparatus

3.1.1 Liquid chromatography (LC): Agilent 1290 Infinity LC consists of vacuum degassing, binary pump, agilent jet weaver, autosampler and column oven, Agilent Technologies, CA, U.S.A.

3.1.2 Mass spectrometry detector (MSD): 6490 triple quadrupole mass spectrometer with an eletrospray ionization (ESI) interface and MassHunter software processing, Agilent Technologies, CA, U.S.A.

3.1.3 Milli-Q, Ultrapure water system, with Millipak® 40 Filter unit 0.22 µm, model Millipore ZMQS5VOOY, Millipore, Billerica, MA, U.S.A.

3.1.4 LC column: ZIC®-HILIC, 2.1 x 100 mm, 3.5 µm, SeQuant ™, Merck, Darmstadt, Germany.

3.1.5 LC column: Zorbax RRHD Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm, Agilent Technologies, CA, U.S.A.

3.1.6 LC column: Bio-rad Aminex® HPX-87P 7.8 x 300 mm, 9 µm, Bio-Rad laboratories, California, U.S.A.

3.1.7 LC column: Dionex IonPac™ AS11 2 x 250 mm, 13 µm with a guard column Dionex IonPac™ AG11 2 x 50 mm, Thermo Scientific, Sunnyvale, CA.

3.1.8 Vacuum pump with pressure regulator, Model 9499241M002, Varian, Leini, Torino, Italy.

3.1.9 Nitrogen gas generator, Model MINIGAS2 NCBLN, Parker Hannifin Itd., Dukesway, England.

3.1.10 Air compressor for nitrogen gas generator, Model PO-3.7MA5, Hitachi, Tokyo, Japan.

3.1.11 Nitrogen gas, ultra high purity grade (99.999% purity), Thai special gas CO., LTD, Bangkok, Thailand.

3.1.12 A glass filter holder set (300 mL funnel, 1 L flask, glass base with tube cap, and 47 mm spring clamp) for LC mobile phase filtration, Millipore, Billerica, MA, U.S.A.

3.1.13 Air pump, Model DOA-P504-BN, Gast®, Michigan, U.S.A.

3.1.14 Vortex mixer, Model G-560E, Scientific Industries, Bohemia, New York, U.S.A.

3.1.15 Centrifuge, Model Sorvall ST16R, Thermo Scientific, Sunnyvale, CA.

3.1.16 Analytical balance (5 digits), Model XS105 dualrange, Mettler-Toledo, Inc., OH, U.S.A.

3.1.17 pH meter, Model 744, Metrohm, Herisau, Switzerland.

3.1.18 Micropipettes 0.5-10 µL, 10-100 µL, 100-1,000 µL and tips, Eppendorf, Hamburg, Germany.

3.1.19 Nylon membrane filters 47 mm, 0.2 µm, Whatman International Ltd., Maidstone, England.

3.1.20 Nylon syringe filter, 13 mm, 0.2 µm, Chrom Tech, MN, U.S.A.

3.1.21 PP short thread micro-vials 0.3 mL, La-Pha-Pack®, GmbH, Germany.

3.1.22 PP cap silicone white/ PTFE red, La-Pha-Pack®, GmbH, Germany.

3.1.23 Polypropylene tube 50 mL

3.1.24 Beaker 10, 25, 50 mL

3.1.25 Graduated cylinder 10.0, 25.0 mL.

3.1.26 Spatulas

3.1.27 Dropper

All experimental glasswares were cleaned with detergents and then rinsed with deionized water.

3.2 Chemicals

3.2.1 Standard compounds

Glyphosate (96%) and AMPA (99%) reference standards were purchased from Aldrich (WI, USA). Glufosinate-ammonium (97.5%) and glufosinate-*N*-acetyl (98%) were obtained from Dr Ehrenstorfer (Augsburg, Germany). 3-MPPA was purchased from Fluka (Buchs, Switzerland).

3.2.2 Organic solvents and other chemicals

Methanol (MeOH) and acetonitrile (MeCN) were LC/MS grade from J.T. Baker (Deventer, The Netherlands). Ammonium acetate and ammonium hydroxide (NH_4OH) were analytical grade from J.T. Baker. Ammonium formate, citric acid monohydrate, triethylamine (TEA) and dimethylamine (DMA) were analytical grade from Fluka. Sodium hydroxide (NaOH) was analytical grade from Carlo Erba Reagents (Rodano, Italy). Acetic acid and formic acid were analytical grade from Merck (Damstadt, Germany) and Fisher Scientific (Leicestershire, UK), respectively. Water was purified from Milli-Q purification system (Millipore, Billerica, MA, USA) at 18.2 MΩ /cm resistivity.

3.3 Preparation of Standard Solutions

3.3.1 Preparation of stock standard solutions

Stock standard solutions of individual analyte at the final concentration of approximately 100 mg/L were prepared in Milli-Q water. Individual standard solution was prepared by weighing 1.0 mg of each standard and dissolved in 10.00 mL Milli-Q water. All stock standard solutions were stored in plastic bottles at 4 °C in a refrigerator.

3.3.2 Preparation of intermediate standard solutions

An intermediate mixture standard solution at 10 mg/L was prepared by pipetting 20 µL of each stock standard solution (section 3.3.1) and adjusted the volume to 200 µL with MeCN for ZIC-HILIC column analysis, MeOH for Zorbax RRHD Eclipse Plus C18 and Dionex IonPac AS11 column analysis and Milli-Q water for Bio-rad HPX-87P column analysis, in a plastic vial. These standards were prepared daily and stored at 4 °C in a refrigerator.

3.3.3 Preparation of working standard solutions

3.3.3.1 ZIC-HILIC® column analysis (HILIC mode)

The working standard solution at 1 mg/L was prepared by pipetting 25 µL of intermediate standard solution (section 3.3.2) and made volume to 250 µL with MeCN in a plastic vial. These standards were prepared daily and stored at 4 °C in a refrigerator.

3.3.3.2 Zorbax RRHD Eclipse Plus C18 column analysis (RPLC mode)

The working standard solution at 1 mg/L was prepared by pipetting 25 µL of intermediate standard solution (section 3.3.2) and made volume to 250 µL with 0.2 M formic acid in water in a plastic vial. These standards were prepared daily and stored at 4 °C in the refrigerator.

3.3.3.3 Bio-rad Aminex® HPX-87P column analysis (IC mode)

The working standard solution at 1 mg/L was prepared by pipetting 25 µL of intermediate standard solution (section 3.3.2) and made volume to 250 µL with Milli-Q water in a plastic vial. These standards were prepared daily and stored at 4 °C in the refrigerator.

3.3.3.4 Dionex IonPac™ AS11 column analysis (IC mode)

The working standard solution at 1 mg/L was prepared by pipetting 25 µL of the intermediate standard solution (section 3.3.2) and made volume to 250 µL with MeOH in a plastic vial. These standards were prepared daily and stored at 4 °C in the refrigerator.

3.3.4 The standard solution for tuning

An individual standard solution at 10 mg/L was prepared by pipetting 20 µL of each stock standard solution (section 3.3.1) and made volume to 200 µL with MeOH in a plastic vial. These standards were prepared daily and stored at 4 °C in the refrigerator.

3.4 The Optimum Instrumental Analysis Conditions

LC-MS/MS was supported by Agilent Technologies. A 1290 Infinity series separation module interfaced with an electrospray ionization 6490 triple quadrupole mass spectrometer was operated. The data was acquired and processed using the MassHunter software.

3.4.1 MS/MS optimization

6490 Triple quadrupole mass spectrometer is used *via* ionization interface of electrospray (ESI) in the positive (ESI+) and negative modes (ESI-). Parameters were optimized in each separation mode (HILIC, RPLC and IC mode) such as precursor ion, product ion, collision energy (CE), cell accelerator energy (CAV),

capillary voltage, nozzle voltage, gas temperature, gas flow, nebulizer pressure, sheath gas temperature and sheath gas flow.

Each standard tuning solution (section 3.3.4) was directly injected into the ESI source. Multiple reactions monitoring mode (MRM) with the two most sensitive transitions were performed for quantification and qualification of analysis.

3.4.2 LC optimization

The LC conditions for separation of a mixture of 5 standard solutions were developed in HILIC, RPLC and IC modes. In each separation mode type, concentration and pH of mobile phase were optimized.

3.4.2.1 HILIC mode

A ZIC®-HILIC column (2.1 x 100 mm, 3.5 μm) was used to separate mixture standard solutions. Parameters used in this study were as follows:

3.4.2.1.1 Types and pH of mobile phase

- Ammonium acetate (mobile phase A) at 5, 10, 15, and 20 mM and pH 3, 4, 5, 6, 7 and 8
- Ammonium formate (mobile phase A) at 5, 10, 15, and 20 mM and pH 3, 4, 5, 6, 7 and 8
- 10 mM ammonium acetate (mobile phase A)-MeCN (mobile phase B) added a modifier solution in the mobile phase A, B and A-B

3.4.2.1.2 Types of solvent for diluting prior to LC analysis

- MeCN
- Water
- MeCN: water (1: 1)

- 10 mM Ammonium acetate in water
- 0.05, 0.1, 0.5% Acetic acid in MeCN
- 0.01, 0.05, 0.1, 0.5, 1% DMA in MeCN
- 0.5, 1 mM NH_4OH in MeCN
- 0.1% TEA in MeCN

3.4.2.2 RPLC mode

A Zorbax RRHD Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm) was used to separate mixture standard solutions. Parameters used in this study were as follows:

3.4.2.2.1 Types of mobile phase

- 10 mM Ammonium acetate (mobile phase A)
- 10 mM Ammonium formate (mobile phase A)
- 0.1% Formic acid in water (mobile phase A)
- 0.2 M Formic acid in water (mobile phase A)
- MeCN (mobile phase B)
- MeOH (mobile phase B)

3.4.2.3 IC mode

3.4.2.3.1 Bio-rad Aminex® HPX-87P column

A Bio-rad Aminex® HPX-87P column (7.8 x 300 mm, 9 µm) was used to separate mixture standard solutions. Parameters used in this study were as follows:

3.4.2.3.1.1 Types of mobile phase

- Water
- MeCN

3.4.2.3.2 Dionex IonPac™ AS11

A Dionex IonPac™ AS11 (2 x 250 mm, 13 μm) was used to separate mixture standard solutions. Parameters used in this study were as follows:

3.4.2.3.2.1 pH of mobile phase

- pH 9.6, 10.5 and 11.0 of 1 mM citric acid (mobile phase B)

3.4.2.3.2.2 Types of solvent for diluting prior to LC analysis

- Water
- MeOH
- MeCN
- 0.1 % formic acid in MeOH
- 0. 1 % formic acid in MeCN

3.5 Extraction Method Optimization

Sample preparation was developed for simultaneous determination of glyphosate, glufosinate, and their metabolites using a procedure that is simple, fast and with no derivatization step. A liquid extraction was used for rice samples. The sample extracts were analyzed by LC-MS/MS (for optimization in section 3.4).

3.5.1 The procedure of extraction solvent and volume optimization

The experiments studied types and volume of solvents in the extraction process as follows:

3.5.1 .1 A blank sample was prepared by weighing 5.00 g of homogenized rice into a polypropylene tube.

3.5.1.2 5 mL of water and 5 mL of MeOH were added.

3.5.1.3 The mixture solution was shaken by hand for 1 min and votexed for 1 min.

3.5.1.4 The mixture solution was centrifuged at 5000 rpm for 10 min at 25 °C.

3.5.1.5 The sample solution was filtered through a 0.2 μm nylon syringe filter prior to LC-MS/MS analysis.

3.5.1.6 A spiked sample was prepared by adding 1 mg/L mixture standard solution into a 5.00 g rice sample.

3.5.1.7 Each spiked sample in step 3.5.1.6 was extracted by following the same procedure described in step 3.5.1.2- 3.5.1.5

The black highlights refer to varied parameters.

3.6 Method Validation [30-33]

Reliability of the method was evaluated using standard calibration curve, matrix-matched calibration curve, matrix effect, linearity, accuracy, precision and lowest calibration level.

3.6.1 Standard calibration curve

Standard calibration curves were prepared using mixture standard solutions. The concentration ranges of each compound were 0.05-0.5 mg/L. Each level was prepared and analyzed in three replicates.

3.6.2 Matrix-matched calibration curve

Matrix-matched calibration curves were prepared using the extraction procedure in section 3.5 (3.5.1.1-3.5.1.7). The concentration ranges of each compound were studied in the same range of the standard calibration curves for study of the matrix effect. Each level was prepared and analyzed in three replicates.

3.6.3 Matrix effect

Comparison of standard calibration curve and matrix-matched calibration curves was studied for the matrix effect and used *t*-test for comparison.

3.6.4 Linearity

Linearity of the method was prepared using the extraction procedure in section 3.5 (3.5.1.1-3.5.1.7). The concentration ranges of each compound were 0.05-2.8 mg/kg with three replicates of each level. The linearity shows the relationship of peak area and concentration. The slope, y-intercept and correlation coefficient (R^2) represents the linearity.

3.6.5 Accuracy

Accuracy refers to closeness between analysis values and truth values. The percentage of recovery was reported for accuracy. The concentrations of spiking level at MRL, 5-MRL and 10-MRL (0.1, 0.5 and 1 mg/L) were studied through the

extraction procedure in section 3.5 (3.5.1.1-3.5.1.7). Each level was prepared in 10 replicates.

3.6.6 Precision

The precision refers to closeness among independent test results obtained under the same conditions. Two divisions of precision are intra-assay precision and intermediate precision. The intra-assay precision uses the same method to repeat analysis on the same day, whereas the intermediate precision uses the same method but repeats analysis on different days. The percentage of relative standard deviations (%RSD) was reported for intra-assay precision and intermediate precision. The concentrations of spiking level at MRL, 5-MRL and 10-MRL (0.1, 0.5 and 1 mg/L) were studied through the extraction procedure in section 3.5 (3.5.1.1-3.5.1.7) on three different days. Each level was prepared in 10 replicates.

3.6.7 Lowest calibration level (LCL)

The lowest calibration level is the lowest concentration of analyte detectable and measured using the calibration curve method.

3.7 Application to Real Samples

The developed method was evaluated for analysis of real rice samples. Seven rice samples were taken from local fresh markets and supermarkets and analyzed using the optimized method.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The Optimization of MS/MS Conditions

Parameters of MS/MS were optimized by considering maximum sensitivity. For manual tuning, standard solution was injected directly into the ion source (ESI) using a full scan mode. The manual tuning studied both negative and positive modes and selected mode with the highest sensitivity. The full scan mode was optimized for identification of a precursor ion ($[M-H]^-$ or $[M+H]^+$). Then a precursor ion induced fragmentation using collision energy, which is called collision-induced dissociation (CID). The two of product ions were from CID. The two most sensitivity transitions were performed for quantification and qualification of analysis. After that, the precursor ion and product ion were presented then the mode of analysis was set up to MRM. Parameters were optimized in each separation mode (HILIC, RPLC and IC mode) such as CAV, CE, nozzle voltage, gas temperature, gas flow, nebulizer pressure, sheath gas temperature and sheath gas flow.

4.1.1 HILIC mode

The MS instrument was performed by electrospray ionization in a positive mode (ESI+) for glufosinate and a negative mode (ESI-) for glyphosate, AMPA, *N*-acetyl glufosinate and 3-MPPA. The MS conditions used in this study were as follows: capillary voltage of +1500 V and -1000 V, nozzle voltage of 0 V, gas temperature of 200 °C, gas flow of 19 L/min, nebulizer pressure of 50 psi, sheath gas temperature of 400°C, sheath gas flow of 12 L/min, fragmentor of 380 V and dwell time 100 ms for each MRM transition. The optimizations of CE and CAV for each compound are shown in Table 4.1.

Table 4.1 Molecular weights (M.W.), ion transition and optimized MS/MS parameters in HILIC mode.

Compounds	M.W.	Quantification Transition (m/z)	CE (V)	CAV (V)	Qualification Transition (m/z)	CE (V)	CAV (V)
glyphosate	169.07	168.1 > 149.9	8	3	168.1 > 63.0	23	3
glufosinate	181.13	182.1 > 136.0	11	5	182.1 > 56.1	29	5
AMPA	111.04	109.9 > 62.9	20	3	109.9 > 79.0	29	3
N-acetyl glufosinate	223.16	222.1 > 59.0	5	5	222.1 > 136.1	5	5
3-MPPA	152.09	151.1 > 133.0	8	3	151.1 > 107.0	14	3

4.1.2 RPLC mode

The MS instrument was performed by electrospray ionization in positive mode (ESI+) for glufosinate and negative mode (ESI-) for glyphosate, AMPA, *N*-acetyl glufosinate and 3-MPPA. The MS conditions used in this study were as follows: capillary voltage of ±4000 V, nozzle voltage of ±500 V, gas temperature of 150 °C, gas flow of 19 L/min, nebulizer pressure of 50 psi, sheath gas temperature of 400 °C, sheath gas flow of 12 L/min, fragmentor of 380 V and dwell time 50 ms for each MRM transition. The optimizations of CE and CAV for each compound are shown in Table 4.2.

Table 4.2 Molecular weights (M.W.), ion transition and optimized MS/MS parameters in RPLC mode.

Compounds	M.W.	Quantification Transition (m/z)	CE (V)	CAV (V)	Qualification Transition (m/z)	CE (V)	CAV (V)
glyphosate	169.07	168.1 > 149.9	8	3	168.1 > 63.0	23	3
glufosinate	181.13	182.1 >56.1	29	5	182.1 > 136.0	11	5
AMPA	111.04	109.9 > 62.9	20	3	109.9 > 79.0	29	3
N-acetyl glufosinate	223.16	222.1 > 136.1	5	5	222.1 > 59.0	5	5
3-MPPA	152.09	151.1 > 133.0	8	3	151.1 > 107.0	14	3

4.1.3 IC mode

4.1.3.1 Bio-rad Aminex® HPX-87P column

The MS instrument was performed by electrospray ionization in negative mode (ESI-) for all compounds. The MS conditions used in this study were as follows: capillary voltage of -1000 V, nozzle voltage of 0 V, gas temperature of 200 °C, gas flow of 19 L/min, nebulizer pressure of 50 psi, sheath gas temperature of 400 °C, sheath gas flow of 12 L/min, fragmentor of 380 V and dwell time 100 ms for each MRM transition. The optimizations of CE and CAV for each compound are shown in Table 4.3.

Table 4.3 Molecular weights (M.W.), ion transition and optimized MS/MS parameters in IC mode on Bio-rad Aminex® HPX-87P column.

Compounds	M.W.	Quantification Transition (m/z)	CE (V)	CAV (V)	Qualification Transition (m/z)	CE (V)	CAV (V)
glyphosate	169.07	168.1 > 149.9	8	3	168.1 > 63.0	23	3
glufosinate	181.13	180.1 > 136.1	14	5	180.1 > 85.1	17	5
AMPA	111.04	109.9 > 79.0	29	3	109.9 > 62.9	20	3
N-acetyl glufosinate	223.16	222.1 > 59.0	5	5	222.1 > 136.1	5	5
3-MPPA	152.09	151.1 > 133.0	8	3	151.1 > 107.0	14	3

4.1.3.2 Dionex IonPac™ AS 11

The MS instrument was performed by electrospray ionization in negative mode (ESI-) for all compounds. The MS conditions used in this study were as follows: capillary voltage of -4000 V, nozzle voltage of -500 V, gas temperature of 200 °C, gas flow of 17 L/min, nebulizer pressure of 50 psi, sheath gas temperature of 400 °C, sheath gas flow of 12 L/min, fragmentor of 380 V and dwell time 100 ms for each MRM transition. The optimizations of CE and CAV for each compound are shown in Table 4.4.

Table 4.4 Molecular weights (M.W.), ion transition and optimized MS/MS parameters in IC mode on Dionex IonPac™ AS 11 column.

Compounds	M.W.	Quantification Transition (m/z)	CE (V)	CAV (V)	Qualification Transition (m/z)	CE (V)	CAV (V)
glyphosate	169.07	168.1 > 149.9	8	3	168.1 > 63.0	23	3
glufosinate	181.13	180.1 > 136.1	14	5	180.1 > 85.1	17	5
AMPA	111.04	109.9 > 62.9	20	3	109.9 > 79.0	29	3
N-acetyl glufosinate	223.16	222.1 > 59.0	5	5	222.1 > 136.1	5	5
3-MPPA	152.09	151.1 > 133.0	8	3	151.1 > 107.0	14	3

In each separation mode, there is a difference in the capillary voltage due to various types and ratios of the mobile phase. RPLC and IC modes use aqueous solution of the mobile phase more than the HILIC mode and each mode uses a different type of mobile phase, in which the polar structure of the analytes are different too. The value of the capillary voltage with the highest sensitivity was selected for the simultaneous determination of all compounds.

4.2 The Optimization of LC Conditions

The LC conditions were optimized for the separation of glyphosate, glufosinate and their metabolites (AMPA, N-acetyl glufosinate and 3-MPPA). A comparative study of various chromatographic separation modes such as hydrophilic interaction liquid chromatography (HILIC), reversed phase liquid chromatography (RPLC), and ion chromatography (IC) were studied. In each separation mode, type, concentration and pH of mobile phase and types of solvent for diluting prior to LC analysis were optimized. The shape of peak and the highest sensitivity were considered for LC optimization.

4.2.1 HILIC mode

The LC separation was performed on a ZIC®-HILIC column (2.1 x 100 mm, 3.5 µm) (SeQuant™ Merck, Darmstadt, Germany). The stationary phase is silica-based carrying a covalently bonded, permanently zwitterionic, functional group of sulfobetaine type. The column was kept at 35 °C. The injection volume was 10 µL and the flow rate of mobile phase was 0.3 mL/min. A mobile phase gradient program started at 70% B (held for 4.5 min), 20% B at 5 min (held for 5 min). The analysis time was 10 min and post time was 5 min. Parameters used in this study were as follows:

4.2.1.1 Types and pH of mobile phase

The mobile phase (MP) should be used a volatile and dissolve in mobile phase to prevent precipitation. MeCN was used as a mobile phase B due to its good miscibility with water, providing good HILIC retention and suitable viscosity. For mobile phase A, ammonium acetate and ammonium formate were chosen for optimization. Ammonium acetate was tested at approximately pH 3, 4, 5, 6, 7 and 8 with ion strengths of 5, 10, 15, and 20 mM, respectively. Ammonium acetate at pH 6 was not adjusted, but acidic pH was adjusted with acetic acid and basic pH was adjusted with NH_4OH. It was observed that the retention time increased with increasing ionic strength and the optimum sensitivity was observed at 10 mM. The mechanism of analytes and the stationary phase may be related to partitioning or electrostatic interaction or dipole-dipole interaction. The retention increase may be related to the hydrophilic partitioning on the stationary phase. The partitioning presents a water-enriched layer on the surface. If the mobile phase has a higher buffer (salt) concentration in the partitioning process, the higher salt concentration will drive more salt ions into the water-enriched layer. This result increases the concentration, leading to increased retention time.

For pH optimization of mobile phase, poor peak separation was observed at pH 3 and 4 for all compounds, except glufosinate at pH 3 and 4 and *N*-acetyl glufosinate at pH 4. The mobile phase at pH 6 had a higher sensitivity than pH 7 and 8. Optimum

separation was observed at pH 6, however, the shape of peaks was broad and tailing was observed in Table 4.5. The column is recommended to be in the range of pH 3 to 8 due to the optimum pH of the mobile studied covered the range. At pH 3 to 8, the analytes are zwitterions in which the stationary phase is the zwitterionic functional group of sulfobetaine type. The analytes might interact between the zwitterionic or anion functional group of the stationary phase due to tailing and broad peaks of analytes.

The ammonium formate was tested at pH 3, 4, 5, 6, 7 and 8 and at ion strength of 5, 10, 15, and 20 mM, respectively. Poor peak separation was observed at pH 3 and 4 for all compounds. The results were the same as the results obtained ammonium acetate, but the sensitivity is lower (Table 4.6). A modifier solution was then added to the 10 mM ammonium acetate (mobile phase A)-MeCN (mobile phase B). The mobile phase was studied such as mobile phase A (adding acetic acid)-MeCN, mobile phase A (adding 0.001% DMA)-MeCN, mobile phase A-MeCN (adding 0.001% DMA) and mobile phase A (adding 0.001% DMA)-MeCN (adding 0.001% DMA). Results showed that the separation was not improved when adding a modifier solution in Table 4.7.

Table 4.5 Chromatograms of standard solutions using varies pH of ammonium acetate analyzed on ZIC®-HILIC column in HILIC mode.

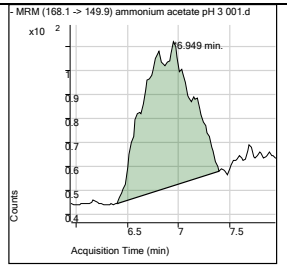
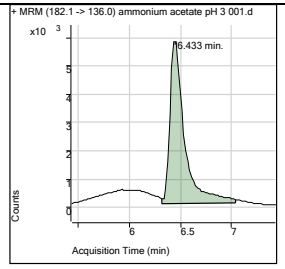
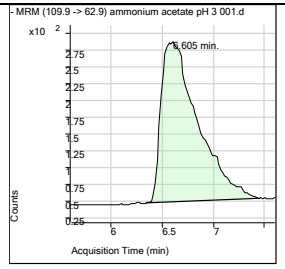
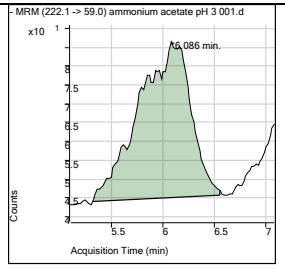
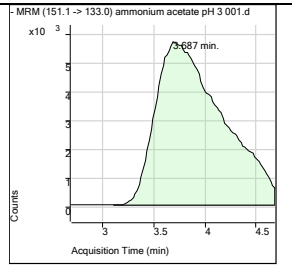
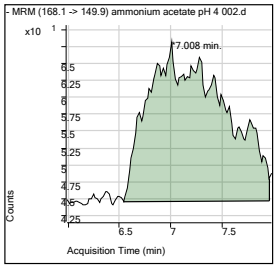
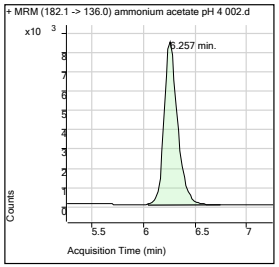
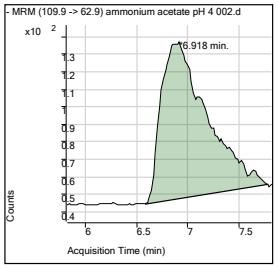
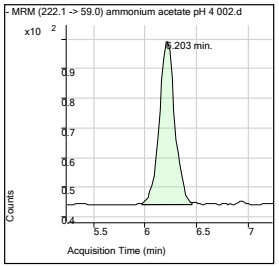
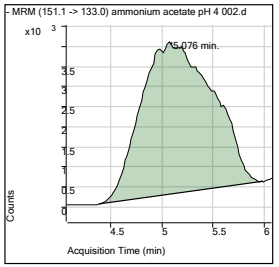
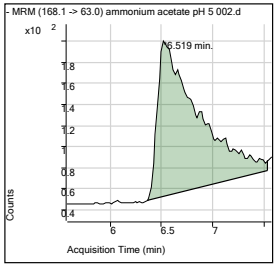
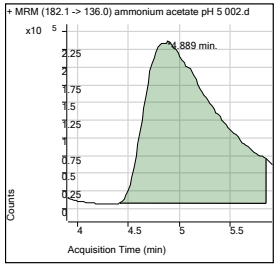
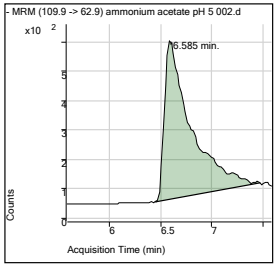
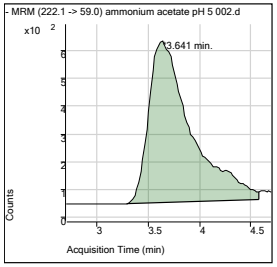
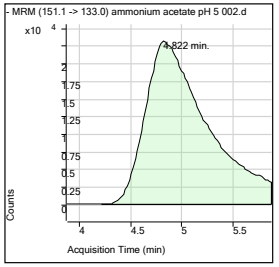
pH	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
3					
4					
5					

Table 4.5 (continued)

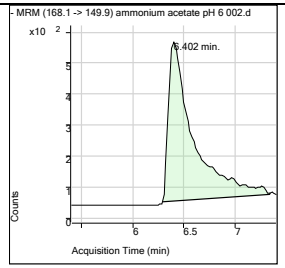
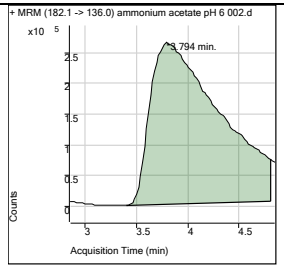
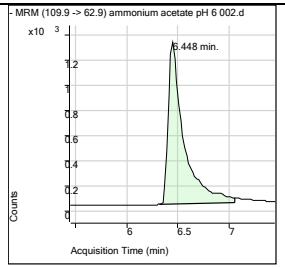
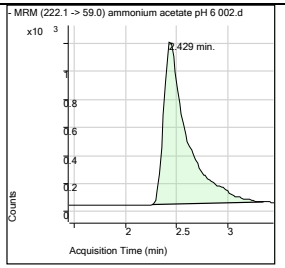
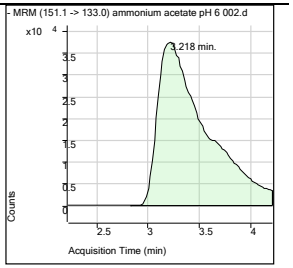
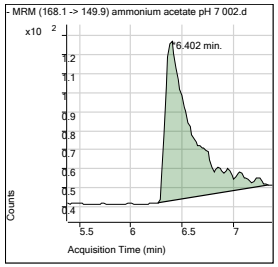
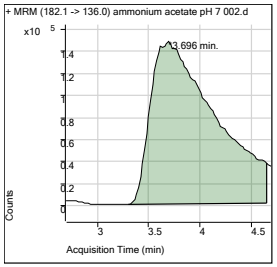
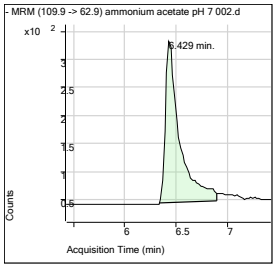
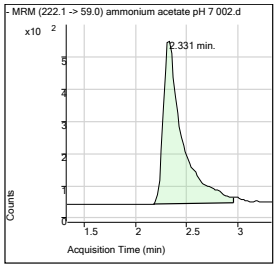
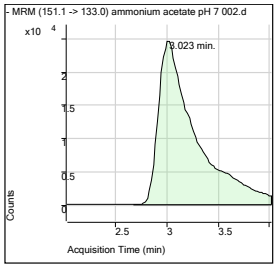
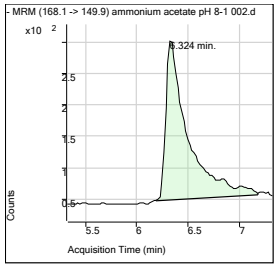
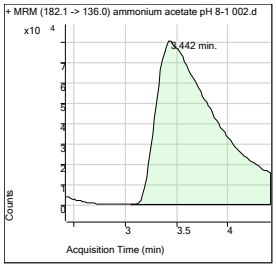
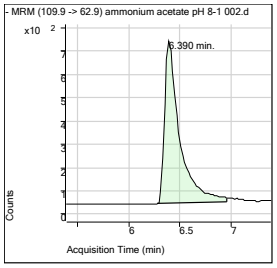
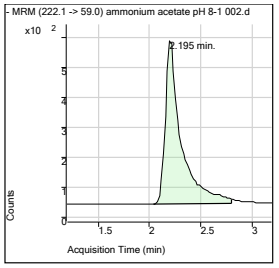
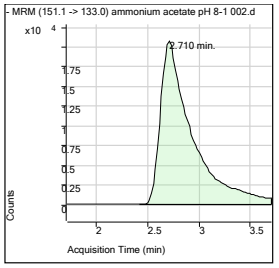
pH	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
6					
7					
8					

Table 4.6 Chromatograms of standard solutions using varies pH of ammonium formate analyzed on ZIC®-HILIC column in HILIC mode.

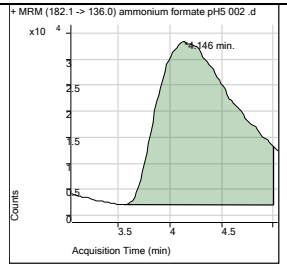
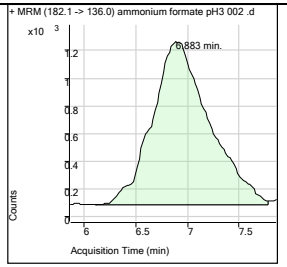
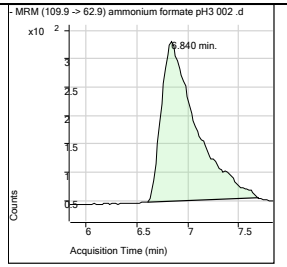
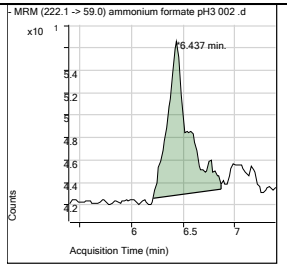
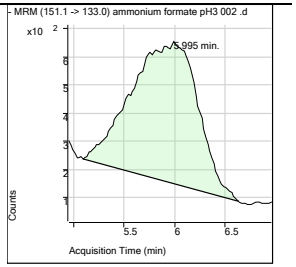
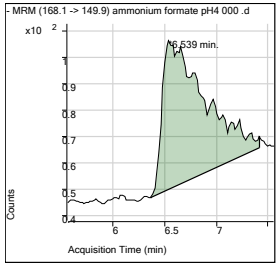
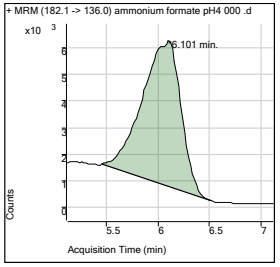
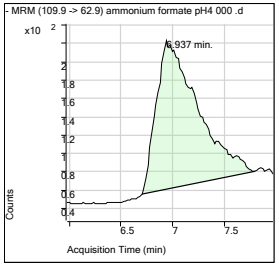
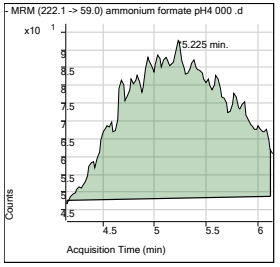
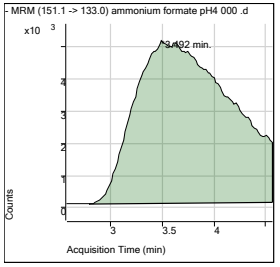
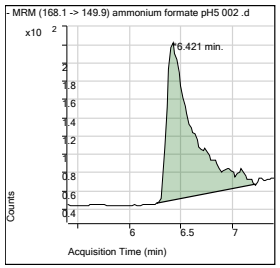
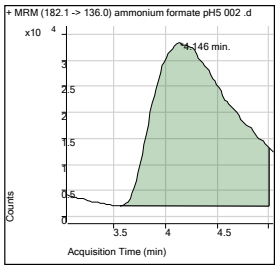
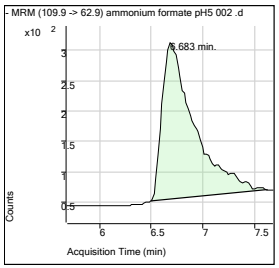
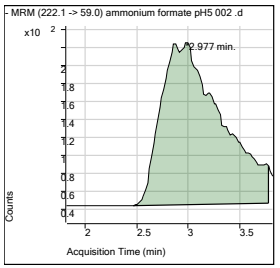
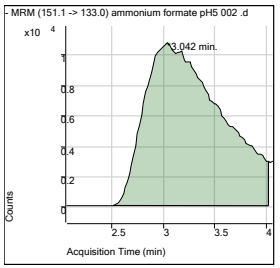
pH	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
3					
4					
5					

Table 4.6 (continued)

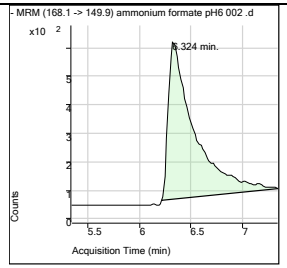
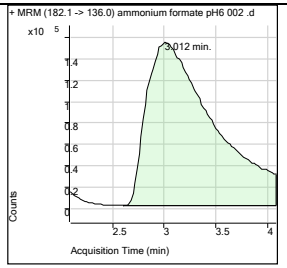
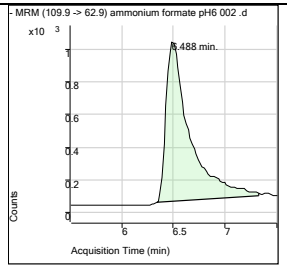
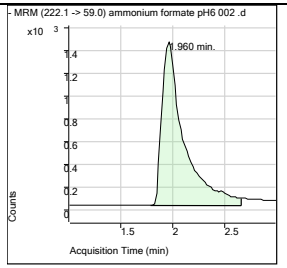
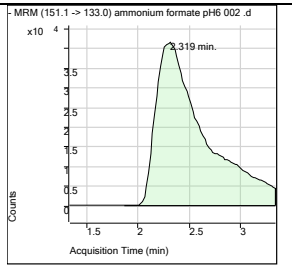
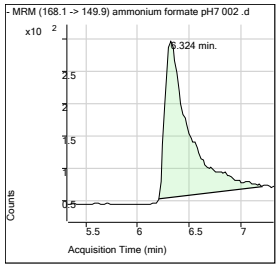
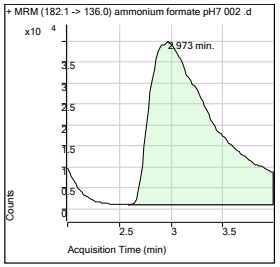
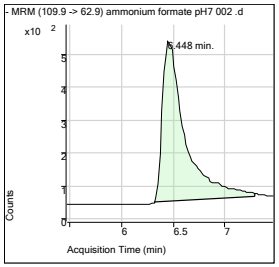
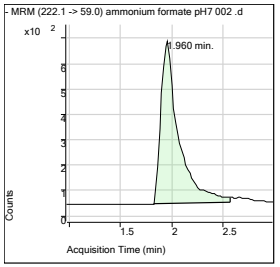
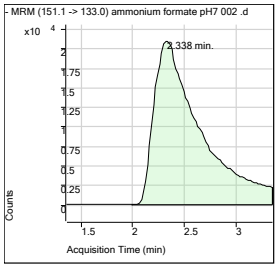
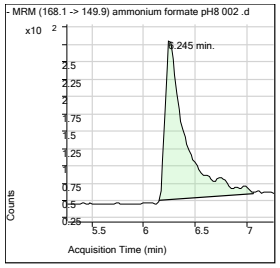
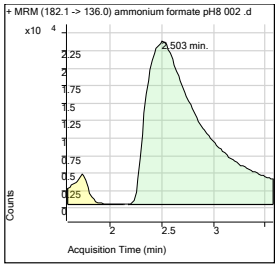
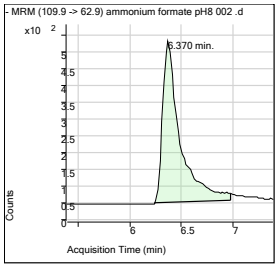
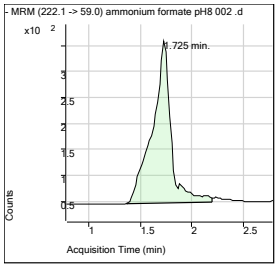
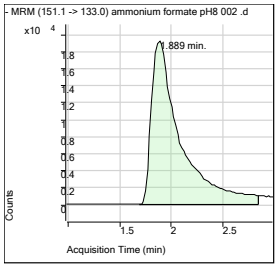
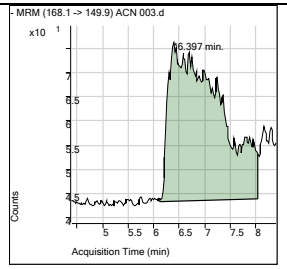
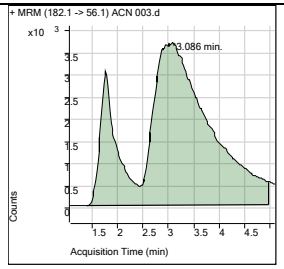
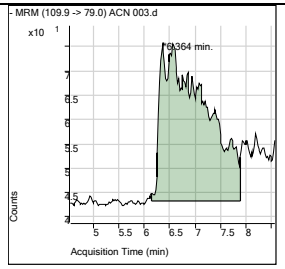
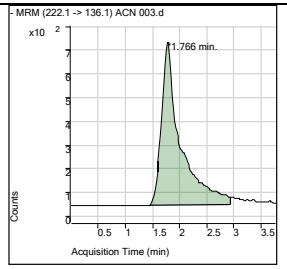
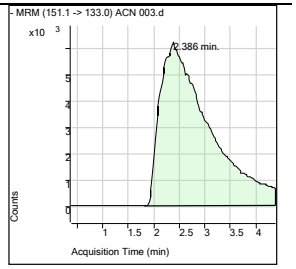
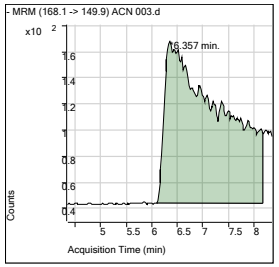
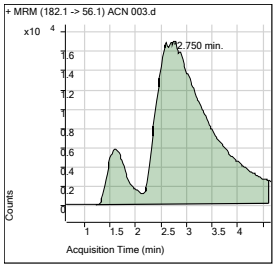
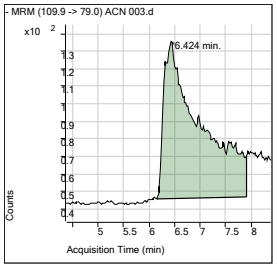
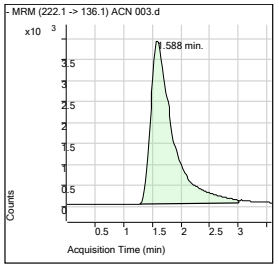
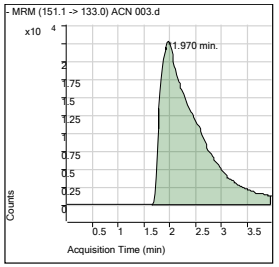
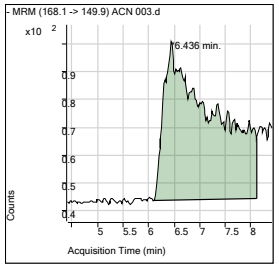
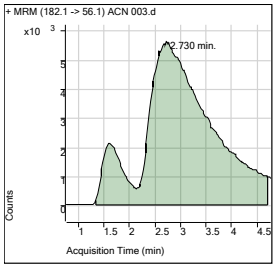
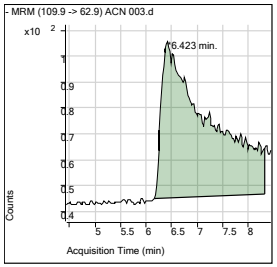
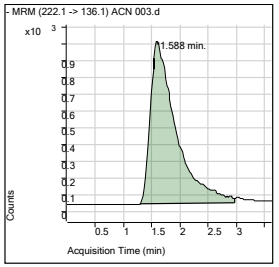
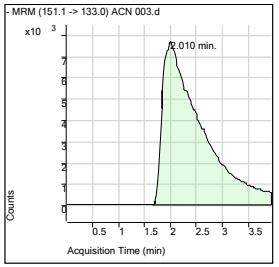
pH	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
6					
7					
8					

Table 4.7 Chromatograms of standard solutions adding a modifier solution in 10 mM ammonium acetate (mobile phase A)-MeCN (mobile phase B) analyzed on ZIC®-HILIC column in HILIC mode.

Mobile phase	Compounds				
	Glyphosate	Glufosinate	AMPA	N-acetyl glufosinate	3-MPPA
A+ 0.001% DMA: B					
A: B+ 0.001% DMA					
A+ 0.001% DMA: B+ 0.001% DMA					

4.2.1.2 Types of solvent for diluting prior to LC analysis

Results from optimization of mobile phase were unsatisfactory. The optimizations of types of solvent for diluting prior to LC analysis were studied as follows:

1) MP: 10 mM ammonium acetate (mobile phase A)-MeCN

   Solvent for diluting: MeCN, water, 10 mM ammomium acetate in water, 0.5% acetic acid in MeCN, 0.01, 0.05, 0.1, 0.5 and 1% DMA in MeCN, 0.5 and 1 mM NH_4OH in MeCN and 0.1% TEA in MeCN

2) MP: mobile phase A (adding acetic acid)-MeCN,

   Solvent for diluting: MeCN, 0.01, 0.05, 0.1, 0.5 and 1% DMA in MeCN and 0.5 and 1 mM NH_4OH in MeCN

3) MP: mobile phase A (adding 0.001% DMA)-MeCN

   Solvent for diluting: MeCN and 0.05 and 0.1% acetic acid in MeCN

4) MP: mobile phase A-MeCN (adding 0.001% DMA)

   Solvent for diluting: MeCN and 0.05 and 0.1% acetic acid in MeCN

5) MP: mobile phase A (adding 0.001% DMA)-MeCN (adding 0.001% DMA)

   Solvent for diluting: MeCN and 0.05 and 0.1% acetic acid in MeCN

In the optimum solvent for diluting, it was expected to change the solvent strength and pH of the analytes due to the change caused directly to the analyte. However results showed that the separation was not improved for peak shape and separation.

From optimization in HILIC mode, the column did not effective for separation of all compounds.

4.2.2 RPLC mode

The LC separation was performed on a Zorbax RRHD Eclipse Plus C18 (2.1 x 50 mm, 1.8 μm) (Agilent Technologies, CA, USA). The Eclipse Plus C18 packing is made by chemically bonding a dense monolayer of dimethyl-n-octadecylsilane stationary phase. The stationary phase is less polar than the mobile phase so polar compounds will elute first. The column was kept at 35 °C. The injection volume was 5 μL and the flow rate of mobile phase was 0.3 mL/min. A mobile phase ioscratic program was 95:5 %v/v. The analysis time was 2 min. The parameters used in this study were as follows:

4.2.2.1 Types of mobile phase

For the mobile phase, 10 mM ammonium acetate-MeCN and 10 mM ammonium formate-MeCN were studied whereby results from glyphosate and AMPA showed poor separation. When using 0.1% formic acid-MeCN as a mobile phase, AMPA was better separated than using ammonium acetate and ammonium formate. The formic acid is more acidic than ammomium acetate and ammonium formate. At pH of ammomium acetate and ammonium formate, the analytes presented more anions than formic acid. The stationary phase is octadecyl (C18) chains, which is nonpolar stationary phase. So the analytes in the formic acid (mobile phase) are better retained in the stationary phase. In addition, 0.2 M formic acid-MeOH was tested which changed both of the mobile phase A (0.2 M formic acid) and B (MeOH). The mobile phase A is changed by increasing the concentration of formic acid because the mobile phase as 0.1% formic acid can give a better separation of AMPA. For the mobile phase B, MeOH is used instead of MeCN due to MeCN having a higher solvent strength than MeOH. Decreasing the solvent strength may retain the analyte better. Results showed that the compounds can separate on the column but glyphosate had less broad peak shapes (Table 4.8).

Table 4.8 Chromatograms of standard solutions using varies types of mobile phase analyzed on Zorbax RRHD Eclipse Plus C18 column in RPLC mode.

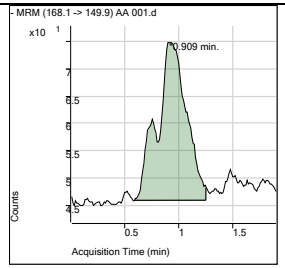
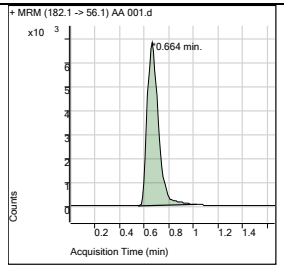
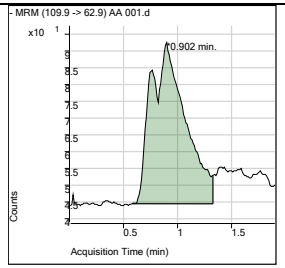
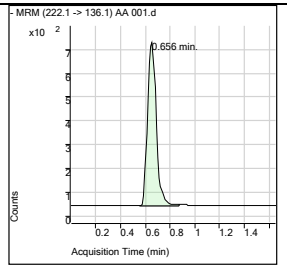
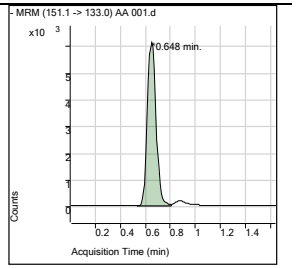
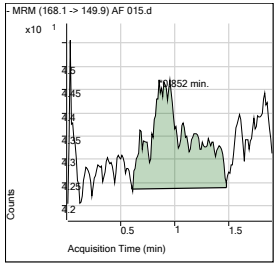
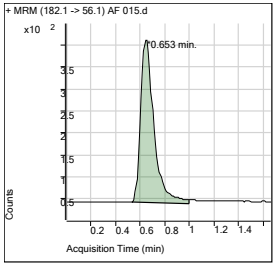
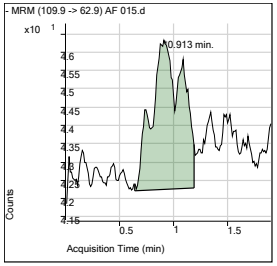
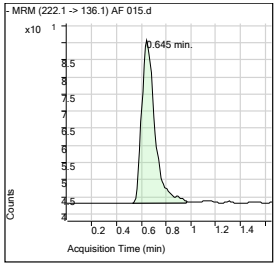
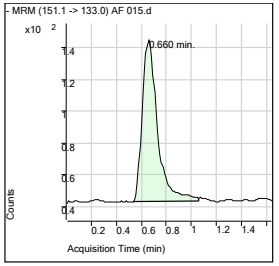
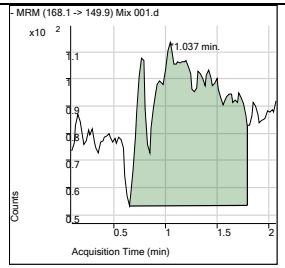
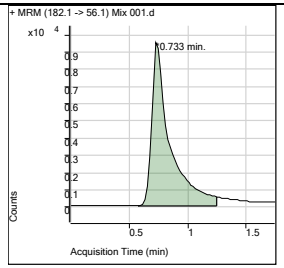
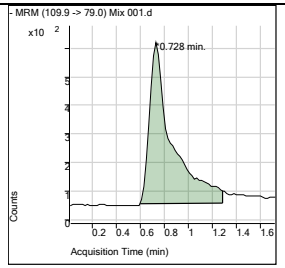
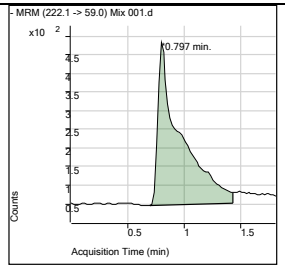
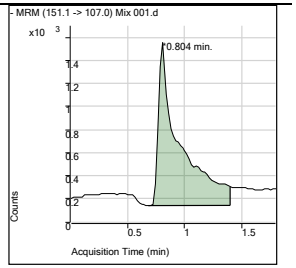
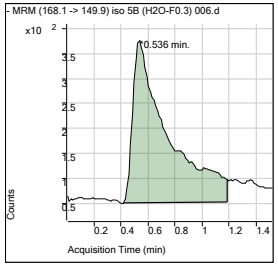
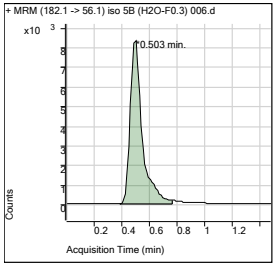
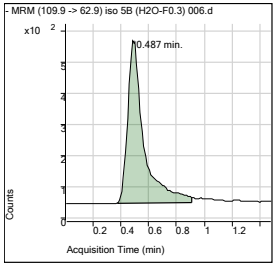
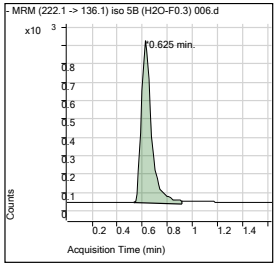
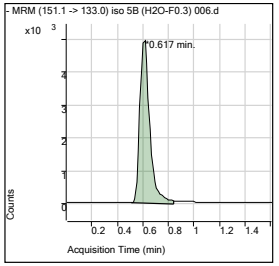
Mobile phase	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
10 mM ammonium acetate-MeCN					
10 mM ammonium formate-MeCN					

Table 4.8 (continued)

Mobile phase	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
0.1% formic acid-MeCN					
0.2 M formic acid-MeOH					

4.2.3 IC mode

4.2.3.1 Bio-rad Aminex® HPX-87P column

The LC separation was performed on a Bio-rad Aminex® HPX-87P (7.8 x 300 mm, 9 µm) (Bio-Rad laboratories, Califonia, USA). The column is based on a sulfonated divinyl benzene-styrene copolymer and the resin ionic form is lead. The column was kept at 85 °C. The injection volume was 10 µL and the flow rate of mobile phase was 0.6 mL/min. A mobile phase isocratic program was used for separation conditions. Parameters used in this study were as follows:

4.2.3.1.1 Types of mobile phase

In IC mode, the mobile phase should be an aqueous solution such as water or buffer for suitable separation. Generally, a column for IC mode is based on polymer, if an organic solvent is used for mobile phase, be the stationary phase will probably swell and lose its ability to separate. For this column, mobile phase such as water is recommended and a small amount of organic modifier can be added to improve the separation. The first mobile phase used was water, but glyphosate and AMPA cannot be eluted. After that, water (mobile phase A)-MeCN (mobile phase B) (95:5 %v/v) was used to separate the compounds, but glyphosate and AMPA cannot elute either. The stationary phase may be not suitable for separation of glyphosate and AMPA.

4.2.3.2 Dionex IonPac™ AS11 column

The LC separation was performed on a Dionex IonPac™ AS11 column (2 x 100 mm, 13 µm) with a guard column Dionex IonPac™ AG11 (2 x 50 mm) (ThermoScientific, Sunnyvale, CA). The column is composed of ethylvinylbenzene crosslinked with 55% divinylbenzene. The anion-exchange layer is functionalized with quaternary ammonium groups. The column was kept at 35 °C. The injection volume was 20 µL and the flow rate of mobile phase was 0.3 mL/min. The optimized mobile phase was water (mobile phase A) and 1 mM citric acid in water pH 11 (adjusted with DMA). A mobile phase gradient program started

at 0% B, and then was increased to 50% B at 8 min (held for 2 min). The analysis time was 10 min and post time 7 min. Parameters used in this study were as follows:

4.2.3.2.1 pH of mobile phase

In this column, the stationary phase is cation (quaternary ammonium groups) therefore the mobile phase generated anions of analytes and it should be basic. The separation mechanism is ion-exchange. Generally, the mobile phase is sodium hydroxide or potassium hydroxide, which can cause a corrosive effect on fused silica capillary and some parts of MS which are made from stainless steel. Therefore, this method used basic solvent instead of sodium hydroxide or potassium hydroxide.

For the mobile phase, water (mobile phase A)- 1 mM citric acid pH 9.6, 10.5 and 11.0 (mobile phase B) (adjusted with DMA) were studied. Poor peak shapes were observed when the mobile phase pH was below 11 due to the mobile phase not being strong enough to elute analytes and the analytes are formed zwitterions. Results from water (mobile phase A)- 1 mM citric acid pH 11.0 (mobile phase B) provided good resolutions and symmetric peak shapes for all compounds. At pH 11, the analytes are anions, so the interaction between the analyte ions and the cationic stationary phase can be improved.

4.2.3.2.2 Types of solvent for diluting prior to LC analysis

The solvent for diluting prior to LC, analysis such as water, MeOH, MeCN, 1% formic acid in MeOH, and 1% formic acid in MeCN, were investigated for improving sensitivity and separation. Adding 1% formic acid (1% formic acid in MeOH, and 1% formic acid in MeCN) is not suitable for analysis because formic acid is acidic and the separation mechanism is an anion-exchange. The acidity may change the pH that effects to charge of the analytes. MeOH provided the greatest sensitivity and peak shape than other solvent (Table 4.9).

Table 4.9 Chromatograms of standard solutions using varies types of solvent for diluting to LC analysis on Dionex IonPac™ AS11 column in IC mode.

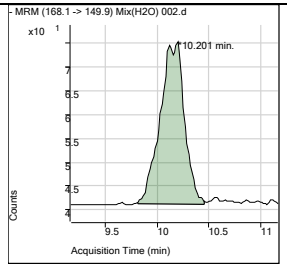
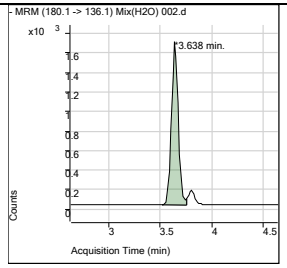
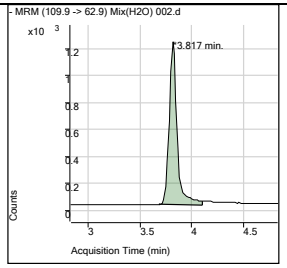
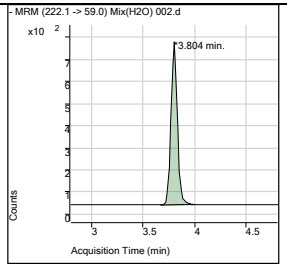
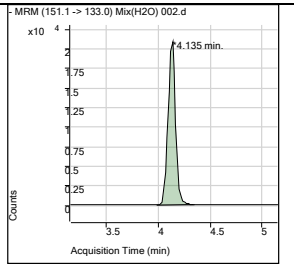
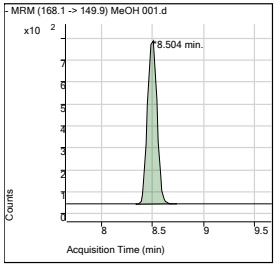
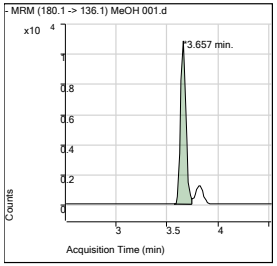
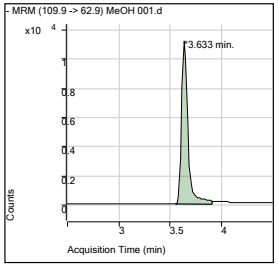
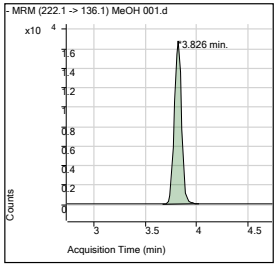
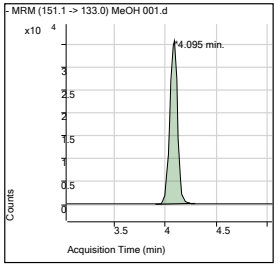
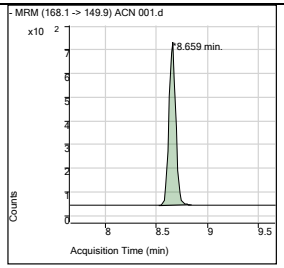
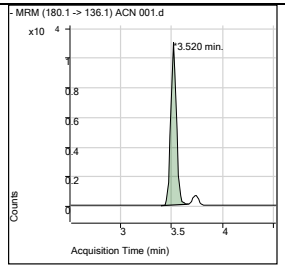
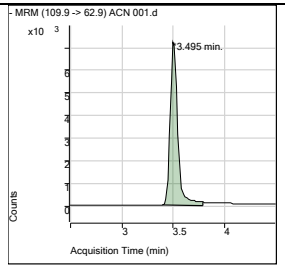
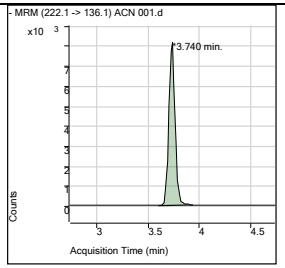
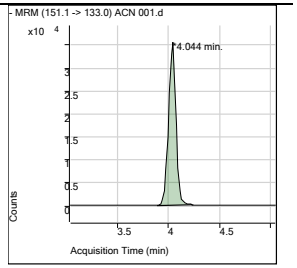
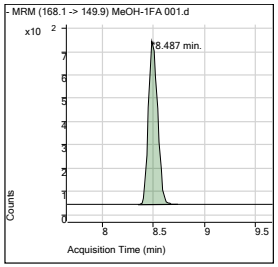
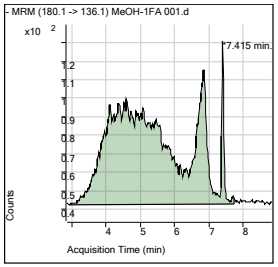
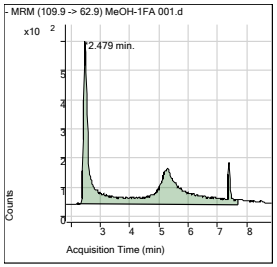
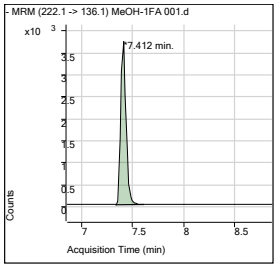
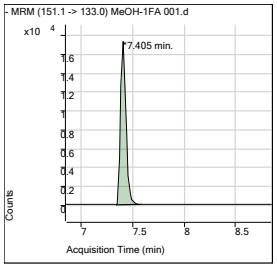
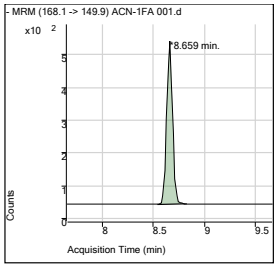
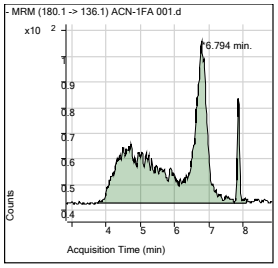
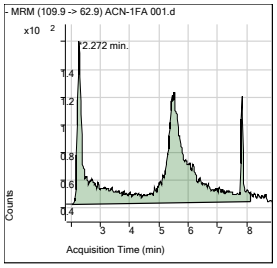
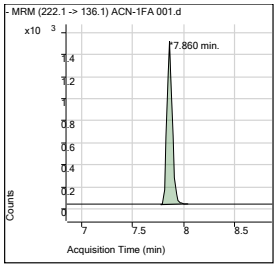
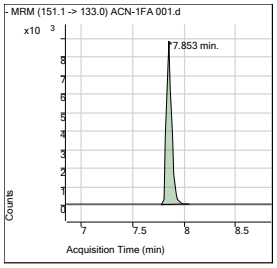
Solvent	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
water					
MeOH					

Table 4.9 (continued)

Solvent	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
MeCN					
1% formic acid in MeOH					
1% formic acid in MeCN					

From the comparative study of the separation modes, the analytes had poor peak shapes and tailing in HILIC mode. Glyphosate had less broad peak shapes in RPLC. In IC mode, Glyphosate and AMPA were not eluted on Bio-rad Aminex® HPX-87P column. In Dionex IonPac™ AS11 column was selected and was the best chromatographic separation of all compounds. Good resolutions and symmetric peak shapes were observed (Figure 4.1) and the condition is shown in Table 4.10. It should be noted that the column should be flushed after 100-150 injections with 30 mM NaOH for 1 hour at a flow rate of 0.3 mL/min. After flushing, NaOH should be removed from the system to protect the MS ion source by flushing mobile phase at a flow rate of 0.3 mL/min for 30 min.

Table 4.10 The LC chromatographic condition using Dionex IonPac™ AS11 column in IC mode for analysis of all compounds.

Parameters	Conditions	
Analytical column	Dionex IonPac™ AS11 (2 x 250 mm, 13 μm)	
Guard column	Dionex IonPac™ AG11 (2 x 50 mm)	
Mobile phase	A: water	
	B: 1 mM citric acid pH 11 (adjusted with DMA)	
Analysis time:	Time (min)	%B
Gradient program	0.0	0.0
	8.0	50.0
	10.0	50.0
Post time	7 min	
Flow rate	0.3 mL/min	
Injection volume	20 μL	
Column temperature	35 °C	
Detector	Tandem mass spectrometry detector	
	Using the MS/MS parameters as shown in section 4.1.3.2 and Table 4.4	

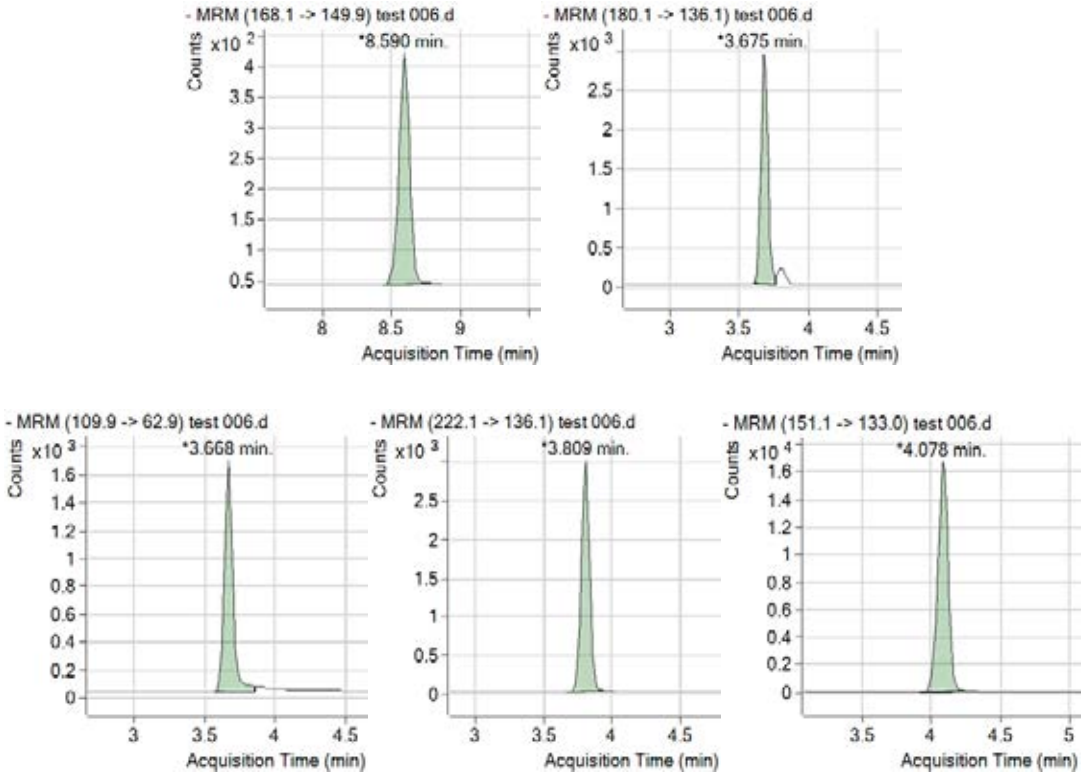


Figure 4.1 Chromatograms of standard solution of all compounds analyzed on Dionex IonPac™ AS11 column in IC mode. (from top left to bottom right are glyphosate, glufosinate, AMPA, *N*-acetyl glufosinate and 3-MPPA, respectively)

4.3 The Optimization of Extraction Procedure

Sample preparation for glyphosate, glufosinate and their metabolites was a liquid extraction for rice samples in which derivatization was not required. Sample extracts were analyzed by a Dionex IonPac™ AS11 column in IC mode.

4.3.1 The results of extraction solvent and volume optimization

The extraction procedure in section 3.5 (3.5.1.1-3.5.1.7) were studied for rice sample extraction. The types and volume of solvents (section 3.5.1.2) were optimized. Parameters used in this study were as follows in Table 4.11.

Table 4.11 Types and volume of solvents extraction for the extraction procedure.

Solvent	H_2O (mL)	MeOH (mL)	MeCN (mL)	1% formic acid in MeOH (mL)
A	10	10	-	-
B	10	-	-	10
C	10	-	-	-
D	-	10	-	-
E	-	-	10	-
F	5	5	-	-
G	5	-	5	-
H	5	4	1	-
I	5	2.5	2.5	-
J	5	2	3	-

The solvent B has formic acid added whereby the acidity may change the pH then effect the charge of the analytes, therefore the separation is not good. The solvent D (MeOH) and E (MeCN) are organic solvent but the analytes prefer a solute in an aqueous solution. The result (solvent D and E), the analytes are not retained and have a low sensitivity and the solvent is not able to extract the analyte from the solution phase. The solvent C (water), the separation is better than B, D and E due to the analyte preferring the solute in the aqueous solution but have low sensitivity due to the rice which has a powder and sugar matrix whereby the matrix may cause ion suppression in the analyte. The solvent A is mixed with an aqueous solution (water) and an organic solvent (MeOH), each solvent is 10 mL. The separation and sensitivity is good whereas the analytes like the water and the MeOH can help the matrix with the ion suppression where the water and MeOH are miscible. Following this, the solvent F has the same composition as the solvent A but differ in the volume of solvent (each solvent 5 mL). The mixing of the solvent provided good results, so the mixed solvent water, MeOH and MeCN namely the solvent H, I and J. The solvent H, I and J did not show improved sensitivity and separation. Therefore, 5 mL of water and 5 mL of MeOH (solvent F) showed better peak shapes of all the compounds and had highest sensitivity of the other solvent. The results are shown in Table 4.12

Table 4.12 Chromatograms of standard solutions using varies extraction solvent.

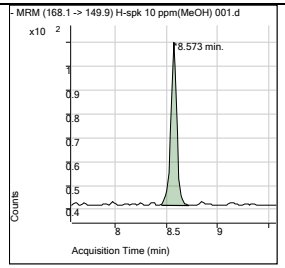
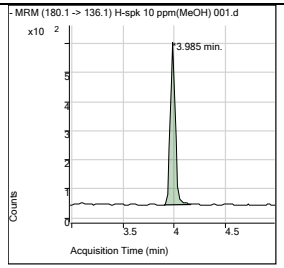
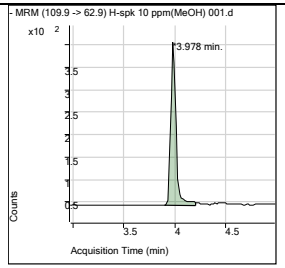
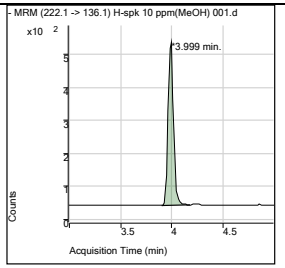
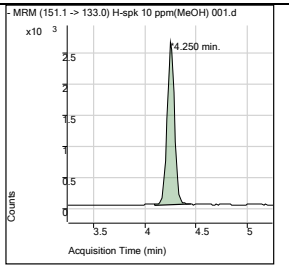
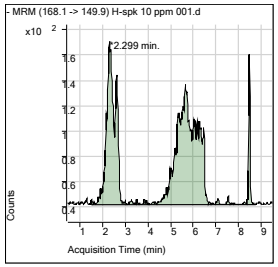
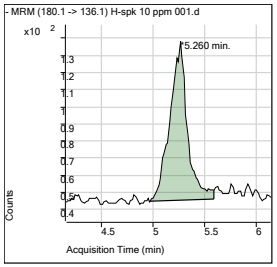
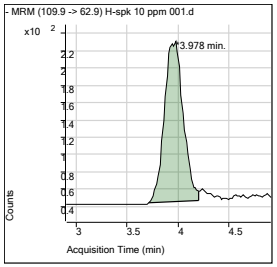
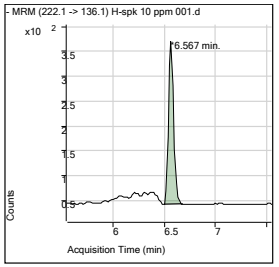
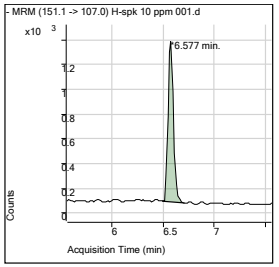
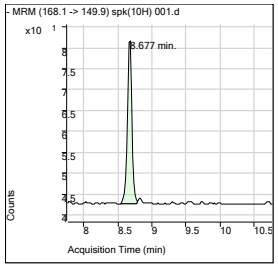
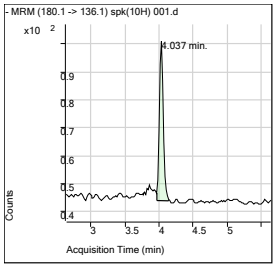
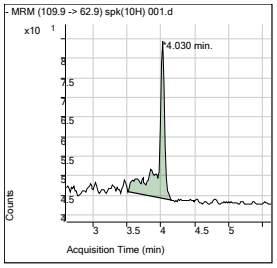
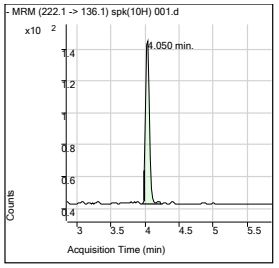
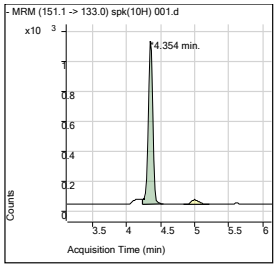
Solvent	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
A					
B					
C					

Table 4.12 (continued)

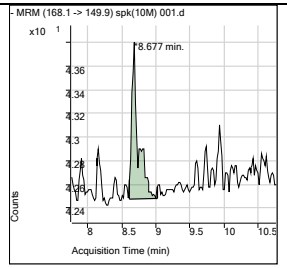
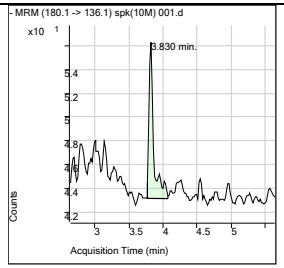
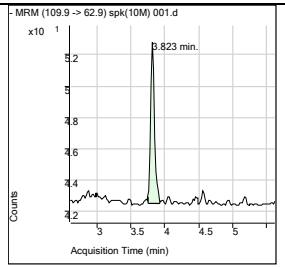
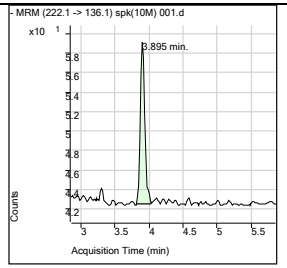
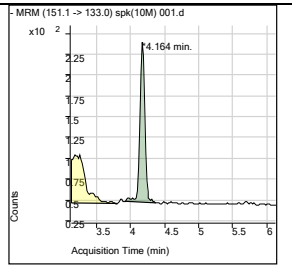
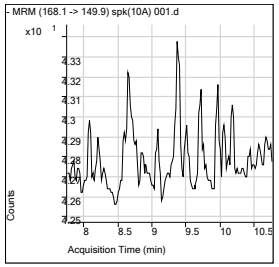
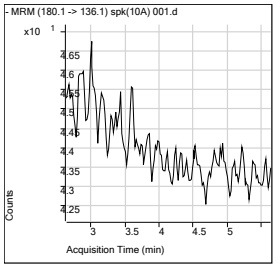
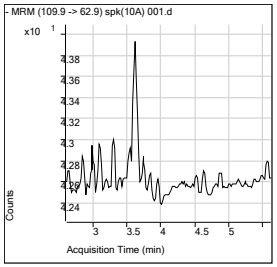
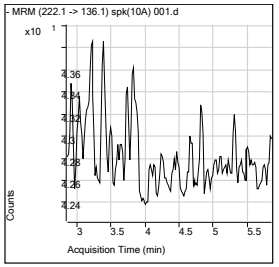
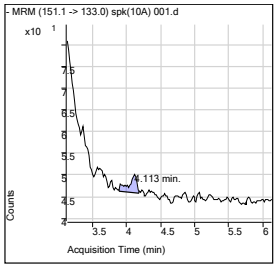
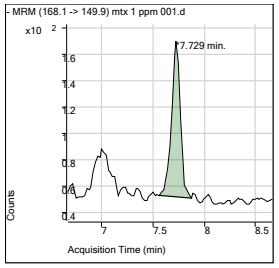
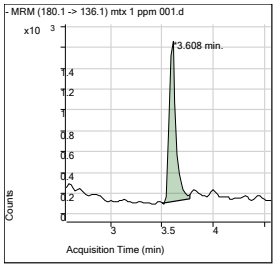
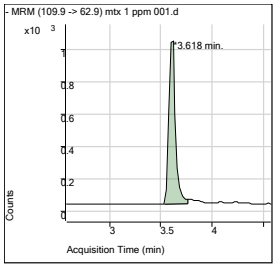
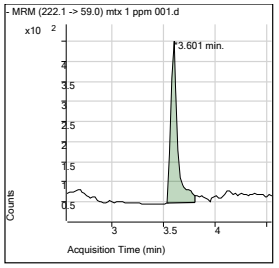
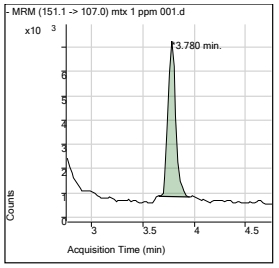
Solvent	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
D					
E					
F					

Table 4.12 (continued)

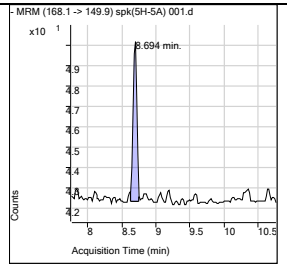
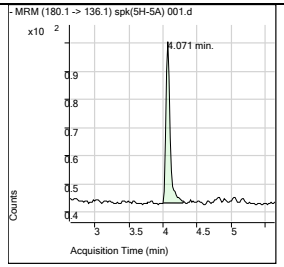
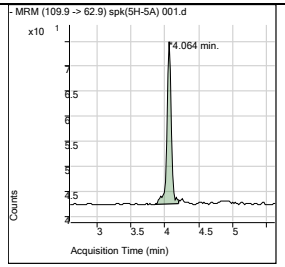
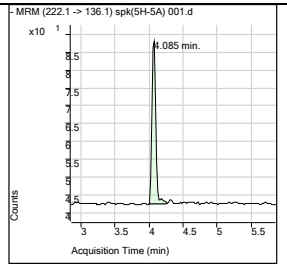
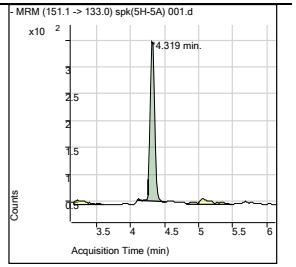
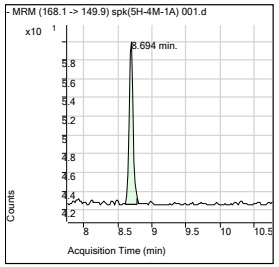
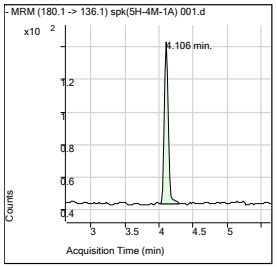
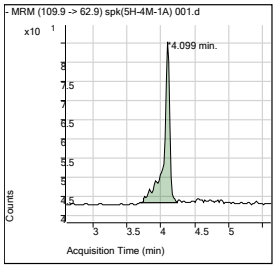
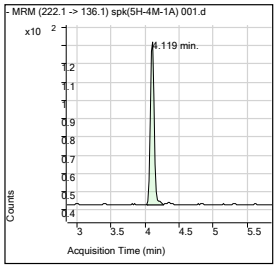
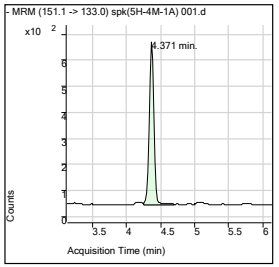
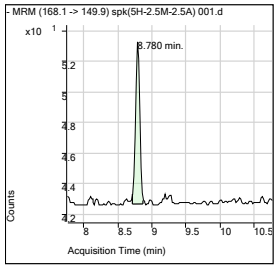
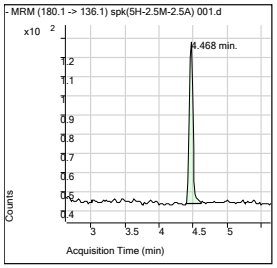
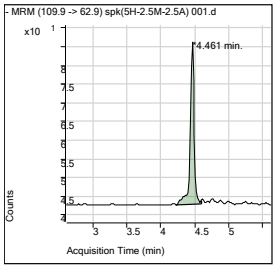
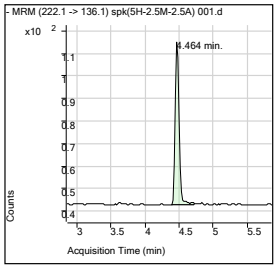
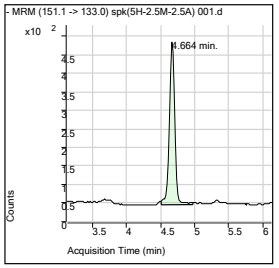
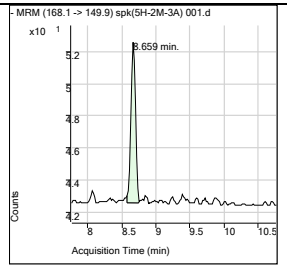
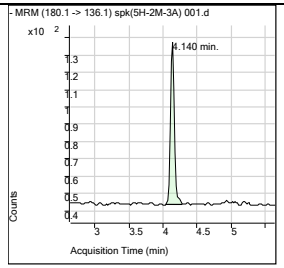
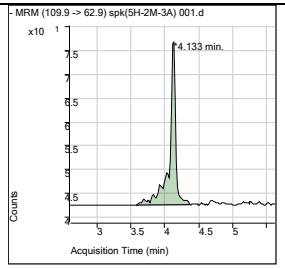
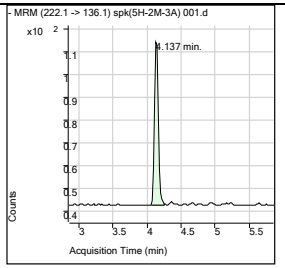
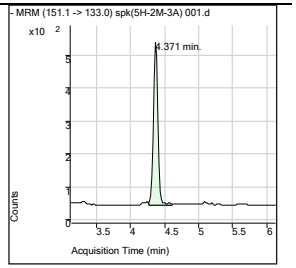
Solvent	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
G					
H					
I					

Table 4.12 (continued)

Solvent	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
J					

4.4 Method Validation

4.4.1 Standard calibration curve

The standard calibration curves were investigated in a range from 0.05-0.5 mg/L. Standard calibration curves were prepared using mixture standard solutions. The calibration curves were prepared at 5 concentration levels with three replicates for each level. The linear regression plots between peak area and concentration are shown in APPENDIX A. Results of slope, y-intercept and correlation coefficient (R^2) are summarized in Table 4.13.

Table 4.13 Linear least-squares regression coefficients of herbicides in the mixture standard solution.

Compounds	Slope	y-Intercept	R^2
glyphosate	77,586	-974.06	0.9836
glufosinate	1E+06	-9,205.6	0.9944
AMPA	1E+06	-30,733	0.9981
N-acetyl glufosinate	290,378	-2,538.82	0.9933
3-MPPA	5E+06	8,488.2	0.9859

The correlation coefficients of the determination higher than 0.98 showed that this method offer good linearity over the concentration range of all compounds.

4.4.2 Matrix-matched calibration curve

The matrix-matched calibration curves were prepared by using the extraction procedure in section 3.5 (3.5.1.1-3.5.1.7). The concentration ranges of each compound were studied in the same range (0.05-0.5 mg/L) of standard calibration curves. The calibration curves were prepared at 5 concentration levels with three replicates for each level. The linear regression plots between peak area and concentration are shown in APPENDIX B. Results of slope, y-intercept and correlation coefficient (R^2) are summarized in Table 4.14.

Table 4.14 Linear least-squares regression coefficients of herbicides in matrix solution.

Compounds	Slope	y-Intercept	R^2
glyphosate	911.39	183.33	0.9516
glufosinate	4,181.9	707.23	0.9482
AMPA	3,177.9	243.83	0.9335
N-acetyl glufosinate	1,256.9	160.80	0.9098
3-MPPA	29,385	879.67	0.9854

The correlation coefficients of the determination higher than 0.90 showed that this method offer good linearity over the concentration range of all compounds.

4.4.3 Matrix effect

In the study of sample there will be a difference between sample and standard solution. The matrix in the sample can have an effect on the analysis such as increased or decreased signal and interference. Therefore the study of the matrix effect is important by comparison between of standard calibration curves and matrix-matched calibration curves that using paired *t*-test with mean of 95% confidence limit. The results from *t*-test are shown in Table 4.15 and APPENDIX C.

Table 4.15 Calculated values of *t*-test with paired to sample for means of 95% confidence limit.

Compounds	Concentration (mg/L)	Peak area		Paired *t*-test	
		Standard solution	Matrix solution	$\lvert t \rvert_{calculated}$	$t_{critical}$
glyphosate	0.050	1,818	204	3.98	2.14
	0.075	4,429	238		
	0.10	6,657	307		
	0.25	21,290	426		
	0.50	36,582	629		

Table 4.15 (continued)

Compounds	Concentration (mg/L)	Peak area		Paired *t*-test	
		Standard solution	Matrix solution	$\lvert t \rvert_{calculated}$	$t_{critical}$
glufosinate	0.050	51,995	732	4.17	2.14
	0.075	78,908	978		
	0.10	115,777	1,370		
	0.25	336,409	1,766		
	0.50	622,519	2,768		
AMPA	0.050	32,652	264	3.77	2.14
	0.075	58,233	418		
	0.10	86,961	736		
	0.25	272,130	1,118		
	0.50	571,809	1,781		

Table 4.15 (continued)

Compounds	Concentration (mg/L)	Peak area		Paired *t*-test	
		Standard solution	Matrix solution	$\lvert t \rvert_{calculated}$	$t_{critical}$
N-acetyl glufosinate	0.050	10,333	157	4.13	2.14
	0.075	17,337	222		
	0.10	26,711	356		
	0.25	75,916	540		
	0.50	140,128	754		
3-MPPA	0.050	237,640	1,916	4.35	2.14
	0.075	359,469	3,076		
	0.10	537,223	4,235		
	0.25	1,532,043	8,352		
	0.50	2,625,795	15,470		

From the statistical analysis, the absolute value of t calculation ($\lvert t \rvert_{calculated)}$ were higher than t critical ($t_{critical}$) for all compounds indicating the significant difference. This informed that the matrix affects to the analysis. Therefore, the matrix-matched calibration curves of all compounds were used for this study.

4.4.4 Linearity

The linearity was performed using the matrix-matched calibration curves investigated in a range from 0.05-2.8 mg/kg. The calibration curves were prepared at 10 concentration levels with three replicates for each level. The linear regression plots between peak area and concentration were shown in APPENDIX D. The results of slope, y-intercept and correlation coefficient (R^2) are summarized in Table 4.16.

Table 4.16 Linear least-squares regression coefficients of herbicides in matrix solution.

Compounds	Slope	y-Intercept	R^2
glyphosate	277.80	-9.7925	0.9698
glufosinate	3,030.2	422.97	0.9804
AMPA	2,055.4	-171.75	0.8940
N-acetyl glufosinate	738.79	238.03	0.9589
3-MPPA	13,573	1,732.7	0.9531

The correlation coefficients of the determination higher than 0.89 showed that this method offer good linearity over the concentration range of all compounds and were acceptable for quantitative analysis.

4.4.5 Accuracy

The accuracy of the method was studied using spiked blank rice samples at MRL, 5-MRL and 10-MRL (0.1, 0.5 and 1 mg/L) on three different analysis days. Each concentration level was prepared with 10 replicates. The percentages of recovery were reported for accuracy and are shown in Table 4.17.

Table 4.17 %Recovery of herbicides at MRL, 5-MRL and 10 MRL (n=10).

Compounds	% Recovery ± SD		
	MRL	5-MRL	10-MRL
glyphosate	101±13	95±11	100±8
glufosinate	108±15	90±5	98±4
AMPA	109±11	98±4	110±3
N-acetyl glufosinate	98±17	90±6	105±6
3-MPPA	95±10	87±4	104±3

The recoveries of the spiked rice matrix at MRL level in the range of 95-109%, 87-98% at 5-MRL level and 98-110% at 10-MRL level were obtained. These recovery values are acceptable according to the AOAC International that recommends the acceptable recovery values of the 100 ppb (µg/L) and 1 ppm (mg/L) between 80-110% [32, 33].

4.4.6 Precision

The precision of this method was studied using intra-assay precision and intermediate precision. The intra-assay precision (within-day precision) was determined using spiked blank rice samples in one day with 3 concentration levels which are MRL, 5-MRL and 10-MRL in 10 replicates for each level. The intermediate precision (between-day precision) was determined using spiked blank rice samples over three days with 3 concentration levels as MRL, 5-MRL and 10-MRL in 10 replicates for each level. The percentages of relative standard deviations (%RSD) were reported for intra-assay precision and intermediate precision.

For the intra-assay precision, the acceptable values of precision were decided using the Horwitz equation [32, 33]. %RSD of the Horwitz equation was 22.6% and 16% at 100 ppb (µg/L) and 1 ppm (mg/L), respectively. For the intermediate precision, the acceptable values of precision were decided by a one way analysis of variance (ANOVA) at 95% confidence limit which is determined using F-value.

4.4.6.1 Results of method precision at MRL level

4.4.6.1.1 Intra-assay precision

The %RSD of spiked rice matrix at MRL level within the same day were in the range of 13-23% on the first day, 10-17% on the second day and 8-21% on the third day and the data is showed in Table 4.18-4.20. These %RSD values are acceptable according to the Horwitz equation because they are less than or equal to the recommend values.

4.4.6.1.2 Intermediate precision

The %RSD of the spiked rice matrix at MRL level on three different days are shown in Table 4.21 and were in the range of 11-19%. From the statistical analysis, the value of F calculation ($F_{calculated}$) was lower than F critical for glyphosate, glufosinate and *N*-acetyl glufosinate with no significant difference between %RSD values on the different days except for AMPA and 3-MPPA.

However, the %RSD values are acceptable according to the Horwitz equation. Therefore the results of the method are reliable in the intermediate precision.

4.4.6.2 Results of method precision at 5-MRL level

4.4.6.2.1 Intra-assay precision

The %RSD of the spiked rice matrix at 5-MRL level within the same day were in the range of 4-10% on the first day, 4-12% on the second day and 3-9% on the third day and the data is shown in Table 4.18-4.20. These %RSD values are less than the recommend values and acceptable according to the Horwitz equation.

4.4.6.2.2 Intermediate precision

The %RSD of the spiked rice matrix at 5-MRL level on three different days are shown in Table 4.21 and were in the range of 4-10%. From the statistical analysis, the value of F calculation ($F_{calculated}$) was lower than F critical for glyphosate, with no significant difference between %RSD values on the different days except for glufosinate, AMPA, *N*-acetyl glufosinate and 3-MPPA. However, the %RSD values are acceptable according to the Horwitz equation. Therefore the results of the method are reliable in the intermediate precision.

4.4.6.3 Results of method precision at 10-MRL level

4.4.6.3.1 Intra-assay precision

The %RSD of the spiked rice matrix at 10-MRL level within the same day were in the range of 5-14% on the first day, 3-8% on the second day and 2-6% on the third day and the data is shown in Table 4.18-4.20. These %RSD values are less than the recommend values and acceptable according to the Horwitz equation.

4.4.6.3.2 Intermediate precision

The %RSD of the spiked rice matrix at 10-MRL level on three different days are shown in Table 4.21 and were in the range of 3-9%. From the statistical analysis, the value of F calculation ($F_{calculated}$) was lower than F critical for glyphosate, with no significant difference between %RSD values on the different days except for glufosinate, AMPA, *N*-acetyl glufosinate and 3-MPPA. However, the %RSD values are acceptable according to the Horwitz equation. Therefore the results of the method are reliable in the intermediate precision.

Table 4.18 %Recovery and %RSD of standards spiked into rice matrix at MRL, 5-MRL and 10-MRL on the first day (n=10).

Level	Compounds	% Recovery												
		1	2	3	4	5	6	7	8	9	10	Mean	SD	%RSD
MRL	glyphosate	88	125	128	68	82	73	99	78	80	94	92	21	23
	glufosinate	63	103	112	78	132	128	112	105	92	96	102	21	21
	AMPA	75	91	78	89	89	112	118	107	85	101	95	14	15
	N-acetyl glufosinate	101	123	109	92	81	103	123	95	102	110	104	13	13
	3-MPPA	127	72	106	74	95	90	111	101	95	90	96	16	17
5-MRL	glyphosate	120	95	98	96	95	96	11	100	96	95	100	8	8
	glufosinate	102	104	100	106	111	104	102	107	112	102	105	4	4
	AMPA	99	102	96	102	101	108	98	102	94	96	100	4	4
	N-acetyl glufosinate	112	116	112	97	93	115	97	103	95	90	103	10	10
	3-MPPA	101	104	98	104	106	101	95	93	99	95	100	4	4
10-MRL	glyphosate	119	118	120	115	99	96	91	82	89	90	102	15	14
	glufosinate	113	114	109	112	110	116	106	103	103	102	109	5	5
	AMPA	117	111	115	107	106	102	99	98	101	98	105	7	7
	N-acetyl glufosinate	126	116	125	107	102	100	107	98	93	100	107	11	11
	3-MPPA	123	123	124	120	122	118	114	114	121	112	119	4	4

Table 4.19 %Recovery and %RSD of standards spiked into rice matrix at MRL, 5-MRL and 10-MRL on the second day (n=10).

Level	Compounds	% Recovery												
		1	2	3	4	5	6	7	8	9	10	Mean	SD	%RSD
MRL	glyphosate	91	123	114	105	106	102	78	98	105	87	101	13	13
	glufosinate	109	114	99	75	123	117	98	109	119	121	108	15	13
	AMPA	122	129	118	94	97	104	100	104	114	110	109	11	10
	N-acetyl glufosinate	120	84	130	98	89	101	73	88	104	96	98	17	17
	3-MPPA	100	102	97	101	81	90	114	91	93	84	95	10	10
5-MRL	glyphosate	106	83	87	76	104	106	107	101	90	94	95	11	12
	glufosinate	90	95	98	95	85	91	84	86	85	89	90	5	5
	AMPA	96	94	95	97	104	97	94	100	99	104	98	4	4
	N-acetyl glufosinate	81	94	87	86	97	93	88	88	99	88	90	6	6
	3-MPPA	89	91	86	83	91	81	83	90	90	83	87	4	5
10-MRL	glyphosate	101	111	108	89	99	91	107	106	94	97	100	8	8
	glufosinate	102	95	92	102	100	103	99	96	97	98	98	4	4
	AMPA	106	105	112	113	109	113	111	112	114	108	110	3	3
	N-acetyl glufosinate	105	111	112	105	98	109	107	105	106	92	105	6	6
	3-MPPA	103	98	100	105	107	107	108	103	105	100	104	3	3

Table 4.20 %Recovery and %RSD of standards spiked into rice matrix at MRL, 5-MRL and 10-MRL on the third day (n=10).

Level	Compounds	% Recovery												
		1	2	3	4	5	6	7	8	9	10	Mean	SD	%RSD
MRL	glyphosate	86	83	119	119	121	68	71	99	88	81	94	20	21
	glufosinate	93	83	113	108	105	114	104	108	117	105	105	10	10
	AMPA	116	126	126	128	115	105	108	101	116	107	115	10	8
	N-acetyl glufosinate	97	103	67	86	124	115	115	103	131	111	105	19	18
	3-MPPA	86	68	87	84	66	66	75	107	89	93	82	13	16
5-MRL	glyphosate	102	103	86	115	91	96	84	97	104	102	98	9	9
	glufosinate	103	92	109	100	100	91	99	103	102	96	100	5	5
	AMPA	114	115	109	110	111	107	110	108	107	105	110	3	3
	N-acetyl glufosinate	104	100	105	107	93	89	102	103	87	92	98	7	7
	3-MPPA	113	113	107	110	115	109	112	108	106	107	110	3	3
10-MRL	glyphosate	106	109	107	104	105	89	100	105	97	106	103	6	6
	glufosinate	109	109	114	104	112	107	108	108	101	106	108	4	3
	AMPA	114	113	111	113	111	116	110	114	104	115	112	3	3
	N-acetyl glufosinate	111	118	116	114	115	115	116	111	118	114	115	2	2
	3-MPPA	97	100	97	101	101	99	96	103	100	95	99	3	3

Table 4.21 Overall %RSD of standards spiked into rice matrix at MRL, 5-MRL and 10-MRL on three different days.

Level	Compounds	%RSD				F-value	
		Day 1	Day 2	Day 3	Over all	$F_{calculated}$	$F_{critical}$
MRL	glyphosate	23	13	21	19	0.74	3.35
	glufosinate	21	13	10	15	0.39	3.35
	AMPA	15	10	8	11	7.67	3.35
	N-acetyl glufosinate	13	17	18	16	0.50	3.35
	3-MPPA	17	10	16	14	3.42	3.35
5-MRL	glyphosate	8	12	9	10	0.60	3.35
	glufosinate	4	5	5	5	25.62	3.35
	AMPA	4	4	3	4	29.07	3.35
	N-acetyl glufosinate	10	6	7	8	7.06	3.35
	3-MPPA	4	5	3	4	92.78	3.35
10-MRL	glyphosate	14	8	6	9	0.16	3.35
	glufosinate	5	4	3	4	19.17	3.35
	AMPA	7	3	3	4	5.11	3.35
	N-acetyl glufosinate	11	6	2	6	4.57	3.35
	3-MPPA	4	3	3	3	90.27	3.35

4.4.7 Lowest calibration level (LCL)

The LCL is defined as the lowest concentration of the matrix-matched calibration curve. The LCL was 0.05 mg/kg of all compounds.

4.5 Application to Real Samples

The developed method was evaluated to analyze real rice samples. Seven rice samples were analyzed using the optimized method in the IC mode and results showed no herbicides in the real rice samples because the retention times did not match the matrix-matched standard (Table 4.22).

Table 4.22 Chromatograms of real rice samples analyzed on Dionex IonPac™ AS11 column in IC mode.

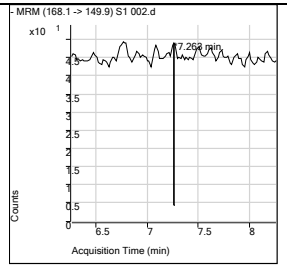
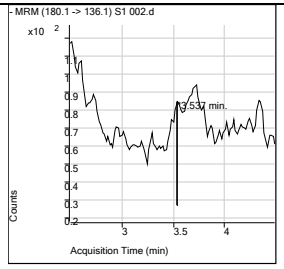
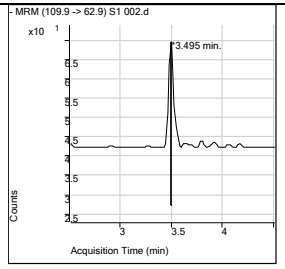
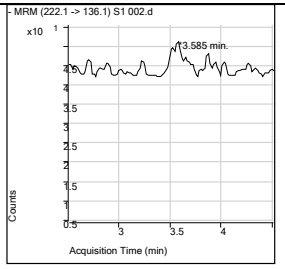
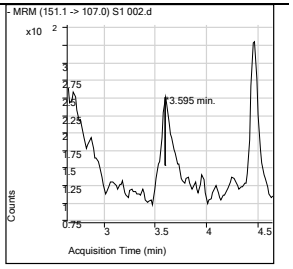
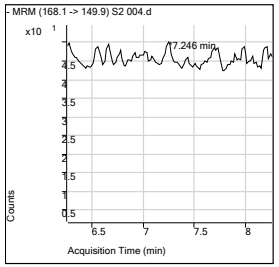
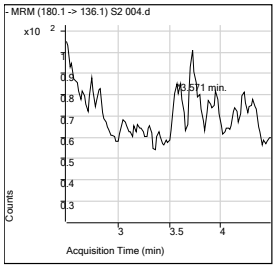
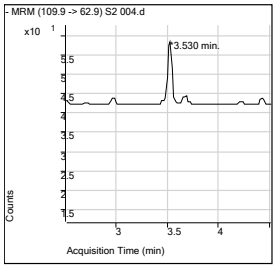
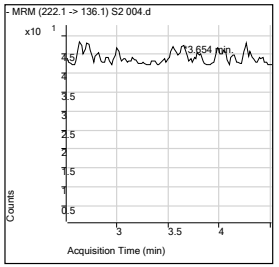
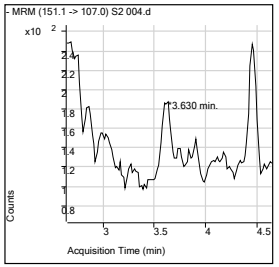
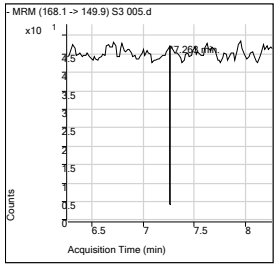
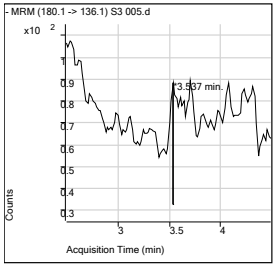
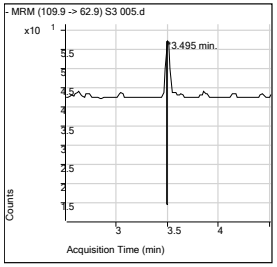
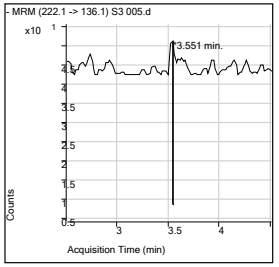
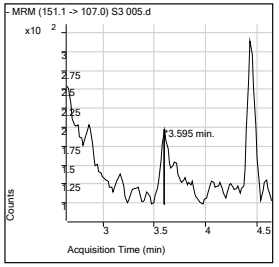
Sample	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
S1					
S2					
S3					

Table 4.22 (continued)

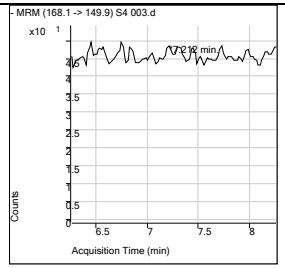
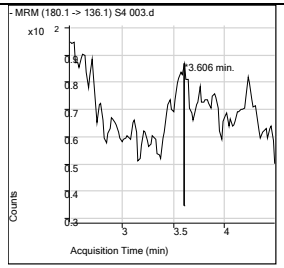
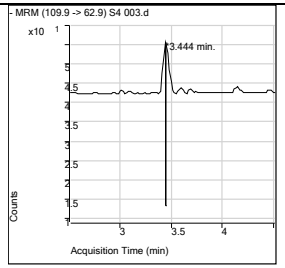
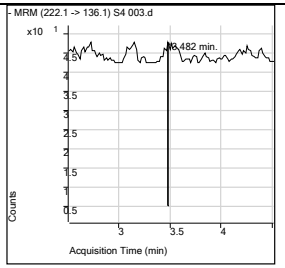
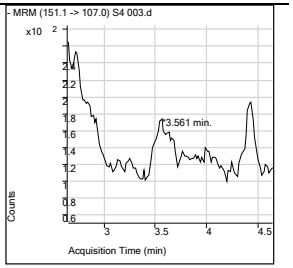
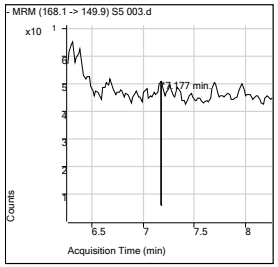
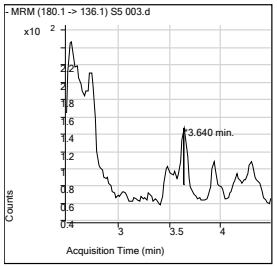
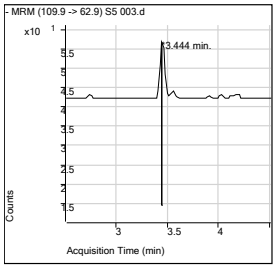
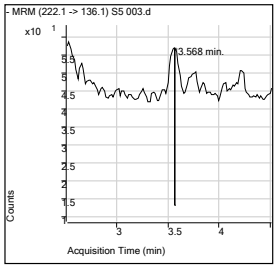
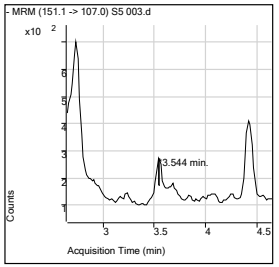
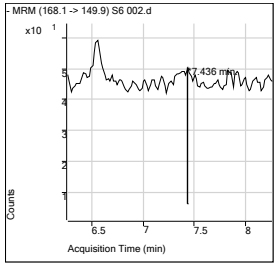
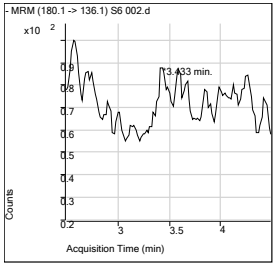
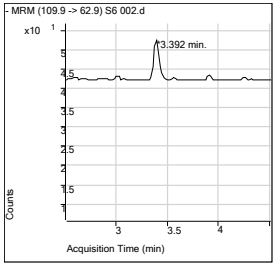
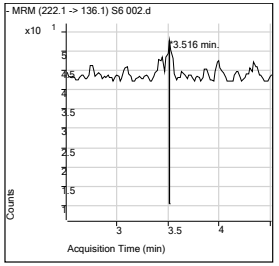
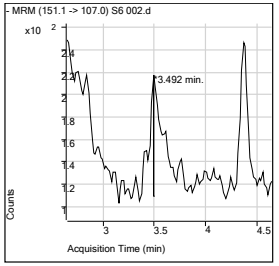
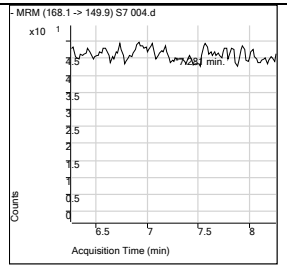
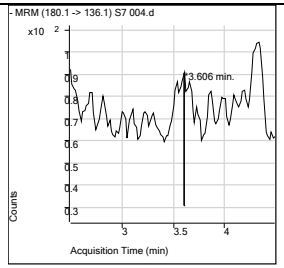
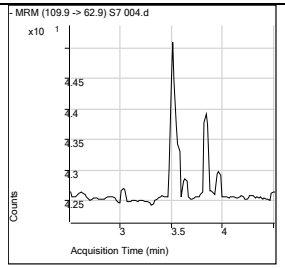
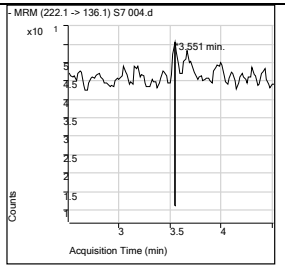
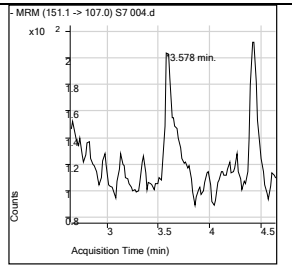
Sample	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
S4					
S5					
S6					

Table 4.22 (continued)

Sample	Compounds				
	Glyphosate	Glufosinate	AMPA	*N*-acetyl glufosinate	3-MPPA
S7					

CHAPTER V

CONCLUSIONS

The simultaneous determination of glyphosate, glufosinate, and their metabolites (AMPA, *N*-acetyl glufosinate and 3-MPPA) using liquid chromatography–tandem mass spectrometry was successful for development. In a comparative study of various chromatographic separation modes, ion chromatography mode showed better separation than hydrophilic interaction liquid chromatography and reversed phase liquid chromatography. Therefore ion chromatography mode was used for the separation of the analytes.

The MRM transitions as the two most sensitive transitions were performed for quantification and qualification of analysis that is shown in Table 5.1. Moreover the optimizations of MS/MS conditions for analysis are shown in Table 5.2.

Table 5.1 Ion transition and optimized MS/MS parameters in IC mode on Dionex IonPac™ AS 11 column.

Compound	Quantification Transition (m/z)	CE (V)	CAV (V)	Qualification Transition (m/z)	CE (V)	CAV (V)
glyphosate	168.1 > 149.9	8	3	168.1 > 63.0	23	3
glufosinate	180.1 > 136.1	14	5	180.1 > 85.1	17	5
AMPA	109.9 > 62.9	20	3	109.9 > 79.0	20	3
N-acetyl glufosinate	222.1 > 59.0	5	5	222.1 > 136.1	5	5
3-MPPA	151.1 > 133.0	8	3	151.1 > 107.0	14	3

Table 5.2 The optimization of MS/MS conditions for analysis of all compounds.

Parameters	Conditions
Polarity	negative mode (-ESI)
Capillary voltage	-4000
Nozzle voltage	-500
Gas temperature	200 °C
Gas flow	17 L/min
Nebulizer pressure	50 psi
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
Fragmentor	380 V
Dwell time	100 ms

From a comparative study of various chromatographic separation modes, separation using ion chromatography was achieved on an anion exchange column (Dionex IonPac™ AS11). The optimizations of chromatographic conditions for analysis are shown in Table 5.3.

Table 5.3 The optimization of chromatographic conditions for analysis of all compounds.

Parameters	Conditions	
Analytical column	Dionex IonPac™ AS11 (2 x 250 mm, 13 μm)	
Guard column	Dionex IonPac™ AG11 (2 x 50 mm)	
Mobile phase	A: water	
	B: 1 mM citric acid pH 11 (adjusted with DMA)	
Analysis time:	Time (min)	%B
Gradient program	0.0	0.0
	8.0	50.0
	10.0	50.0
Post time	7 min	
Flow rate	0.3 mL/min	
Injection volume	20 μL	
Column temperature	35 °C	

From the development of the extraction procedure, liquid extraction was employed for extraction of rice samples and the method was simple, fast, used less chemicals and had no derivatization step. The extraction procedure is described as follows:

1. Weigh 5.00 g of homogenized rice into a polypropylene tube.
2. 5 mL of water and 5 mL of MeOH were added.

3. The mixture solution was shaken by hand for 1 min and votexed for 1 min.
4. The mixture solution was centrifuged at 5000 rpm for 10 min at 25 °C.
5. The sample solution was filtered through a 0.2 µm nylon syringe filter prior to the LC-MS/MS analysis.

The analysis techniques and the sample preparation were optimized for determination of glyphosate, glufosinate, and their metabolites after that the optimum procedures were validated for reliability of the method. The rice matrix has an effect on the analysis. Therefore, the matrix-matched calibration curves of all compounds were used for this study.

Good figures of merit were obtained in this study. The correlation coefficients of the determination higher than 0.89 showed that this method offer good linearity over the concentration range of all compounds. The results of the method performance are shown in Table 5.4.

The accuracy and precision were evaluated at three concentration levels as MRL, 5-MRL and 10-MRL with 10 replicates of each level in the rice sample. The precision was studied as intra-assay precision (within-day precision) and intermediate precision (between-day precision). The intra-assay precision values were in the range of 8-23% at MRL level, 3-12% at 5-MRL level and 2-14% at 10-MRL level for which the values are acceptable according to the Horwitz equation. The intermediate precision on three difference analysis days, the %RSD values are accepted by Horwitz equation which is less than the recommend values. The statistical analysis, the value of F calculation were lower than F critical at 95% confidence limit except for AMPA and 3-MPPA at MRL level, glufosinate, AMPA, *N*-acetyl glufosinate and 3-MPPA at 5-MRL and 10-MRL level. However, the %RSD values are acceptable according to the Horwitz equation. Therefore the result of the method was reliable in the intermediate precision. The mean recovery was in the range of 95-109% at MRL level, 87-98% at 5-MRL level and 98-110% at 10-MRL level for which the recovery values are accepted by the AOAC International.

Table 5.4 The method performance of retention time (t_R), linearity, coefficient of determination (R^2) and lowest calibration level (LCL).

Compounds	t_R (min)	Linearity (mg/kg)	R^2	LCL (mg/kg)
glyphosate	7.759±0.058	0.05-2.8	0.9698	0.05
glufosinate	3.603±0.019	0.05-2.8	0.9804	0.05
AMPA	3.605±0.012	0.05-2.8	0.8940	0.05
N-acetyl glufosinate	3.608±0.028	0.05-2.8	0.9589	0.05
3-MPPA	3.774±0.010	0.05-2.8	0.9531	0.05

In the real sample analysis, the seven rice samples were taken from local fresh markets and supermarkets. Results showed no herbicides in the real rice samples.

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APPENDICES

APPENDIX A

A. Standard calibration curve

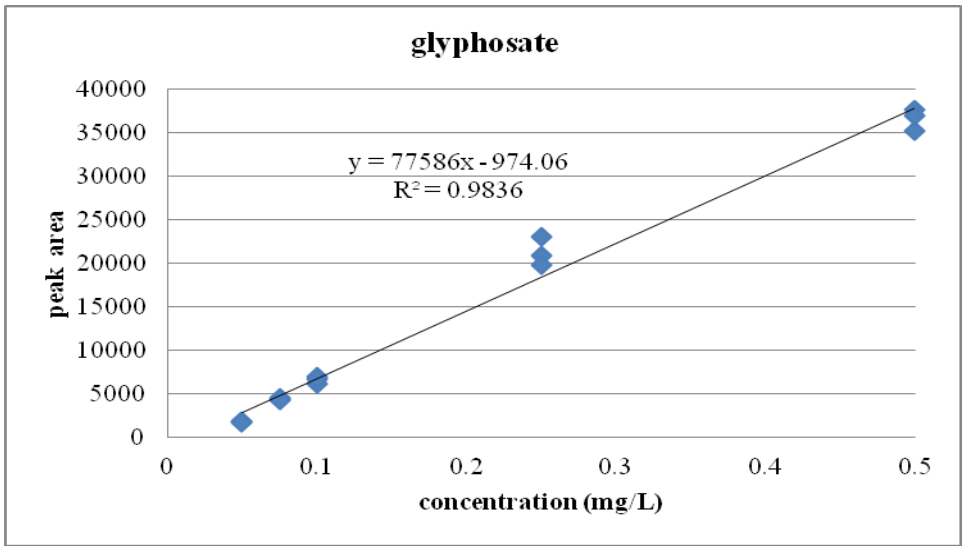

Figure A-1 The standard calibration curve of glyphosate.

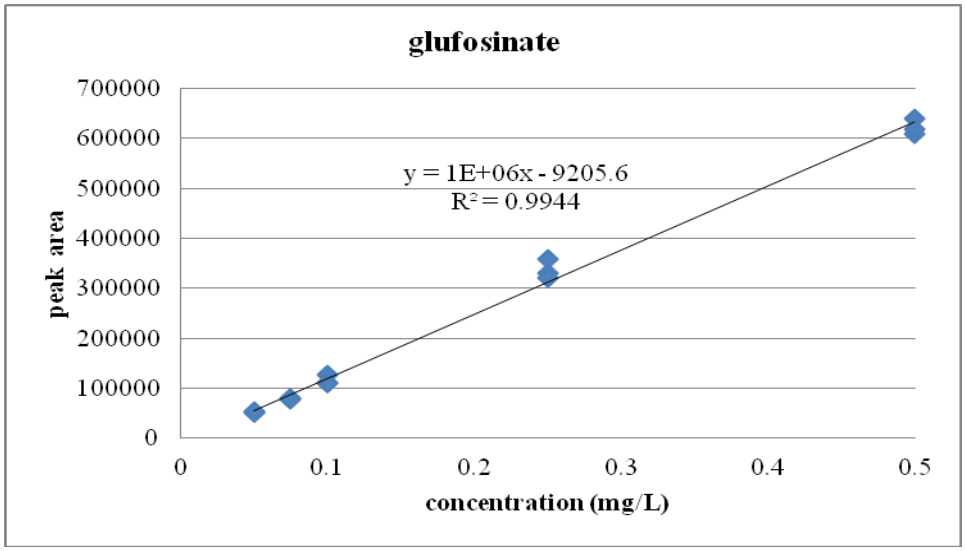

Figure A-2 The standard calibration curve of glufosinate.

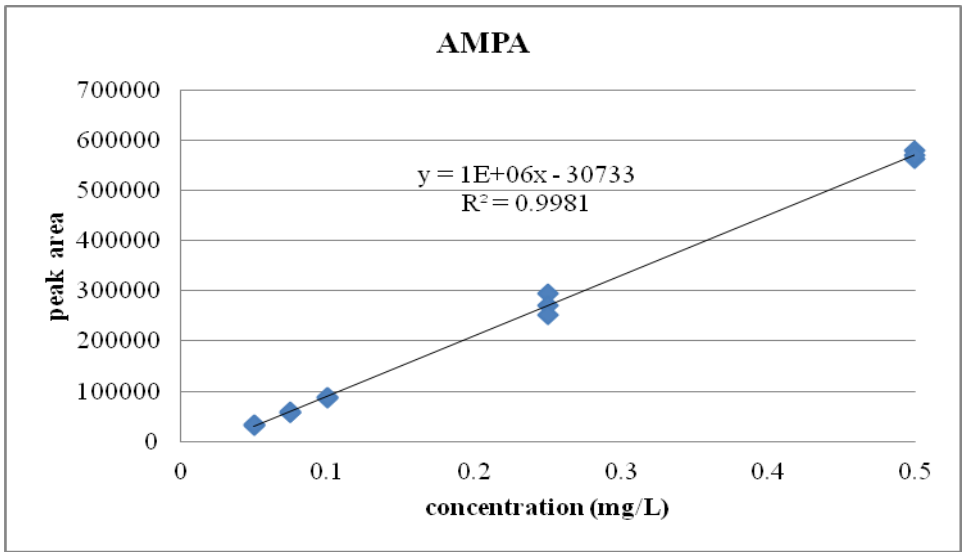

Figure A-3 The standard calibration curve of AMPA.

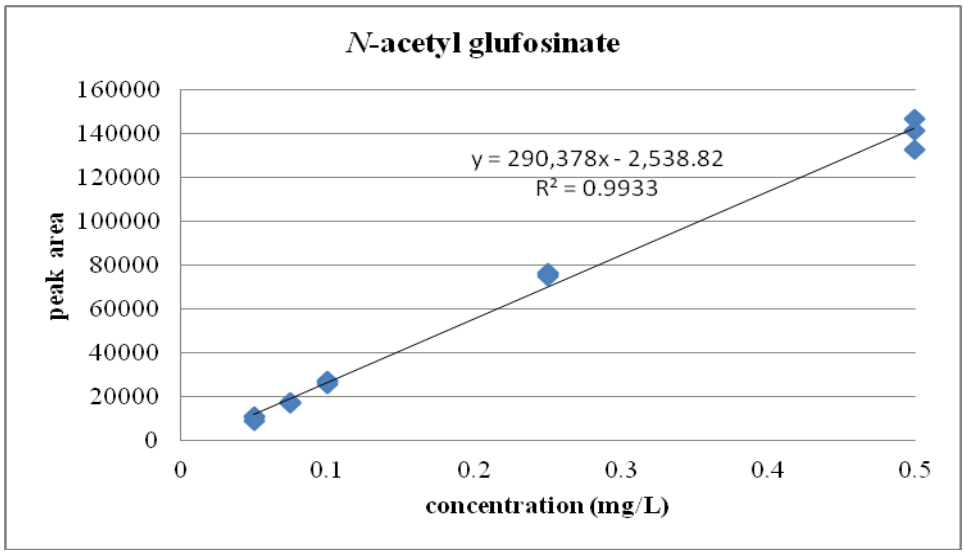

Figure A-4 The standard calibration curve of *N*-acetyl glufosinate.

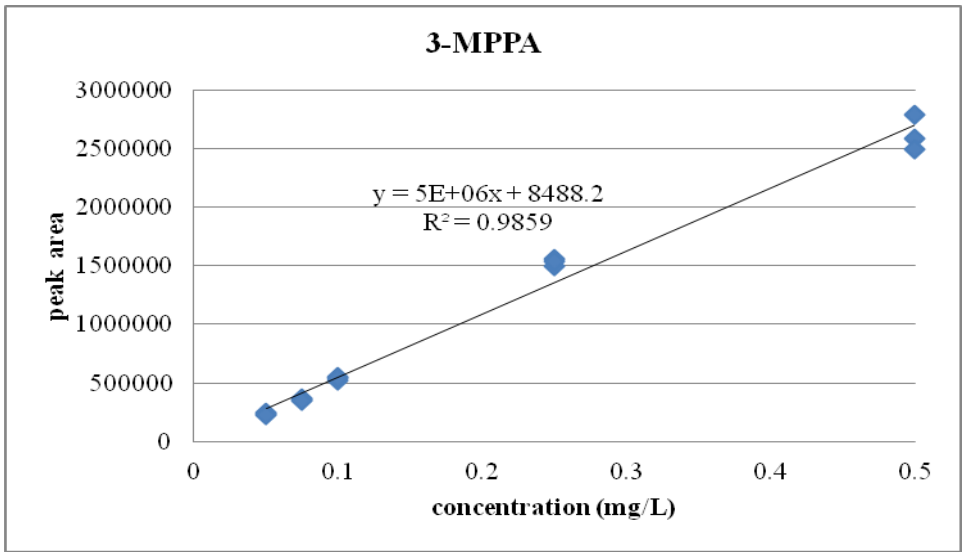

Figure A-5 The standard calibration curve of 3-MPPA.

APPENDIX B

B. Matrix-match calibration curve

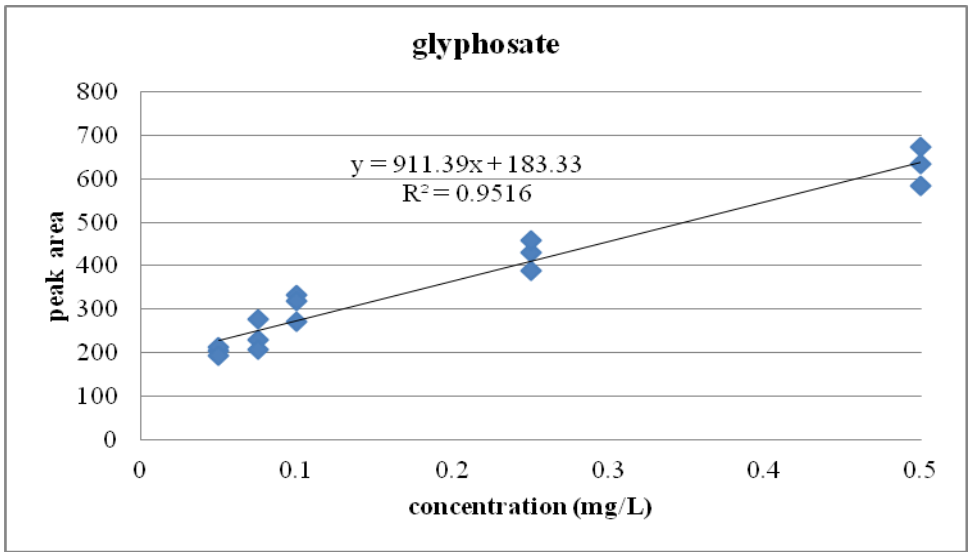

Figure B-1 The matrix-match calibration curve of glyphosate.

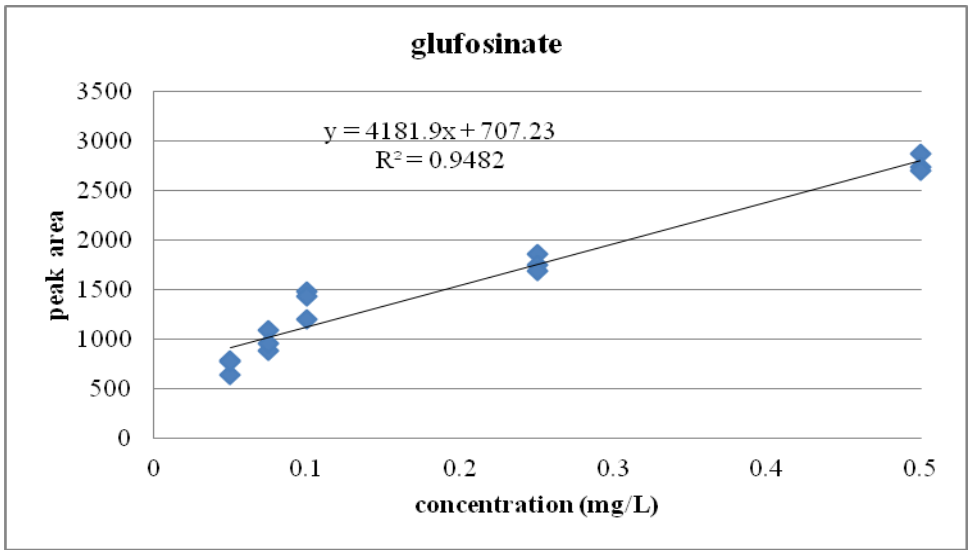

Figure B-2 The matrix-match calibration curve of glufosinate.

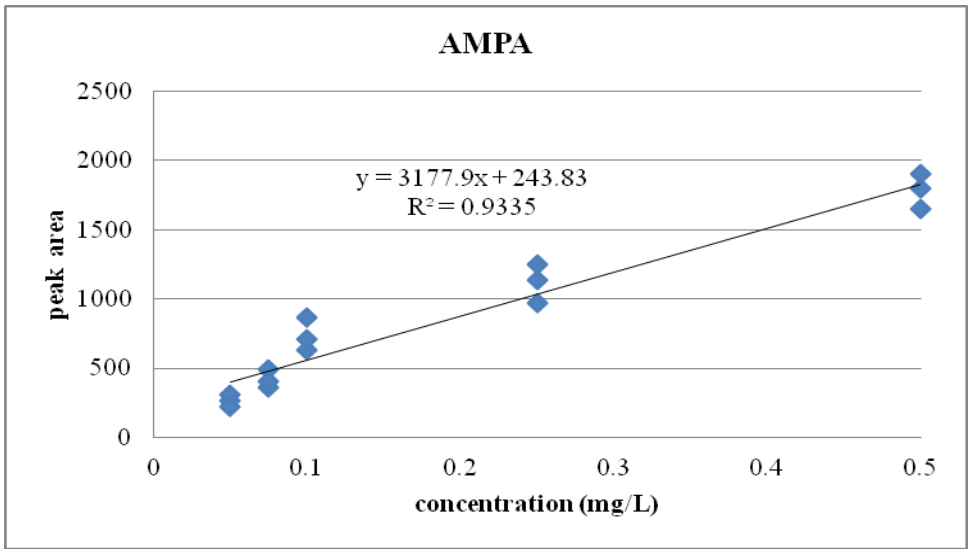

Figure B-3 The matrix-match calibration curve of AMPA.

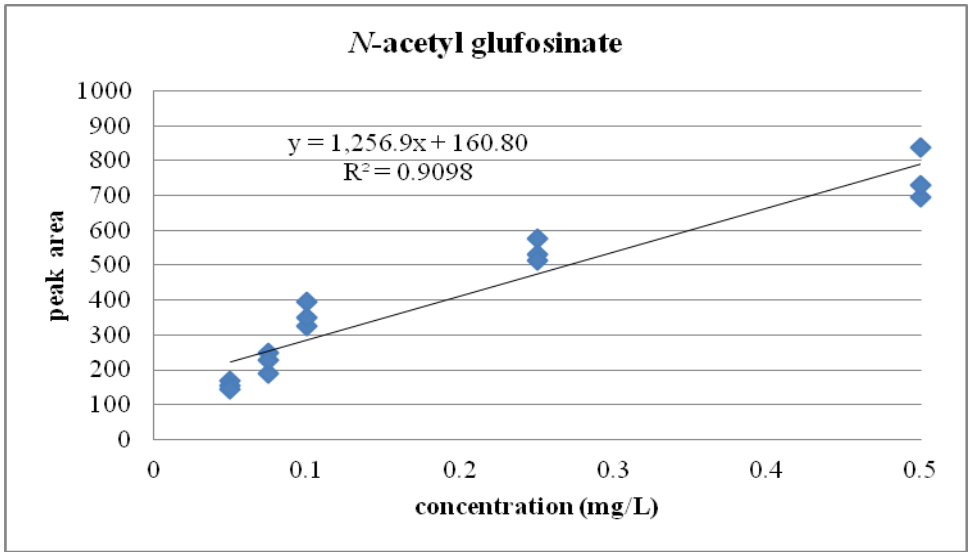

Figure B-4 The matrix-match calibration curve of *N*-acetyl glufosinate.

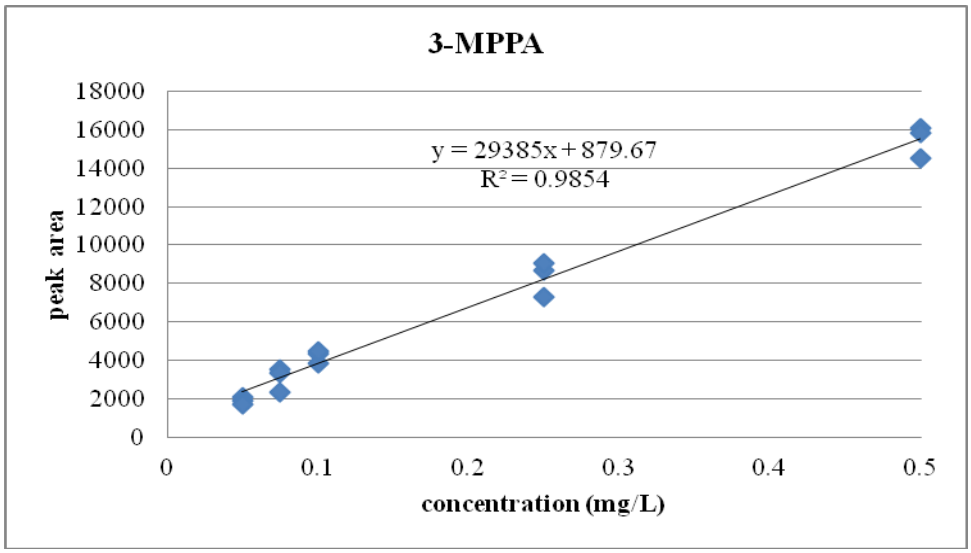

Figure B-5 The matrix-match calibration curve of 3-MPPA.

APPENDIX C

C. Matrix effect

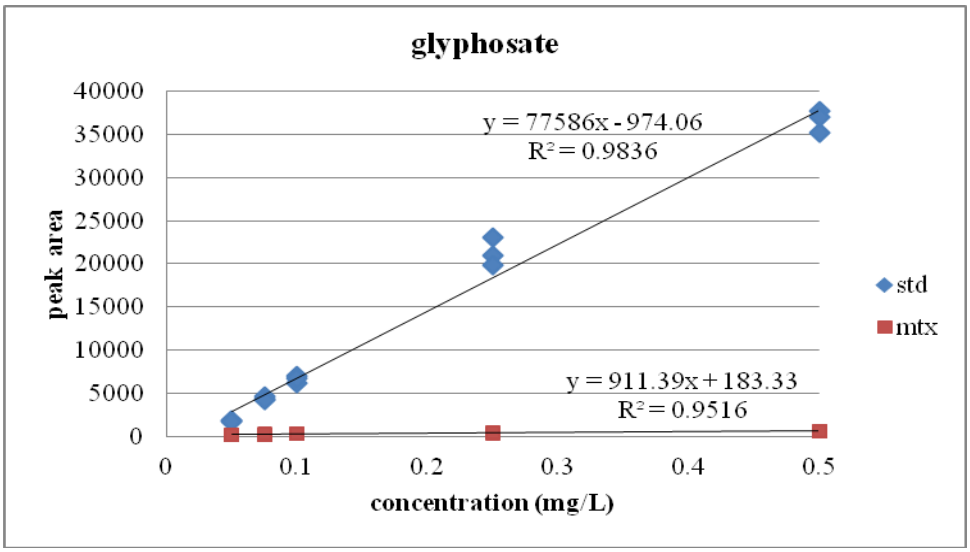

Figure C-1 The standard calibration curve and matrix-match calibration curve of glyphosate.

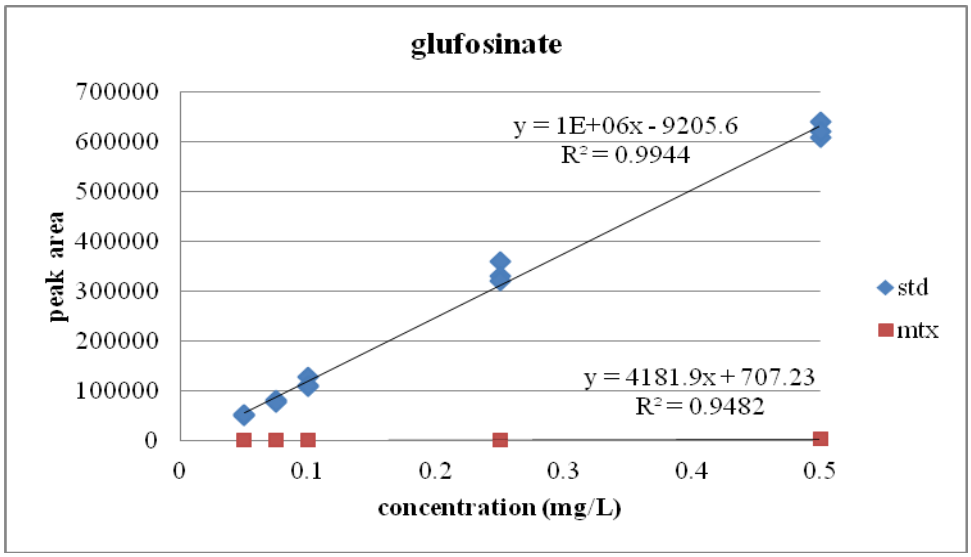

Figure C-2 The standard calibration curve and matrix-match calibration curve of glufosinate.

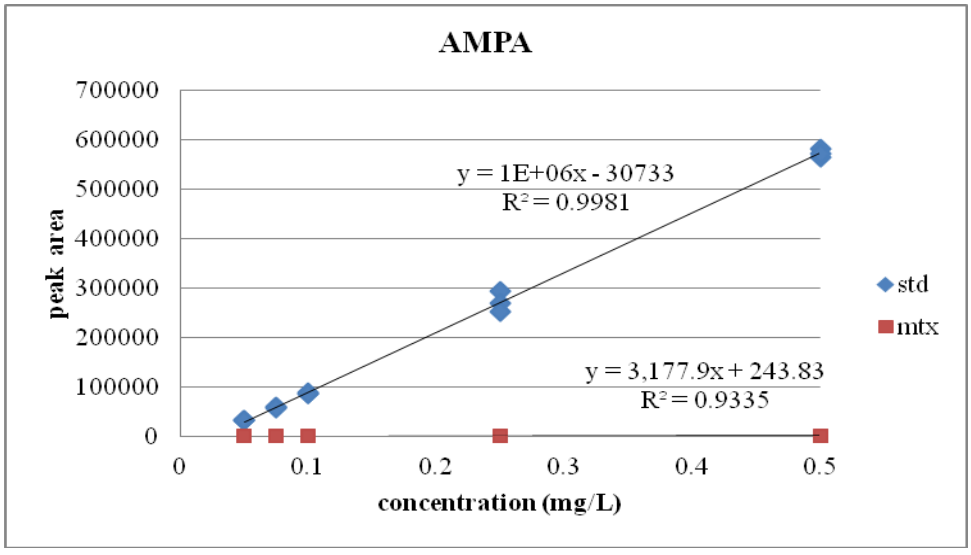

Figure C-3 The standard calibration curve and matrix-match calibration curve of AMPA.

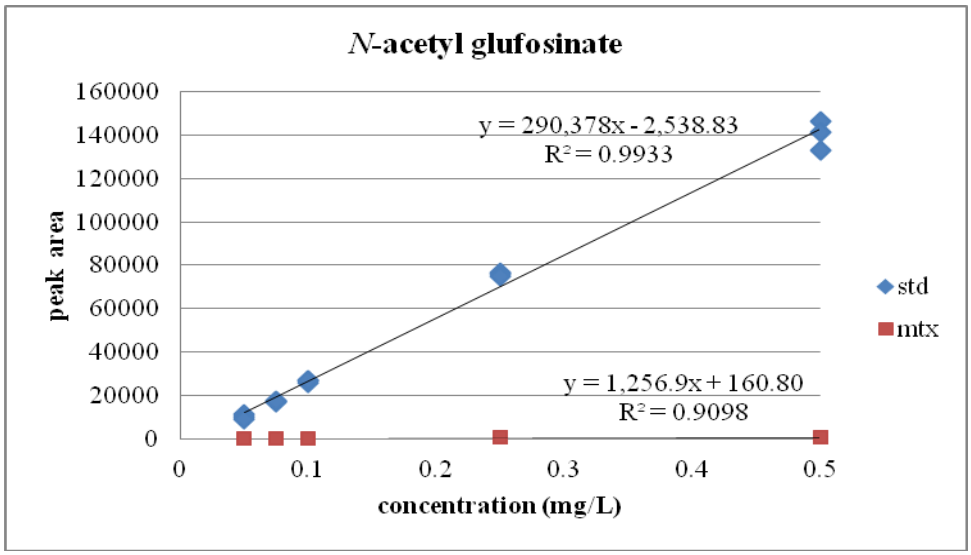

Figure C-4 The standard calibration curve and matrix-match calibration curve of *N*-acetyl glufosinate.

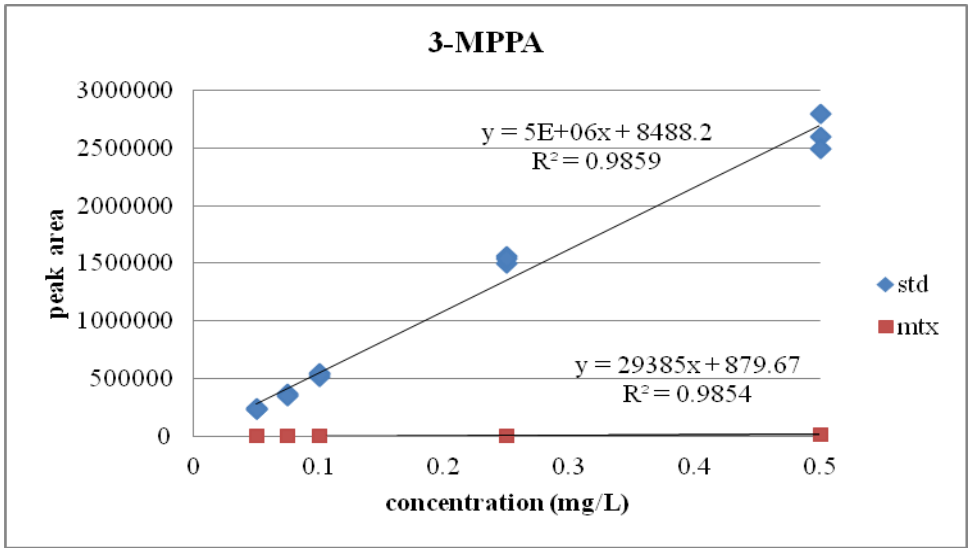

Figure C-5 The standard calibration curve and matrix-match calibration curve of 3-MPPA.

Table C-1 The result of *t*-test for paired two samples for means of glyphosate for analysis of matrix effect.

glyphosate	*std*	*mtx*
Mean	14155.17396	361.052343
Variance	184254068	26280.91772
Observations	15	15
Pearson Correlation	0.977997346	
Hypothesized Mean Difference	0	
df	14	
t Cal	3.982284204	
P(T<=t) one-tail	0.000681294	
t Critical one-tail	1.761310115	
P(T<=t) two-tail	0.001362589	
t Critical two-tail	2.144786681	
|t Cal|>|t Crit| : **Significant difference**		

Table C-2 The result of *t*-test for paired two samples for means of glufosinate for analysis of matrix effect.

glufosinate	*std*	*mtx*
Mean	241121.5506	1522.695023
Variance	49892519333	555281.0553
Observations	15	15
Pearson Correlation	0.975384473	
Hypothesized Mean Difference	0	
df	14	
t Cal	4.168002994	
P(T<=t) one-tail	0.000473872	
t Critical one-tail	1.761310115	
P(T<=t) two-tail	0.000947743	
t Critical two-tail	2.144786681	
|t Cal|>|t Crit| : **Significant difference**		

Table C-3 The result of *t*-test for paired two samples for means of AMPA for analysis of matrix effect.

AMPA	*std*	*mtx*
Mean	204356.8388	863.5124269
Variance	43840814278	325703.7017
Observations	15	15
Pearson Correlation	0.962072387	
Hypothesized Mean Difference	0	
df	14	
t Cal	3.773954964	
P(T<=t) one-tail	0.001026899	
t Critical one-tail	1.761310115	
P(T<=t) two-tail	0.002053798	
t Critical two-tail	2.144786681	
|t Cal|>|t Crit| : **Significant difference**		

Table C-4 The result of *t*-test for paired two samples for means of *N*-acetyl glufosinate for analysis of matrix effect.

N-acetyl glufosinate	*std*	*mtx*
Mean	54085.07373	405.8983005
Variance	2555851096	52279.54971
Observations	15	15
Pearson Correlation	0.96273781	
Hypothesized Mean Difference	0	
df	14	
t Cal	4.13027043	
P(T<=t) one-tail	0.000510028	
t Critical one-tail	1.761310115	
P(T<=t) two-tail	0.001020055	
t Critical two-tail	2.144786681	
|t Cal|>|t Crit| : **Significant difference**		

Table C-5 The result of *t*-test for paired two samples for means of 3-MPPA for analysis of matrix effect.

3-MPPA	*std*	*mtx*
Mean	1058434.095	6609.754487
Variance	8.85327E+11	26380766.96
Observations	15	15
Pearson Correlation	0.990423668	
Hypothesized Mean Difference	0	
df	14	
t Cal	4.353025844	
P(T<=t) one-tail	0.000331058	
t Critical one-tail	1.761310115	
P(T<=t) two-tail	0.000662115	
t Critical two-tail	2.144786681	
|t Cal|>|t Crit| : **Significant difference**		

APPENDIX D

D. Linearity

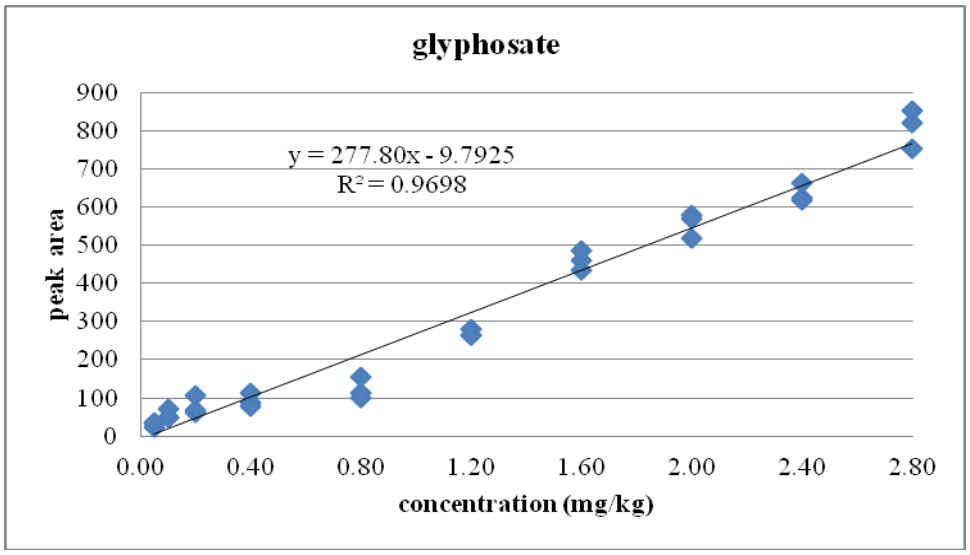

Figure D-1 The matrix-match calibration curve of glyphosate.

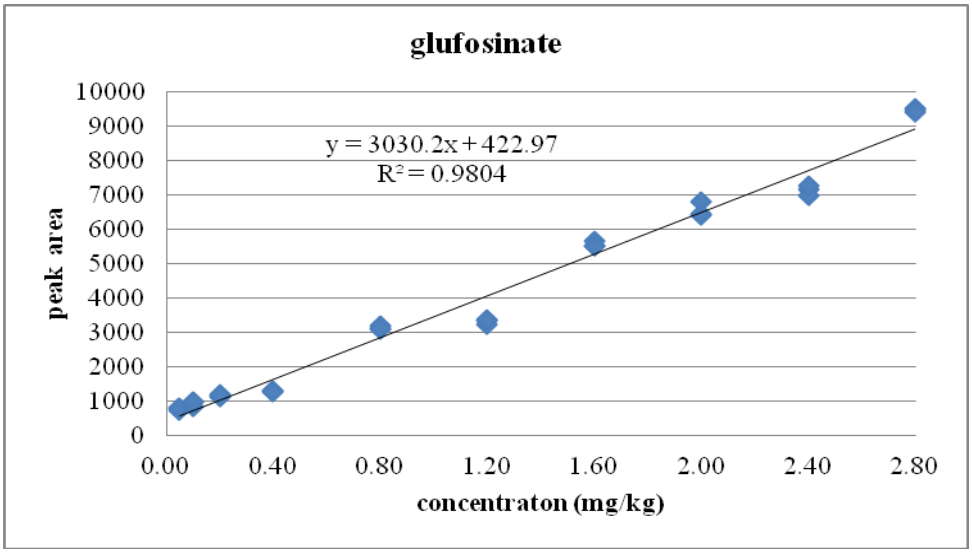

Figure D-2 The matrix-match calibration curve of glufosinate.

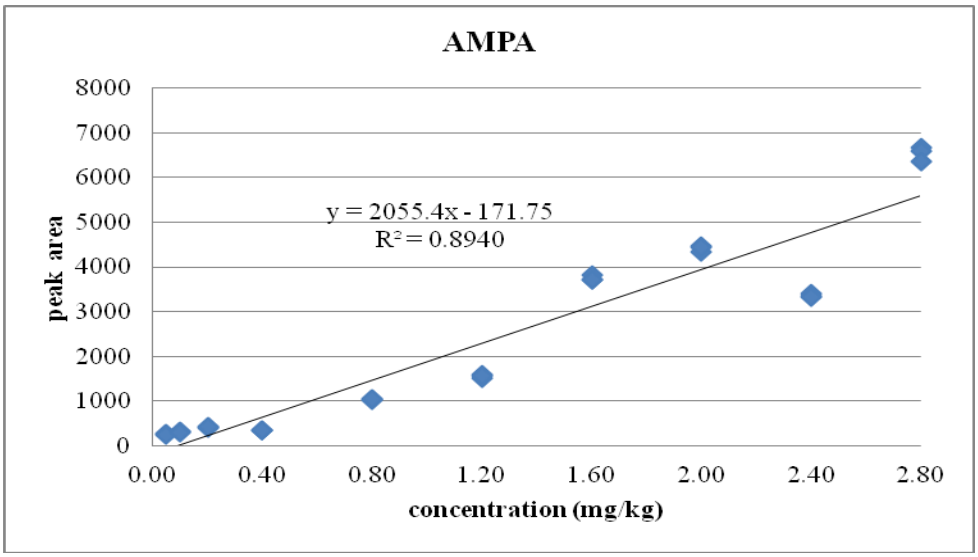

Figure D-3 The matrix-match calibration curve of AMPA.

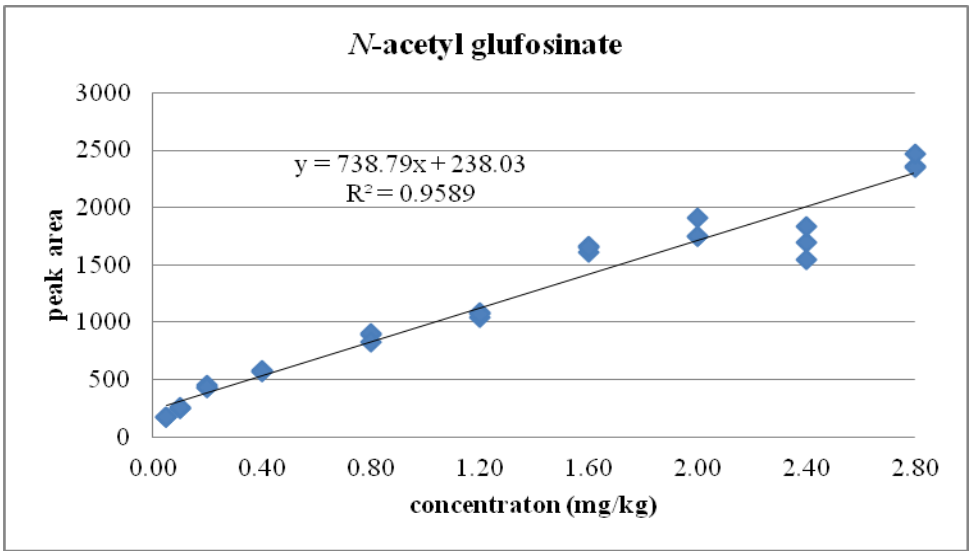

Figure D-4 The matrix-match calibration curve of *N*-acetyl glufosinate.

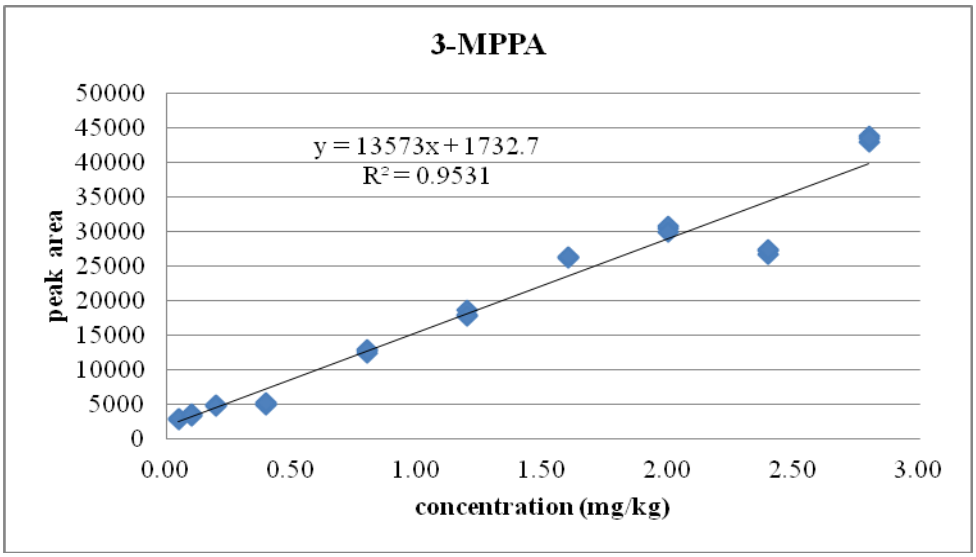

Figure D-5 The matrix-match calibration curve of 3-MPPA.

APPENDIX E

E. Precision: statistical analysis

Glyphosate

Anova: Single Factor MRL glyphosate

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	915	91.5	425.389
day 2	10	1009	100.9	171.656
day 3	10	935	93.5	399.611

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	490.4	2	245.2	0.73807	0.4874346	3.3541
Within Groups	8969.9	27	332.2185			
Total	9460.3	29				

Anova: Single Factor 5-MRL glyphosate

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	1001	100.1	69.6556
day 2	10	954	95.4	121.822
day 3	10	980	98	86.2222

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	110.87	2	55.43333	0.59885	0.5565793	3.3541
Within Groups	2499.3	27	92.56667			
Total	2610.2	29				

Anova: Single Factor 10-MRL glyphosate

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	1019	101.9	212.989
day 2	10	1003	100.3	57.5667
day 3	10	1028	102.8	35.5111

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	32.067	2	16.03333	0.15716	0.8553474	3.3541
Within Groups	2754.6	27	102.0222			
Total	2786.7	29				

Glufosinate

Anova: Single Factor MRL glufosinate

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	1021	102.1	446.544
day 2	10	1084	108.4	211.378
day 3	10	1050	105	104

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	198.87	2	99.43333	0.39151	0.6798112	3.3541
Within Groups	6857.3	27	253.9741			
Total	7056.2	29				

Anova: Single Factor 5-MRL glufosinate

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	1050	105	16
day 2	10	898	89.8	24.1778
day 3	10	995	99.5	29.1667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1184.6	2	592.3	25.6243	5.774E-07	3.3541
Within Groups	624.1	27	23.11481			
Total	1808.7	29				

Anova: Single Factor 10-MRL glufosinate

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	1088	108.8	25.5111
day 2	10	984	98.4	12.2667
day 3	10	1078	107.8	13.7333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	658.4	2	329.2	19.1726	6.577E-06	3.3541
Within Groups	463.6	27	17.17037			
Total	1122	29				

AMPA

Anova: Single Factor MRL AMPA

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	945	94.5	208.056
day 2	10	1092	109.2	130.622
day 3	10	1148	114.8	91.2889

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2198.5	2	1099.233	7.66966	0.0023034	3.3541
Within Groups	3869.7	27	143.3222			
Total	6068.2	29				

Anova: Single Factor 5-MRL AMPA

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	998	99.8	16.6222
day 2	10	980	98	13.7778
day 3	10	1096	109.6	9.82222

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	779.47	2	389.7333	29.0685	1.848E-07	3.3541
Within Groups	362	27	13.40741			
Total	1141.5	29				

Anova: Single Factor 10-MRL AMPA

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	1054	105.4	49.1556
day 2	10	1103	110.3	9.78889
day 3	10	1121	112.1	11.6556

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	240.47	2	120.2333	5.10907	0.0131288	3.3541
Within Groups	635.4	27	23.53333			
Total	875.87	29				

_N_-acetyl glufosinate

Anova: Single Factor MRL *N*-acetyl glufosinate

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	1039	103.9	172.322
day 2	10	983	98.3	284.233
day 3	10	1052	105.2	349.956

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	268.87	2	134.4333	0.50006	0.6120041	3.3541
Within Groups	7258.6	27	268.837			
Total	7527.5	29				

Anova: Single Factor 5-MRL *N*-acetyl glufosinate

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	1030	103	97.7778
day 2	10	901	90.1	30.3222
day 3	10	982	98.2	52.6222

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	850.2	2	425.1	7.05669	0.0034248	3.3541
Within Groups	1626.5	27	60.24074			
Total	2476.7	29				

Anova: Single Factor 10-MRL *N*-acetyl glufosinate

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	1074	107.4	129.378
day 2	10	1050	105	36
day 3	10	1148	114.8	5.95556

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	521.87	2	260.9333	4.56887	0.0195406	3.3541
Within Groups	1542	27	57.11111			
Total	2063.9	29				

3-MPPA

Anova: Single Factor MRL 3-MPPA

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	961	96.1	271.656
day 2	10	953	95.3	92.9
day 3	10	821	82.1	177.433

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1236.3	2	618.1333	3.42147	0.0473795	3.3541
Within Groups	4877.9	27	180.663			
Total	6114.2	29				

Anova: Single Factor 5-MRL 3-MPPA

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	996	99.6	19.1556
day 2	10	867	86.7	15.3444
day 3	10	1100	110	9.55556

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2724.9	2	1362.433	92.776	7.992E-13	3.3541
Within Groups	396.5	27	14.68519			
Total	3121.4	29				

Anova: Single Factor 10-MRL 3-MPPA

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	10	1191	119.1	18.9889
day 2	10	1036	103.6	11.6
day 3	10	989	98.9	6.54444

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2234.6	2	1117.3	90.2666	1.103E-12	3.3541
Within Groups	334.2	27	12.37778			
Total	2568.8	29				

VITAE

Miss Walaiporn Chanchanasophon was born on November 7, 1987 in Nakhon Ratchasima, Thailand. She had graduated a Bachelor's degree of Science in the Chemistry from Chulalongkorn University in 2009. In 2010, she continued her academic education for Master degree at Department of Chemistry, Faculty of Science, Chulalongkorn University. She has presented her work in the topic "Comparative study of chromatographic separation mode for the determination of glyphosate, glufosinate, and their metabolites by liquid chromatography-tandem mass spectrometry" at Proceedings of Pure and Applied Chemistry international Conference 2013 (PACCON 2013), The Tide Resort, Chonburi, Thailand, January 23-25, 2013. She had completed the program in May 2013 and received her Master's degree of Science in Analytical Chemistry in July 2013.