การวิเคราะห์อย่างรวดเร็วและสภาพไวสูงของสารตกค้างอีทอกซีควินในกุ้งด้วยอัลตราเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟีแทนเดมแมสสเปกโทรเมตรี

นางสาวสงกรานต์ ชิกะกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ปีการศึกษา 2554

RAPID AND HIGHLY SENSITIVE ANALYSIS OF ETHOXYQUIN RESIDUES IN SHRIMP USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
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Thesis Title	Rapid and highly sensitive analysis of ethoxyquin residues in shrimp using ultra performance liquid chromatography-tandem mass spectrometry
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Field of Study	Chemistry
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อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.ณัฐชนัญ ลีพิพัฒน์ไพบูลย์, 106 หน้า.

งานวิจัยนี้เป็นการพัฒนาวิธีการวิเคราะห์อย่างรวดเร็วและสภาพไวสูง สำหรับตรวจวัดชนิดและปริมาณอีทอกซีควินตกค้างในกุ้ง การพัฒนาวิธีการเตรียมตัวอย่างอาศัยเทคนิค QuEChERS พบว่าการสกัดอีทอกซีควินในตัวอย่างที่เหมาะสม คือ การสกัดด้วยสารละลาย 2% กรดอะซิติกในแอซิโทไนไทรล์ และทำการกำจัดสิ่งรบกวนด้วยวัฏภาคของแข็งดูดซับ (dispersive solid-phase extraction) primary secondary amine 50 มิลลิกรัม และgraphitized carbon black 10 มิลลิกรัม จากนั้นเจือจางสารละลายตัวอย่างด้วยแอซิโทไนไทรล์ต่อน้ำ อัตราส่วน 80 ต่อ 20 ตรวจวัดด้วยเทคนิคอัลตราเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟีแทนเดมแมสสเปกโทรเมตรีตรวจวัดประจุบวก ใช้เวลา 2 นาที งานวิจัยที่นำเสนอนี้ได้ตรวจสอบความใช้ได้ของวิธีการตามข้อกำหนดการวิเคราะห์สารตกค้างยาฆ่าแมลงในผลิตภัณฑ์อาหารและอาหารสัตว์ (NO.SANCO/10684/2009) ค่าร้อยละการคืนกลับของอีทอกซีควินในกุ้งที่ระดับความเข้มข้น 1.5 ถึง 50 ไมโครกรัมต่อกิโลกรัมอยู่ในช่วง 82 ถึง 92% และค่าเบี่ยงเบนมาตรฐานสัมพันธ์อยู่ในเกณฑ์ดี (น้อยกว่า 6.2%) เมื่อเทียบกับค่าที่คำนวณได้จาก Horwitz equation ให้ค่าความเป็นเส้นตรงของการทำปริมาณวิเคราะห์ที่ระดับความเข้มข้นในช่วง 0.8-64 ไมโครกรัมต่อกิโลกรัม มีค่า R^2 มากกว่า 0.995 มีขีดจำกัดต่ำสุดของการตรวจวัดเท่ากับ 0.45 ไมโครกรัมต่อกิโลกรัม และได้นำไปประยุกต์กับตัวอย่างกุ้งจากตลาดในกรุงเทพมหานครและกุ้งส่งออกในประเทศไทย ซึ่งตรวจพบอีทอกซีควินตกค้างอยู่ในช่วง 1.5 ถึง 350 ไมโครกรัมต่อกิโลกรัม แสดงถึงความเหมาะสมของการนำงานวิจัยนี้ไปประยุกต์กับงานวิเคราะห์ประจำในห้องปฏิบัติการได้อย่างรวดเร็ว

ภาควิชา.....เคมี..... ลายมือชื่อนิสิต.....

สาขาวิชา....เคมี..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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SONGKRAN CHIKAKUL: RAPID AND HIGHLY SENSITIVE ANALYSIS OF ETHOXYQUIN RESIDUES IN SHRIMP USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY.
THESIS ADVISOR: ASST. PROF. NATCHANUN LEEPIPATPIBOON, Dr.,rer.nat, 106 pp.

A rapid, sensitive method for quantitative and qualitative analysis was developed for the determination of ethoxyquin residues in shrimp. The method involved a new sample preparation process based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) methodology. Ethoxyquin was extracted from homogenized samples with optimized condition using 2% acetic acid in acetonitrile, followed by a cleanup step of dispersive solid-phase extraction using 50 mg of primary secondary amine and 10 mg of graphitized carbon black and then dilution of an extract with acetonitrile: water (80:20). The detection of ethoxyquin was achieved by ultra performance liquid chromatography–tandem mass spectrometry within 2 min retention using positive electrospray ionization mode. The validation method has been performed according to guideline of pesticide residues analysis in food and feed NO.SANCO/10684/2009. Good performance data were obtained with recovery of ethoxyquin from the spiked shrimp samples at 1.5–50.0 µg/kg equal to 82-92% and relative standard deviations were in acceptable range (<6.2%) when compared with the value from Horwitz equation. Good linear calibration in the range of 0.8–64 µg/kg was yielded with coefficient of determination above 0.995. The limit of detection was 0.45 µg/kg. The optimized method has been successfully applied to real shrimp sample which can be detected ethoxyquin in a range of 1.5-350 µg/kg from local fresh markets and supermarkets, Bangkok, Thailand and the shrimp farm for export in Thailand. The method can also be employed for shrimp analysis in routine laboratory with high sample throughput.

Department: Chemistry Student's Signature .....
Field of Study: : Chemistry Advisor's Signature .....
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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
ºC	degree Celsius
µg/kg	microgram per kilogram
µL/min	microliter per minute
µm	micrometer
ADI	acceptable daily intake
APCI	atmospheric pressure chemical ionization
BEH	bridged ethylene hybrid
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
C18	octadecyl
CE	collision energy
CI	chemical ionization
DAD	diode array detector
DC	direct current
ESI	electrospray ionization
EU	the European Union
eV	electron volt
FAB	fast atom bombardment
FDA	food and drugs administration
FLD	fluorescence detection
g	gram
g/mol	gram per mole
GC	gas chromatography
GCB	graphitized carbon black
GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
HSS	high strength silica
IPs	identification points
kV	kilovolt
L/hr	liter per hour
LC	liquid chromatography

LC-FLD	liquid chromatography with fluorescence detector
LC-MS	liquid chromatography–mass spectrometry
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LC-TOF	liquid chromatography- time of flight mass spectrometry
LLE	liquid- liquid extraction
LOD	limit of detection
LOQ	limit of quantification
m/z	mass per charge ratio
mg	milligram
mg/kg	milligram per kilogram
mg/mL	milligram per milliliter
MHLW	ministry of health, labour and welfare
min	minute
mL	milliliter
mM	millimolar
mm	millimeter
MRLs	the maximum residue limits
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS^n	mass spectrometry
NaOH	sodium hydroxide
NH_2	aminopropyl
nm	nanometer
pKa	power of acid dissociation constant
PSA	primary secondary amine
psi	pound per square inch
PTFE	polytetrafluoro ethylene
QC	quality control
QuEChERS	quick, easy, cheap, effective, rugged, safe
R^2	correlation coefficient
RF	radio frequency
RSD	relative standard deviation
S/N	signal to noise ratio

SD	standard deviation
sec	second
SFE	super critical fluid extraction
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
t_R	retention time
TSP	thermospray
UPLC	ultra performance liquid chromatography
UPLC-MS/MS	ultra performance liquid chromatography-tandem mass spectrometry
UV	ultra violet
V	volt
v/v	volume by volume
v/v/v	volume by volume by volume

CHAPTER I

INTRODUCTION

1.1 Problem Definition

Ethoxyquin is widely used in feeds because of its excellent antioxidant properties and high stability than other antioxidant chemicals. The increasing use of ethoxyquin results from a significant concern for rancidity and auto–oxidation in product quality and residuals management in feed industry. Ethoxyquin is the most predominantly used antioxidants in the feed industry, where it primarily stops the propagation of chemical oxidation initiated by air, light, or transition metals in unsaturated hydrocarbons such as lipid, carotenes, and vitamin A and E. The use of ethoxyquin as a food additive in animal feed is strictly controlled since it may be remained in farm animals and possibly transferred to human by consumption. In usages with high concentration, these ethoxyquin can leave residues in edible tissue and poses a potential risk to health. The daily consumption of food containing high levels of ethoxyquin may affect genetic material (mutagenic) and chromosomal damage in human lymphocytes. Despite its importance as a preservative, there is another concern about ethoxyquin utilization in feeds because it may leave harmful residues in meat tissue, inducing food safety problems for the consumer. To ensure the safety of food for human consumption, many countries including European Union (EU) and US Food and drugs Administration (FDA) and Japan Ministry of Health, Labour and Welfare (MHLW) became aware of this problem and introduced a positive list system for control of agricultural chemicals remaining in food (MHLW, Notifications 497 and 499, 2005) to protect human health from potentially harmful ethoxyquin residues. This legislation mandates about ethoxyquin level that foods shall not be produced, imported, processed, used, cooked or stored for sale with exceeded ethoxyquin level more than 10 µg/kg.

In Thailand, shrimp is an important food product for both domestic consumption and exporting. The value of Thai shrimp is totally 17,023.51 billion baht in January to June 2012. [1] The largest export markets for Thai shrimp are the United States, Japan and EU. Recently, there is the international trading block case between Thailand and Japan, owing to the detection of sulfonamide in imported frozen sushi shrimp, cooked headless tail from Thailand. Furthermore, it has been lately reported that ethoxyquin was found in imported frozen chicken from Brazil at 130 µg/kg to Japan in 2008. Nowadays, preventive action for exported products has become an extremely important

issue. Therefore, the availability of simple and reliable systems for the detection of ethoxyquin residue is an essential tool for assuring the safety of food products.

Traditionally, analytical methods have been employed to determine ethoxyquin, such as thin layer chromatography (TLC) provides only semi-quantitative measurements and incomplete data leads to lack of specificity and precision for modern regulatory purpose. As the ethoxyquin residues in food are always present in very small quantities, the analytical method for determining ethoxyquin residue should be sensitive, accurate, reliable and rapid. Therefore, higher sensitive detection methods are required.

Chromatographic techniques for quantitative and qualitative analysis are principally based on different retention times of each compound. Liquid chromatography (LC) is a method of choice for determining the residues of pesticides, feed additives and veterinary drugs. There are several studies in chemical residue analysis using the instrument with relatively low selectivity such as high performance liquid chromatography (HPLC) with ultraviolet (UV), diode array (DAD), fluorescence (FLD), or electrochemical detection. Due to the lack of selectivity from sample preparation which often includes a lengthy clean up procedure, the result from this analysis needs to be confirmed by selective and sensitive chemical analysis.

Currently, the gas chromatography (GC) coupled with mass spectrometry (GC-MS) and liquid chromatography coupled with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) have become valuable techniques in the pesticide residue analysis. They provide the most efficient confirmatory tools that can discriminate the residues at ultra-trace level with reliable and sensitive detection to ensure food safety.

Sample preparation is one of the most important and crucial steps in the whole analytical process. Commonly, several sample preparation methods have been developed, including thin layer chromatography (TLC), liquid-liquid extraction (LLE), supercritical fluid extraction (SFE). Moreover, these methods use large amount of organic solvent, tedious, time-consuming, and also lack of sufficient selectivity, which are important consideration for trace analysis. Recently, the QuEChERS method (quick, easy, cheap, effective, rugged, and safe) have been developed as an attractive alternative method for sample preparation and have received the attraction as an official method for using in the detection of multiple pesticide residues in fruits and vegetables. The QuEChERS method is particularly popular for determination of polar, middle polar

and non-polar pesticide in various food matrices because of its simplicity, inexpensive, amenable to high throughput, and relatively high efficient results with a minimal number of steps.

Despite the worldwide extensive use of ethoxyquin in animal feed and the restrictive regulation on ethoxyquin the maximum residue limits (MRLs) in food, it is surprising that no specific method have been reported for the analysis ethoxyquin in shrimp. Because of the ethoxyquin polarity, the low residue levels and the polar and non-polar components in shrimp matrices (fat, protein and pigment), the ethoxyquin extraction is difficult. As a result, an effective and reliable sample preparation and sensitive analysis method are extremely required.

In this study, a rapid and effective method was newly developed and then validated for the analysis of the low concentration of ethoxyquin residue in shrimp.

1.2 Structure and chemistry

Ethoxyquine (6-ethoxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline) is a quinoline-based chemical, containing aromatic nitrogen compound characterized by double ring structure with a benzene fused to pyridine at two adjacent carbon atoms. (pyridine is a ring structure compound of five carbon atoms with a nitrogen atom). Quinoline itself is the simplest member of the quinoline. It is a hygroscopic, yellowish and oily liquid. It is commonly used as a preservative in animal feed and a pesticide (under commercial name such as “stop –scald”)

Table 1.1 Properties of ethoxyquin

	Properties
IUPAC name	6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline
Chemical formula	$C_{14}H_{19}NO$
Molecular weight	217.31 g/mol
Chemical group	quinoline
Melting point	<25ºC
Boiling point	123-125°C at 2 mmHg
Specific gravity	1.028 – 1.032
Vapor density	7.48
Solubility in water	<1 mg/mL at 20ºC
Stability	Stable, combustible, incompatible with oxidizing agents, strong acids, polymerizes if heated, may polymerize upon exposure to light and air

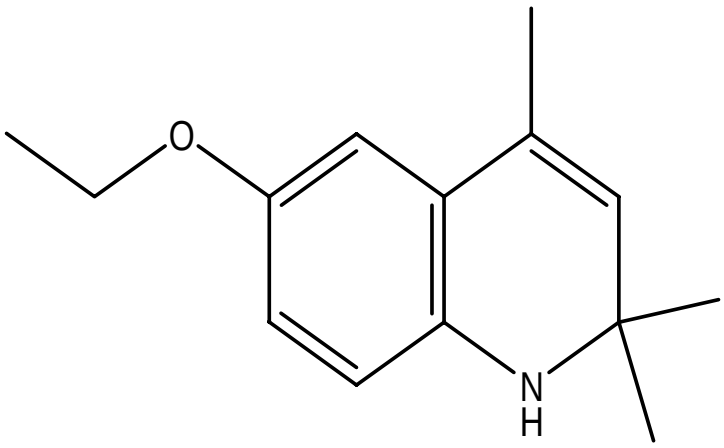

Figure 1.1 Chemical structure of ethoxyquin

1.3 Regulation of ethoxyquin in food

The regulatory regarding the control of ethoxyquin is given in Council Directive 70/524/EEC concerning additives in feeding stuffs. The regulatory limit for the use of ethoxyquin, alone or in combination with butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA), on feeds is less than 150 mg/kg. [2] In Japan, the Ministry of health, labour and Welfare (MHLW) introduced a positive list system for agricultural chemical remaining in foods, which is a system to prohibit the distribution of foods containing agricultural chemical levels exceed the MRLs. Moreover, the Minister of MHLW has been legislated the limitation of agricultural chemical residues (including pesticides, feed additives, and veterinary drugs) in foods by shall not be produced, imported, processed, used, cooked, stored for sale, or sold, and the control level of residues is 0.01 mg/kg [3,4]. Food and drug Administration (FDA) has specifically established a tolerance limit of 0.5 mg/kg for ethoxyquin residues in or on the uncooked meat and meat by products (including milk and eggs) and 5 mg/kg for cooked meat and meat by products of animals fed forage crops or feed supplements treated with ethoxyquin. For uncooked liver and fat of poultry feed treated feed, a limit of 3 mg/kg has been established. Tolerance residues of 3 mg/kg have also been set for apples and pears. [5] The maximal residual level of ethoxyquin tolerated in fillet of farmed has not been yet regulated, leaving the acceptable daily intake (ADI) as the only legislated value to guide the industry and the ADI for ethoxyquin is 0-0.005 mg/kg body weight. [6]

On the basis of Commission Decision 2002/657/EC, when MS detection is performed by fragmentography, the pseudomolecular ion shall preferably be selected as a precursor ion and a system of identification points (IPs) shall be used to interpret the data. For confirmation of substances, a minimum of 4 IPs is usually required as targeted compounds. The number of IPs is useful for the confirmation of an analyte and also depends on the type of mass analyzer and its resolution grades. A triple quadrupole is a low resolution mass spectrometer, when working in multiple reactions monitoring (MRM) mode. In this case, 1 IPs is earned for the precursor ion and 1.5 IPs for each product ion. Each ion should only be counted once. The selection of two MRM transition allows the earning of 4 IPs, ensuing substance compounds confirmation. [7]

1.4 Literature Review

In 1970, EU recommended that ethoxyquin concentration in feed should be lower than 150 µg/kg when use as single chemical or in combination with other legislated antioxidants.

Traditionally, analysis methods for ethoxyquin were based on thin layer chromatography (TLC) but it often lacks selectivity and precision for regulatory purposes. Therefore, TLC is not suitable for quantitative analysis of ethoxyquin in feed animal and animal tissue. To overcome these problems, chromatographic method has been utilized for analysis of ethoxyquin. Liquid chromatography is commonly coupled with spectrophotometric detections such as ultraviolet (UV) or fluorescence detection (FLD) to determine ethoxyquin in feed animal and food producing animal.

In 1996, Schreier *et al*. reported the use of acetonitrile for extraction for ethoxyquin in pet food. Supernatant is analyzed by isocratic liquid chromatographic with LC-FLD. The detection limit of ethoxyquin was 0.5 µg/kg and used fewer solvents. This method provides enough sensitive for manufacturers to be able detect ethoxyquin in extruded pet foods are labeled natural. [8]

High-performance liquid chromatography with FLD detection was also used to determine ethoxyquin residue and ethoxyquin dimer in ocean farmed salmons by Ackman *et al*. [9] The samples were submitted to hydrolyze with 50% NaOH, and then the lipid and antioxidant residues were extracted with hexane. After solvent removal and recovery with acetonitrile, the ethoxyquin was separated on a CSC-2 column using an isocratic elution with mixture of 0.02 ammonium acetate and acetonitrile (80:20). The ethoxyquin determination was monitored at 360 nm excitation wavelength and 440 nm emission wavelengths. The recoveries ranged 83-93%. This method was carried out to confirm the occurrence of ethoxyquin and ethoxyquin dimer by GC-MS.

In 2001, Brannegan *et al*. was studied the extraction of ethoxyquin in lean beef matrices. SFE with methanol modified 1, 1, 1, 2-tetrafluoroethane was used in the extraction of ethoxyquin in lean beef and beef fat. The dynamic time of 60 min was used for all extraction. For separation of ethoxyquin, an ODS column was used in isocratic with the mixture of methanol and water (75:25, v/v). Ethoxyquin was determined by HPLC-UV. This method has limit of quantification (LOQ) at 0.5 µg/kg. This SFE method provided a very clean extraction.

In 2006, EU and MHLW has set legislation and the maximum residue limits (MRLs) of the use of ethoxyquin in food animal and food stuff. [3] There are several works

attempting to determine the residue of ethoxyquin with highest effective analysis methods.

Berdikova *et al.* determined ethoxyquin and ethoxyquin metabolites in salmon. Sample saponified in ethanol–NaOH was extracted with hexane and determined by HPLC-FLD. The separation was achieved on tandem coupled phenyl hexyl and C18 columns by two phase gradient elution with acetonitrile containing ascorbic acid and diethyl amine containing acetic acid. The total runs time was within 23.5 min. The ethoxyquin determination was monitored at excitation wavelength set at 358 nm and the emission wavelength at 433 nm. The repeatability and inter-day precision of the method in salmon muscle were in the range of 2.3-10.6% and 10.4-20.9%, respectively. The within-day precision of quantitation in tissue was in the range of 0.4-16%. Accuracy was 96-99%. The reported operative range for the determination of ethoxyquin dimer in salmon was 0.2-10 µg/kg. [10]

Aoki *et al*. presented the determination of ethoxyquin in food products of animal origin using HPLC-FLD with simple and rapid sample preparation. The samples were extracted by methanol and isopropanol and then evaporated to dryness. The dry residue was dissolved in acetonitrile and water. HPLC was performed using a silica octadecylsilane column and butyl hydroxyl toluene-acetonitrile-water (0.05:800:200 (v, v, v) as mobile phase, and detection was performed at excitation and emission wavelengths of 370 and 415 nm, respectively. HPLC-MS was used to confirm for positive sample. The LOQ of foods was 0.01 µg/g. Residue levels of ethoxyquin in 33 commercially available food products of animal origin purchased on the west side of the Tokyo metropolitan area were surveyed. Contents of ethoxyquin residues in three chicken fat samples by this method were 0.03, 0.04 and 0.08 µg/g. All detecting amount was less than the MRL (5µg/g). [11]

In 2010, Koesukwiwat *et al.* was investigated the high throughput analysis of 150 pesticides in fruits and vegetables using QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) and low pressure gas chromatography time of flight mass spectrometry. The method is simple, rapid and reliable determination for nearly 150 pesticides residues in fruits. [17]

Additionally, there is the method using liquid chromatography coupled to time of flight mass spectrometry (LC-TOF) for the quantitative analysis of pesticides. The samples were extracted using QuEChERS extraction. The separation of pesticide was achieved on Acquity UPLC HSS T3 column, is based on high resolution column which provides

strong retention for polar analyte. The limit of quantification was less than 10µg/kg. [37]

1.5 Purpose of the study

Since ethoxyquin was regulated by EU and MHLW due to health risk assessment, many researchers were paid attention to find a method that obtains limits of detection below the MRLs. From literature review, ethoxyquin in feed and animal origin (cattle, cow, chicken, salmon and pig) were analyzed with various sample preparation and detection techniques. HPLC-UV and HPLC-FLD has become a common detection technique because of high sensitivity. However, the analysis needs to be confirmed by HPLC-MS because the most reliable and acceptable chromatographic technique used for confirmation of pesticides and food additive residues in food and food stuffs, is the LC-MS/MS. The combination of LC and MS/MS allows both quantification and identification of analyte. The requirement for quantitative results with high selectivity and specificity at trace level in food matrices need for a powerful analytical technique, therefore tandem MS detection completely fulfills these criteria. The sample preparation is normally a necessary tool to reach the low limits of detection, which are required in the analysis of ethoxyquin in food from animal origin. Most extraction methods of ethoxyquin from animal tissue consist of extract by LLE. However, common extraction processes require an additional step of filtration and evaporation. These methods use large amount of organic solvent, time-consuming and also lack of sufficient selectivity, which are an important consideration for residue analysis. Recently, the QuEChERS method has been developed as an attractive method for sample preparation. The QuEChERS method provides significant advantages over traditional methods, including high recoveries for wide range of pesticide, high sample throughput, minimal labor, time saving, limited solvent usage, and low waste production. In addition, the method is manually accommodating which has made QuEChERS a very popular methodology for the analysis of pesticide residues in recent years.

In this work, the modified QuEChERS approach was used for the sample preparation of ethoxyquin residues in shrimp. Their residues usually exist in low amounts and induce the difficulty to extract from complex matrices. The structure and property of this ethoxyquin are shown in Table 1.1.

In modified QuEChERS, this method entailed using an acidified acetonitrile extraction, partitioning with a magnesium sulfate, followed by a dispersive solid phase extraction (d-SPE) clean up and sample solution was then diluted with organic solvent. After extraction, the extraction solution was directly injected to UPLC-MS/MS. The related parameters were optimized such as the concentration acidified acetonitrile, different types of dispersive solid phase extraction (d-SPE) sorbent, various amounts of d-SPE and dilution solution ratio. The optimized modified QuEChERS method was applied with various extraction methods for extraction of ethoxyquin in shrimp obtained from local fresh market and super markets, Bangkok, Thailand and shrimp farm for export in Thailand.

CHAPTER II

THEORY

2.1 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) [20, 21, 22, 23, 24]

Liquid chromatography (LC) is worldwide technique of chromatographic separation which is based on the difference in the surface interaction of the compound between two phases on the difference in the surface; these are called a stationary phase and a mobile phase. High performance liquid chromatography (HPLC) is the term commonly used to describe liquid chromatography. HPLC is very effective in separating a polar, non-volatile and thermally labile compound. Unfortunately, the lack of sensitive and selective LC detectors had, just until recently, prevented the widespread of this technique in food residue analysis. This drawback has been solved by combining LC with mass spectrometry (MS). MS is the detection system that can overcome this limitation and also offers high sensitivity and selectivity of analysis. MS can provide absolute identification molecular weight, structure, identity, quantity and purity of a sample.

2.1.1 High performance liquid chromatography (HPLC)

Chromatography is a technique, in which a mixture sample is separated into components, so can be identified and measured. HPLC, principle is separation of mixtures by passage of the sample through a column containing a stationary solid by means of a pressurized flow of a liquid mobile phase; components migrate through the column at different rates dues to different relative affinities for the stationary and mobile phases based on adsorption, size or charge. A schematic diagram of a typical HPLC instrument is shown in Figure 2.1

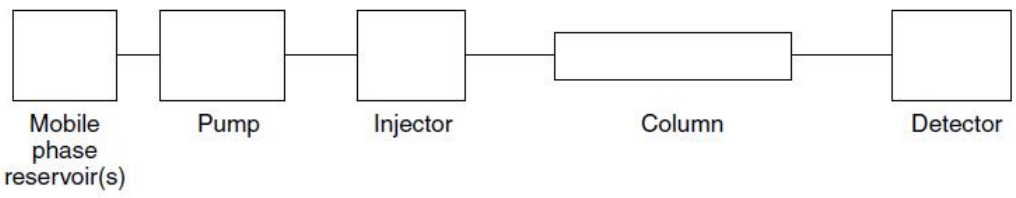

Figure 2.1 Schematic diagram of a typical HPLC instrument [20]

2.1.1.1 Mobile phase reservoir and mobile phase

The mobile phase reservoir can be any clean, inert container that it is made with glass or stainless steel and can contain 200 to 2000 mL of mobile phase and it should have a cap that allows the tubing inlet line to the pumping system. The mobile phase should be degas solvents before used because the dissolved gases in the mobile phase can collect in other components, particularly in the pump heads and the detector. Mobile phase in HPLC, the relative interaction of analyte with both the mobile phase and stationary phases determines its retention characteristics. Hence, it is the varying degrees of interaction of different analytes with the mobile phase and stationary phase. The solvents chosen affect the elution of solute that HPLC requires a mobile phase by which the analytes are soluble. It is not always possible to achieve an adequate separation by using a single solvent as a mobile phase, therefore, mobile phase are usually mixture of two or more individual solvents with or without additional additive or modifiers. Separation involving a mobile phase of constant composition is called isocratic elution, while change in mobile phase composition during the separation is called gradient elution. Gradient elution mode reduces analysis time and increase resolution for complex mixtures. Buffer is used in HPLC to control the degree of ionization of the analyte and thus the tailing of response and the reproducibility of retention. Solvent are typically chosen based on a compound of interest is solubility and compatibility with various ionization techniques used in LC-MS. Volatility and the solvent's ability to donate a proton are important in electrospray ionization (ESI) and other atmospheric ionization techniques. A range of buffers is available but those most widely used are inorganic, and thus non-volatile, materials, such as potassium or sodium phosphate.

Solvent considerations

- Solvent in the gas phase limits ionization by ESI to molecules more basic than the solvent. The exception is photoionization (which is not acid/base ionization) but nonetheless mediated by solvent.
- Removing solvent and water vapor from the ionization region increases types of compounds that can be ionized at atmospheric pressure.
- Reducing liquid volume relative to the sample or analyte of interest contained in the liquid improves ESI performance (i.e., lower flow rates).

- Useful Solvents
- Water
- Acetonitrile
- Methanol
- Ethanol
- Propanol
- Isopropanol
- Acceptable additives
- Acetic acid , pKa 4.76 (Maximum buffering obtained when used with ammonium acetate salt, Used in 0.1-1.0% range)
- Formic acid, pKa 3.75 (Maximum buffering obtained when used with ammonium formate salt. Used in 0.1-1.0% range.
- Ammonium hydroxide
- Ammonium formate, pKa 9.2 (Buffer range 8.2-10.2, Used in the 1-10 mM range.)
- Ammonium acetate, pKa 9.2 (Buffer range 8.2-10.2, Used in the 1-10 mM range)
- Nonvolatile salts (phosphate, borate, citrate, etc.)
- Can deposit in source and plug capillaries thus requiring more cleaning and maintenance operations
- Modern source designs can handle non-volatiles better than older designs
- Surface-active agents (surfactants/detergents) suppress electrospray ionization
- Inorganic acids are corrosive
- Trifluoroacetic acid (TFA), pKa 0.3 (Ion pair additive, can suppress MS signal, used in the 0.02-0.15 range.
- To some extent suppresses positive-ion electrospray at levels exceeding 0.01%.
- Greatly suppressed negative-ion electrospray.
- Triethylamine (TEA)
- Suppresses positive ion electrospray of less basic compounds.

- Tetrahydrofuran (THF)
- 100% THF is highly flammable, so at morphemic pressure chemical ionization (APCI) and most interface techniques use nitrogen as the nebulizer gas. (Using air creates an explosion hazard).
- Reacts with PEEK® tubing.

2.1.1.2 Pump

The pump is to deliver mobile phase solvents from their reservoir. There the number of difference types of pumps that can provide the pressure and flow rate required. Most HPLC pumps are based on a reciprocating piston design. A driven motor pulls the piston back and forth in the pump head. The pump must provide stable flow rates from 0.1-2 mL/min with LC-MS requirement dependent upon the interface being used and diameter of the HPLC column.

2.1.1.3 Sample introduction (Injector)

The purpose of the introduction system is to apply the sample extract onto the column in a narrow band. The sample is introduced using a loop injection (Figure 2.2). Sampling loops are interchangeable and available with volumes ranging from 0.5-2 mL. In the load position, the sampling loop is isolated from the mobile phase and is open to the atmosphere. The syringe with a capacity several times that of a sampling loop is used to place the sample in the loop. Any extra sample beyond that needed to fill the sample loop exits though the waste line. After looping the sample, the injector is turned to the inject position. In the position the mobile phase is directed though the sampling loop and the sample is swept onto the column.

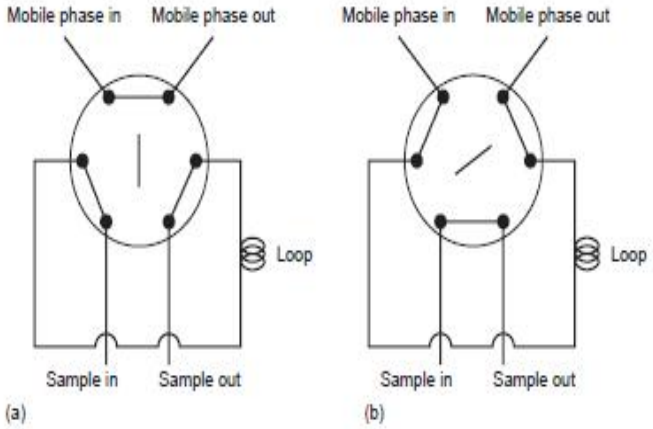

Figure 2.2 Schematic diagram of loop injection in the (a) load and (b) inject position [22]

2.1.1.4 Stationary phase (Column)

The column is a very important of the HPLC instrument as the separation occurs here. The most widely used columns contain a chemically modified silica stationary phase, in which the chemical modification determines the polarity of the column. A very popular stationary phase is one with a C 18 alkyl group is bonded to the silica surface.

2.1.1.5 Detector

Detector is to monitor the solute as they are eluted from the column. The choice of detector depends upon the characteristics and concentration of the compounds which need to be separated and analyzed. A number are in routine method use, including the UV/Vis, fluorescence, electrochemical, conductivity, refractive index and mass spectrometer detectors. The most popular HPLC, detectors based on spectroscopic measurement are UV/visible and fluorescence detectors. The analytical wavelength is selected using appropriated filters to essentially a modified spectrophotometer equipped with flow cell. When using a UV/Vis detector, the resulting chromatogram is a plot of absorbance as a function of wavelength and elution time. Fluorescence detectors provide additional selectivity when solutes can fluorescence. The resulting chromatogram is a plot of fluorescence intensity as a function of time. Nowadays, mass spectrometry (MS) is commonly used as a chromatographic detector. MS determination can be definitive, providing information on analyte retention and concentration, while simultaneously confirming analyte identity.

2.1.2 Ultra performance liquid chromatography (UPLC)

UPLC has been developed with the same practicality and principle as HPLC. One of the primary drivers for the growth of this technique has been evaluation of packing materials used to affect the separation. The underlying principles of this evaluation are governed by the van deemter equation (Figure 2.3), which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency). Since particle size is one of the variables, a van deemter curve can be used to investigate chromatographic performance. According to the van deemter equation, as the particle size decreases to less than 2.5 µm, not only is there a significant gain in efficiency, but the efficiency does not diminish at increased flow rate or linear velocity. By using smaller particles, speed and peak capacity (number of peak resolved per unit time in gradient separations) can be extended to new limits, commercialized by WATERS Corporation (Milford, Massachusetts) as ACQUITY UPLC™ or Ultra Performance Liquid Chromatography. The technology takes full advantage of chromatographic principle to run separations using columns packed with smaller particles and/or higher flow rate for increased speed, with superior resolution and sensitivity. For these characteristics, UPLC provides high speed separation with low injection volume and proposes high sensitivity from less band spreading during migration through a column. A UPLC column is illustrated in Figure 2.4

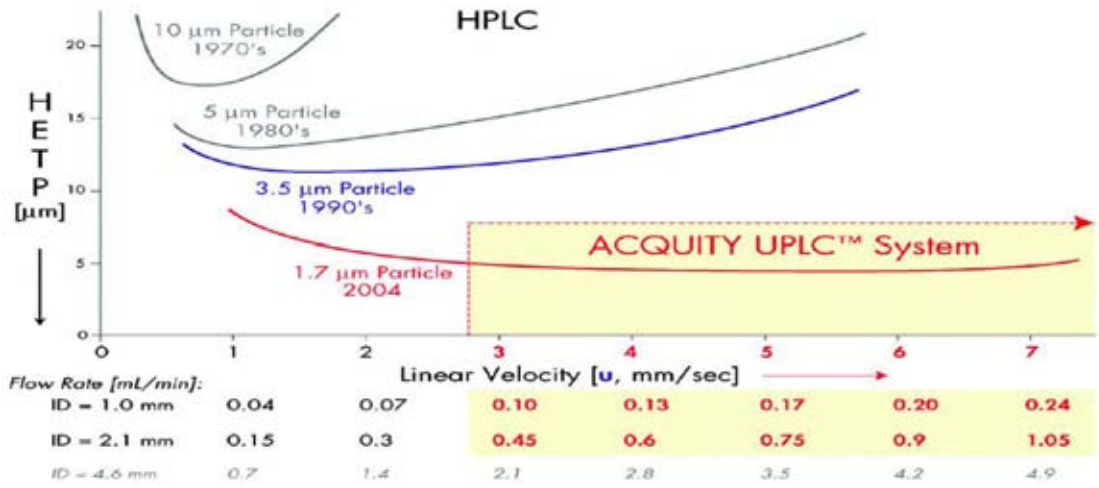

Figure 2.3 Van deemter plot [37]

Figure 2.4 Acquity UPLC® column [24]

The Acquity UPLC® columns have been created for widely used pH range and pressure tolerant, capable at operating at pressures up to 15000 psi (1000 bar). Acquity UPLC® columns are available in two particle substrate Ethylene Bridged Hybrid (BEH) and High Strength Silica (HSS). The hybrid organic-inorganic particles are prepared via co-polymerizations which were designed to retain silica's mechanical strength while overcoming pure silica's tendency to undergo column-damaging hydrolysis in alkaline environments (greater than pH 8). The column chemistries include C8, C18, phenyl and hilic etc. Moreover, eCord™ technology (Figure 2.4) is installed for unique information and used record of each column type.

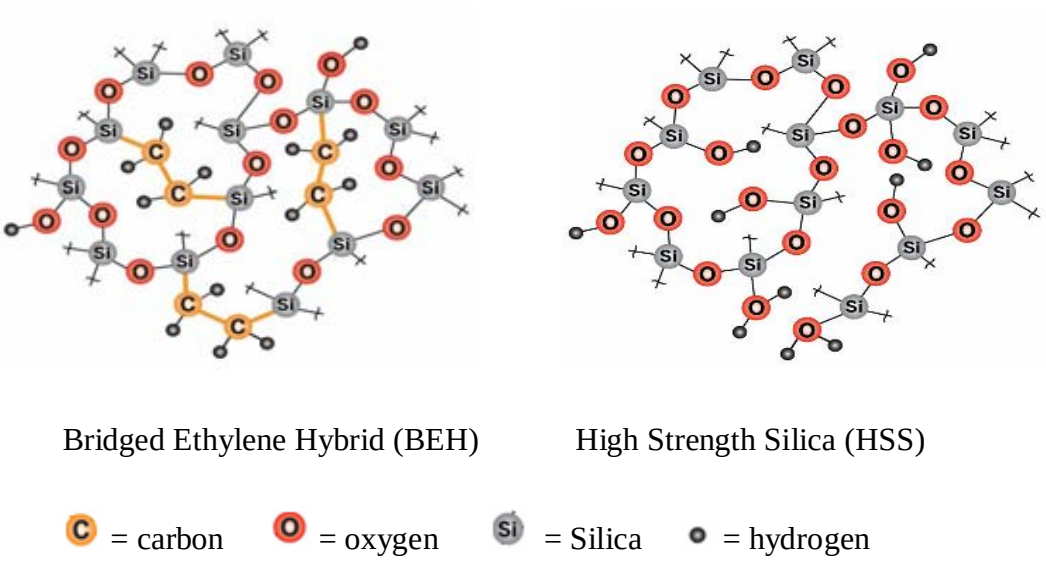

Figure 2.5 The particle profile of Acquity UPLC column [26]

The Acquity UPLC particle created was the 1.7 µm Bridged Ethylene Hybrid (BEH) particles which contain C-C bridges between pairs of silica atoms. (Figure 2.5) The hybrid organic-inorganic particles, which are prepared via copolymerization of TEOS and bis (triethoxysilyl) ethane, were designed to retain silica's mechanical strength while overcoming pure silica's tendency to undergo column-damaging hydrolysis in alkaline environments (greater than pH 8). BEH's covalently bonded Si-C-C-Si units render the hybrid material chemically stable up to a pH of 12. The second Acquity UPLC column type is the particle 1.8 µm High Strength Silica (HSS) particle which is 100% silica particle specially designed for tolerable pressure up to 15,000 psi or 1,034 bar (Figure 2.5). HSS particle technology is also available in HPLC particle sizes (3.5 and 5 µm) in the HSS family of HPLC columns, enabling seamless transfer between HPLC and UPLC technology platforms. Therefore the UPLC application requires a better pumping system than HPLC and detector for UPLC must have a high sampling rate for sensitive detection and reliable quantification of the narrow peaks produced.

2.1.3 Mass spectrometry (MS) [26, 27, 28, 29, 30]

Mass spectrometry (MS) is an analytical technique that measures the mass to charge ratio of charge particles. It is used for determining masses of particles for determining the elemental composition of a sample or molecules, and elucidating the chemical structure of molecules. MS worked by ionizing chemical compounds to generate charge molecules or molecule fragment and measuring their mass to charge ratios. A diagram of a typical mass spectrometer scheme is depicted in Figure 2.6. The simplest form of an MS system should perform the following fundamental tasks.

1. Vaporize compounds of varying volatility. This is accomplished in the inlet system. Introduction of the sample is done by direct insertion probe, reservoir inlet, following a chromatographic separation (HPLC and UPLC). As mentioned earlier, to introduce the LC flow to the mass spectrometer on line, we need an appropriate interface. Development of appropriate interfaces was the utmost for evolution of the LC-MS coupling.

2. The components of the sample are ionized by one of a variety of methods. This takes place in the ion source. As can be seen in Figure 2.6, there are several modes of ion source. Of those, the most used in LC-MS are

chemical ionization (CI), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).

3. The ions are separated according to their mass to charge ratio (m/z ratio). This takes place in the mass analyzer. The most used analyzer is the quadrupole, either as single or as triple quadrupole, combined with a soft ionization.

4. The ions are detected, usually by a quantitative method.

5. The ions signal is processed into mass spectra.

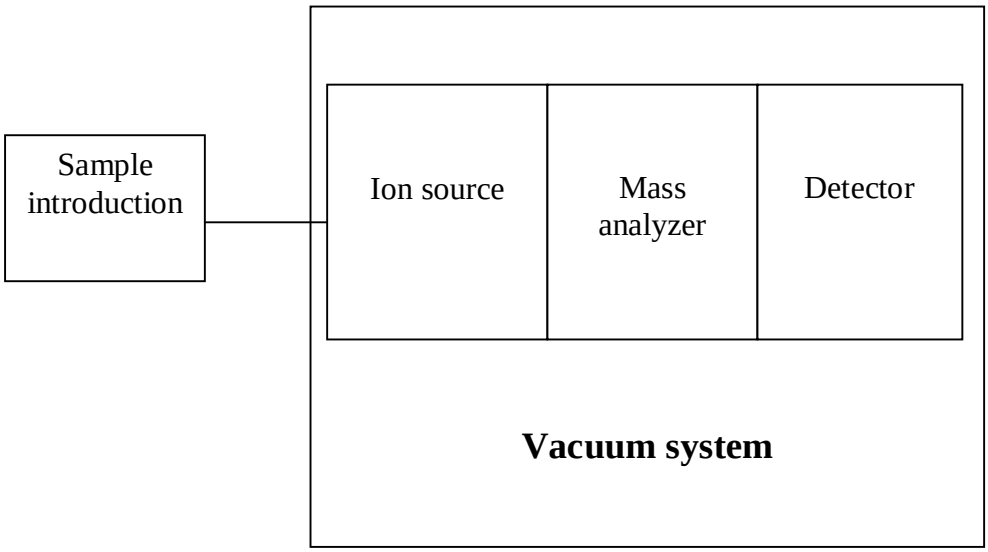

Figure 2.6 Schematic diagram of MS system

2.1.3.1 Ionization methods

Ionization methods that may be utilized in LC-MS include chemical ionization (CI), electron ionization (EI), fast atom bombardment (FAB), thermospray (TSP), electrospray (ESI) and atmospheric pressure chemical ionization (APCI). There have two ionization interface, ESI and APCI are available with most LC-MS instruments. Choice of an LC-MS interface for a particular application depends on polarity and molecular mass of the analyte.

2.1.3.1.1 Electrospray ionization (ESI)

ESI is a useful ionization technique to analyze sample that become single or multiple charge depending on their molecular structure. In ESI, as in Figure 2.7, ESI source as both an LC interface and a source of generating ions. The heart of the ESI source is a stainless steel capillary tube through which a solvent flows continuously at rate of 2-5 µL/min. The flow is assisted by an infusion syringe or an LC pump. A high voltage is applied to the tip of a capillary to produce an electrostatic field sufficiently strong to disperse the emerging solution into a fine mist of charge droplets. The ions are sampled through a conical nozzle and a skimmer into regions maintained at progressively reduced pressures, where most of the neutrals are removed by auxiliary pumping. The free ions then enter the mass analyzer for subsequent mass analysis. The formations of negative and positive ions depend on the capillary bias. ESI is capable of analyzing small to very large compound. The basis of high mass analysis is the formation of a series of multiple charged ions of a molecule. The benefit of multiple charging is to reduce significantly the m/z ratio of the intact analyte bringing very high mass analytes with in the usable mass range of a mass spectrometer. Several parameters must be controlled to obtain a stable spray of the emerging solvent. Flow rate, conductivity and surface tension of solvent profoundly influence operation of the electrospray process. A flow rate of 1-10 µL/min is optimum for maintaining a stable spray. At higher flow rate, larger droplets are formed, leading to electrical breakdown. This technique can be analyzed by a wide variety of mass analyzers, including quadrupole, ion trap and time of flight.

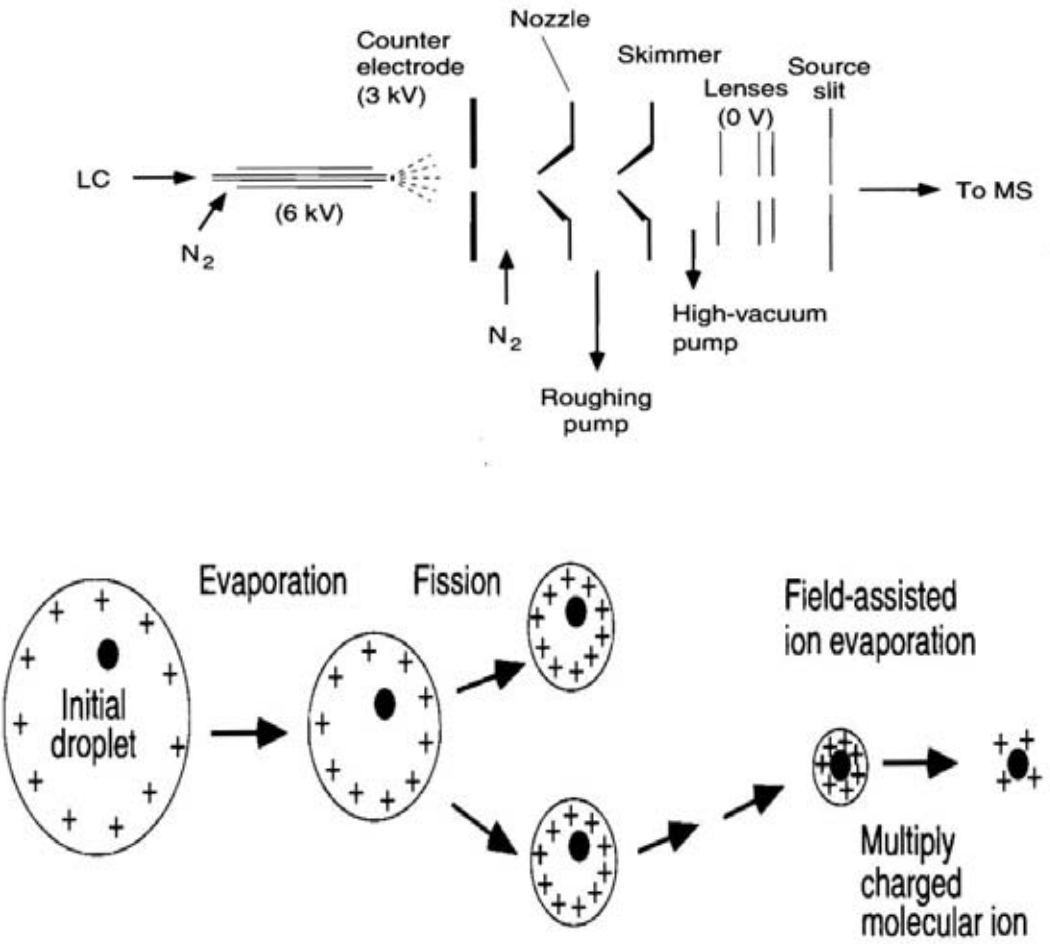

Figure 2.7 Schematic of the atmospheric pressure electrospray ionization process [30]

2.1.3.1.2 Atmospheric pressure chemical ionization (APCI)

APCI is an ionization method because it generates ions directly from solution and it is capable of analyzing relatively polar and nonpolar compounds, as with ESI, the liquid effluent of APCI (Figure 2.8), liquid is pumped though a capillary and nebulized at the tip. A corona discharge takes place near the tip of the capillary, initially ionizing gas and solvent molecules present in the ion source. However, the similarity stops there. The droplets are not charge and the APCI source contains a heated vaporizer, which facilitates rapid desolvation/vaporization of the droplet. Vaporized sample molecules are carried through an ion molecule reaction region at atmospheric pressure. The technique is capable of dealing with flow rates between 0.5-2 mL/min and so making it directly compatible with 4.6 mm columns and is much more tolerant to a range of buffers.

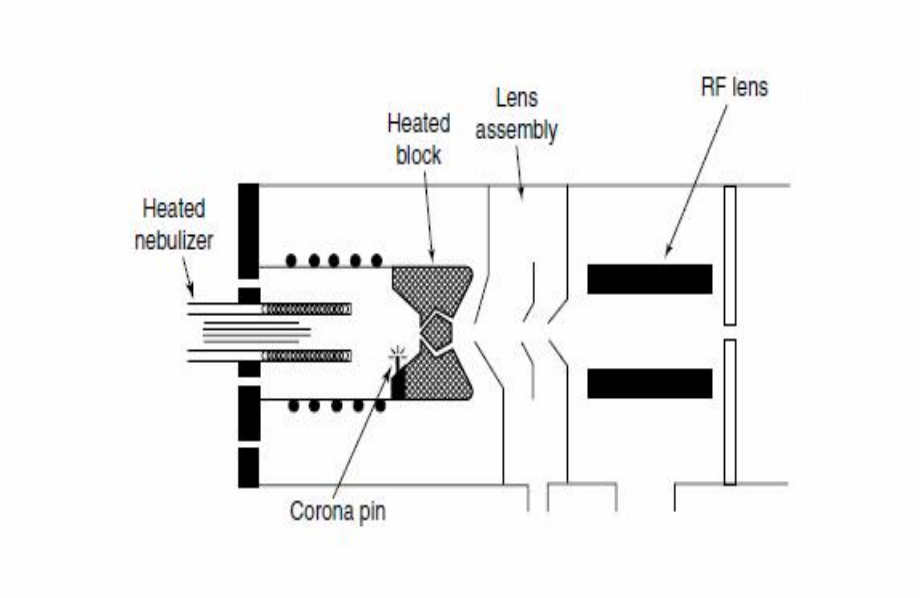

Figure 2.8 Schematic of the atmospheric pressure chemical ionization process [29]

2.1.3.2 Mass analyzer

The mass analyzer separates ions by their mass to charge ratio (m/z) in space or in time. After ions are formed in the ion source region, they are accelerated into the mass analyzer. The mechanism is performed with electric and magnetic fields, sometimes including RF fields. There should be some ion focusing device to prevent the spread of ions from the ion source. The selection of the mass analyzer depends on the resolution, scan rate, mass range and detection limits required for the application. Each analyzer has different operating characteristics, and an additional instrument. In hyphenated LC-MS, quadrupole, quadrupole ion trap and time of flight (TOF) are widely used mass analyzer. These techniques are considered as ion transmission system.

2.1.3.2.1 Quadrupole mass analyzer

The quadrupole mass spectrometer is a most common mass analyzer because of its compact size, high transmission efficiency, fast scan rate and moderate vacuum requirements. The quadrupole analyzer consists of a set of four parallel metal rods or electrodes which must be precisely straight and parallel. Two parallel rods are connected to direct current (DC), while the other are connected to radio frequency (RF). When the beam of ions directed axially between the quadrupole, both DC and RF are chosen to filter ions according to their m/z, only ions of selected m/z or resonance ions pass through quadrupole analyzer. (Figure 2.9) A combination of constant and varying

radio frequency (RF) voltages allows the transmission of a narrow band of m/z values along the axis of the rods. By varying the voltages with time, it is possible to scan across a range of m/z values, resulting in a mass spectrum and scan speeds up to 1000 m/z per second or more are common. They usually operate at unit mass resolution meaning that the mass accuracy is seldom better than 0.1 m/z. As an alternative to scanning, the quadrupole can be set to monitor a specific m/z value, the set to monitor another m/z value, and so on. This is achieved by stepping the voltages. This technical is useful in improving the detection limits of targeted analytes.

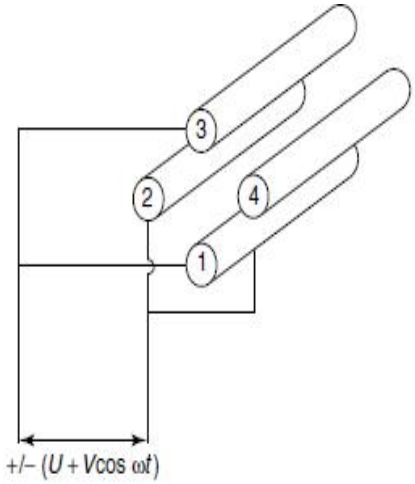

Figure 2.9 Schematic of the quadrupole mass analyzer [29**]**

2.1.3.2.2 Quadrupole ion trap mass analyzer

The quadrupole ion trap consists of a ring shaped electrode with curved caps on the top and bottom. (Figure 2.10) Ions are injected form the source through one of the caps, and by applying a combination of voltages to the ring and capping electrodes, the ions can be trapped in complicated three dimensional orbits. The electric field is constructed in such a way that the force on an ion is proportional to its distance from the center of the trap. A constant pressure of helium is usually maintained in the cell to remove excess energy from the ions, which would otherwise repel other to the extent their trajectories became unstable causing loss of ion from the trap. Application of a resonant frequency allow selection expulsion of ions of a given m/z ratio, generating a mass spectrum.

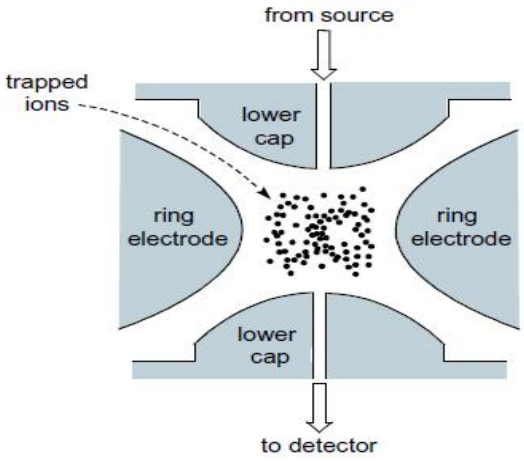

Figure 2.10 Schematic of the quadrupole ion trap mass analyzer [31**]**

2.1.3.2.3 Time of flight (TOF) mass analyzer

Time of Fight (TOF) analysis operates based on accelerating ions though a high voltage. The velocity of the ions and hence the time taken to travel down a flight tube to reach the detector, depends on their m/z values. The resolution of the TOF analyzer is dependent upon the ability to measure the very small difference in the times required for ions of similar m/z to reach the detector. TOF analyzer can acquire spectra extremely quickly with high sensitivity. It also has high mass accuracy, which allows a molecular formula to be determined for small molecules. (Figure 2.11)

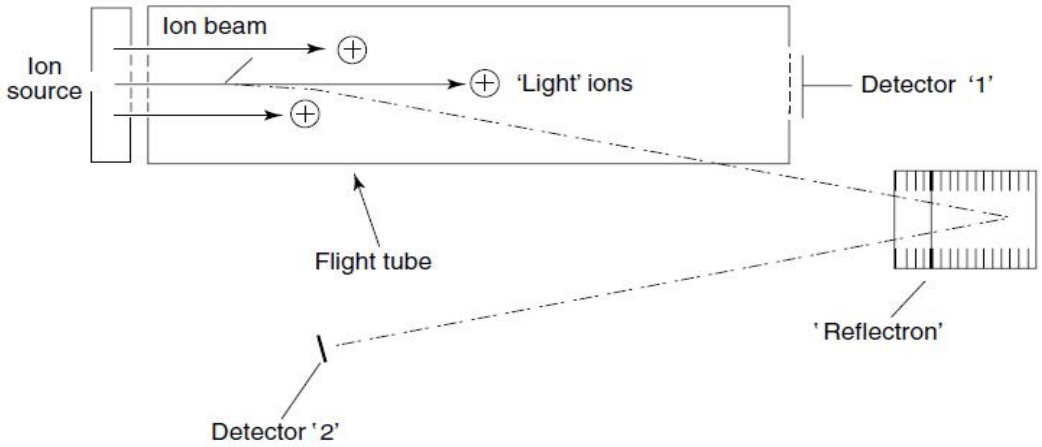

Figure 2.11 Schematic of the time of flight (TOF) mass analyzer [29]

2.1.3.3 Detector

The detector is used to measure the ions leaving from the mass analyzer by converting the ions into an electrical current or other forms of signal, processing and recording into ion mass spectrum. A detector is selected by speed, dynamic range, gain and geometry. Most detectors currently used to amplify the ion signal are electron multiplier tube (Figure 2.12) and photo multiplier tube (Figure 2.13). Electron multiplier tube offers electron from surface of tube for analyte ions. Analyte ions are attracted to the entrance of tube and collide with tube surface, and then the inner surface coated with electron emission material releases electrons. These electrons re accelerated to hit another portion of tube electrostatic force and the surface loses more electrons in every collisions. Amplified electrons are counted by an electrical circuit and displayed as signal intensity. The photo multiplier tube comprises a photocathode and a series of dynodes. In the high voltage tube, incident photo strikes the photo cathode and emits electrons due to the photoelectric effect. These electrons are accelerated towards a series of additional electrodes called dynodes. At the dynodes, the amount of electrons is increased at every collision. This creates an amplified signal that is finally collected and measure at the node.

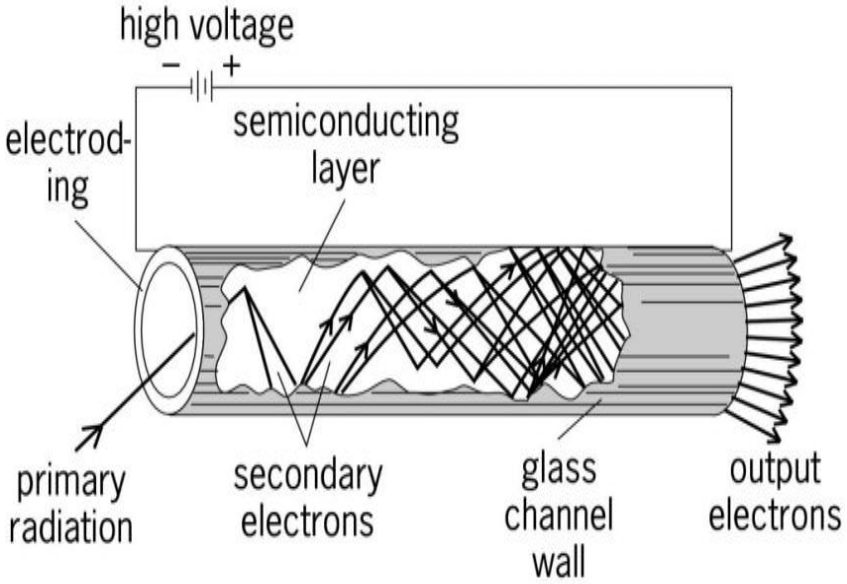

Figure 2.12 Electron multiplier tube [32]

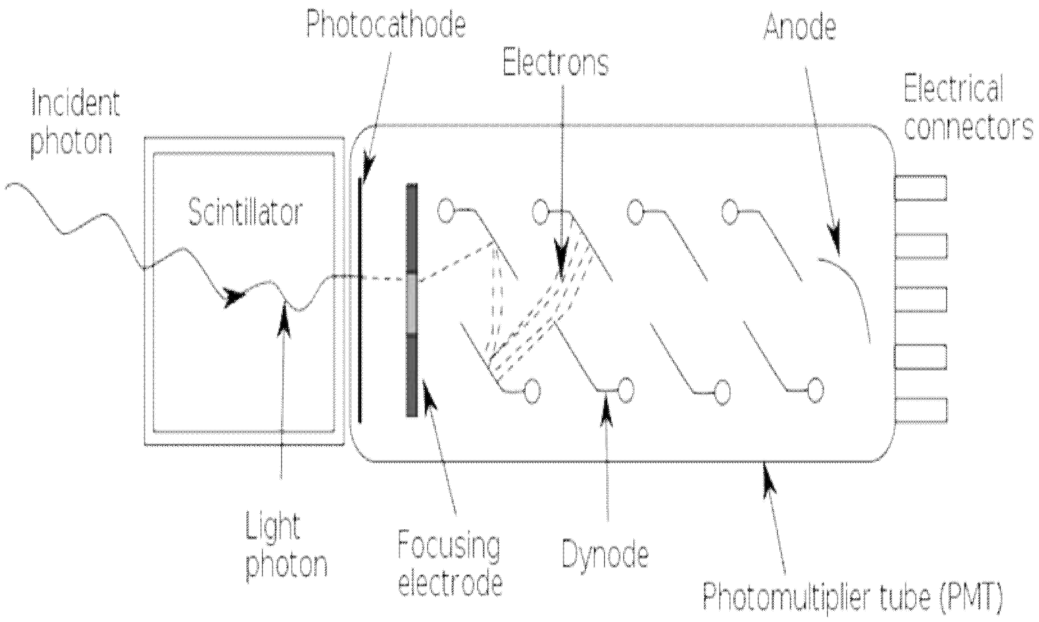

Figure 2.13 Photo multiplier tubes [33]

2.1.4 Tandem mass spectrometry (MS/MS)

Tandem mass spectrometry (MS/MS) is term which covers a number of techniques in which one stage of mass spectrometry, not necessarily the first, is used to isolate an ion of interest. The second stage is then used to probe the relationship of this ion with other from which it may have been generated or which it may generate on decomposition. These two stages of mass spectrometry are related in specific ways in order to provide the desired analytical information. There is a large number of difference MS/MS experiment that can be carried out the four most popular are the precursor ion scan, product ion scan, the neutral loss scan and selected reaction monitoring. There are many operating modes as described in section 2.1.4.1 to 2.1.4.4 below.

2.1.4.1 Precursor ion scan mode

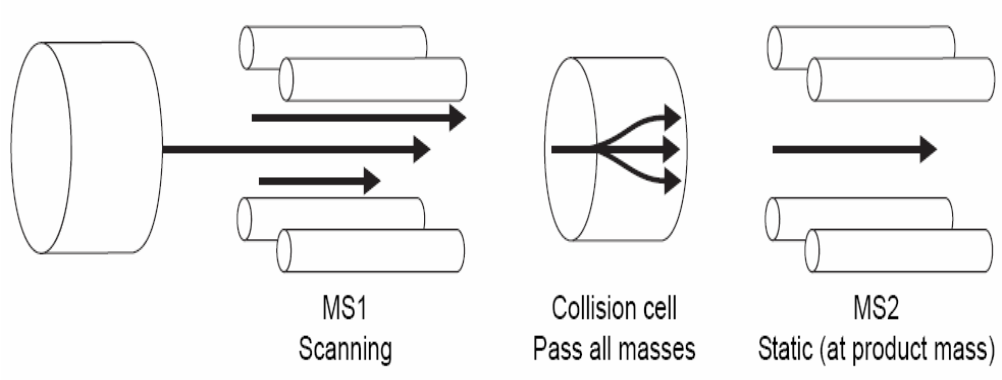

Figure 2.14 Schematic of precursor ion scan mode [34]

This mode is used to determine what higher mass has a similar fragment ion. The second quadrupole isolates a specific fragment ion and the first quadrupole is scanned to pass a wide range of ions to the collision cell for fragmentation.

2.1.4.2 Product ion scan mode

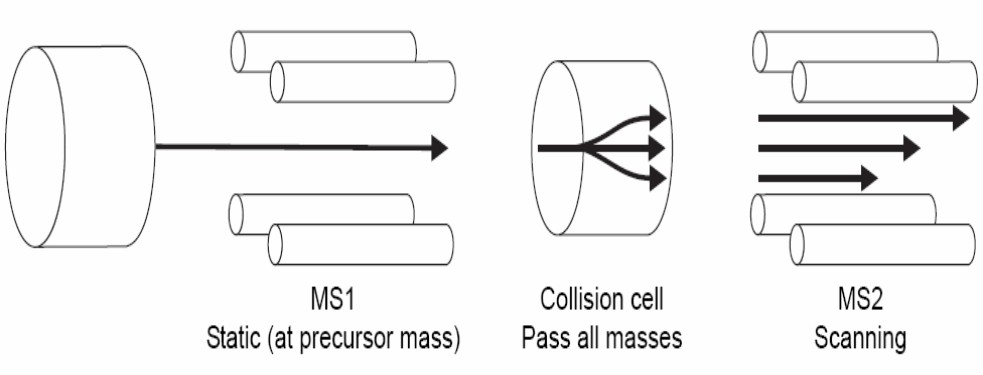

Figure 2.15 Schematic of product ion scan mode [34]

The first quadrupole is static, passing a selected m/z into the collision cell. A voltage is applied in the collision cell to accelerate the ion into the collision gas (argon); this process results in fragmentation. The ions from the fragmentation pass into the second quadrupole which is scanned to acquire all of the ions.

2.1.4.3 Constant neutral loss

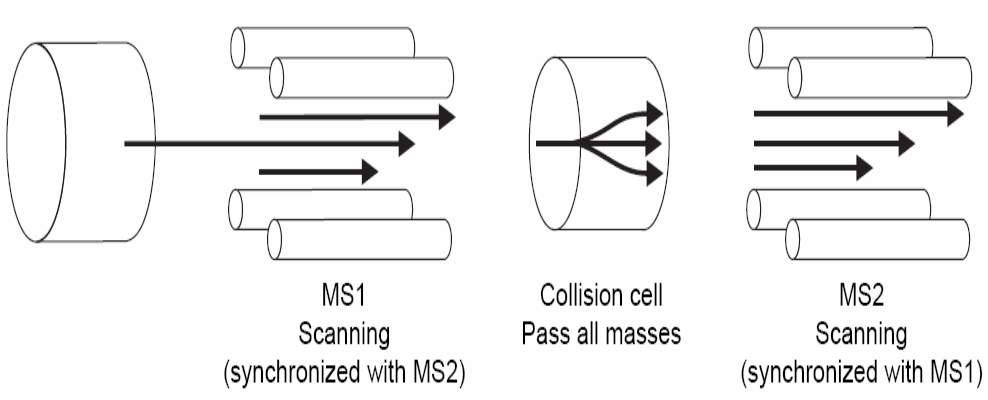

Figure 2.16 Schematic of constant neutral loss [34]

This analysis mode detects the loss of a specific neutral fragment or functional group from an unspecified precursor. Both quadrupoles are operated in scanning mode. Constant neutral loss mode is useful in determining which compounds have a similar fragmentation pattern.

2.4.1.4 Multiple reactions monitoring (MRM)

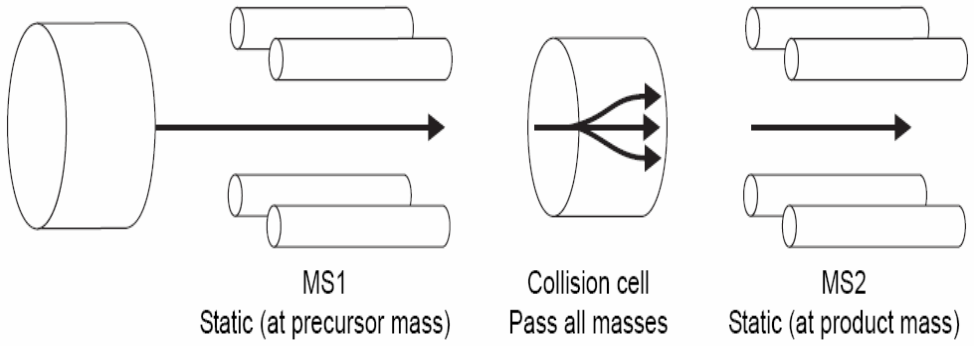

Figure 2.17 Schematic of multiple reactions monitoring [34]

This mode has no scanning taking place during Multiple Reaction Monitoring (MRM). The Q1 (MS1) and Q2 (MS2) quadrupoles only allow transmission of a specified precursor ion which gives a specified product ion to be monitored. The data is usually viewed as a chromatogram over time, rather than a summed spectrum. This is the most selective and sensitive mode because only a specific ion which fragments to produce the specific product ion will be monitored for the whole of the scan time cycle rather than part of it. Moreover, a greater dwell time on the ions of interest is possible and

therefore better sensitivity is achieved. For this reason, the sensitivity is raised up as well. MRM can eliminate the interference of matrix in the samples, reducing the amount of sample preparation required before analysis. MRM is the primary method of data recording used by triple quadrupole mass spectrometer. It is used for analysis of pharmaceutical drugs, or pesticides in environmental studies as well as many other areas of analysis.

The triple quadrupole is probably the most widely used MS/MS instrument. The hardware, as the name suggests, consists of three sets of quadrupole rods in series. (Figure 2.18) The second set of rods is not use as a mass separation device but as a collision cell, where fragmentation of ions transmitted by the first of quadrupole rods is carried out, and as a device for focusing any product ions into the third set of quadrupole rods. Both sets of rods may be controlled to allow the transmission of ions of single m/z ratio or a range of m/z values to give the desired analytical information.

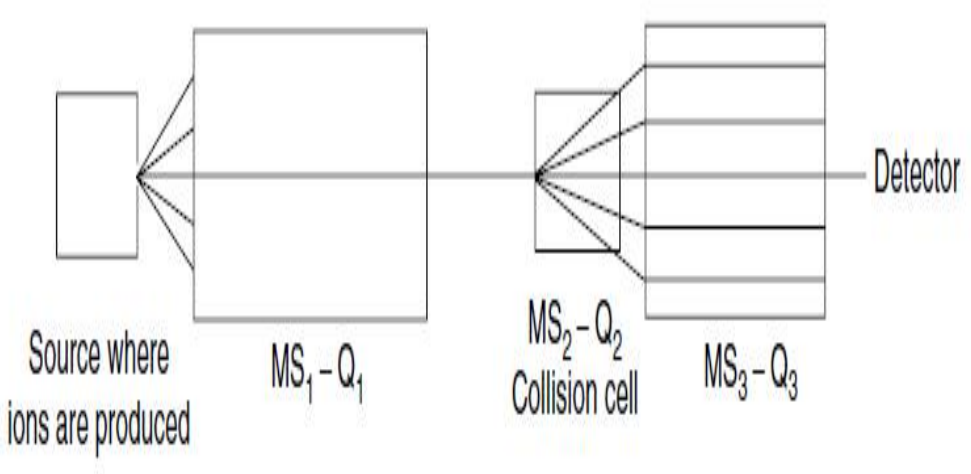

Figure 2.18 Schematic of the triple quadrupole mass spectrometer [29]

Ion trap mass spectrometer is capable of performing tandem mass spectrometry (MS/MS), in which a certain ion is selected for fragmentation. This can help to identify particular elements in a molecule. During fragmentation, bonds in the molecule break, thereby producing fragment ions that are characteristic for certain chemical moieties. In some cases, highly labile bonds are present and fragmentation will yield only little compositional information. Ion trap mass spectrometer can perform multi stage mass spectrometry (MS^n or MS/MS/MS…) where the number of successive reaction or

fragmentation stages can be very large. This can be useful for elucidating ionic structure.

In contrast to an ion trap, time of flight (TOF) is not capable of performing tandem mass spectrometry. On the other hand, it can determine molecular masses of ionized compounds with much higher accuracy than the ion trap. Accurate mass determination can also aid in resolving the elemental composition of an unknown compound.

2.2 Sample preparation technique - clean up

Sample clean-up is the procedure for removal of substance in the sample extract which may interfere in the sample of the analysis. Usually, the target analyte is preconcentrated by reduction of the solvent amount in order to obtain enough analytes's concentration for detection.

2.2.1 QuEChERS method [40-49, 52-53]

The QuEChERS method is a portmanteau word formed "Quick, Easy, Cheap, Effective, Rugged and Safe" for the preparation of pesticides in foods and agricultural samples. The technique uses simple glassware, a minimal amount of organic solvent and various salt/buffer additives to partition analytes into an organic phase for clean up by dispersive solid phase extraction (d-SPE), there have been several modifications of the technique depending on analytes, matrices, instrumentation and analyst preference. Basically, there are three steps of the sample extraction which are followed according to the steps below.

Step 1: Sample preparation and extraction – Commodities are uniformly crushed and although other non-halogenated solvents such as acetone and ethyl acetate may be used, acetonitrile is the recommended solvent for QuEChERS because, upon the addition of salts, it is separated more easily from water than acetone. Ethyl acetate has the advantage of partial miscibility with water but it co-extracts lipids and waxes, obtains lower recoveries for acid–base pesticides, and provides less clean up in d-SPE. Acetonitrile extracts less of the lipophilic materials. However, samples with high sugar content, acetonitrile and water can form two phases. Internal standards can be added to monitor extraction efficiencies.

Addition of salts-The purpose of salt addition is to induce phase separation. The salting out effect also influences analyte partition, which is dependent upon the solvent used for extraction. The concentration of salt can influence the percentage of water in the organic phase and can adjust its "polarity". In QuEChERS, acetonitrile alone is often sufficient to perform excellent extraction efficiency without the need to add nonpolar co-solvents that dilute the extract and make the extracts too nonpolar**.** In some instances, the pH of the extraction must be controlled. Most, but not all, pesticides are more stable at lower pH. For certain problematic pesticides, such as those that are strongly protonated at low pH, the extraction system must be buffered in the range of pH 2–7 for successful extractions. Of course, the pH at which the extraction is performed can also influence the coextraction of matrix compounds and pesticide stability.

Step 2: Extract cleanup -A subsample of solvent extract is cleaned up using d-SPE, a key improvement incorporated in the QuEChERS technique. Small polypropylene centrifuge tubes are prefilled with precise weights of magnesium sulphate ($MgSO_4$) and d-SPE adsorbents to remove excess water and unwanted contaminants from the extracted samples. After agitation and centrifugation, the cleaned extracts are ready for analysis. At this stage, the d-SPE is generally mentioned to primary secondary amine (PSA), aminopropyl (NH_2), octadecyl (C18) and graphitized carbon back (GCB)

2.2.1.1 Primary secondary amine (PSA)

Primary secondary amine (PSA) is a polymeric-based sorbent that contains both primary and secondary amines. The structure (in Figure 2.19) performs as weak anion exchanger sorbent with two pKa's 10.1 and 10.9. The PSA functional group is a very good bi-dentate ligand, making PSA an excellent sorbent for chelation. Its higher carbon content makes it a more non-polar sorbent than NH_2 and thus a better choice for very polar compounds that retain too strongly on NH_2 sorbent.

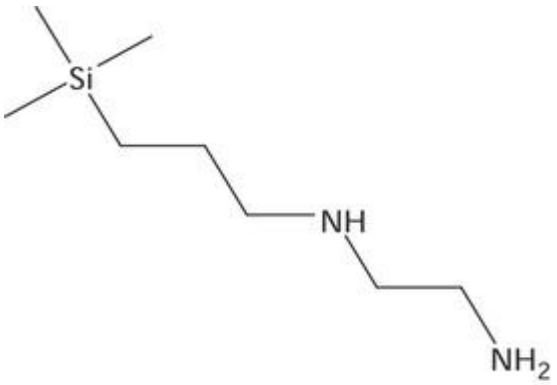

Figure 2.19 The chemical structure of primary secondary amines [40]

It has a strong affinity and high capacity for removing fatty acids, organic acids, lipids, some polar pigment, sugars and some other matrix co-extractives that from hydrocarbon that might act as instrumental interferences.

2.2.1.2 Aminopropyl (NH_2)

Aminoproply (NH_2) is a very polar sorbent. It can utilize both hydrogen bonding and anion exchange. Since the pKa of the NH_2 sorbent is 9.8, at any pH below 9.8 the majority of the functional groups are positively charged (shown in figure 2.20). NH_2 is a weak anion exchanger because it is a quaternary amine sorbent that is always charged and it is therefore a better sorbent choice for retention of very strong anions, such as sulfonic acids. Because an ethyl group supports the NH_2 functionality, it can be used for non-polar isolations from polar samples, but its strong polarity is its primary characteristic like diol and silica (Si). NH_2 is excellent for separation of structural isomers.

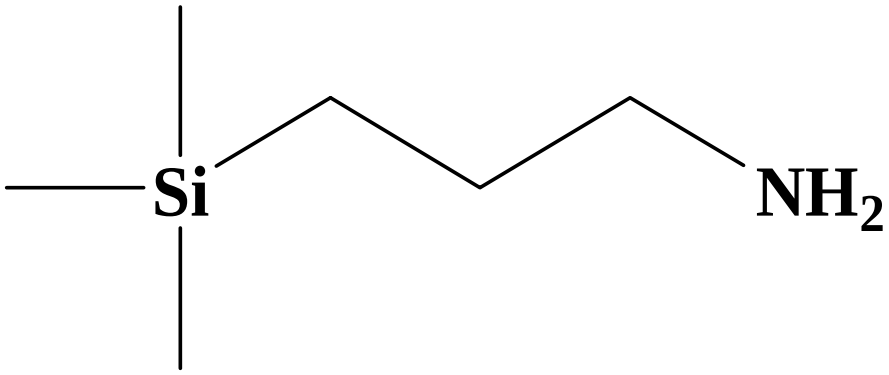

Figure 2.20 The chemical structure of aminopropyl [46]

PSA and NH_2 interact with chemicals by hydrogen bonding, and removed similar types of compounds, including fatty acids, other organic acids, and to some extent various sugars and pigments. PSA removed more matrix co-extractives than NH_2 per given quantity because PSA has higher capacity due to the presence of amines.

2.2.1.3 Octadecyl (C18)

Octadecy (C18) is the most hydrophobic silica-based sorbent available (shown in Figure 2.21). It is the most popular SPE sorbent because of its extremely retentive nature for non-polar compounds. C18 is generally regarded as the least selective silica-based sorbent, since it retains most organic analytes from aqueous matrices. The potential for polar interactions between analytes and sorbent is less significant with C18 than with any other sorbents because of the predominant effect of the long hydrocarbon chain. C18 is suggested to use for removing non-polar interferences such as fat and long chain fatty compound

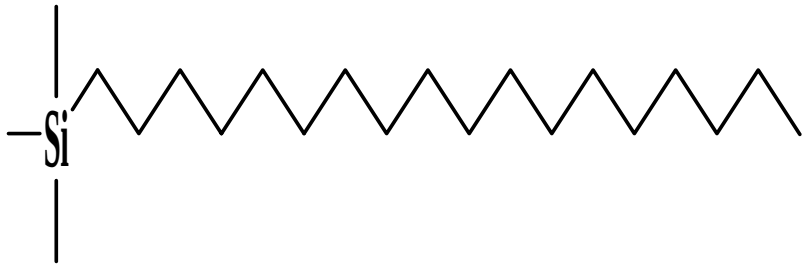

Figure 2.21 The chemical structure of octadecyl [47]

2.2.1.4 Graphitized carbon black (GCB)

Graphitized carbon sorbent (GCB) is non-porous (Figure 2.22). Consequently, surface interaction depends solely on dispersion forces. GCB exhibits hydrophobic surface characteristics, meaning that small, polar molecules such as water are not adsorbed. Therefore, analyte displacement by water is significantly reduced, allowing them to be effectively used to trap organic compounds despite high humidity. GCB for removing pigments, polyphenols and other polar compounds.

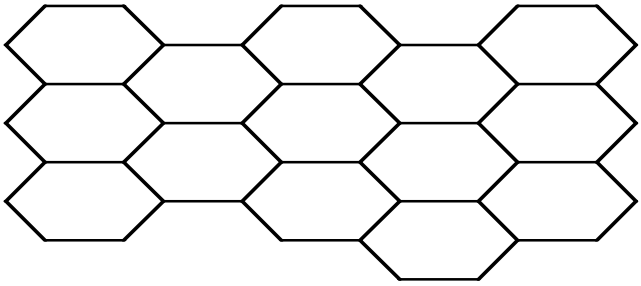

Figure 2.22 The chemical structure of graphitized carbon black sorbent [49]

Step 3: Sample analysis

pH of samples may be adjusted to protect sensitive analytes to improve analysis by either GC/MS or LC/MS. Internal standards can be added.

CHAPTER III

EXPERIMENTAL

3.1 Instrumental and Apparatus

3.1.1 Ultra Performance Liquid Chromatography (UPLC): Water Acquity UPLC™ consists of an automatic degasser, a binary high pressure mixing pump, an autosampler and a column thermostat from Waters Corporation, Milford, Massachusetts, U.S.A.

3.1.2 Mass spectrometry detector (MSD): Quattro Premier™ XE with electrospray ionization interface from Micromass WATERS Corporation, Milford, Massachusetts, U.S.A.

3.1.3 UV-vis spectrophotometer Agilent 8453 (G1103A), Wilmington, DE, U.S.A.

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

3.1.5 The Edwards XDS series DRY Vacuum scroll Pump conversion by BOC Edwards, Wilmington, MA, U.S.A.

3.1.6 Nitrogen gas, ultra high purity grade (99.999% purity), Chatakorn lab center CO., LTD, Bangkok, Thailand.

3.1.7 Argon gas, ultra high purity grade (99.999% purity), Chatakorn lab center CO., LTD, Bangkok, Thailand.

3.1.8 Column: Acquity UPLC® BEH Shield RP18 column, 2.1x100 mm, 1.7 µm from WATERS Corporation, Milford, Massachusetts, U.S.A.

3.1.9 ACQUITY UPLC® BEH C18 VanGuard™ Pre-column, 2.1x5mm, 1.7 µm from WATERS Corporation, Milford, Massachusetts, U.S.A.

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

3.1.11 Vacuum pump with pressure regulator, Model DOA-P504-BN, Gast®, Michigan, U.S.A.

3.1.12 Vortex mixer, Model G-5605, Scientific Industries, Bohemia, New York, U.S.A.

3.1.13 Centrifuge model Allegra™ x-12 from Beckman Coulter Inc., Brea, CA, U.S.A.

3.1.14 Microcentrifuge, Microfuge®18, Beckman.

3.1.15 Micropipettes: volume 0.1-10, 10-100, 25-200, 100-1000 µL and tips, Eppendorf, Hamburg, Germany.

3.1.16 Electronic balance 2 digits and 4 digits from Mettler Toledo, Prague, Czech Republic.

3.1.17 N_2 evaporator model N-EVAP™ 12, organomation associates, Inc., MA, U.S.A.

3.1.18 Ultra sonic bath model 8200 from Branson Ultrasonic Corporation, Danbury, CT, U.S.A.

3.1.19 Shaker from Gerhardt GmbH & Co., KG, Germany.

3.1.20 Microtubes, 2-mL clear MCT-200-c, Axygen, CA, U.S.A.

3.1.21 Filter membrane 47 mm, 0.2 µm, type Nylon, Whatman international Ltd., Maidstone, England.

3.1.22 Syringe filters, 13 mm, 0.2 µm, ChromTech, MN, U.S.A.

3.1.23 HPLC amber vials 2-mL, agilent Technologies, CA, U.S.A.

3.1.24 Preslit cap, La-Pha-Pack® GmbH, Germany.

3.1.25 Volumetric flask volume class A 10, 25, 50, 100-mL.

3.1.26 Beakers 100, 250, 1000-mL.

3.1.27 Graduated cylinders 10, 50, 100-mL.

3.1.28 Stirring rod.

3.1.29 Polypropylene centrifuge tube 50-mL.

3.1.30 Test tube 15-mL with screw cap

3.1.31 Dispersive solid phase extraction (d-SPE)

- PSA powder and NH_2 powder, 40 µm from Varian, Oxfordshire, UK.
- C18 powder, 40 µm from Merck, Darmstadt, Germany
- Graphitized carbon black (GCB, 120/140 mesh, 100 m^2g^{-1}), Supelco, Bellefonte, PA, USA.

All experimental glassware was washed sequentially with detergents and follow by rinsed with deionized water and acetone before used.

3.2 Chemicals

3.2.1 Standard compound

Ethoxyquin was purchased from Dr.Ehrenstorfer (Augsburg, Germany) with purity of 96.50%.

3.2.2 Organic solvents and other chemicals

Acetonitrile HPLC grade for sample preparation and LC/MS grade for analysis were purchased from J.T. baker (Deventer, the Netherlands), ammonium acetate and glacial acetic acid of analytical grade were supplied by J.T. baker (Deventer, the Netherlands). Sodium chloride (RFCL, New Delhi, India) and anhydrous magnesium sulfate were obtained from Panreac (Barcelona, Spain). A Milli-Q water purification system from Millipore (Billerica, MA, USA) with 18.2MΩ/cm resistivity was used.

3.3 Preparation of standard solutions

3.3.1 Preparation of stock standard solution at 100 mg/L

The primary standard of 100 mg/L ethoxyquin solution was prepared by dissolving 0.0100 g (to nearest 0.0005 g) of ethoxyquin in 100-mL volumetric flasks with acetonitrile. The stock standard solution below –20^{o}C in an amber glass bottle in a freezer and keep for 2 years.

3.3.2 Preparation of standard solution at 1000 µg/L

The 1000 µg/L standard solution was prepared by pipetting 100 µL of 100 mg/L ethoxyquin stock solution into a 10-mL volumetric flask and diluting with acetonitrile. The standard solution was transfer to an amber glass bottle with Teflon screw cap. This standard was prepared daily and stored at 4^{o}C in refrigerator until use.

3.3.3 Preparation of working standard solution at 100 µg/L

The 100 µg/L standard solutions was prepared by pipetting 1 mL of of stock standard solution 1000 µg/L, as detailed in section 3.3.2 into a 10-mL volumetric flask and diluting with acetonitrile. This standard was prepared daily and stored at 4^{o}C in refrigerator until use.

3.3.4 The standard solution for UPLC-MS/MS tuning

A standard solution containing 10 mg/L was prepared by pipetting 100 µL of stock standard solution 100 mg/L, as detailed in section 3.3.1 into a 10-mL volumetric flask and diluting with acetonitrile. This standard was preparing daily and stored at 4^{o}C in refrigerator until use.

3.4 The optimum UPLC-MS/MS condition

In this research, the studied ethoxyquin was measured using a UPLC-MS/MS system consisting Water; Acquity UPLC^{TM} coupled to a Micromass Quattro Premier^{TM} XE benchtop tendem quadrupole mass spectrometer (Milford, MA, USA). Electrospray ionization (ESI) was used as an ionization source in positive mode.

3.4.1 MS/MS optimization

The most abundant ESI-MS/MS transition for ethoxyquin was monitored in the multiple reaction monitoring (MRM) modes to obtain the highest quantitative sensitivity. The choice of fragmentation product for analyte based on the most intense signal and the optimization of cone voltage, energy collisions and other instrument parameter were individually investigated for compound in the combined flow state mode through direct infusion of standard solutions in acetonitrile (10 mg/L). This was performed by UPLC where the mobile phase pumped directly into the MS via a switching valve. The optimized MS settings employed for ethoxyquin was develop and maintained at the following: capillary voltage, extractor voltage, 120^{o}C source temperature, 350^{o}C desolvation temperature, 50 L/hr cone gas (nitrogen) flow, 1000 L/hr desolvation gas (nitrogen) flow, and argon gas was used as the collision gas at $3.5x10^{-3}$ mbar. Instrument control and data processing was evaluated using the MassLynx 4.1 software package from MicromassTM (Waters, MA, USA).

3.4.2 UPLC optimization

In the UPLC system, chromatographic separation was performed on Acquity UPLC® BEH Shied RP18 (2.1x100 mm, 1.7 µm) column from WATERS Corporation, the column and sample temperature was controlled at 40 and 15ºC, respectively. The flow rate was set at 0.5 mL/min and the injection volume was 5 µL using partial loop with needle overfill loop mode. The following mobile phase gradient program and mobile phase optimization. In the first stage, the type of mobile phase was evaluated in order to optimize the conditions of UPLC.

3.4.2.1 The mobile phase optimization

In the first stage, the two different buffer and acidified types were tested, ammonium acetate and acetic acid. The concentration of different buffer and acidified types in that study are summarized in Table 3.1. During this test, 5 µg/L of ethoxyquin standard solution was injected and mobile phase isocratic mode was composed of 20% constituent in mobile phase A and 80% in mobile phase B.

Table 3.1 Mobile phase type

No.	Mobile phase	
	A	B
1	5 mM ammonium acetate	acetonitrile
2	10 mM ammonium acetate	acetonitrile
3	10 mM ammonium acetate containing 0.1% acetic acid	acetonitrile containing 0.1% acetic acid
4	10 mM ammonium acetate containing 0.2% acetic acid	acetonitrile containing 0.2% acetic acid
5	10 mM ammonium acetate containing 0.3% acetic acid	acetonitrile containing 0.3% acetic acid
6	10 mM ammonium acetate containing 0.4% acetic acid	acetonitrile containing 0.4% acetic acid

3.5 Extraction method optimization

The sample preparation in this study based on the modified QuEChERS extraction method. Shrimp sample blank (sample blank determination means the complete analytical procedure applied to test portion taken from a sample from which the analyte is absent). The standard ethoxyquin solution (section 3.3.2) was spiked into the sample at the first stage of extraction procedure with concentration levels 50 µg/kg. These concentration levels were used for the entire investigation. Five replicates were done in each test method. Parameter affecting procedure such as extraction solution, type and amount of dispersive solid phase extraction (d-SPE) sorbent and dilution solution ratio can be described as the following method below:

3.5.1 The procedure of extraction solution optimization

Indeed, the principle of QuEChERS method is based on the LLE technique for the extraction of ethoxyquin. Acetonitrile is introduced due to the benefit over other solvents. In this study, the extraction procedure, at stage 3.5.1.3 was varied by concentrations acetic acid in acetonitrile (%)**,** ranging from 0.1, 0.5, 1, 2 and 3 were investigated in five replicated. The process of extraction was performed as follows:

3.5.1.1 5.00±0.05 g of Shrimp sample was weighed into the polypropylene centrifuge tube. For each sample blank, prepared fortified sample at concentration level 50 µg/kg by adding 250 µL of standard ethoxyquin solution 1000 µg/L (section 3.3.2)

3.5.1.2 A 2 mL of water was transferred to centrifuge tube and then vortexed for 45 sec.

3.5.1.3 A 20 mL of ***acidified acetonitrile*** added into the sample and vortexed for 45 sec.

3.5.1.4 The mixture was then mechanically shaken for 10 min.

3.5.1.5 A 5 g of anhydrous magnesium sulfate was added into each tube, which was then vigorously shaken by hand for 45 sec.

3.5.1.6 The mixture solution was centrifuged at 3,400 rpm for 5 min.

3.5.1.7 A 0.5 mL of sample solution was diluted to 1 mL with acetonitrile: water (80: 20, v/v).

3.5.1.8 The extract solution was filtered through a 0.2 µm nylon membrane syringe filter prior to UPLC-MS/MS analysis.

3.5.1.9 The final concentration of ethoxyquin was calculated and reported as percent recovery of analyte.

3.5.2 The procedure of dispersive solid phase extraction (d-SPE) sorbent for cleanup method optimization

The acidified acetonitrile was used for extraction. The co-extractive would be presented in the extraction solution. Consequently, the cleanup steps to get rid of the interferences or the co-extractives are important in order to protect the instrument from becoming dirty. At this stage, dispersive solid phase extraction (d-SPE) sorbent (i.e. PSA, NH_2, C18, and GCB) were employed to absorb any interferences. A single type of d-SPE sorbent, a combination of d-SPE sorbent and amount of d-SPE sorbent were studied for efficiency of clean up.

3.5.2.1 The effect of a single type of various d-SPE clean up optimization

In this study, the procedure of dispersive solid phase extraction (d-SPE) sorbent, at stage 3.5.2.1.7 was varied by 50 mg of PSA, NH_2, C18, GCB sorbent and without d-SPE sorbent were investigated in five replicates. The process extraction was performed as follows:

3.5.2.1.1 5.00±0.05 g of Shrimp sample was weighed into the polypropylene centrifuge tube. For each sample blank, prepared fortified sample at concentration level 50 µg/kg by adding 250 µL of standard ethoxyquin solution 1000 µg/L (section 3.3.2)

3.5.2.1.2 A 2 mL of water was transferred to centrifuge tube and then vortexed for 45 sec.

3.5.2.1.3 A 20 mL of 2% acetic acid in acetonitrile was added into the sample and vortexed for 45 sec.

3.5.2.1.4 The mixture was then mechanically shaken for 5 min.

3.5.2.1.5 A 5 g of anhydrous magnesium sulfate was added into each tube, which was then vigorously shaken by hand for 45 sec.

3.5.2.1.6 The mixture solution was centrifuged at 3,400 rpm for 5 min.

3.5.2.1.7 A 1 mL aliquot of extract was transferred to a 2-mL microcentrifuge tube containing ***type and amount of d-SPE***.

3.5.2.1.8 The sample solution was vortexed mixing for 30 sec and centrifuged at 14000 rpm for 3 min.

3.5.2.1.9 A 0.5 mL of sample solution was diluted to 1 mL with acetonitrile: water (80: 20, v/v).

3.5.2.1.10 The extract solution was filtered through a 0.2 µm nylon membrane syringe filter prior to UPLC-MS/MS analysis

3.5.2.1.11 The final concentration of ethoxyquin was calculated and reported as percent recovery of analyte.

3.5.2.2 The effect of various amount of PSA sorbent optimization

In this study the procedure was perform similar to 3.5.2.1, except that the state 3.5.2.1.7 was varied amount of PSA sorbent, ranging from amounts 5, 10, 20, 30, 40, 50 and 100 mg were investigated in five replicates.

3.5.2.3 The effect of various amount of GCB sorbent optimization

In this study the procedure was perform similar to 3.5.2.1, except that the state 3.5.2.1.7 was varied amount GCB sorbent, ranging from amounts 5, 10, 20, 30, 40 and 50 mg were investigated in five replicates.

3.5.3 The effect of dilution solution ratio optimization

In this study the procedure was perform similar to 3.5.2.1, except that the state 3.5.2.1.9 was varied dilution solution ration, ranging from 0, 20, 40, 60, 80 and 100 acetonitrile in water were investigated in five replicates.

3.6 Method validation

Validation of the presented method in this research has been performed according to the requirements defined by the method validation quality control procedures for pesticide residues analysis in food and feed No. SANCO/10684/2009, establishing the performance criteria for the analytical residue method. [45]

3.6.1 Selectivity

An analytical method is claimed as selective technique if the ability given can measure the accurate response of an analyte in the presence of interferences. This is achieved by mass selectivity of the detector via electrospray ionization in positive and negative modes. Selectivity was studied by analyzing 10 blank shrimp samples. The identification of all compounds was described by determination of retention time (t_R), and the mass ion ratio value. The ratio value is a ratio of the area of qualitative ion over the area of quantitative ion which is the most significant factors in terms of the confirmation of questioned analytes. The matrix-matched standard (in section 3.6.3.1.2) was performed as a chromatographic retention time and the mass ion ratio values.

3.6.2 Specificity

The purpose of specificity for method is to ensure that the measured signal was obtained from the compound of interest, and there is no interference for experiment or no degradation products and impurities. Specificity was studied by analyzing 10 blank shrimp samples. The samples were confirmed to be free of target compound residues in shrimp by UPLC-MS/MS after sample preparation with the developed procedure.

3.6.3 Linearity

The linearity of a test procedure is its ability (within a given range) to obtain test results proportional to the concentration of analyte in the sample. Linearity of this method was obtained from calibration range of ethoxyquin analyte. Correlation coefficient (R^2) represents the linearity of the proposed method. Under optimized UPLC-MS/MS conditions. The linear calibration range for ethoxyquin analyte was 0.8-64 µg/kg and matrix blanks were prepared for evaluation. Calibration curve spike in matrix blanks were made at levels of 0.8, 4, 8, 24, 32, 40, 48, 56 and 64 µg/kg for ethoxyquin analyte with three replicated of each level. The calibration curves were plotted as concentration over peak area of ethoxyquin analyte. The slope, y-intercept and correlation coefficient (R^2) of ethoxyquin.

3.6.3.1 Matrix effect

A measurement defines a relation between the instrumental response (peak area of quantitative ion) and the standard concentration. According to the theory, the matrix might lead to significant signal suppression effect on analytes in mass spectrometry technique because “the matrix effect” must be used for a consequent analytical (linearity) error. Thus, two types of standard calibration curves were compared for the significant difference between the dilution in mobile phase (see section 3.6.3.1.1) and sample extract solution (see section 3.6.3.1.2) by preparation of standard calibration curve with the same concentration range. Each level was done by triplicate injection. The preparation of calibration solution and the matrix-matched standards solution can be described as the following below

3.6.3.1.1 The preparation of calibration solution standard (the solution of the analyte used for calibration of the determination system) in the mobile phase was prior to the UPLC-MS/MS analysis. The mixture standard solutions (section 3.3.3) were diluted in the acetonitrile: water (80:20) which covered the concentration range as shown in Table 3.2

3.6.3.1.2 The preparation of matrix-matched standards solution (calibration was intended to compensate for interference, if present) were prepared prior to UPLC-MS/MS analysis. The mixture standard solutions (section 3.3.3) were diluted in a blank extract of a matrix similar to that analyzed which covered the concentration range as shown in Table 3.2.

Table 3.2 The concentration level of standard calibration curve.

Level	Volume of standard 100 µg/L (µL)	Volume of final solution (1000 µL)	Calibration level of standard curve (µg/L)
1	1	999	0.1
2	5	995	0.5
3	10	990	1
4	20	980	2
5	30	970	3
6	40	960	4
7	50	950	5
8	60	940	6
9	70	930	7
10	80	920	8

3.6.4 Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) of a method is the smallest amount or concentration of an analyte that can be reliably distinguished from the absence of analyte (a blank value) and be positively identified according to predetermined criteria and/or levels of confidence. The LOD values were calculated at 3 times of standard deviation at the smallest fortification level. The limit of quantitation (LOQ) of the method is often defined as the lowest concentration of analyte that can be determined with the measurable levels. Usually, the recommendation is to quote the LOQ as 10 times of standard deviation from the calculation of the smallest fortification level. In this study, matrix-matched calibration curve at concentration levels in the range of 0.1–1.0 µg/kg. The 0.8 µg/kg fortified samples were found to have sufficient signal to noise for

analytes from ten replicate samples and the standard deviation calculated. The LOD and LOQ were calculated, based on the following equations:

LOD = 3×SD

LOQ = 10×SD

3.6.5 Accuracy

The method accuracy refers to the closeness of agreement between the observed results from method and the true value of the analyte in the sample. The recovery experiments were carried out at four levels in independent sample at 1.5, 5, 25 and 50 µg/kg by adding 7.5, 25, 125, 250 µL of standard ethoxyquin solution 1000 µg/L (section 3.3.2) in blank shrimp samples. Fortification at each level was repeated ten times.

3.6.6 Precision

The precision is the closeness of agreement between independent test results obtained under same condition. The two categories of precision are repeatability and intermediate. The repeatability is the precision derived from repeated tests on the same method with single analytical runs, while the intermediate precision acquired from repeated tests on the same method with different analytical runs, with difference in calibration curves or different times. In this work, the repeatability precision was calculated from the analysis of 10 blank shrimp samples fortified with ethoxyquin at four specified fortification levels 1.5, 5, 25 and 50 µg/kg by adding 7.5, 25, 125, 250 µL of standard ethoxyquin solution 1000 µg/L (section 3.3.2). Intermediate precision was obtained by following the same protocol but performing the analysis in three different days with four specified fortification levels 1.5, 5, 25 and 50 µg/kg, respectively. The determination of 10 replicates was made and the method precision was represented in the percentage of relative standard deviations (%RSD). Precision is usually expressed in terms of standard deviation or relative standard deviation. Both repeatability and intermediate are generally dependent on the analyte concentration, and should be determined at various concentrations and if relevant, the relationship between precision and analyte concentration should be established. The standard deviation (SD) of each concentration is used for calculation of the relative standard deviation (RSD_r). The RSD_r is the acceptance limitations for RSD_r of the replicate results. RSD_r was calculated from the modified Horwitz Equation [34]

$$\%RSD_r = 0.66 \times 2^{(1-0.5 \log C)}$$

When: RSD_r is the relative standard deviation calculated from the results obtained from repeatability conditions (within laboratory)

C is the concentration of fortified sample (analyte /sample in g/g)

To evaluate the intermediate precision, the two-tailed F test was employed determine the significant difference of results obtained.

Intermediate precision from ANOVA, If ANOVA indicates “in significant difference” during 3 days of analysis (P > 0.05); calculate the relative standard deviation, %RSD from SD where you treat the results as a single data set, using the equation below:

$$SD = \sqrt{within \quad group \quad mean \quad square}$$

$$\% \text{ RSD} = \frac{SD}{mean} x100$$

Intermediate precision from ANOVA, If ANOVA indicates “significant difference” among 3 days of analysis (P <0.05); calculate the relative standard deviation, %RSD from the ANOVA using the equation below

$$S_{within} = \sqrt{within \quad group \quad MS}$$

$$\boldsymbol{S_{between}} = \sqrt{\frac{between \quad group \quad MS \quad - within \quad group \quad MS}{n}}$$

$$SD = \sqrt{S^2_{within} \quad + \quad S^2_{between}}$$

$$\%RSD = \frac{SD}{mean} x100$$

When: n = number of each within group

3.7 Application to real samples

The developed method was applied to the analysis in real shrimp samples. The 100 shrimp samples were taken in various local fresh market and supermarkets, Bangkok, Thailand and shrimp farm for export in Thailand and then analyzed under the optimized condition. Within each batch of 10 samples, the solvent standard at 1 µg/L was injected to create a calibration curve at concentration from 0.1 to 8 µg/L before and after batch samples. In every batch of samples, Quality control (QC) was prepared fortified level at 5 µg/kg by adding 25 µL of standard ethoxyquin solution 1000 µg/L (section 3.3.2) in blank shrimp sample. These blank samples were previously analyzed to confirm the absence of the analytes. Satisfactory QC recoveries (%) were obtained for ethoxyquin (between 70 to 120%)

CHAPTER IV

RESULTS AND DISCUSSION

Ethoxyquin is widely used as pesticide in food and a preservative in feeds for farmed animal. Despite ethoxyquin importance as a preservative, there is concern that utilization of ethoxyquin in animal feed may impair animal health and ethoxyquin residues in food producing animal may pose health risk to human. In this study, ethoxyquin residues in shrimp were determined by using UPLC-MS/MS. Parameters related to a sample preparation method, such as extraction solution optimization, dispersive solid phase extraction (d-SPE) sorbent, type of d-SPE clean up, various amount of d-SPE sorbent, and sample dilution ratio and UPLC-MS/MS such as MS/MS conditions and UPLC conditions were optimized. The validation of the method was proved and confirmed by the statistical values. The applicable method was efficiently applied for routine analysis in real shrimp samples from local fresh market and super markets, Bangkok, Thailand and shrimp farm for export in Thailand.

4.1 The optimization of UPLC-MS/MS conditions

UPLC, provides improved resolution, speed and sensitivity. The main advantages of MS/MS detections are the reduction of background noise and the better selectivity. The sensitivity in detection and the confidence level of compounds can be improved. Therefore, the combination of ultra-performance liquid chromatography and tandem mass spectrometry (UPLC-MS/MS) is preferred for analysis of samples with complex matrices. In this study, the hyphenated technique UPLC-MS/MS was selected for the determination of ethoxyquin residue in shrimp at trace level. The MS/MS parameter and UPLC condition were optimized.

4.1.1 The optimization of MS/MS parameters

The important parameters of the MS/MS system were optimized to effectively determine ethoxyquin in samples. Firstly, the manual tuning of the ethoxyquin was performed to achieve maximum sensitivity of The MS/MS by using syringe infusion pump in the positive ESI mode. Then, the most intensive transition of the compound was selected for quantification and the second transition was for the confirmation of the compound. The compound produces the protonated ion, $[M+H]^+$ as a precursor ion for

ethoxyquin. The cone voltage was adjusted to its highest response at the first quadrupole of the mass spectrometer. After that, the product ion spectra were recorded at various collision energies in order to find the two most intense transitions for ethoxyquin. The appropriate MRM method was created to assess the maximum sensitivity of ethoxyquin signals and the compound should be detected within adequate and shot dwell times. The optimized MRM transitions and UPLC-MS/MS conditions as shown in Table 4.1 were subsequently applied in further studies. The solution standard at the concentration level of 5 µg/L was injected for checking the effectiveness of chromatographic condition. (see Figure 4.1)

Table 4.1 MS/MS parameters for ethoxyquin

Parameters	ESI $^{+}$
Capillary voltage (kV)	1
Extractor (V)	3
Source temperature (ºC)	120
Desolvation temperature (ºC)	350
Cone gas flow (L/hr)	1000
Low MS 1 Resolution	14
High MS 2 Resolution	14
Ion energy 1	0.3
Low MS 2 Resolution	14
High MS 2 Resolution	14
Parameters	ESI $^{+}$
Ion Energy 2	0.5
Collision gas flow (ml/min)	0.18

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Table 4.1 *(continued)*

Parameters	ESI $^{+}$
Multiplier	675
Molecular weight	217.31
Cone voltage (V)	40
1st transition quantification	218.15>160.10
Collision energy (eV)	32
2nd transition quantification	218.15>148.10
Collision energy (eV)	30

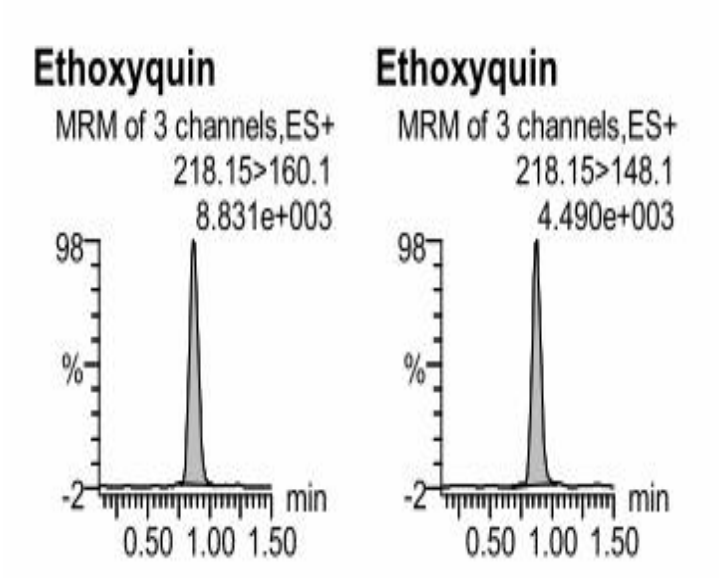


Figure 4.1 The chromatogram of 5 µg/L ethoxyquin under optimum UPLC-MS/MS Condition

4.1.2 Mass fragmentation pathway of targeted compound

The mass fragmentation pathways of ethoxyquin were studied using ESI, the soft ionization technique which provides the molecular ions as the precursor ions. At suitable fragmentor voltage, common fragmentation ions of targeted compound which have already been discussed in previous studies [16, 17, 19, 36-37] are observed. Ethoxyquin was provided the accurate product ion mass spectrum by isolating the protonated molecule $[M+H]^+$ as precursor ion (218.15 m/z), fragmenting it in the collision cell and studying the product ions whether the different fragment ions match with the precursor compound. The product ion, relative abundances and the sensitivity of analysis were chosen depend on the collision energy (CE). The production ion mass spectra were selected at 32 eV for 1st transition quantification (218.15>160.10) and 30 eV for 2nd transition quantification (218.15>148.10) because the analysis of ethoxyquin at these energies was highly sensitive. In this work, the characteristic fragment ions of ethoxyquin were 160.10 m/z $(M\text{-}C_3H_5OH+H)^+$ and 148.10 m/z $(M\text{-}C_4H_7OH+2H)^+$. The proposed fragmentation pathways (Figure 4.2) were supported for an unambiguous identification in the determination of ethoxyquin.

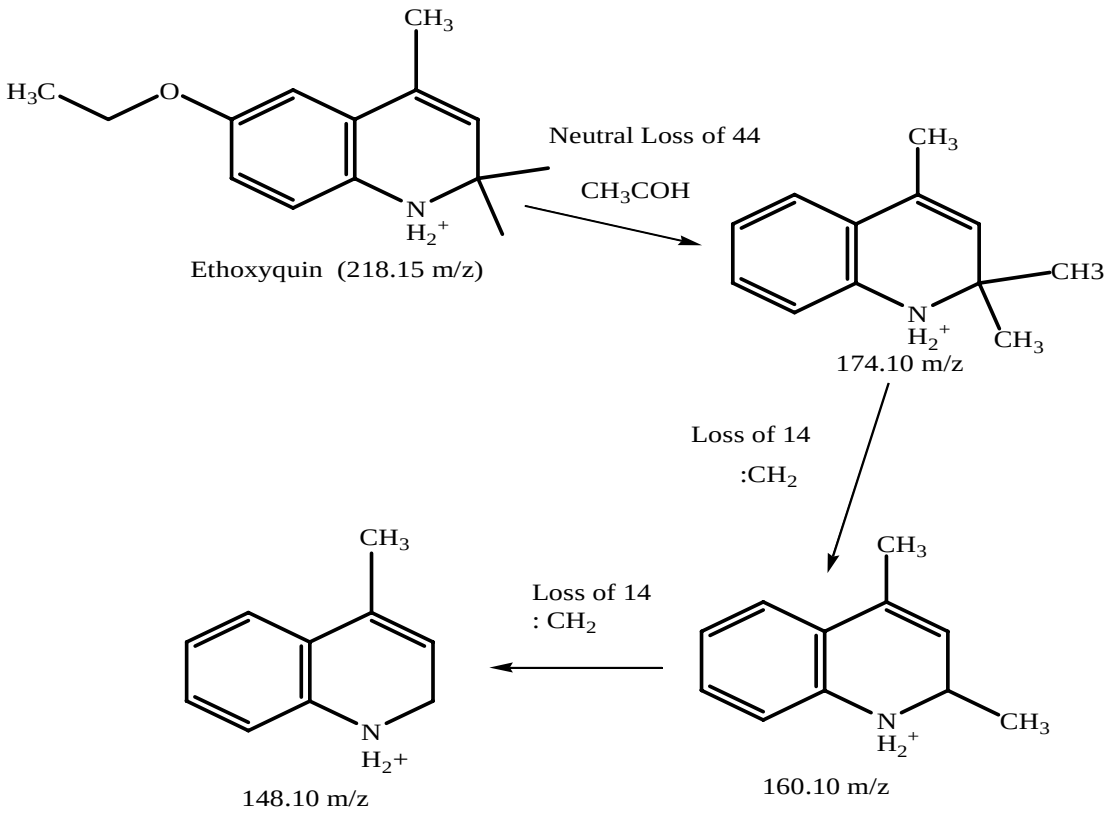


Figure 4.2 The fragmentation pathways of ethoxyquin

For the ethoxyquin using UPLC-MS/MS detection, time windows during a UPLC analysis, while maintaining an optimum number of data points is required to improve precision of peak area and sensitivity. The optimized MRM time window for this method is shown in Table 4.2

Table 4.2 Time window for ethoxyquin residue analysis

Compound	Precusor ion (m/z)	Product ion (m/z)	Dwell (s)	Cone (V)	Collision (V)
Ethoxyquin	218.15	160.10	0.2	40	32
	218.15	148.10	0.2	40	30

4.2 The optimization of UPLC condition

Although complete separation of compound is not necessary for the selective MS/MS detection, the improvement of sensitivity and the reduction of ion suppression effect are required. The most widely used organic modifiers for those purposes in LC-ESI/MS are methanol and acetonitrile. Even methanol cannot be certainly claimed to enhance the analytical performance in LC/ESI/MS, it has been preferred as an organic modifier in most LC-MS/MS application. [50] Methanol offered slightly better ESI efficiency than did acetonitrile, but the peak shape of the ethoxyquin was further destroyed. Therefore, acetonitrile was then chosen as a mobile phase solvent for separation and applied to this work. The use of a buffer or acidification of mobile phase was recommended in order to obtain sufficient retention times, even though it causes the reduction of signal intensities from ion suppression effect in the MS interface. For the LC-MS/MS analysis, easily volatile compounds such as ammonium formate, ammonium acetate, formic acid and acetic acid were preferred as mobile phase additives.

4.2.1 The optimization of mobile phase

The development of the chromatographic parameters was aimed to maximize sensitivity and minimizing runtime of analysis. For the ethoxyquin, the sensitivity was further improved by adding acetic acid, ammonium acetate buffer to the mobile phase. The recommended operating pH range for Acquity UPLC® BEH Shied RP18 column is pH 1-12 and ammonium acetate was commonly used in the range 1-10 mM and acetic acid has a maximum buffering effect when used with ammonium acetate salt 0.1-1%. [51] As the result in Table 4.3, the mobile phase composition was carefully optimized to achieve efficient ionization and separation of ethoxyquin. Frist, several experiments were performed testing different mobile phase consisting of 5 mM ammonium acetate or 10 mM ammonium acetate as buffer and acetonitrile with different concentrations of acetic acid (from 0.1 to 0.4%). When acetonitrile was used as in mobile phase have a retention time decrease and sharp peaks, so acetonitrile was selected for the separation of the selected ethoxyquin in further experiments. On the other hand, 10 mM ammonium acetate as buffer provided better results than 5 mM ammonium acetate and it was used to improve the ionization efficiency but low sensitivity. Then analysis of ethoxyquin using various mobile phase additives and a combination of ammonium acetate and acetic acid, the effect of various concentration of acetic acid form 0.1-0.4 %. Sensitivity increased with increasing acetic acid content to 0.3%, then the reduced sensitivity and tailing peak at 0.4%. Finally, the best result obtained when the optimum mobile phases was used 10 mM ammonium acetate containing 0.3% acetic acid (mobile phase A), and acetonitrile containing 0.3% acetic acid (mobile phase B). The short runtime was obtained, leading to sharp peaks, peak symmetry and high sensitivity.

Table 4.3 Comparison of mobile phase

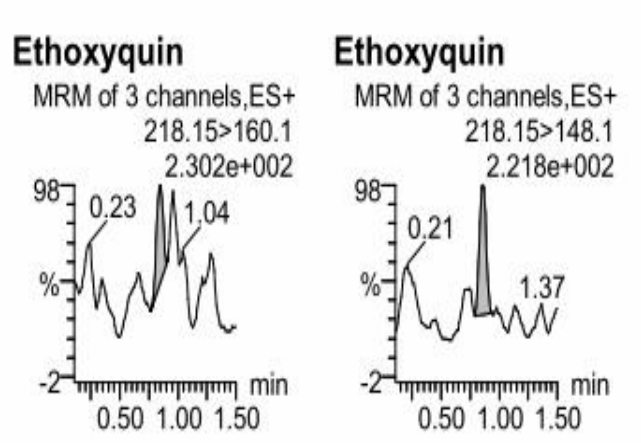
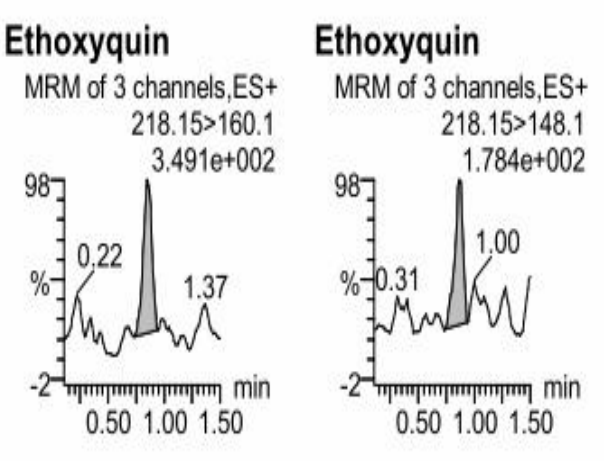
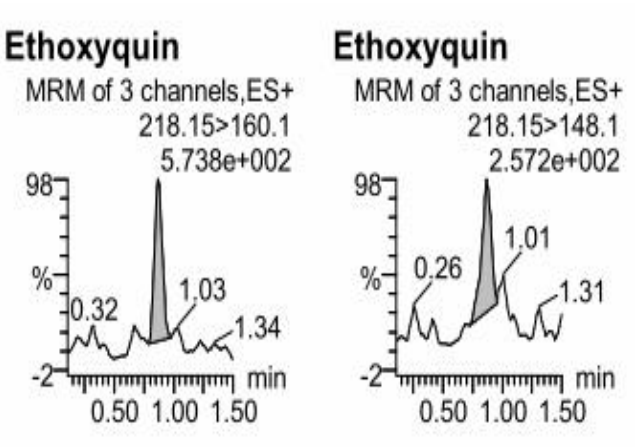
No.	Mobile phase		Representative chromatogram of ethoxyquin at 5 µg/L
	A	B	
1	5 mM ammonium acetate	acetonitrile	
2	10 mM ammonium acetate	acetonitrile	
3	10 mM ammonium acetate containing 0.1% acetic acid	acetonitrile containing 0.1% acetic acid	

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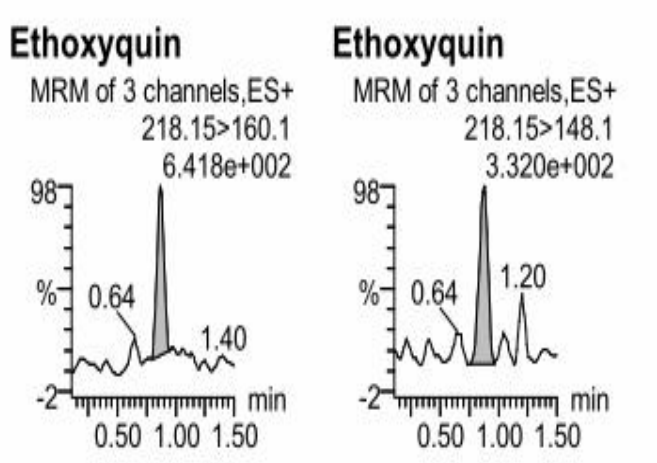
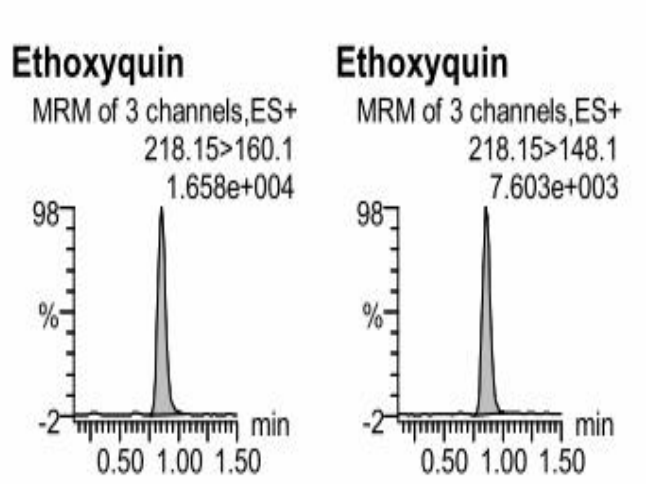
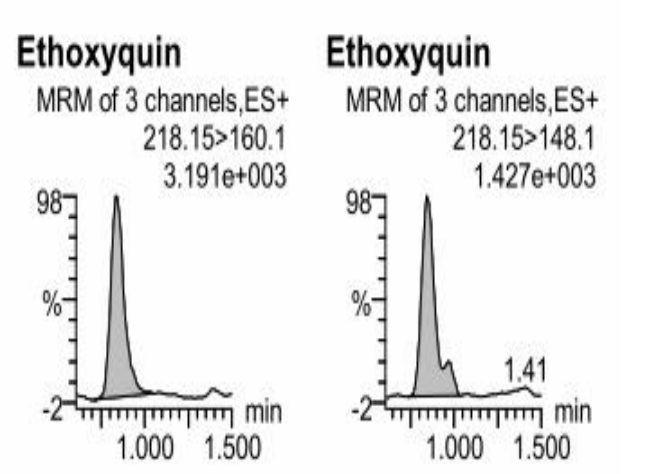
Table 4.3 *(continued)*

No.	Mobile phase		Representative chromatogram of ethoxyquin at 5 µg/L
	A	B	
4	10 mM ammonium acetate containing 0.2% acetic acid	acetonitrile containing 0.2% acetic acid	
5	10 mM ammonium acetate containing 0.3% acetic acid	acetonitrile containing 0.3% acetic acid	
6	10 mM ammonium acetate containing 0.4% acetic acid	acetonitrile containing 0.4% acetic acid	

In the UPLC system, chromatographic separation was performed on Acquity UPLC® BEH Shield RP18 (2.1x100 mm, 1.7 µm) column from WATERS Corporation, the column and sample temperature was controlled at 40 and 15 ºC, respectively. The flow rate was set at 0.5 mL/min and the injection volume was 5 µL using partial loop with needle overfill loop mode. The following mobile phase gradient was applied: 10 mM ammonium acetate containing 0.3% acetic acid, pH 4 (mobile phase A) and acetonitrile containing 0.3% acetic acid (mobile phase B). The separation of ethoxyquin was achieved within 2 min in the following gradient program as shown in Table 4.4

Table 4.4 Optimized gradient elution profile for column separation

Time	Mobile Phase	
(min)	A	B
0.0	20	80
1.1	5	95
1.4	5	95
1.5	20	80

Where: A = 10 mM ammonium acetate containing 0.3% acetic acid, pH 4
B = Acetonitrile containing 0.3% acetic acid

Run time: 2 mins

4.3 The optimization of extraction procedure

Method development is the process of design and preliminary assessment of the characteristics of a method, including ruggedness. Ethoxyquin is relatively strong acid (pka 4.56) [38] and is more stable at low pH. Therefore, the pH of the extraction is also important and should be suitably controlled. However, shrimp is composed of high protein, lipid and pigment (hemocyanin). Then, the analysis of ethoxyquin in complicated matrix is very difficult due to their chemical and physical nature. In this study, the proposed extraction method was based on the extraction, liquid-liquid partition and dispersive solid phase clean up procedure. Several factors have been shown to affect the performance in QuEChERS such as the nature of the solvent addition, the nature of solid phase sorbent, pretreatment or modification of the nature of the sample (pH adjustment), most specifically for the nature of the sample matrices and analytes separation were also studied. Each parameter is described as below:

4.3.1 The effect of extraction solution

In sample preparation optimization, this step was the selection of a suitable extraction solvent. Acidified acetonitrile was selected as the extraction solvent by adding acetic acid solution into acetonitrile because it offers satisfactory yield of residues with low levels of matrix co-extractives and effective denaturing proteins. The acidified acetonitrile was usually employed for extraction in the presence of anhydrous magnesium sulfate. The addition of salt induces phase separation of the solvent from the aqueous phase. The residues of analytes and matrix co-extractives were separated into the relevant liquid phase based on their polarity. The residues were partitioned into the organic phase while the matrix co-extractives were partitioned into the aqueous phase.[52-53] In this experiment, the concentration of acetic acid in acetonitrile was varied from 0.1, 0.5, 1, 2 and 3%. Five different concentrations of acetic acid were tested to evaluate which of the extraction solvents offered good yield recovery of the analyte. Table 4.5 and Figure 4.3 show the recovery of ethoxyquin. The recovery was increased and directly proportional to the concentration of acetic acid in range of 0.1-2%, then the recovery was decreased at 3% acetic acid. It can be assumed that the degradation of analytes may occur in a highly acidic condition. Therefore, 2% acetic acid was selected to extract ethoxyquin and to enhance the effectiveness of protein precipitation by eliminating turbidity of the solution.

Table 4.5 Effect of acetic acid concentration in acetonitrile (%) on the recovery (%)

(n = 5)

No.	% Recovery (n=5)				
	Concentration of acetic acid in acetonitrile (%)				
	0.1	0.5	1	2	3
1	77	84	86	93	87
2	78	86	87	92	86
3	81	82	85	95	87
4	80	81	84	94	86
5	79	74	85	90	79
Average	79	81	85	93	85
SD	1.6	4.6	1.1	1.9	3.4
RSD (%)	2.0	5.6	1.3	2.1	4.0

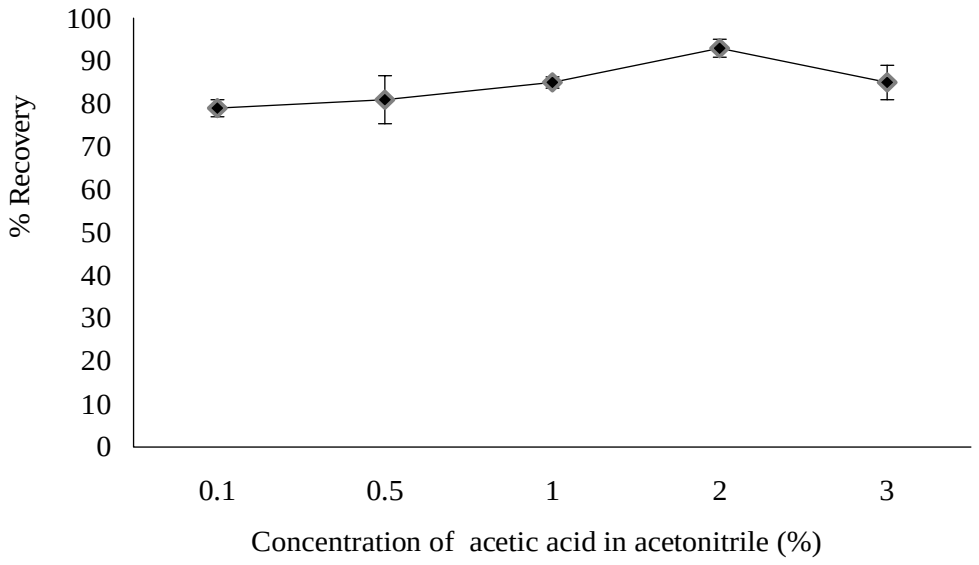


Figure 4.3 Effect of acetic acid concentration in acetonitrile (%) on the recovery (%) of ethoxyquin for the spiked shrimp sample at 50 µg/kg (n = 5)

4.3.2 The effect of dispersive solid phase extraction (d-SPE) sorbent for extraction method

Development of additional efficient clean up step was necessary to remove the remaining matrix. Dispersive solid phase extraction (d-SPE) which is being increasingly used for sample cleanup in ethoxyquin residues analysis was evaluated with various adsorbents such as type and amount of d-SPE.

4.3.2.1 The effect type of dispersive solid phase extraction (d-SPE)

The method development was the evaluation of a suitable sorbent to provide selective and complete isolation of the co-extracted compounds from acidified acetonitrile extracts. Even though pigment and lipids are not very soluble in acetonitrile, a certain amount a fat will be co-extracted, which should be removed prior to chromatographic analysis. This step in the modified of the QuEChERS method for shrimp sample was to reevaluate the effect of cleanup in the sample preparation procedure. In this study, different commercially available sorbents were compared. Table 4.6, Figure 4.4 show the extracting solution after cleanup with different types of sorbent, including C18, NH_2, GCB and PSA sorbent when the amount 50 mg per 1 mL of shrimp extract were used for cleanup. The best recovery for ethoxyquin was obtained using PSA sorbent**.** However, the result shown in Figure 4.5 demonstrates that only C18 and GCB sorbent can obviously remove the pigment from shrimp sample extract. Only GCB provide satisfactory recovery. Therefore, GCB was selected for the further optimization procedure.

Table 4.6 Effect of without sorbent and different sorbent types in d-SPE cleanup on the recovery (%) of ethoxyquin (n=5)

No.	% Recovery (n=5)				
	No clean up	C18	NH_2	GCB	PSA
1	50	67	73	88	90
2	55	58	76	84	98
3	48	63	77	84	90
4	52	68	76	80	98
5	50	64	72	74	98
Average	51	64	75	82	95
SD	2.6	3.9	2.2	5.3	4.4
RSD (%)	5.2	6.2	2.9	6.5	4.6

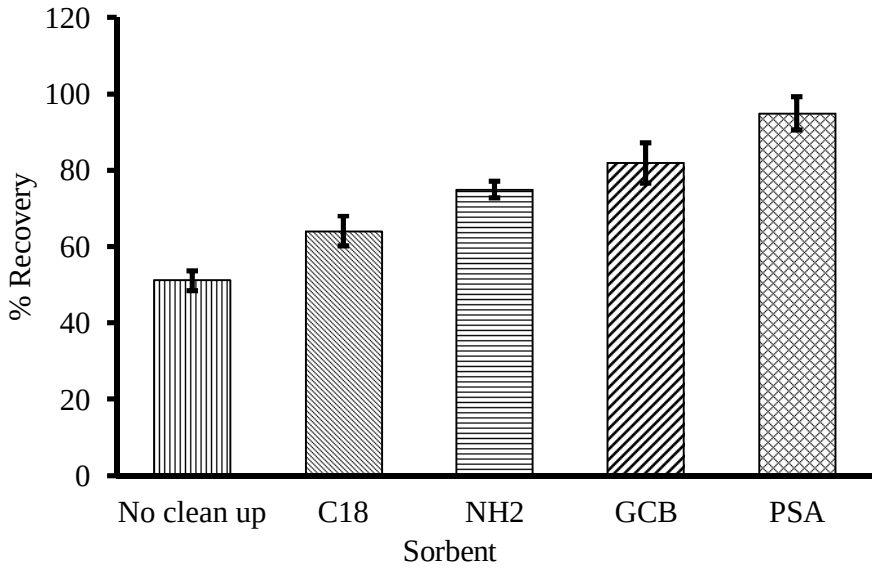


Figure 4.4 Effect of different sorbent types in d-SPE cleanup on the recovery (%) of ethoxyquin for the spiked shrimp sample at 50 µg/kg (n = 5)

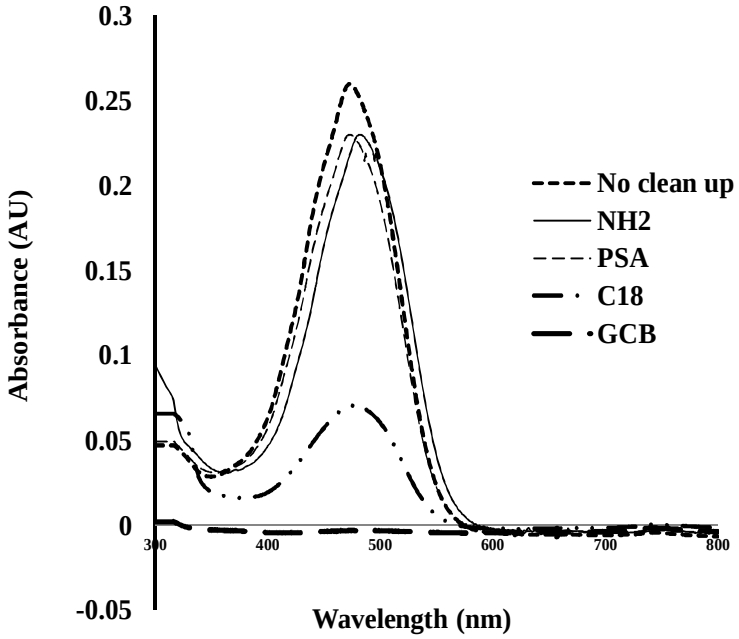


Figure 4.5 The spectrum of pigment (hemocyanin) sample extracts after cleanup with different d-SPE sorbents using UV-visible spectrophotometer

4.3.2.2 The effect of various amount of PSA sorbent

The PSA sorbent is a polymeric-based sorbent that contains both primary and secondary amines. The structure (see Figure 2.19) performs as weak anion exchanger sorbent with pKa of 10.1 and 10.9. The PSA functional group is a very good bi-dentate ligand, making PSA an excellent sorbent for chelation. Its higher carbon content makes it a more non-polar sorbent and thus a better choice for very polar compounds.[40] Then PSA could remove polar matrices from the sample extract. In this experiment, the amount of PSA sorbent was varied from 5 -100 mg per 1 mL of shrimp extract were used for cleanup. Table 4.7 and Figure 4.6 show the effect of various amounts of PSA sorbent on recovery (%) for ethoxyquin. The recovery was increased to the amount of PSA at 50 mg, and then the recovery remains constant.

Table 4.7 Effect of various amount PSA sorbent (5–100 mg) on the recovery (%) for the spiked shrimp sample at 50 µg/ kg (n = 5).

No.	% Recovery (n=5)						
	PSA sorbent (mg)						
	5	10	20	30	40	50	100
1	80	84	79	86	85	90	90
2	79	80	86	79	85	90	83
3	78	80	79	78	85	85	91
4	78	81	74	83	84	85	91
5	74	80	86	81	82	89	87
Average	78	81	81	81	84	88	88
SD	2.3	1.7	5.2	3.2	1.3	2.6	3.4
RSD (%)	2.9	2.1	6.4	3.9	1.60	3.0	3.9

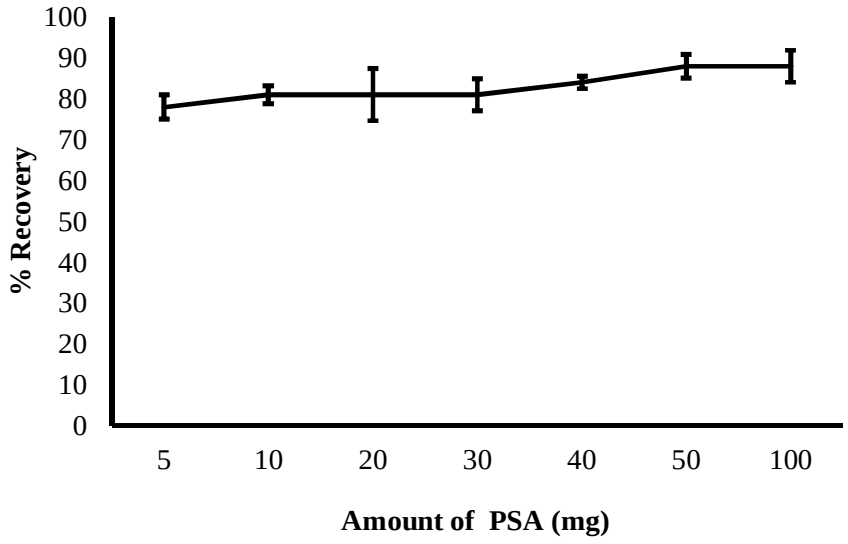


Figure 4.6 Effect of various amount of PSA sorbent per 1 mL of sample extract on the recovery (%) of ethoxyquin for the spiked shrimp sample at 50 µg/ kg (n = 5)

4.3.2.3 The effect of various amount of GCB sorbent

The PSA could remove polar matrixes from the sample extract but could not remove pigment (hemocyanin) thus select other d-SPE combined with PSA for cleanup. The graphitized carbon sorbent (GCB) is non-porous (Figure 2.22). Consequently, surface interaction depends solely on dispersion forces. GCB exhibits hydrophobic surface characteristics, meaning that small, polar molecules such as water are not adsorbed. Therefore, analyte displacement by water is significantly reduced, allowing them to be effectively used to trap organic compounds despite high humidity and GCB has a strong affinity for planar molecules, and thus GCB effectively removes pigment [46, 47]. The different amounts of GCB 5–50 mg per 1 mL of shrimp extract were used for cleanup. The results of the experiment showed that the use of a combination of 50 mg PSA and 10 mg GCB could remove pigment (hemocyanin) and provided excellent recovery of ethoxyquin, then the recoveries were decreased and remain constant when the amount of GCB higher than 20 mg. The results are shown in Table 4.8 and Figures 4.7 and 4.8

Table 4.8 Effect of various amount GCB sorbent (5–50 mg) with 50 mg PSA on the recovery (%) for the spiked shrimp sample at 50 µg/kg (n = 5)

No.	% Recovery (n=5)					
	GCB (mg)					
	5	10	20	30	40	50
1	117	93	70	66	67	67
2	126	105	72	63	63	66
3	111	90	67	63	65	63
4	126	96	66	76	62	58
5	126	93	68	70	57	62
Average	121	95	69	68	63	63
SD	6.9	5.8	2.4	5.5	3.8	3.6
RSD (%)	5.7	6.0	3.5	8.1	6.0	5.6

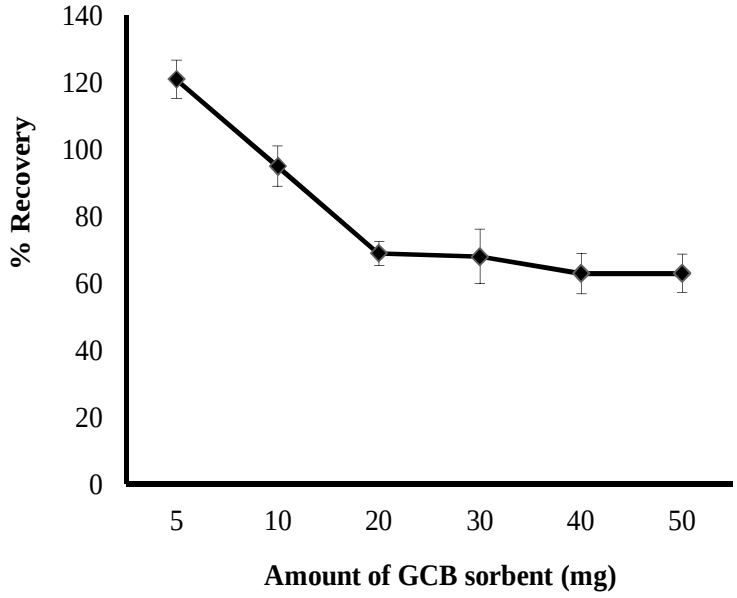


Figure 4.7 Effect of various amount of GCB sorbent (5–50 mg) with 50 mg PSA on the recovery (%) for the spiked shrimp sample at 50 µg/kg (n = 5)

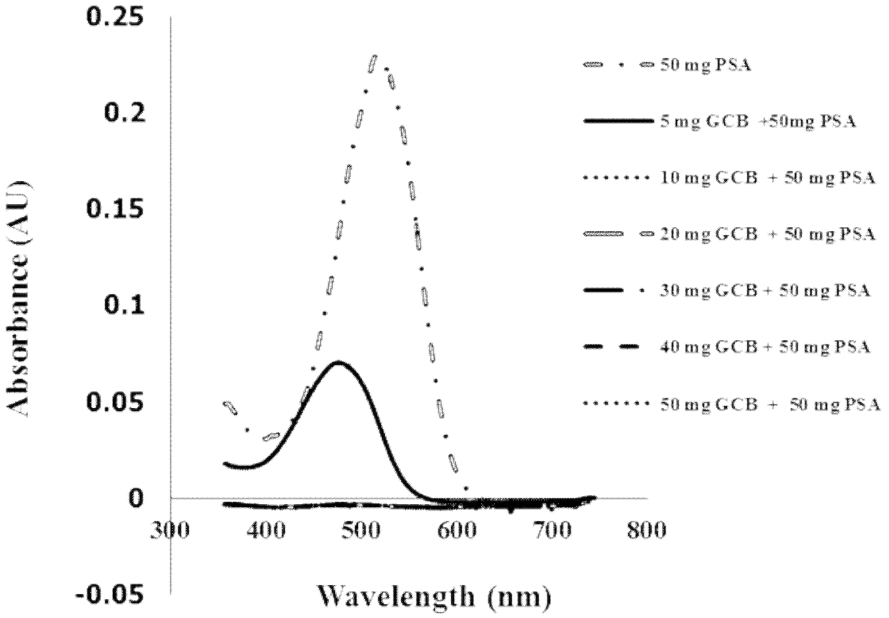


Figure 4.8 The spectrum of pigment (hemocyanin) sample extracts after cleanup by various amounts of GCB sorbent (5–50 mg) with 50 mg PSA using UV-visible spectrophotometer

4.3.3 The effect of dilution solution ratio

In this study, properties of the extracted solution before injection into UPLC-MS/MS should be considered because low sensitivity of ethoxyquin determination was obtained from the direct injection of acetonitrile sample extract. In general, the dilution of samples can sufficiently reduce the signal suppression from the matrix effect. Therefore, the dilution ratio studied were 0, 20, 40, 60, 80 and 100% solutions of acetonitrile in water. The results (Tables 4.8 and 4.9, Figure 4.9) indicates that 80% acetonitrile in water is the most suitable ratio for sample dilution, providing the highest sensitivity and best peak shape, because the selected dilution solution is similar to the initial composition of the mobile phase.

Table 4.9 The effect of dilution solution ratio

No.	Peak area (n=3)					
	Acetonitrile in water (100 mL)					
	0	20	40	60	80	100
1	4321	4710	4400	4685	5483	4713
2	4131	4375	4199	4411	5418	4366
3	3211	4584	4383	4517	5498	5164
Average	3888	4556	4327	4538	5466	4748
SD	590	170	110	140	40	400
RSD (%)	15	3.7	2.6	3.0	0.78	8.4

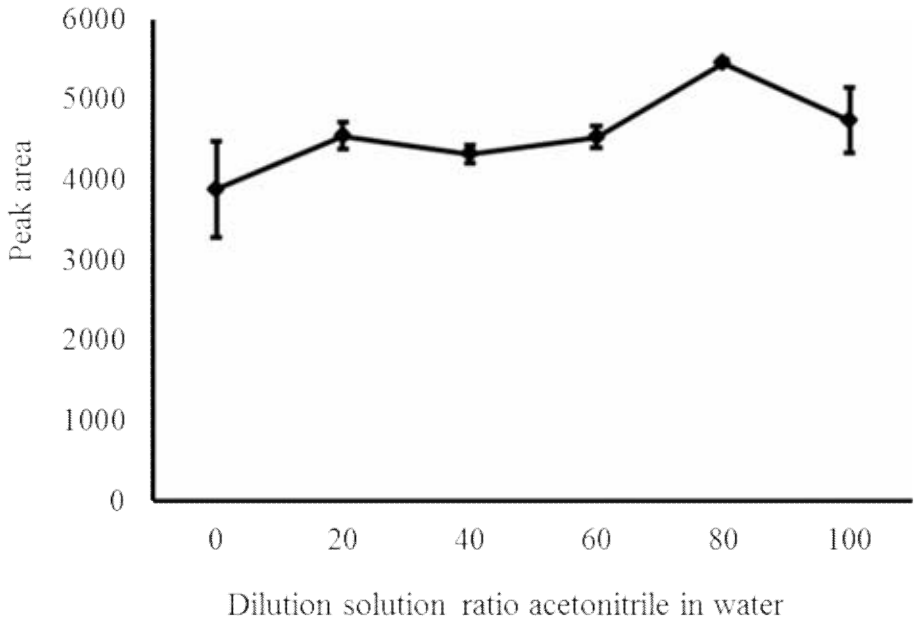


Figure 4.9 The effect of dilution solution ratio

Table 4.10 The various dilution solution ratio of matrix match standard at 5 µg/kg

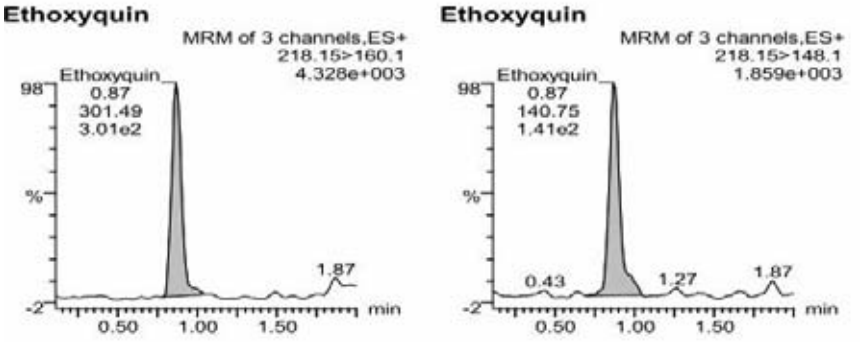
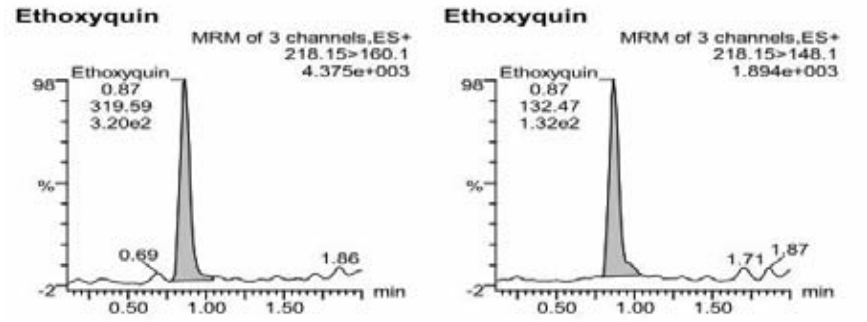
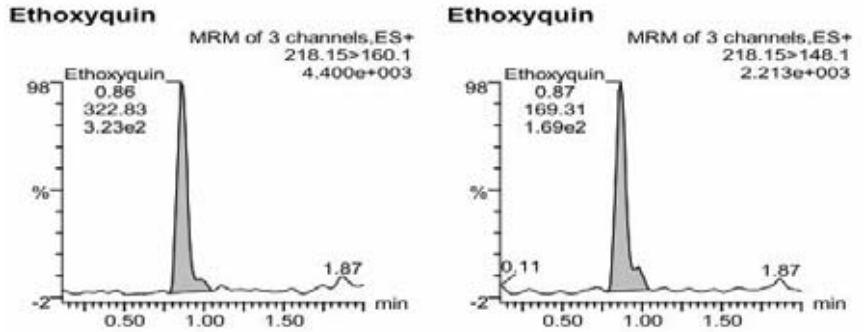
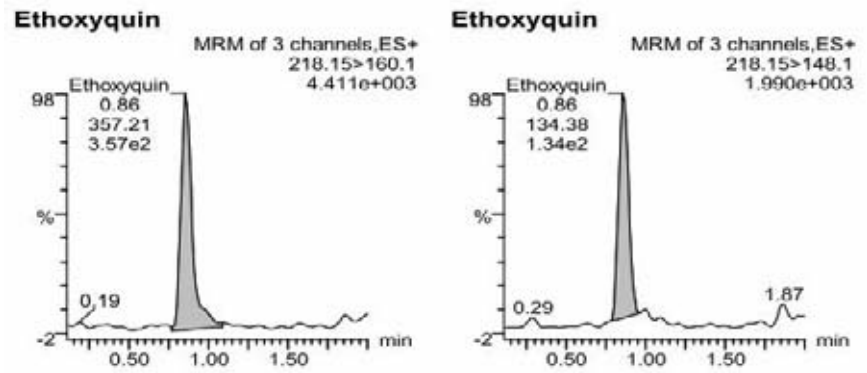
The dilution solution ratio acetonitrile in water	The representative of chromatogram ethoxyquin from the various dilution ratio of matrix match standard at 5 µg/kg
0	
20	
40	
60	

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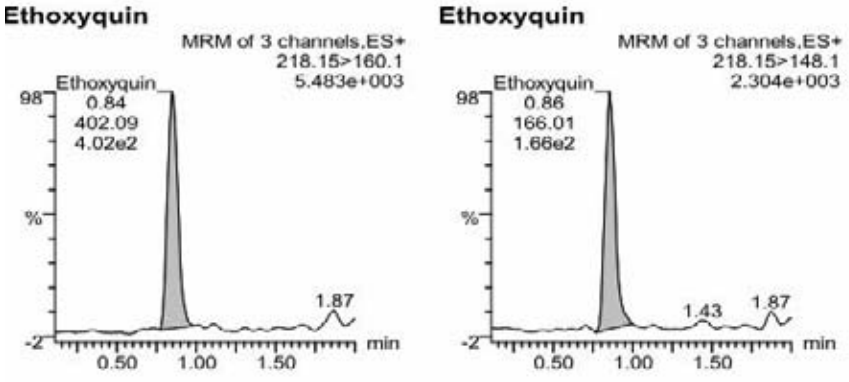
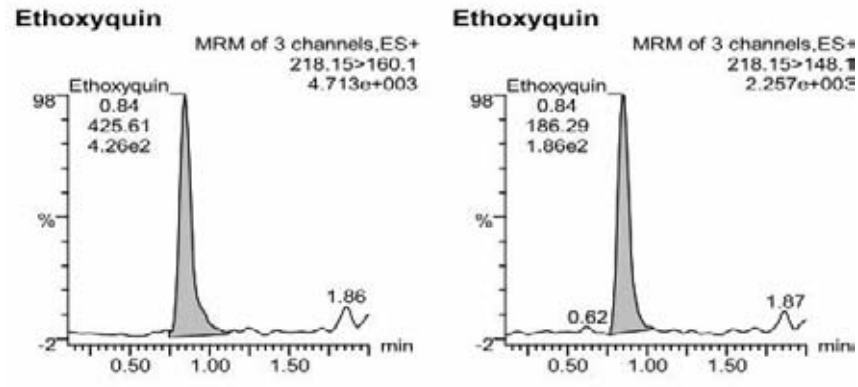
Table 4.10 *(continued).*

The dilution solution ratio acetonitrile in water	The representative of chromatogram ethoxyquin from the various dilution ratio of matrix match standard at 5 µg/kg
80	
100	

4.4 Method validation

The applicability of the developed method was tested following the accepted criteria for analytical method validation and estimation of selectivity, specificity, linearity, limit of detection, limit of quantification, accuracy, precision of the method is based on the analysis of spiked shrimp sample. Method precision was expressed as relative standard deviation (RSD). Method accuracy was determined by mean of percentage recoveries of the initial analyte concentrations in spiked shrimp matrix. Method validation was evaluated statistically.

4.4.1 Method selectivity

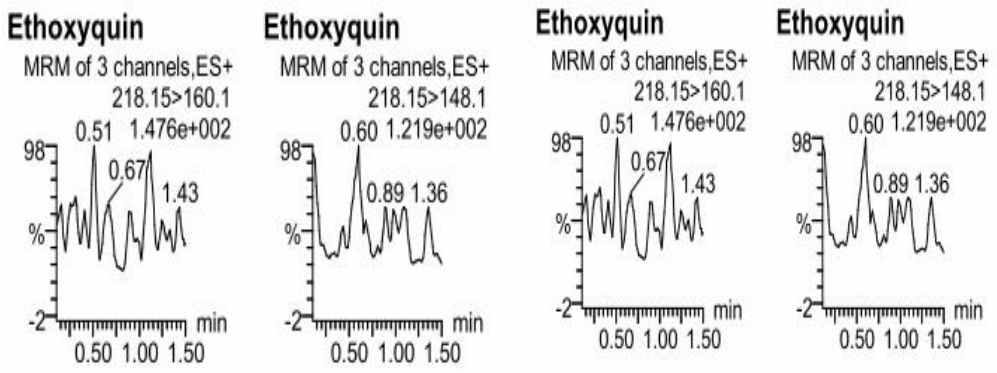
The selectivity of a method is important for the differentiation and identification of the existing target analytes in shrimp samples. Ethoxyquin was identified by matching peaks retention times (t_R) with the values of the corresponding standard analyzed under the same experimental conditions and the ion ratio of standard (ratio of the area of qualitative ion over the area of quantitative ion) as shown in Table. 4.11. Regarding the confirmation of ethoxyquin, the ion ratio was used for selection of the accurate analytes. The ion ratio is one of the most significant factors in terms of the confirmation of questioned analytes. The acceptable range of the ion ratio values complied with 2002/657/EC concerning the performance of analytical methods and the interpretation of results. [7] (Table 4.1) was used for distinguishing the analyte from the matrix interference thus allowing greater evidence in compound identification. The developed LC-MS/MS method is capable of separating ethoxyquin under the given gradient condition within 2 min.

Table 4.11 The demonstration of retention time and ion ratio

No.	t_R (min)	Fortified level (µg/kg) (n=10)		
		5	25	50
		Ion ratio		
1	0.86	0.44	0.46	0.54
2	0.87	0.50	0.54	0.51
3	0.86	0.47	0.48	0.50
4	0.87	0.44	0.46	0.51
5	0.86	0.49	0.49	0.49
6	0.87	0.53	0.54	0.45
7	0.87	0.50	0.54	0.55
8	0.87	0.45	0.46	0.42
9	0.86	0.50	0.55	0.46
10	0.87	0.52	0.41	0.46
Average	0.87		0.49	
SD	0.005		0.039	
3SD	0.01		0.12	

4.4.2 Method specificity

The specificity was assessed by analyzing solvent blank and blank shrimp sample. The absence of background peaks at the signal to noise ratio equal to 3 implied that the method is free of endogenous interferences at the retention time of the target compounds. The chromatogram of solvent blank and blank shrimp sample was shown in Figures 4.10 and the chromatogram of spiked shrimp sample at 1.5 µg/kg is shown in Figure 4.11.



(A) **(B)**

Figure 4.10 Representative chromatogram of (A) solvent blank and (B) blank shrimp sample

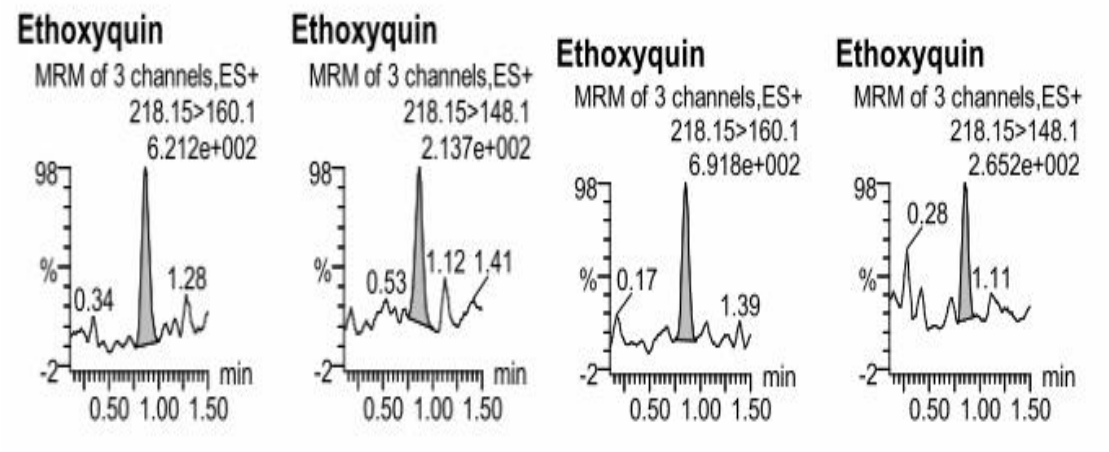


Figure 4.11 Representative chromatogram of spiked shrimp sample at 1.5 µg/kg

4.4.3 Method linearity

The linearity was demonstrated for ethoxyquin analyte by establishing a ten-point calibration curve at concentration levels of 0.8-64 µg/kg with three replicate analysis. Results were then fitted by the correlation coefficient (R^2). The slope and y-intercept in Table 4.12 and Figure 4.12 were demonstrated that the linearity were linear over the investigated concentration range. The sensitivity of the compound was determined by the slope of linear equation.

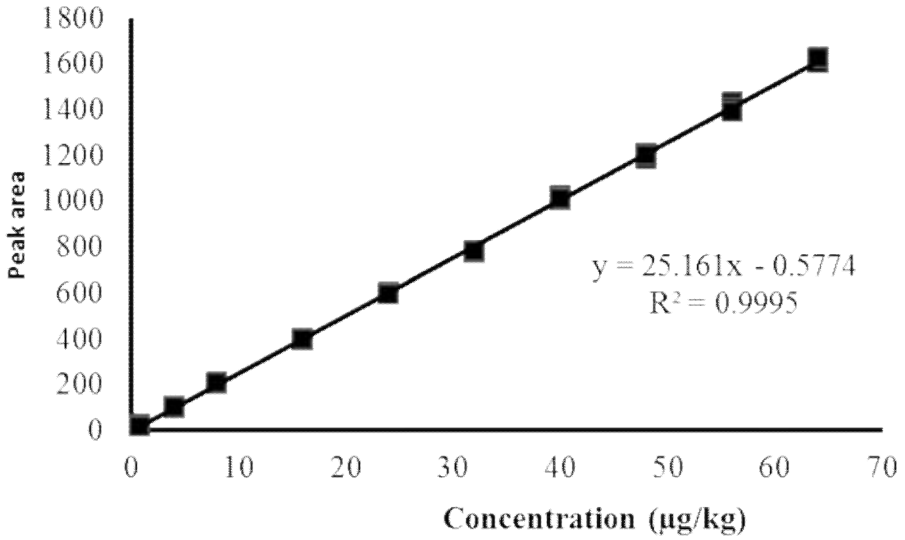


Figure 4.12 The linearity of ethoxyquin residue at concentration of 0.8-64 µg/kg

Table 4.12 The linear equation and coefficient of linearity analysis of ethoxyquin residue

The linearity of ethoxyquin residue	
Concentration range (µg/kg)	0.8-64
Linear equation	y=25.611 x-0.5774
Correlation coefficient (R^2).	0.9995

4.4.3.1 The standard calibration curve

The standard mixture (section 3.3.3) was prepared by dilution standard solution in acetonitrile: water (80:20, v/v) (see Table 3.2) prior to UPLC-MS/MS analysis. The coefficient of determination, R^2 more than 0.99 indicates the method linearity over the concentration range. The linear regression plots are shown in Figure 4.13. The results showed in Table 4.13 demonstrated that the standard curves were linear over the investigated concentration range. The sensitivity of the compound was determined by the slope of linear equation. The comparison between the standard calibration curve and matrix-matched calibration curve was performed for matrix effect by statistical *t* values (*t*-test: Paired two samples for means) at 95% confidential level.

4.4.3.2 The matrix-matched calibration curve

According to the theory, the matrix might have a significant signal on analytes in a mass-spectrometry technique. As the co-eluting matrix peaks rarely interfere with compounds of interest in the MS/MS technique, the matrix standard was selected as a standard of choice for mitigation of the ion on signal effect. Blank shrimp sample is a used for the matrix-matched standard dilution. The standard mixture (section 3.3.3) was prepared by diluting standard solution in matrix matched solution (standards added to blank extracts) prior to transfer of an aliquot for UPLC-MS/MS analysis. The coefficient of determination, R^2 more than 0.99 was represented the method linearity over the concentration range. The linear regression plots are shown in Table 4.13 and Figure 4.13. The results of matrix effect on UPLC-MS/MS quantitation, matrix-matched standard solution have the slope less than standard solvent solution. It has suppressing the ionization of ethoxyquin in shrimp sample.

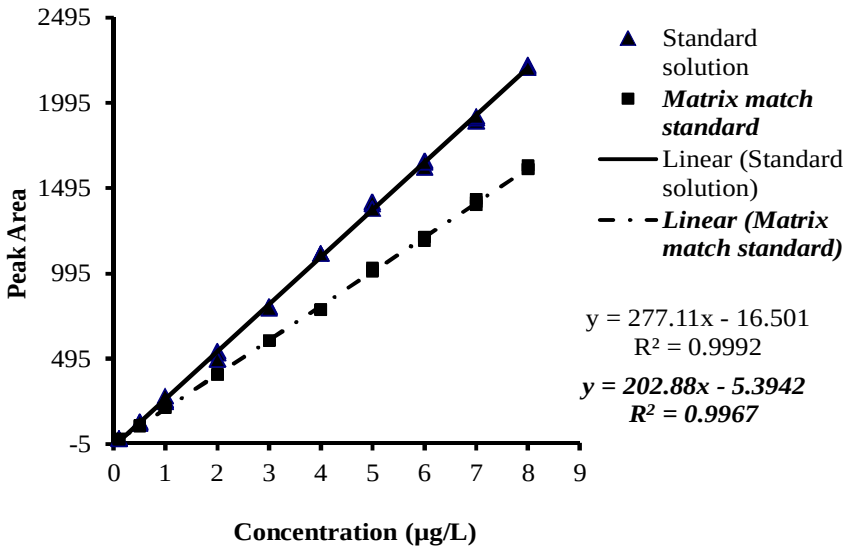


Figure 4.13 Solvent standard calibration and matrix-matched standard calibration curve

Table 4.13 The linear equation and coefficient of determination of standard calibration curve

Ethoxyquin	Solvent standard calibration	Matrix matched standard
Concentration range (µg/L)	0.1-8	0.1-8
Linear equation	y= 277.11x-16.501	y= 202.88x-5.3942
Correlation coefficient (R^2)	0.9992	0.9967

4.4.3.3 The evaluation of matrix effect

The assessment of matrix effect was investigated from the significant difference value. The t-test for paired two samples for means at 95% confident level was employed for the evaluation of significant difference. If the absolute value of t critical (the statistical *t*-test) is less than the absolute value of t calculation, there is significant difference between the dilution of standard in solvent and in matrix extract solution. On the other hand, if the absolute t value of calculation is less than the absolute t critical value (the statistical *t*-test), there is insignificant difference which means that there is no effect from the matrix that indicated that standard solution can be prepared in any solution. As a result in Table 4.14, ethoxyquin showed that the absolute value of t-critical (the statistical paired *t*-test) is less than the absolute value of t-calculation (t cal). Therefore, there have the significant difference between the dilution of standard in solvent and in matrix extract solution. Moreover, the determination of the matrix effect was proved that there was the matrix effect suppression for ethoxyquin. Consequently, the matrix-matched standard calibrations have to be served for this analysis because the matrix effect is almost occurred on MS/MS.

Table 4.14 The result of paired *t*-test for investigation of matrix effect

Analyte	Concentration range (µg/L)	Slope		Paired t-test	
		Standard solution	Matrix matched standard	[t cal]	[t crit]
Ethoxyquin	0.1-8	277.11	202.88	7.16	2.05

4.4.4 Limit of detections (LOD) and limit of quantification (LOQ)

The method limits of detection were calculated from 3 times of standard deviation of fortified level at 0.8 µg/kg and the chromatographic signal (peak area) at three times higher than background noise (S/N=3). The lowest spiked concentration of ethoxyquin at 0.8 µg/kg by a factor of 0.125 give 0.1 µg/kg under optimized condition method was employed to calculate LOD and the study was done in ten replicates. In the same way, the method limit of quantification was calculated from ten times of standard deviation of fortified level at 0.8 µg/kg and the chromatographic signal (peak area) but estimated ten times higher than background noise (S/N = 10). The results for the study are shown in Table 4.15.

Table 4.15 The result and standard deviation (SD)

No.	Result (µg/kg)
1	0.432
2	0.840
3	0.592
4	0.432
5	0.424
6	0.776
7	0.544
8	0.552
9	0.632
10	0.744
average	0.60
SD	0.15

The LOD and LOQ are calculated as follows:

LOD = 3SD

LOD = 3×0.15 = 0.45µg/kg

LOQ = 10SD

LOQ = 10×0.15 = 1.5 µg/kg

The result of LOD and LOQ are 0.45, 1.5 µg/kg, respectively.

4.4.5 Method accuracy

Accuracy is the closeness of agreement between a test result and the accepted reference value. To determine the accuracy of the method, shrimp samples were fortified at four concentrations level (1.5, 5, 25, 50 µg/kg) in ten replicates for each concentration level. After sample preparation and UPLC/MS/MS analysis, the accuracy was expressed as recovery (%). All recoveries were determined by comparing the peak areas obtained from fortified sample with peak areas resulting from direct injection of the matrix matched standards. The result of method accuracy on the percent recoveries of ethoxyquin and relative standard deviation (RSD) were show in Table 4.16.

Table 4.16 The recovery (%) and relative standard deviation (RSD) of the fortified sample (n=10)

No.	% Recovery (n=10)			
	Fortified level (µg/kg)			
	1.5	5	25	50
1	87	94	88	92
2	81	89	84	90
3	80	81	83	90
4	82	80	92	93
5	82	81	92	93
6	82	84	89	89
7	85	82	98	84
8	80	80	96	98
9	81	82	90	92
10	82	84	100	95
Average	82	84	91	92
SD	2.2	4.5	5.6	3.7
RSD (%)	2.7	5.4	6.2	4.1

The results for the fortification at 1.5, 5, 25, 50µg/kg were in range of 82-92%. The acceptable of % recovery range was shown in SANCO/10684/2009 said that "Acceptable recovery is a function of the concentration and the purpose of the analysis" determine average % recovery for both spike levels within range 70-120% [45] and AOAC Guidelines for single laboratory validation of chemical method for dietary supplements and botanicals [44] said that "Acceptable recovery is a function of the concentration and the purpose of the analysis" which are as below:

Concentration	Recovery limits
0.01%	85-110%
10 µg/g (ppm)	85-115%
1 µg/g	75-120%
10 µg/kg	70-125%

As a result, ethoxyquin were given the %recovery within this range. The results obtained from the above studies were indicated that the developed extraction method provided good accuracy for the analysis of ethoxyquin residues in shrimp.

4.4.6 Method precision

The precision is defined as the function of **repeatability and intermediate.** Ethoxyquin were studied and each batch was prepared by fortifying negative control of shrimp sample with solution standard (section 3.3.2) at the concentration level 1.5, 5, 25, 50 µg/kg. Method precision at 1.5, 5, 25, 50 µg/kg were studied by repeating experiment in the same day and three consecutive days. The fortified shrimp samples were analyzed with the optimization method. Fortification at each level was repeated ten times in Table 4.17. The results of the method precision are summarized and the Horwitz equation, the RSD values of ethoxyquin at 1.5, 5, 25, 50 µg/kg were calculated as shown in Table 4.18.

The assessment of repeatability

The percentage of recovery from ten times replicates was calculated by comparing the area of matrix-matched standard. The percentage recoveries obtained during the experiment are described in Table 4.17. Indeed, the repeatability standard deviation varies with concentration. Consequently, the standard deviation (SD) of each concentration is used for calculation of the $\%RSD_r$. This is used for comparison to $\%RSD_r$ target. The $\%RSD_r$ target is the acceptance limitations for the relative standard deviation (RSD_r) of the replicate results. It is calculated from the modified Horwitz Equation.:

$$: \%RSD_r < 0.66 \times 2^{(1- 0.5 \log C)}$$

Where: C is concentration of fortified sample (analyte /sample, g/g)

RSD_r is the relative standard deviation calculated from the results obtained from repeatability conditions (within laboratory)

Concentration level, µg/kg	$\%RSD_r$ target
1.5	28%
5	23%
25	18%
50	16%

The assessment of reproducibility

The measured parameter**,** reproducibility precision is commonly termed the intermediate precision. The percentage of recovery from ten times replicates was calculated by comparing the area of matrix-matched standard. The percentage recoveries obtained during the experiment are described in Table 4.17. Indeed, the reproducibility and intermediate standard deviation varies with concentration. Consequently, the standard deviation (SD) of each concentration is used for calculation of the $\%RSD_R$. This is used for comparison to % RSD_R target. The % RSD_R target is the acceptance limitations for the relative standard deviation (RSD_R) of the replicate results. It is calculated from the modified Horwitz Equation.:

: $\%RSD_R < 2^{(1-0.5 \log C)}$

Where: C is concentration of fortified sample (analyte /sample, g/g)

RSD_R is the relative standard deviation calculated from the results obtained from reproducibility conditions (single laboratory or within laboratory)

Concentration level, µg/kg	$\%RSD_R$ target
1.5	43%
5	36%
25	28%
50	25%

Table 4.17 Recovery (%) and RSD (%) of spiked shrimp matrices at 1.5, 5, 25, 50 µg/kg on three day (n=10)

No.	% Recovery (n=10)											
	Day 1				Day 2				Day 3			
	Fortified level (µg/kg)				Fortified level (µg/kg)				Fortified level (µg/kg)			
	1.5	5	25	50	1.5	5	25	50	1.5	5	25	50
1	87	94	88	92	80	87	87	94	84	87	94	92
2	81	89	84	90	80	89	91	95	83	89	87	87
3	80	81	83	90	85	85	84	86	81	85	89	95
4	82	80	92	93	85	90	94	94	87	90	93	91
5	82	81	92	93	80	83	86	83	82	83	90	93
6	82	84	89	89	85	84	82	91	93	84	84	90
7	85	82	98	84	82	84	93	85	90	84	85	85
8	80	80	96	98	87	84	90	99	81	84	93	92
9	81	82	90	92	82	82	92	91	82	82	92	90
10	82	84	100	95	83	84	94	97	80	84	89	90
Average	82	84	91	92	83	85	89	92	84	85	90	91
SD	2.2	4.5	5.6	3.7	2.5	2.6	4.3	5.3	4.3	2.6	3.5	2.9
RSD (%)	2.7	5.4	6.2	4.1	3.0	3.1	4.8	5.8	5.1	3.1	3.9	3.2

Table 4.18 Summarization of RSD (%) of spiked shrimp sample at 1.5, 5, 25, 50 µg/kg

Fortified level (µg/kg)	RSD (%)				P-value	F-value		ANOVA
	Day 1	Day 2	Day 3	Overall		F calculated	F critical	
1.5	2.7	3.0	5.1	3.8	0.33	1.1	3.4	insignificant
5	5.4	3.1	3.1	3.9	0.52	0.66		insignificant
25	6.2	4.8	3.9	5.0	0.61	0.50		insignificant
50	4.1	5.8	3.2	4.4	0.81	0.22		insignificant

When; $P > 0.05$ = insignificant, $P < 0.05$ = significant

F calculated < F critical = insignificant, F calculated > F critical = significant

The repeatability precision of the method on three different days at 1.5, 5, 25, 50 µg/kg as shown in Table 4.18, ranged between 2.7-6.2%. According to the summary results, the % RSD_r of repeatability precisions were calculated and compared to %RSD_r from Horwitz equation. All the %RSD_r values were less than the %RSD_r target (from Horwitz equation).

The intermediate precision of the method on three different days at 1.5, 5, 25, 50 µg/kg as shown in Table 4.18, ranged between 3.8-5.0%. From the statistical analysis (a one way analysis of variance (ANOVA) at 95% confident limit, there is insignificant difference between RSD (%) values of each day except for the concentration at 1.5, 5, 25, 50 µg/kg. However, the RSD (%) values of ethoxyquin were still lower than the acceptable values calculated by Horwitz's equation. Therefore the propose method has reliable intermediate precision at the level of analytes being measured.

The precision of method was expressed as the percentage relative standard deviation (%RSD). On the basis of the Horwitz equation, the acceptable RSD_r (%) ranged between 16-28% and the acceptable RSD_R (%) ranged between 25-43% when compared to RSD this method, the obtained RSD (%) values in Table 4.18 were clearly

illustrated that this method is sufficiently precise at the concentration level of analytes being measured within the same day and difference day .

4.4.7 Application to real sample

To prove that the application of the developed method when applied in real sample, the 100 shrimp samples taken from various local fresh markets and supermarkets, Bangkok, Thailand and from the shrimp farm for export in Thailand were analyzed. The quality control for each batch of shrimp samples consisted of a matrix-matched calibration with a reagent blank, and a spiked blank shrimp sample at 5µg/kg for ethoxyquin analysis. Among the shrimp samples analyzed, trace amount of ethoxyquin (<LOD) was presented in 80 samples, and ethoxyquin was detected at concentrations in the level excess their LOQ (1.5 µg/kg) in 20 samples. The results detected negative sample (ethoxyquin residue below<LOD) and positive sample (ethoxyquin residue at or above the LOD). The results were shown in Table 4.19 and representative of chromatogram obtained from real sample analysis shown in Figure 4.14.

Table 4.19 The analysis of ethoxyquin in real shrimp sample

Sample ID	Sample type	Incurred sample		Matrix –match standard		Ethoxyquin (µg/kg)
		Ion ratio	t_R (min)	Ion ratio	t_R (min)	
11	Fresh shrimp	0.56	0.87	0.55	0.87	5
12	Raw white shrimp	0.56	0.87	0.55	0.87	9
15	Frozen raw shrimp	0.54	0.87	0.56	0.87	8
20	Fresh white shrimp	0.58	0.87	0.58	0.87	130

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Table 4.19 *(continued).*

Sample ID	Sample type	Incurred sample		Matrix –match standard		Ethoxyquin (µg/kg)
		Ion ratio	t_R (min)	Ion ratio	t_R (min)	
26	Fresh white shrimp	0.56	0.86	0.56	0.86	210
34	Vannamei shrimp	0.52	0.86	0.51	0.86	7
40	Fresh white shrimp	0.46	0.86	0.47	0.86	1.5
53	Fresh white shrimp	0.52	0.87	0.51	0.86	137
55	Frozen raw shrimp	0.54	0.87	0.53	0.87	138
56	Fresh white shrimp	0.53	0.87	0.53	0.87	154
63	Raw white shrimp	0.51	0.86	0.53	0.86	213
64	Fresh shrimp	0.49	0.86	0.53	0.86	6
71	Frozen white shrimp	0.57	0.87	0.55	0.87	9

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Table 4.19 *(continued).*

Sample ID	Sample type	Incurred sample		Matrix –match standard		Ethoxyquin (µg/kg)
		Ion ratio	t_R (min)	Ion ratio	t_R (min)	
79	Frozen white shrimp	0.56	0.87	0.55	0.87	260
80	Frozen white shrimp	0.56	0.87	0.55	0.87	350
93	Raw white shrimp	0.60	0.87	0.55	0.87	102
95	Fresh white shrimp	0.60	0.86	0.52	0.86	2
96	Fresh white shrimp	0.60	0.86	0.58	0.86	5
99	Raw white shrimp	0.49	0.87	0.55	0.86	4
100	Fresh white shrimp	0.54	0.87	0.55	0.87	5

The reporting of results refers to SANCO/10684/2009.[45] The positive finding was confirmed by performing two transitions in MS/MS for quantification and identification in compliance with recent EU guidelines. The ion ratio of analytes in incurred samples was confirmed to be existed within the range of permitted tolerance when compared to ions in the matrix-matched standard (±25% of accepted values). The

retention time of the analyte in positive sample was identical within instrumental variation when compared to the retention time of the analyte in the matrix-matched standard (±5% of accepted values), from the data in Table 4.19. Chromatograms of the determination of ethoxyquin in shrimp samples were shown in Figure 4.14

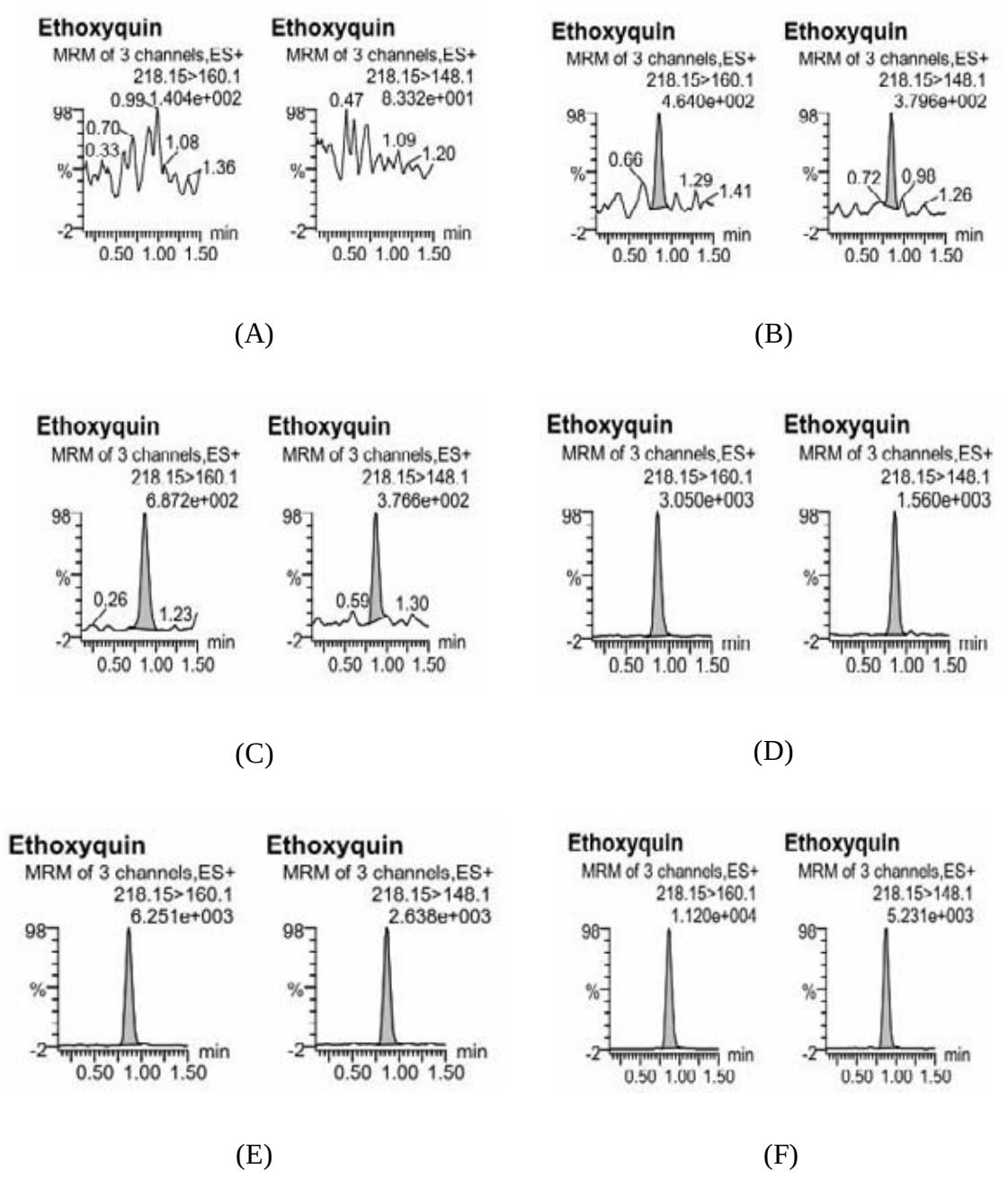

Figure 4.14 UPLC-MS/MS chromatograms of ethoxyquin obtained from real sample analysis: (A) fresh white shrimp (ND), (B) fresh white shrimp (1.5 µg/kg), (C) Fresh shrimp (5 µg/kg), (D) raw white shrimp (102 µg/kg), (E) frozen white shrimp (260 µg/kg) and (F) frozen white shrimp (350 µg/kg)

CHAPTER V
CONCLUSIONS AND SUGGESTION FOR FURTHER STUDY

In this study, the proposed UPLC-MS/MS method was successfully applied to the analysis of ethoxyquin residues. The analysis was performed by using optimized condition of ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with the electrospray ionization (ESI) in positive mode. Acquity UPLC^{TM} BEH C18 column was used for the separation of analyte.

The optimized MS/MS conditions for the analysis of selected compound were shown in Table 5.1.

Table 5.1 The optimum ESI-MS/MS conditions for analysis of ethoxyquin

ESI parameter	Condition
Detection mode	Positive
Capillary voltage (V)	1000
Source temperature (ºC)	120
Desolvation temperature (ºC)	350
Cone gas flow; nitrogen (L/hr)	50
Desolvation gas flow; nitrogen (L/hr)	1000
Collision gas (argon)	3.5×10^{-3}
Molecular weight (g/mol)	217.31
Cone voltage (V)	40
1st transition quantification	218.15>160.10
Collision energy (eV)	32
2nd transition quantification	218.15>148.10
Collision energy (eV)	30

The selection of ionization mode and optimization of various parameters were influenced on MS signal of analyte, including the specific cone and collision energies for ethoxyquin. The method was operated in multiple reactions monitoring (MRM) mode which used for quantification and confirmation. The MRM transitions of ethoxyquin analyte were previously illustrated in Table 5.1. In chromatographic optimization, separation and selectivity of ethoxyquin were achieved by using Acquity UPLC^{TM} BEH C18 stationary phase with gradient elution. The mobile phase containing of a 10 mM ammonium acetate containing 0.3% acetic acid (mobile phase A):

acetonitrile containing 0.3% acetic acid (mobile phase B) were applied. The selectivity of UPLC-MS/MS method was evaluated by the matching of peak retention time and ion ratio of precursor and product ion. If various compounds have same retention time, there can be confirmed by structural information of the compound. The optimum chromatographic conditions in this study are shown in Table 5.2.

Table 5.2 The optimum chromatographic conditions for analysis of ethoxyquin

Parameters	Condition
Analytical column	Acquity UPLC® BEH Shield RP18
Mobile phase	A: 10 mM ammonium acetate containing 0.3% acetic acid
	B: Acetonitrile containing 0.3% acetic acid
Flow rate	0.5 mL/min
Total runtime	2 min
Injection volume	5 µL
Injection mode	the partial loop with needle overfill loop mode
Column temperature	40ºC
Sample temperature	20ºC

The use of Acquity UPLC® BEH Shield RP18 separation technique was successfully completed for short run time of ethoxyquin analysis.

For the sample preparation, the QuEChERS was modified for the determination of these ethoxyquin residues, the optimal procedure was employed liquid extraction with acidified acetonitrile. The concentration of acetic acid is also investigated. Satisfactory results were obtained with 2% acetic acid in acetonitrile which showed the influence in the extraction efficiency and chromatographic separation. 50 mg PSA and 10 mg GCB were used for sample extracts clean up. The sample extracts was then diluted with acetonitrile: water (80:20) in order to reduce signal suppression from matrix effect with the optimum dilution ratio of 1:1 (sample solution: dilution solution). The optimum procedure for sample preparation was summarized in Figure 5.1.

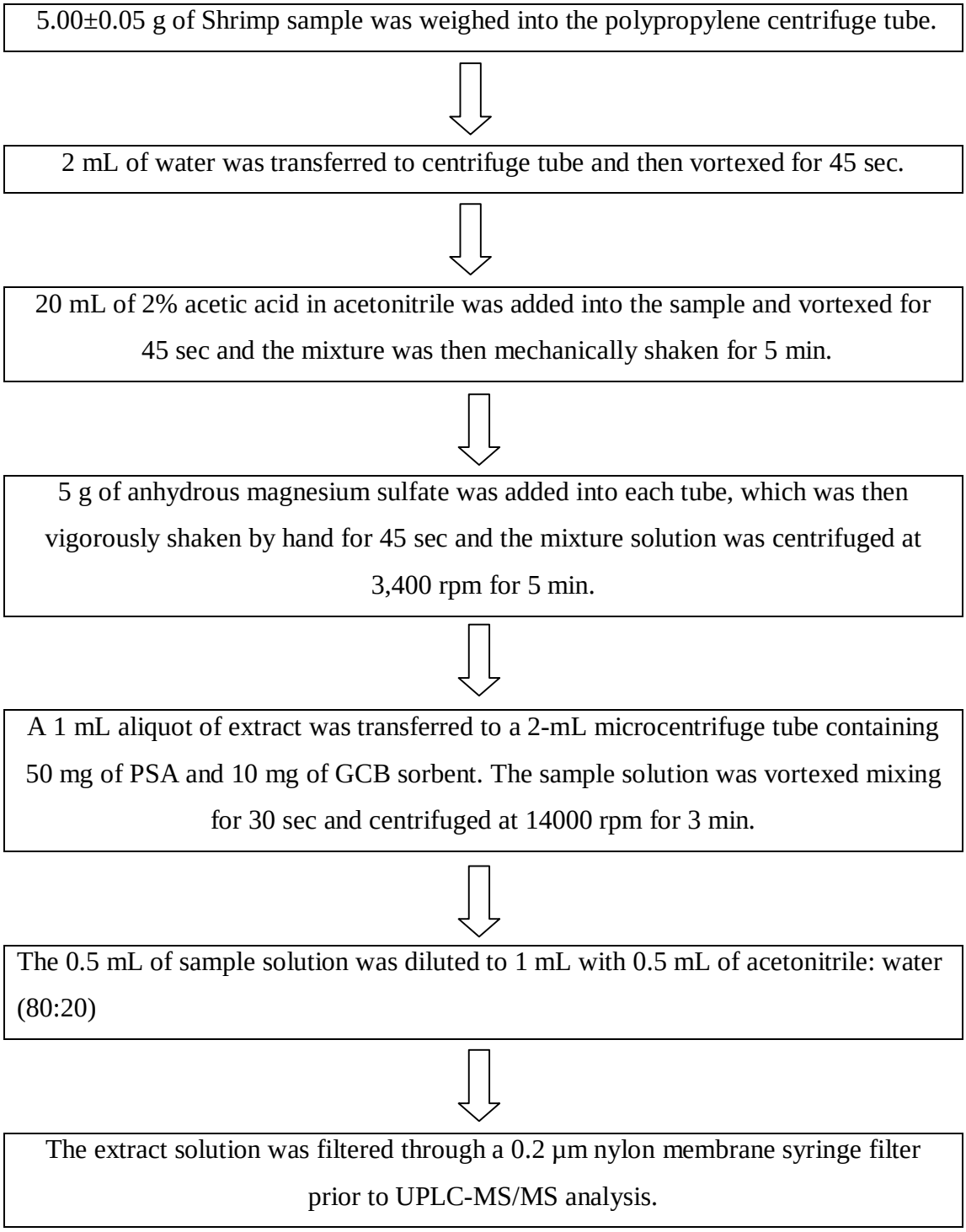

Figure 5.1 Schematic diagram of optimized sample preparation

Signal suppression from the matrix effect was observed during UPLC-MS/MS analysis. To compensate this effect, the matrix-matched standards were used in this study for quantification in order to reach high accuracy.

The present method was validated according to the requirements defined by the method validation quality control procedures for pesticide residues analysis in food and feed No. SANCO/10684/2009. The results showed good linearity over the concentration range of 0.8– 64 µg/kg for ethoxyquin with correlation cefficient (R^2) higher than 0.99. Table 5.3 showed method performance of ethoxyquin analysis.

Table 5.3 Method performance of ethoxyquin analysis

Compound	t_R(min)	R^2	LOD (µg/kg)	LOQ (µg/kg)
Ethoxyquin	0.87±0.01	0.9995	0.45	1.5

The accuracy was evaluated at four concentrations levels at 1.5, 5, 25, 50 µg/kg tests in shrimp sample matrices. The results were in range of 84-97%. The % recoveries were acceptable range. Precision was evaluated at four concentration levels at 1.5, 5, 25, 50 µg/kg for repeatability and intermediate test in shrimp matrices. The repeatability was expressed as relative standard deviation (%RSD_r) for within day precision and The intermediate was expressed as relative standard deviation (%RSD_R) for between day precision at 1.5, 5, 25, 50 µg/kg levels were ranged from 2.7-6.2 and 3.8-5.0%. The satisfactory reproducibility of this method on three different days achieved with standard deviation was lower than the calculated %RSD derived from the Horwitz equation. Good validation parameter such as linearity, accuracy, precision, LOD and LOQ were obtained.

A real shrimp samples were bought from local fresh markets and supermarkets, Bangkok, Thailand and the shrimp farm for export in Thailand and were detected by this developed method for ethoxyquin analysis. The detected amounts in shrimp samples were at or higher than LOQ (1.5µg/kg). The positive finding was confirmed by performing two transitions in MS/MS for quantification and identification in compliance with recent EU guidelines. The technique of detection could be supportive for exported shrimp to EU and harmonization with 2002/657/EC concerning the performance of analytical methods and the interpretation of result. The ion ratio of analytes in incurred samples was confirmed to be within the range of permitted tolerance when compared to ions in the matrix-matched standard (±25% of accepted

values). The retention time of the analyte in positive sample was identical within instrumental variation when compared to the retention time of the analyte in the matrix-matched standard (±5% of accepted values). The results were shown in Table 5.4.

Table 5.4 The analysis of ethoxyquin in real shrimp sample

Commodities	No. of positive sample	Positive range (min-max),µg/kg
Shrimp 100 samples	20	1.5-350

The developed method is a highly sensitive method for quantitation and confirmation for the analysis of ethoxyquin in shrimp. UPLC-MS/MS could be analyzed ethoxyquin within a short chromatographic run time (2 min). The LOQ by UPLC-MS/MS is well below the MRL of ethoxyquin (10µg/kg). Therefore, the modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method can be regarded as a strong alternative method for current extraction techniques by offering fast, simple, inexpensive, real time screening within the MRLs of ethoxyquin and high throughput method (The proposed method is able to extract 20 shrimp samples in less than 30 min and the extracts can be analyzed and interpretation of result in less than 43 min). The results were illustrated that the proposed study is suitable for applying in routine laboratories. This method can be further developed and applied to food products of other animals (cattle, pig, chicken, fish and egg) for the analysis of ethoxyquin residues that the residues in some foods were critical concern at present.

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APPENDICES

APPENDIX A

Table A The result of *t*-test for paired two samples for the mean values of ethoxyquin for investigation of matrix effect.

Ethoxyquin	Standard solvent	Matrix match standard
Mean	1031.172414	760.5862069
Variance	544151.2192	286762.1084
Observations	29	29
Pearson Correlation	0.999291114	
Hypothesized Mean Difference	0	
df	28	
t cal	7.158874469	
P(T<=t) one-tail	4.31291E-08	
t Critical one-tail	1.701130934	
P(T<=t) two-tail	8.62582E-08	
t Critical two-tail	2.048407142	

|t Cal|>|t Crit| : significant

Statistical analysis of ethoxyquin

Anova: Single Factor at 1.5 µg/kg

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	822	82	5
Day 2	10	829	83	6
Day 3	10	843	84	19

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	23	2	11	1.1	0.33	3.4
Within Groups	269	27	10			
Total	291	29				

P > 0.05 = insignificant

Anova: Single Factor at 5 µg/kg

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	837	84	20
Day 2	10	852	85	7
Day 3	10	852	85	7

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	15	2	8	0.66	0.52	3.4
Within Groups	305	27	11			
Total	320	29				

$P > 0.05$ = insignificant

Anova: Single Factor at 25 µg/kg

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	912	91	32
Day 2	10	893	89	18
Day 3	10	896	90	12

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	21	2	10	0.50	0.61	3.4
Within Groups	558	27	21			
Total	579	29				

$P > 0.05$ = insignificant

Anova: Single Factor at 50 µg/kg

SUMMARY

Groups	Count	Sum	Average	Variance
Day 1	10	916	92	14
Day 2	10	915	92	29
Day 3	10	905	91	8

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	7	2	4	0.22	0.81	3.4
Within Groups	457	27	17			
Total	465	29				

$P > 0.05$ = insignificant

APPENDIX B

Table B The analysis of ethoxyquin in real shrimp sample from various local fresh markets supermarket Bangkok, Thailand and the shrimp farm for export in Thailand

Sample ID	Sample Description	Result	Area of purchase
1	Frozen raw headless shell-on vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
2	Frozen raw headless shell on vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
3	Frozen raw headless shell on vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
4	Frozen raw headless shell on vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
5	Fresh shrimp	ND	Klong Toey fresh market
6	Fresh shrimp	ND	Nanglingee fresh market
7	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
8	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
9	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
10	Fresh shrimp	ND	TOPs supermarket at Nanglingee Chongnonsi
11	Fresh shrimp	5	TOPs supermarket at Nanglingee Chongnonsi
12	Raw white shrimp	9	Shrimp farm for export at Samutsakorn
13	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
14	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn

ND = Not Detected

Table B *(continued)*

Sample ID	Sample Description	Result	Area of purchase
15	Frozen raw peeled and deveined tail off vannamei white shrimp	8	Shrimp farm for export at Samutsakorn
16	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
17	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
18	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
19	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
20	Fresh white shrimp	130	TOPs supermarket at Central plaza RAMA III
21	Fresh white shrimp	ND	Bangbon fresh market
22	Fresh white shrimp	ND	Bangbon fresh market
23	Fresh white shrimp	ND	Klong Toey fresh market
24	Fresh white shrimp	ND	Klong Toey fresh market
25	Fresh white shrimp	ND	Klong Toey fresh market
26	Fresh white shrimp	210	Klong Toey fresh market
27	Fresh white shrimp	ND	Tesco Lotus at RAMA II
28	Black tiger shrimp	ND	Tesco Lotus at RAMA II
29	Black tiger shrimp	ND	TOPs supermarket at RAMA II

ND = Not Detected

Table B *(continued)*

Sample ID	Sample Description	Result	Area of purchase
30	Fresh white shrimp	ND	TOPs supermarket at RAMA II
31	Fresh white shrimp	ND	TOPs supermarket at RAMA II
32	Frozen shrimp	ND	Shrimp farm for export at Samutsakorn
33	Frozen shrimp	ND	Shrimp farm for export at Samutsakorn
34	Vanamei shrimp	7	Shrimp farm for export at Samutsakorn
35	Vanamei shrimp	ND	Shrimp farm for export at Samutsakorn
36	Vanamei shrimp	ND	Shrimp farm for export at Samutsakorn
37	Vanamei shrimp	ND	Shrimp farm for export at Samutsakorn
38	Vanamei shrimp	ND	Shrimp farm for export at Samutsakorn
39	Vanamei shrimp	ND	Shrimp farm for export at Samutsakorn
40	Fresh white shrimp	1.5	Shrimp farm for export at Samutsakorn
41	Fresh white shrimp	ND	Big C at RAMA II
42	Fresh black tiger shrimp	ND	Big C at RAMA II
43	Fresh black tiger shrimp	ND	TOPs supermarket at Central plaza RAMA II
44	Fresh white shrimp	ND	TOPs supermarket at Central plaza RAMA II
45	Fresh white shrimp	ND	Shrimp farm for export at Samutsakorn
46	Fresh white shrimp	ND	Shrimp farm for export at Samutsakorn

ND = Not Detected

Table B *(continued)*

Sample ID	Sample Description	Result	Area of purchase
47	Fresh white shrimp	ND	Shrimp farm for export at Samutsakorn
48	Fresh white shrimp	ND	Shrimp farm for export at Samutsakorn
49	Fresh white shrimp	ND	Shrimp farm for export at Samutsakorn
50	Fresh white shrimp	ND	Shrimp farm for export at Samutsakorn
51	Raw pelled deveined tail on, vannamai shrimp	ND	Shrimp farm for export at Samutsakorn
52	Raw pelled deveined tail on, vannamai shrimp	ND	Shrimp farm for export at Samutsakorn
53	Fresh white shrimp	137	Shrimp farm for export at Samutsakorn
54	Fresh white shrimp	ND	Shrimp farm for export at Samutsakorn
55	Frozen white shrimp	138	Shrimp farm for export at Samutsakorn
56	Fresh shrimp	154	Shrimp farm for export at Samutsakorn
57	Fresh shrimp	ND	Shrimp farm for export at Samutsakorn
58	Fresh shrimp	ND	Shrimp farm for export at Samutsakorn
59	Fresh shrimp	ND	Shrimp farm for export at Samutsakorn
60	Raw white shrimp	ND	Shrimp farm for export at Samutsakorn
61	Raw white shrimp	ND	Shrimp farm for export at Samutsakorn
62	Raw white shrimp	ND	Shrimp farm for export at Samutsakorn
63	Raw white shrimp	213	Shrimp farm for export at Samutsakorn

ND = Not Detected

Table B *(continued)*

Sample ID	Sample Description	Result	Area of purchase
64	Fresh shrimp	6	Prakanong fresh market
65	Fresh shrimp	ND	Prakanong fresh market
66	Fresh shrimp	ND	Prakanong fresh market
67	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
68	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
69	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
70	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
71	Frozen white shrimp	9	Shrimp farm for export at Samutsakorn
72	Raw pelled deveined tail on, vannamai shrimp	ND	Shrimp farm for export at Samutsakorn
73	Raw pelled deveined tail on, vannamai shrimp	ND	Shrimp farm for export at Samutsakorn
74	Raw pelled deveined tail on, vannamai shrimp	ND	Shrimp farm for export at Samutsakorn
75	Raw pelled deveined tail on, vannamai shrimp	ND	Shrimp farm for export at Samutsakorn
76	Raw pelled deveined tail on, vannamai shrimp	ND	Shrimp farm for export at Samutsakorn
77	Raw pelled deveined tail on, vannamai shrimp	ND	Shrimp farm for export at Samutsakorn
78	Raw pelled deveined tail on, vannamai shrimp	ND	Shrimp farm for export at Samutsakorn
79	Frozen white shrimp	260	Shrimp farm for export at Samutsakorn
80	Frozen white shrimp	350	Shrimp farm for export at Samutsakorn

ND = Not Detected

Table B *(continued)*

Sample ID	Sample Description	Result	Area of purchase
81	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
82	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
83	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
84	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
85	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
86	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
87	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
88	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
89	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
90	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
91	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
92	Frozen raw pelled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
93	Raw white shrimp	102	Shrimp farm for export at Samutsakorn
94	Frozen raw peeled and deveined tail off vannamei white shrimp	ND	Shrimp farm for export at Samutsakorn
95	Fresh white shrimp	2	Shrimp farm for export at Samutsakorn
96	Fresh white shrimp	5	Shrimp farm for export at Samutsakorn

ND = Not Detected

Table B *(continued)*

Sample ID	Sample Description	Result	Area of purchase
97	Fresh white shrimp	ND	Shrimp farm for export at Samutsakorn
98	Fresh white shrimp	ND	Shrimp farm for export at Samutsakorn
99	Fresh white shrimp	4	Shrimp farm for export at Samutsakorn
100	Fresh white shrimp	5	Shrimp farm for export at Samutsakorn

ND = Not Detected

VITA

Miss Songkran Chikakul was born on February 22, 1981 in Sisaket, Thailand. She had graduated a Bachelor's degree of science in Chemistry from Bansomdejchaopraya Rajabhat University in 2003. Afterwards she has worked for National Food Institute at Bangkok Company since 2003-2006. Work as scientist. Second job at Overseas Merchandise Inspection Co.,Ltd (OMIC) Company since 2006 until now. Work as chemist in 2009, she continued her academic education for Master degree at Department of Chemistry, Faculty of Science, Chulalongkorn University. She had completed the program in July 2012 and received her Master's degree of Science in analytical Chemistry in July 2013.