การสกัดระดับจุลภาคในเฟสของเหลวด้วยเส้นใยกลวงเพื่อการตรวจวัดสารปฏิชีวนะกลุ่มแมกโครไลด์ตกค้างในน้ำและเนื้อสัตว์ปีก

นางสาว โสภารัตน์ ยุทธวรศิษย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมี ภาควิชาเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

HOLLOW-FIBER LIQUID-PHASE MICROEXTRACTION FOR DETERMINATION OF MACROLIDE ANTIBIOTIC RESIDUES IN WATER AND POULTRY MUSCLE

Miss Soparat Yudthavorasit

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
Department of Chemistry
Faculty of Science
Chulalongkorn University
Academic Year 2009

Thesis Title	Hollow-fiber liquid-phase microextraction for determination of macrolide antibiotic residues in water and poultry muscle
By	Miss Soparat Yudthavorasit
Field of Study	Chemistry
Advisor	Assistant Professor Natchanun Leepipatpiboon, Dr.,rer.nat

---

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

S. Hannongbua ..... Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr.,rer.nat)

THESIS COMMITTEE

Sirirat kokpol ..... Chairman

(Associate Professor Sirirat Kokpol, Ph.D.)

Natchanun Leepipatpiboon ..... Thesis Advisor

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

Pakorn Varanusupakul ..... Examiner

(Assistant Professor Pakorn Varanusupakul, Ph.D.)

..... External Examiner

(Prakorn Ramakul, Ph.D.)

โสภารัตน์ ยุทธวรศิษย์ : การสกัดระดับจุลภาคในเฟสของเหลวด้วยเส้นใยกลวงเพื่อการตรวจวัดสารปฏิชีวนะกลุ่มแมกโครไลด์ตกค้างในน้ำและเนื้อสัตว์ปีก. (HOLLOW-FIBER LIQUID-PHASE MICROEXTRACTION FOR DETERMINATION OF MACROLIDE ANTIBIOTIC RESIDUES IN WATER AND POULTRY MUSCLE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.ณัฐชนัญ ลีพิพัฒน์ไพบูลย์, 116หน้า.

การสกัดระดับจุลภาคในเฟสของเหลวด้วยเส้นใยกลวงเป็นเทคนิคในการเพิ่มความเข้มข้นของสารปฏิชีวนะกลุ่มแมกโครไลด์ตกค้างในน้ำและเนื้อสัตว์ปีก โดยนำเส้นใยกลวงซึ่งมีรูพรุนขนาดเล็กและมีราคาถูกมาใช้ในการเพิ่มความเข้มข้นด้วยรูปแบบอย่างง่าย งานวิจัยนี้ศึกษาถึงตัวแปรที่มีผลต่อการเพิ่มความเข้มข้น อาทิเช่น เวลาในการแช่เส้นใยกลวงในตัวทำละลายอินทรีย์ชนิดของตัวทำละลายอินทรีย์ สารละลายตัวให้ สารละลายตัวรับ และเวลาในการสกัด พบว่าเมื่อใช้สารละลาย 20% Aliquat 336ในไดเฮกซิลอีเทอร์บรรจุในรูพรุนของเส้นใยกลวงจุ่มอยู่ในสารละลายภายนอกหรือสารละลายตัวให้ที่มีพีเอช 8.0 จะเกิดการสกัดและเพิ่มความเข้มข้นของสารปฏิชีวนะกลุ่มแมกโครไลด์ทั้งสี่ชนิดเข้าสู่สารละลายภายในเส้นใยกลวงหรือสารละลายตัวรับที่มีพีเอช 4.0 ใช้เวลาในการสกัด 60 นาที ทำการตรวจวัดชนิดและปริมาณด้วยเทคนิคลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมทรี จากการศึกษาในสภาวะที่เหมาะสม เทคนิคนี้สามารถเพิ่มความเข้มข้นได้ในช่วง 12.38 ถึง 36.14 เท่า ที่ความเข้มข้นของสาร 50.0 ไมโครกรัมต่อลิตร โดยมีขีดจำกัดต่ำสุดของการตรวจวัดเท่ากับ 0.07 ถึง 2.28 ไมโครกรัมต่อลิตร ค่าสัมประสิทธิ์สหสัมพันธ์ในช่วงความเข้มข้น 0.50 ถึง 5.00 ไมโครกรัมต่อลิตร เท่ากับ 0.97 ถึง 0.99 ค่าร้อยละการคืนกลับสูงในช่วง 89.90 ถึง 102.99 และค่าเบี่ยงเบนมาตรฐานสัมพัทธ์อยู่ในเกณฑ์ดี (น้อยกว่า1.35) เมื่อเทียบกับค่าที่คำนวณได้จาก Horwitz equation และเมื่อนำสภาวะดังกล่าววิเคราะห์สารในน้ำตัวอย่างและเนื้อสัตว์ปีก พบว่า สามารถเพิ่มความเข้มข้นของสารปฏิชีวนะกลุ่มแมกโครไลด์ทั้งสี่ชนิดได้ 14.15 ถึง 35.81 เท่า และ 3.94 ถึง 7.31 เท่า ตามลำดับ โดยให้ร้อยละของการคืนกลับสูงในช่วง 82.93 ถึง 97.20 และ 71.78 ถึง 90.23 แสดงถึงความเหมาะสมของการนำเทคนิคนี้ในไปใช้วิเคราะห์สารในตัวอย่างจริงได้อย่างมีประสิทธิภาพ

ภาควิชา	เคมี	ลายมือชื่อนิสิต	โสภารัตน์ ยุทธวรศิษย์
สาขาวิชา	เคมี	ลายมือชื่ออ.ที่ปรึกษา	ณัฐชนัญ ลีพิพัฒน์ไพบูลย์
ปีการศึกษา	2552		

507 25443 23 : MAJOR CHEMISTRY

KEYWORDS : LIQUID-PHASE MICROEXTRACTION / HOLLOW-FIBER / MACROLIDE ANTIBIOTIC

SOPARAT YUDTHAVORASIT : HOLLOW-FIBER LIQUID-PHASE MICROEXTRACTION FOR DETERMINATION OF MACROLIDE ANTIBIOTIC RESIDUES IN WATER AND POULTRY MUSCLE. THESIS ADVISOR : ASST. PROF.NATCHANUN LEEPIPATPIBOON, Dr.,rer.nat, 116 pp.

Hollow-fiber liquid-phase microextraction (HF-LPME) has been developed for preconcentration of macrolide antibiotic residues in water and poultry muscle. A low-cost microporous hollow fiber was employed with simple configuration for enrichment process. HF-LPME parameters affecting enrichment factor such as immersion time, organic solvent composition, donor solution, acceptor solution and extraction time were investigated. A few microliters of 20% Aliquat 336 in dihexylether immersed in the hollow fiber pores have induced the extraction and preconcentration of four macrolide antibiotics from outside (donor solution pH 8.0) to inside (acceptor solution pH 4.0) fiber membrane within 60 minutes of extraction time, qualitative and quantitative detection with liquid chromatography-tandem mass spectrometry. In selected HF-LPME condition, macrolide antibiotics can preconcentrated with enrichment factor between 12.38 to 36.14 at concentration 50.00 µg/L. Limit of detection ranged from 0.07 to 2.28 µg/L with correlation coefficient between 0.97 to 0.99 in concentration range of 0.50-5.00 µg/L, the high value of % recovery ranged from 89.90 to 102.99 and the relative standard deviations were in acceptable range (<1.35) when compared with the value from Horwitz equation. This method was effectively applied in real sample with enrichment factor of four macrolides ranged from 14.15 to 35.81 for water sample and 3.94 to 7.31 for poultry sample. The % recoveries of macrolides from water and poultry sample were high in range of 82.93 to 97.20 and 71.78 to 90.23, respectively.

Department : Chemistry Student's Signature Soparat Yudthavorasit

Field of Study : Chemistry Advisor's Signature Natchanun Leepipatpiboon

Academic Year : 2009

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude to my thesis advisor, Assistant Professor Natchanun Leepipatpiboon, for her professionalism, generosity, plentiful guidance, continuous assistance, sincere encouragements, kind patience for my mistake, and critical proofreading throughout this research. I would like to extend my thanks to Assistant Professor Siripastr Jayanta, for her recommendations and critical reading. My appreciation also extends to all thesis committees for their insightful comments and suggestions.

This research was financially supported by the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and Center for Petroleum, Petrochemicals, and Advanced Materials. I would like to thank Miss Chayada Chiaochan for LC-MS/MS support and information in this work. Further thank should also go to Mr.Thorsten Hueffer, for his language assistance.

In addition, warm thanks to all members of the Chromatography and Separation Research Unit (ChSRU) for their helpfulness and suggestions. I would like to thank my friends at Chulalongkorn University for valuable time and supports. Particularly, many thanks go to all my friends from Mahidol University, for their cheerful perspectives and friendship.

Finally, I must thank my beloved parent, my three sisters and brother for their unlimited support, love and understanding.

CONTENTS

PAGE

PAGE

PAGE

LIST OF TABLES

TABLES **PAGE**

LIST OF FIGURES

FIGURES **PAGE**

LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
°C	degree Celsius
ACN	acetonitrile
Aliquat 336	tricaprylmethylammonium chloride
AP-ESI	atmospheric pressure electrospray ionization
APCI	atmospheric pressure chemical ionization
ASE	accelerated liquid extraction
cm	centimeter
CE	capillary electrophoresis
D2EHPA	di(2-ethylhexyl)phosphoric acid
DAD	diode array detector
DC	direct current
DHE	di-n-hexyl ether
EE	extraction efficiency
EF	enrichment factor
ERY	erythromycin
EU	The Europian Union
eV	electron volt
g	gram
GC	gas chromatography
HF-LPME	hollow-fiber liquid-phase microextraction
HPLC	High Performance Liquid Chromatography
I.D.	internal diameter
K	partition coefficient

K_{ow}	octanol-water partition coefficient
kV	kilovolt
L/h	liter per hour
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LIX 84	2-hydroxy-5-nonylacetophenone oxime
LLE	liquid-liquid extraction
LODs	limit of detections
LOQs	limit of quantifications
LPME	liquid-phase microextraction
M	molar
m/z	mass per charge ratio
mg/L	milligram per liter
min	minute
mL	milliliter
mL/min	milliliter per minute
mm	millimeter
MMLLE	microporous membrane liquid-liquid extraction
MRLs	maximum residue limits
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
Pa	Pascal
pKa	power of acid dissociation constant
PLE	pressurized liquid extraction
psi	pound per square inch
PTFE	polytetrafluoro ethylene
QqQ	triple quadrupole mass analyzer

R.S.D.	relative standard deviation
R^2	correlation coefficient
RF	radio frequency
S.D.	standard deviation
S/N	signal to noise ratio
SDME	single-drop microextraction
SIM	selected ion monitoring
SLM	supported liquid membrane
SPE	solid-phase extraction
SPI	spiramycin
TCA	trichloroacetic acid
TIL	tilmicosin
TOF	time-of-flight mass analyzer
TYL	tylosin
UPLC	ultra performance liquid chromatography
UV	ultraviolet
v/v	volume by volume
µg/kg	microgram per kilogram
µg/L	microgram per liter
µL	microliter
µm	micrometer

CHAPTER I

INTRODUCTION

1.1 Problem Definition

In modern animal agriculture, antibiotics are widely used for therapeutic and non-therapeutic purposes with worldwide large-scale consumption. The residual problem of antibiotics as veterinary drugs for food-producing animals is of particularly concern and it is increasing consumer awareness of food safety. Due to the application of antibiotics as feed additives for treatment, prophylactic, and even growth promoter, these drugs can leave residues in edible tissue or transfer to aquatic environment, which can lead to health problems. Even at low concentration, continuous consumption of drug-residue containing meat or water can cause allergic reactions, which are related to the human immune system. Besides all, antibiotic residues may induce resistance of bacteria from promoting bacterial biological mutation and DNA exchange. These new resistant strains of bacteria can transfer from animal to human and, therefore, pose a threat to human health via three ways (i.e., food, working with animal, and environment).

For non-therapeutic purpose, antibiotics are extensively used as feed additives in cattle, swine, sheep, and poultry in low dosage levels to promote growth and prevent infection. As growth-promoter, antibiotics in feed help animals gain weight more efficiently by controlling bacteria that can interfere with animal ability to absorb nutrients. Animals become healthier, grow faster and stronger, and fewer die from disease. In contrast, healthy animals raised on factory farms are regularly fed low dosage levels of antibiotics for extended periods of time, in order to promote faster growth and compensate for overcrowded and unsanitary conditions that may bring on sickness, especially in industrial-scale factory farms. The overuse amount of antibiotics can leave a residue in animal and contaminate in aquatic environment.

In the U.S., it has been reported that meat producer used nearly 25 million pounds or estimate 70% of all antibiotics non-therapeutically in food-producing animal, which are mainly swine, cattle, and poultry. *(1)* To regulate drugs residues, The European Union

(EU) has taken actions in legislation of antibiotic use in feeds and banned antibiotics as growth promoters. Legislation regarding the control of antibiotic residues in live animals and animal products is given in Council Directive 96/23/EC including the prohibition of the use of growth promoting agents. *(2)* Moreover, EU has set the maximum residue limits (MRLs) given in Council Regulation 2377/90 for the use of veterinary drugs in food animal species *(3)* and the method and performance criteria are described in Commission Decision 2002/657/EC. *(4)*

As antibiotic residues in animal foodstuff can also accumulate in every part of the food-chain and endanger human, antibiotics are presently considered as serious emerging contaminants. Antibiotics imply a wide range of substances including natural, semi-synthetic, and synthetic compounds. Classes of antibiotics can be divided by chemical structure or mechanism of action such as macrolides, sulfonamides, tetracyclines, quinolones, β-lactams, aminoglycosides, and others. Macrolide is a one of the most important antibacterial class that has a critical residue problem because of its efficiency against diseases produced by gram-positive bacteria and *Mycoplasma* species in multiple animal species. Poultry is one target of food-producing group that is well known to experience macrolide antibiotic residue in many parts. In 2003, Interscience Conference on Antimicrobial Agents and Chemotherapy scientists reported about the risk of humans acquiring resistant bacteria by eating meat or poultry from animals treated with macrolides that leads to failure in using antibiotic treatment for bee sting. *(5)* Hence, the EU regulates residual macrolides in bovine, porcine, and poultry by setting MRLs as shown in Table 1.1.

Table 1.1 Maximum Residue Limits (MRLs) of macrolide antibiotics in food-producing animal *(3)*.

Macrolide	**MRLs (µg/kg)**
erythromycin	40 – 200
spiramycin	200 – 400
tilmicosin	50 – 1000
tylosin	50 – 200
josamycin	200 – 400
tulathromycin	100 – 3000
tylvalosin	50

However, low amounts of antibiotic residue combined with the complexity of sample matrix lead to difficulties in analysis resulting in a strong need to provide suitable techniques for their determination. Sample preparation step is a powerful tool in solving these analysis problems. Extraction, enrichment, and clean-up are necessary sample preparation processes to improve antibiotic detection in order to follow EU legislation criteria.

The conventional sample preparation techniques, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) still have some drawbacks. LLE is considered as time-consuming, multi-stage operation, and requires large volume of toxic organic solvent. Even though SPE eliminates LLE disadvantage in case of shorter sample preparation time, less organic solvent usages, and easier operation, SPE requires extra step for evaporation, additional device cost, and provides low preconcentration of analytes. It is difficult to determine macrolides with conventional methods because of their similar structures and low-level residues. Therefore, a simple, low-cost, high enrichment, sensitive, and selective method should be developed for macrolide antibiotics residue determination in food-producing animal and water samples.

1.2 Macrolide Antibiotics

Since the discovery in the 1950s, macrolide antibiotics are used for a variety of applications in both human and animal foodstuffs (poultry, cattle, sheep, swine, fish, and companion animals). Macrolides are delivered to the different animals by various routes of administration such as feed, water, injection, tablet, and others. This antibiotic class is used to treat infections of the respiratory tract and genital and gastrointestinal tissue infections because these compounds are biologically active against living microorganisms. Macrolide common mechanism of action is the inhibition of bacterial protein synthesis with the activity against gram-positive bacteria and *Mycoplasma* species. Consequently, macrolides are important in maintaining a healthy livestock and poultry.

1.2.1 Structure and chemistry

Macrolide are characterized by a macrocyclic lactone ring containing 14, 15, or 16 atoms with sugars linked via glycosidic bonds. Macrolide antibiotics are further classified into three groups based on the number of atoms in the lactone ring as described in Table 1.2. Macrolide compounds are produced semi-synthetically or naturally by microorganism. Macrolide are mainly produced by various *Streptomyces* organisms except rosaramicin and mirosamicin, which are isolated from *Micromonospora* species.

Table 1.2 Macrolide antibiotic compounds classification *(6)*.

No. of atom in lactone ring		
14-membered macrolides	**15-membered macrolides**	**16-membered macrolides**
Erythromycin	Azithromycin	Leucomycin
Oleandomycin	Tulathromycin	Josamycin
Clarithromycin		Kitasamycin
Dirithromycin		Rokitamycin
Roxithromycin		Rosaramicin
Flurithromycin		Mirosamicin
		Spiramycin
		Tilmicosin
		Tylosin
		Tylvalosin

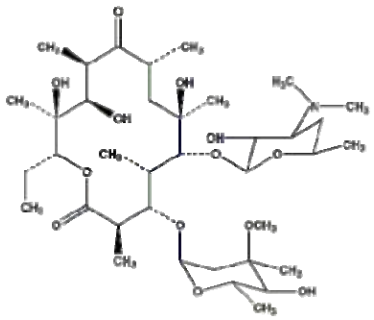
In the group of 14-membered macrolides, the most important compound is erythromycin, a fermentation product produced from *Saccharopolyspora erythraea*. It has been extensively used in many different chemical forms (e.g., free base, salts, and ester) and formulations. It has also been frequently utilized as the chemical starting material for many 14-membered semi-synthetic derivatives, such as clarithromycin, roxithromycin, dirithromycin, and flurithromycin. Another major semi-synthetic derivative is azithromycin, a 15-membered Macrolide, which consists of a heterocyclic nitrogen, is produced from ring expansion process. Although these semi-synthetic derivatives share many common attributes with erythromycin, their individual structural features may also perform some significant difference in their various antimicrobial

activities and biological features. The second largest family is 16-membered macrolide, which is usually divided into two principal sub-families based on differences in the substitution pattern of their structures. Tylosin is the prototype of one sub-family that includes its semi-synthetic compound, tilmicosin. Leucomycin is the other sub-family, which has a unique feature of a second amino sugar in its skeleton. 16-membered macrolides also exhibit their common characteristics with their individual bioactivities. Macrolide compound structures are shown is Figure 1.1. From their structures, macrolides are lipophilic molecules, they are soluble in methanol and are unstable in acid solution. Macrolides are weak bases with pKa values ranging from 7.4 to 9.2.

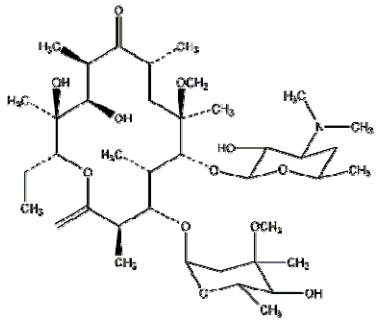
1.2.2 Mechanism of action

All macrolide antibiotics display antibacterial properties and are active against gram-positive and some gram-negative bacteria, and are particularly useful in the treatment of *Mycoplasmas*, *Haemophilus influenzae*, *Chlamydia* species, and *Rickettsia*. Macrolide antibiotics exhibited their antibacterial activity ribosomes. The macrolide mechanism of action inhibits the bacterial protein synthesis via reversibly binding to the 50s ribosomal subunit of bacterial ribosome. A general diagram of macrolide inhibition of bacterial protein synthesis within the ribosome is illustrated in Figure 1.2. There are four modes of macrolide inhibition of protein synthesis: 1) Inhibition of the progression of the initial peptide chain during early steps of translation; 2) Promotion of peptidyl tRNA dissociation from the ribosome; 3) Inhibition of peptide bond formation; and 4) Interference with 50S subunit assembly. All of these mechanisms have some relationship with the location of the macrolide binding site on the ribosome. With macrolide binding, tRNA cannot bind with mRNA and then amino acid of tRNA cannot form peptide bond with another tRNA that inhibits protein production at ribosome of bacteria.

14-membered macrolides

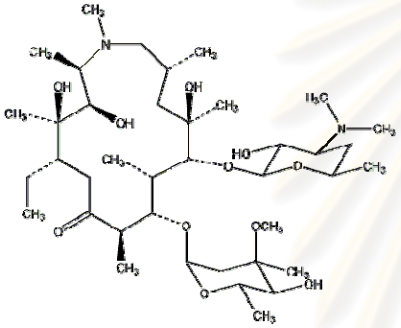


Erythromycin

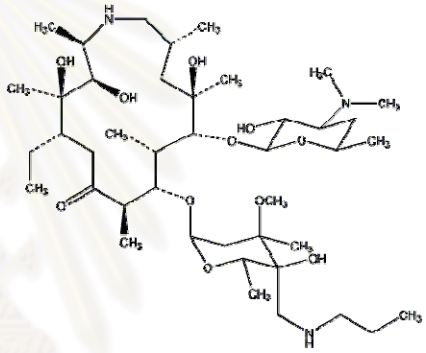


Clarithromycin

15-membered macrolides

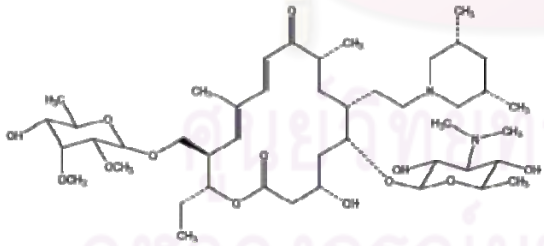


Azithromycin

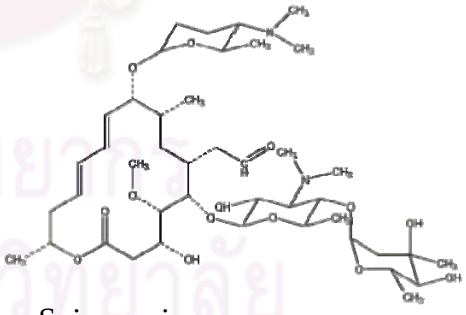


Tulathromycin

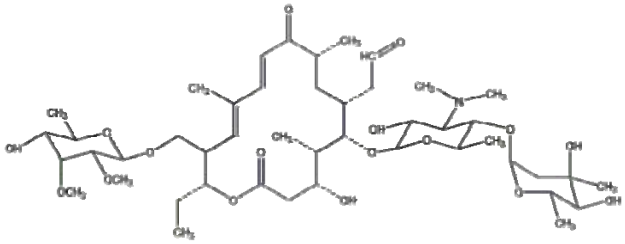
16-membered macrolides



Tilmicosin



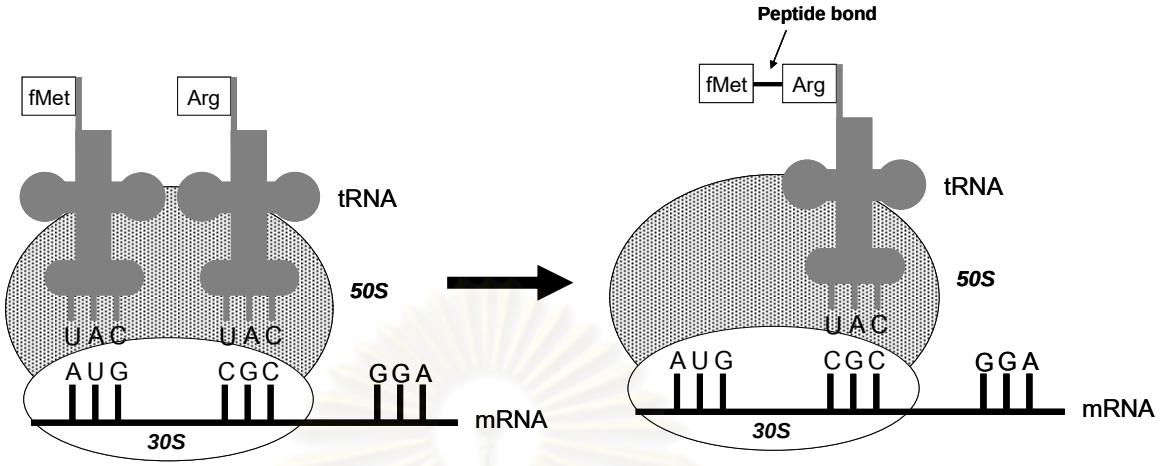
Spiramycin



Tylosin

Figure 1.1 Some macrolide antibiotic chemical structures

(a)



(b)

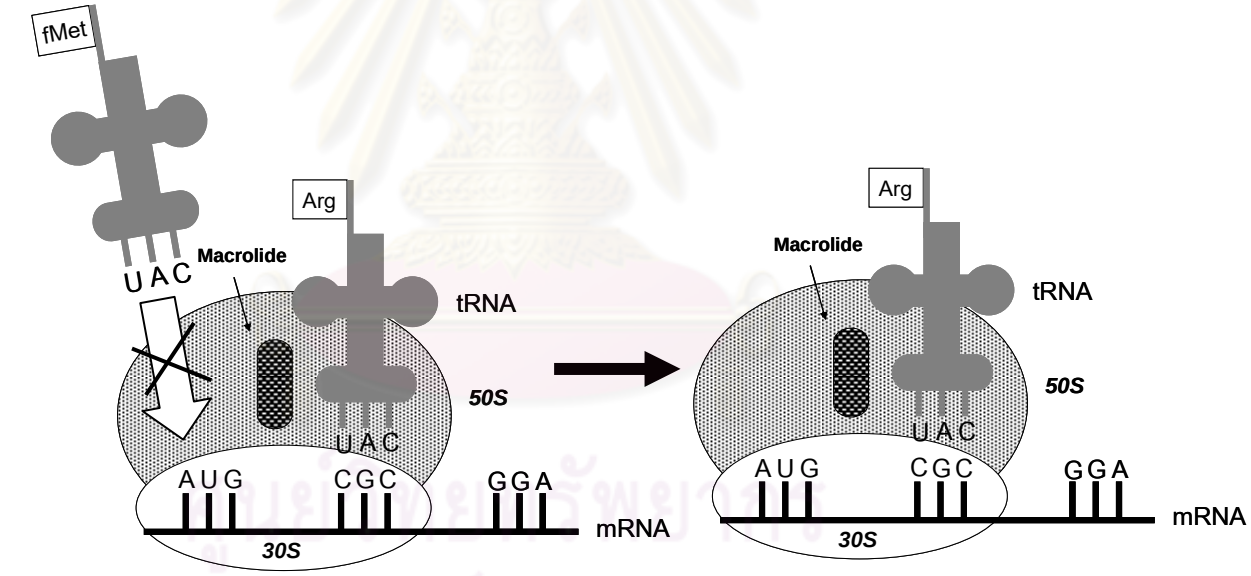


Figure 1.2 Diagram of macrolide inhibition of protein synthesis within the bacterial ribosome (adapted from *(7)*) a) without macrolide b) with macrolide.

1.2.3 Mechanism of resistance

Resistance of macrolides can occur by target site modification, drug inactivation, or drug efflux out of the bacteria cell. Organisms that develop resistance to one macrolide antibiotic may also be resistant to other macrolide antibiotics. Therefore, certain peptides can bind with 50s subunit and continue their protein synthesis processes, which leads to a reduction of antibiotic activities. These macrolide resistance genetics are capable of being transmitted from gram-positive to gram-negative bacteria and vice versa. *(8)* Many of the macrolide-resistance genes have become physically linked to other drug resistance symptoms and result in other drug resistance abilities to other antibiotic classes. Furthermore, the danger of drug resistance also influences human health because of the transportation of these antibiotic-resistance genes from bacteria to human through food, environment, and working with animals that contain resistant bacteria. When human were treated with the antibiotics, drug resistance gene that accumulated in body are affected to the effectiveness of drug in treatment diseases.

1.2.4 Growth promoters

Non-therapeutic applications of antibiotics are growth promotion and disease prevention, whereas most of the concern about human health consequences of antimicrobial use has focused on growth promotion rather than disease prevention purpose because of the economic profits. Macrolide is one antibiotic class commonly added in low doses to the feed of farm animals to improve their growth performance for significant economic benefits such as weight gain and improved feed efficiency. This increasing growth rate depends on the hygiene level on the farm, the age of the animal, and the influence of feed additives. Healthy food-producing animals raised on factory farms such as swine, cattle, and poultry are regularly fed low dosage levels of macrolide antibiotics for extended periods of time, in order to promote faster growth and compensate for overcrowded and unsanitary conditions that may bring on sickness, especially in industrial-scale factory farms. Unfortunately, the use of low dosages of antibiotics over an extended period is one of the best ways to promote the development of antibiotic-resistant bacteria and induce human health at risk. As a result, EU has prohibited the use of antibiotics as growth promoting agents but there still is misuse of antibiotic applications for those purposes**.**

1.3 Literature review

In 1990, EU has set legislation and the Maximum Residue Limits (MRLs) of the use of veterinary drugs in food animal species. *(3)* There are several works that attempt to determine the residue of antibiotics with highest effective analysis methods.

Traditionally, screening methods for antibiotic, including macrolides, are based on microbiological and immunological assays (ELISA) but they often lack selectivity and precision for regulatory purposes. Therefore, in ELISA it is difficult to confirming what kinds of residual antibiotics are found in the animal tissue. To overcome these problems, chromatographic methods have been utilized for many macrolide determinations. Liquid chromatography is common coupled with spectrophotometric detections, such as UV and diode array detector (DAD), to determine macrolide antibiotics in food-producing animal.

High-Performance Liquid Chromatography (HPLC) with UV detection was used to determine five macrolides in swine, cattle, and chicken meat by Horie et al. *(9)* The samples were submitted to LLE using a mixture of 0.3% metaphosphoric acid and methanol followed by a SPE clean-up on Bond Elut SCX cartridges. The separation of the macrolides was performed on a C18 column using a gradient elution with a mixture of phosphate buffer and acetonitrile. The macrolide determination was monitored at two different wavelengths, 232 and 287 nm. The recoveries ranged from 70.8 and 90.4% and the detection limits (LODs) were estimated to 50 µg/kg for each macrolides.

Few years later, Leal et al. were using the same extraction method, employed both LC-UV and LC-DAD detection for the determination of macrolide antibiotics in spiked chicken muscle. *(10)* In LC-UV, the authors achieved the separation of seven macrolides on a C18 reversed phase column using a binary gradient elution of phosphate buffer (mobile phase A), and a phosphate buffer and acetonitrile mixture (mobile phase B). The method was also based on UV detection at different wavelengths and could determine five compounds from the seven tested macrolides in chicken poultry with spiked below their MRLs. The authors tested two different UV detection systems based on absorption, wavelength-programming, and multi-wavelength detection, it was found that the latter system is more suitable. For macrolides determination with LC-DAD, the proposed method was not sensitive enough for

determining some macrolides at the MRL values because of the lack of suitable chromophore groups in macrolide chemical structures. At three spiking levels, recoveries between 60 and 80% were gained. It was found that DAD has an additional advantage over UV system in case of the confirmation of identical analytes by spectra of the eluting peaks.

For confirmatory purpose, mass spectrometry (MS) is the preferred detection system for analyte identification rather than DAD. Due to its high specificity and sensitivity, LC-MS has been widely applied in antibiotic determination, especially in animal tissues. The LC-MS detector can reach low detection limit to determine of all macrolides in their MRLs. Codony et al. determined seven macrolides in poultry muscle with LC-MS using electrospray ionization (ESI). *(11)* The samples were treated like in the previous study, extraction with meta-phosphoric acid followed by clean-up with SPE cartridge. The separation was performed on a C18 column applying a gradient elution with a mobile phase consisting of a mixture of 0.02% aqueous trifluoroacetic acid and acetonitrile. LC-ESI-MS was operated in positive mode and each compound was monitored with selected ion monitoring (SIM) mode for quantification purposes. Recovery ranged from 56 and 93% with RSD lower than 12%. The proposed method was successfully applied for determination macrolides below the MRLs. However, there still are drawbacks of this method due to the concern about the number of analysis ions required for confirmatory purposes according to EU legislation.

Tandem mass spectrometry (MS/MS) overcomes this problem by providing abundant ions for quantitative and qualitative information. LC-MS/MS allows separation and detection compounds that have the same molecular mass but different product ions. For this reason, macrolide antibiotic class, which consists of many compounds with similar structures, can utilize LC-MS/MS for determination. Wang et al. have developed a method for determination of five macrolide antibiotics in honey with the comparison between LC–ESI–MS and LC–ESI–MS/MS systems. *(12)* The samples were extracted with phosphate buffer adjusted to pH 8.0, and then submitted to SPE on Oasis HLB cartridges and filtered before injection into the system. The separation of the macrolides was carried out on a C18 column using a gradient elution with a mixture of acetonitrile, 1% formic acid and water as mobile phase. For LC–ESI–MS, the obtained recoveries were between 97.8 and 109.3% with R.S.D. below 12% and detection limits below 1

µg/kg. For LC–ESI–MS/MS, recoveries ranged from 98.3 to 114.6% with R.S.D. below 13% and the detection limits were between 0.01 and 0.07 µg/kg. This work proved that the sensitivity of MS/MS is higher than single MS system and allows detection of macrolide antibiotics in ng/kg level. LC coupled with MS is proved that it is a necessary tool in many applications for determination the low-level residues.

In water sample analysis, macrolides antibiotics are considered as serious emerging contaminant in every parts of aquatic resource and have several researches in the determination of macrolide antibiotics in diverse water samples with LC-MS and LC-MS/MS detection. Mcardell et al. define seven macrolide antibiotics in wastewater and river samples via filtered sample and clean-up with solid-phase extraction (SPE) followed by both LC-MS and LC-MS/MS detection. *(13)* They claimed that macrolides are mainly contaminated in surface water. This method showed the low detection limit ranged from 0.06 to 0.33 µg/L with the acceptable ranges of relative standard deviation. With single LC-MS detection system, Abuin et al. can determine five macrolide antibiotics in natural water sample with detection limit in very low µg/L and satisfactory recovery ranged from 85 to 115 %. *(14)* The water sample was filtered and clean-up with the same process like previous work. This way to prepared sample is traditional mode in the application with water sample. Therefore, the difficulty between water sample and food-producing animal analysis is the sample preparation process. The animal matrices are complicated sample because of their components and required more preparation step than water sample which required only filtration or some clean-up steps.

Sample preparation is a very important and essential step to improved method analytical performance. Many researchers tried to extract and clean-up macrolide antibiotics from complex sample matrices such as animal sample. As previously described, several works initiated the same extraction and deproteinisation procedure with a mixture of meta-phosphoric acid and methanol followed by a partial evaporation of the extract and a final clean-up step on Oasis SPE HLB cartridges. Horie et al. developed a multiresidue method for eight macrolides in meat and fish with single run LC-ESI-MS. *(15)* The authors modified the sample preparation step with optimization the percentage of metaphosphoric acid in order to reduce the degradation of the macrolides in acidic media while keeping the efficiency of the extraction process. 0.2%

metaphosphoric acid in methanol was found to be the appropriate proportion. The detection limit stated in the method was 10 µg/kg for all the target macrolides. With the same SPE process, Berrada et al. applied different extraction procedure from previous work for seven macrolides determination in animal tissues using LC-DAD and LC-ESI-MS. *(16)* EDTA-McIlvaine's buffer was utilized to extract macrolide before SPE steps. Recovery data were satisfactory with values higher than 67% and R.S.Ds were lower than 13% and 15% for intra-day and inter-day assays. The author claimed that this confirmatory method could efficiently determine macrolides in animal sample according to EU regulation 2002/657/EC.

Another type of liquid extraction for determining seven macrolide antibiotics in meat and fish is pressurized liquid extraction (PLE); combines with LC-ESI-MS it was reported by Berrada et al. *(17)* PLE is an accelerated liquid extraction (ASE) procedure, whereby increased temperature for accelerating the extraction kinetics, and extended pressure to keep the solvent below its boiling point. ASE is reported to use the same aqueous and organic solvents as traditional extraction methods. The extracts were completely transferred for further solid-phase extraction, typically using Oasis HLB cartridge. The advantage of using PLE is the online capability, high specificity, and selectivity in extraction.

For the extraction of three macrolides in milk and bovine tissues, Msagati et al. investigated supported liquid membrane (SLM) as sample pre-treatment and clean-up technique. *(18)* In SLM, an organic liquid is immersed in small pores of a polymer support and held by capillary forces. If the organic solvent is immiscible with water, this polymer membrane separated two aqueous phases, feeding and stripping streams. Macrolides were extracted from sample with ACN-isopropyl alcohol (95:5) and then preconcentrated and clean-up with online-SLM. After extraction, macrolides were dried and dissolved in feeding solution, and continually extracted with organic solvent into pores of membrane and passed through to stripping solution with pH adjustment. With LC-ESI-MS system, the macrolides were detected following extraction at concentration levels between 0.01 and 0.08 µg/kg. In SLM, the membrane is reusable and the organic solvent employed is at minimal amount. Even though, SLM is an environmental friendly technique, there may be carry-over effects, online-SLM requires a flow system and it can extract only one sample at a time.

1.4 Purpose of the study

Since macrolide antibiotics were regulated by the EU 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, macrolide antibiotics in water and food-producing animal were analyzed with various sample preparation and detection techniques. LC-MS and LC-MS/MS have become a common detection technique because of their improved selectivity and high sensitivity. However, despite the high sensitivity of LC coupled with MS system, sample preparation is normally a necessary tool to reach the low limits of detection, which are required in the analysis of antibiotics in food from animal origin and water sample. Most extraction methods of macrolide antibiotics from animal tissue consist of extract and protein precipitation with meta-phosphoric acid in methanol followed by clean-up procedure. However, common extraction processes require an additional step of filtration and evaporation. Traditionally, SPE is the only preconcentration and clean-up method used in the macrolide determination. SPE required sorbent and elution solvent optimization in order to obtain strong interaction with analytes and completely elute all analyte from sorbent. Even if the consumption of organic solvent is relatively low in SPE, high preconcentration of analyte is difficult. In addition, SPE requires an extra step for evaporation and SPE cartridges are expensive.

To detect very low amounts of drug residues in complex matrices, a preconcentration method should be provided and SLM is proved to be an alternative on-line liquid extraction technique to obtain high preconcentration with very low organic solvent consumption. As mentioned, SLM overcomes some SPE drawbacks and polymeric membrane is less expensive than SPE cartridge. However, SLM still has disadvantages by which it remains a carry-over effect, it allows only one sample per extraction, and it includes additional devices for online system.

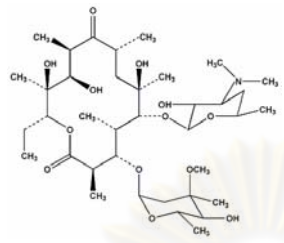
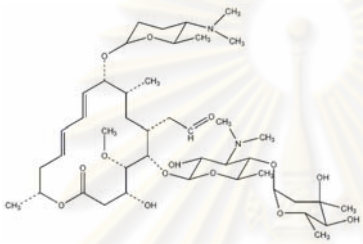
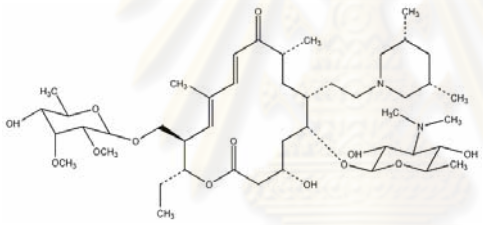
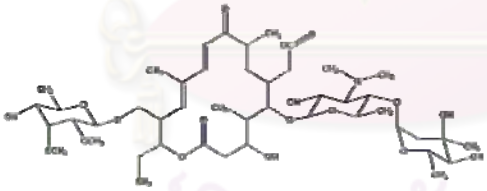
Liquid-phase microextraction (LPME) is termed from the off-line version of SLM and it shares some characteristics with on-line SLM such as the extraction principle, high preconcentration, and clean-up abilities. To overcome on-line SLM drawbacks, LPME plays an important role with regard to carry-over effect, high sample throughput, and almost free of organic solvent use. In addition, the configuration of LPME is generally

simple, inexpensive, and the method is high sensitivity and versatile for various types of samples.

In this work, LPME based on hollow fiber employment, well known as hollow-fiber liquid-phase microextraction (HF-LPME) in three-phase mode was chosen for the determination of four macrolide antibiotis residues in poultry muscle and water. The four macrolides; erythromycin, spiramycin, tilmicosin, and tylosin; are commonly used as veterinary medicine in food-producing animal and easily transfer to aquatic environment. Their residues usually exist in low amounts and induce the difficulty to extract from complex matrices. The structure and property of these four macrolides are shown in Table 1.3.

In HF-LPME, analytes were extracted from aqueous donor or sample solution with organic solvent immersed in the hollow fiber pores and back-extract to aqueous acceptor solution in the hollow fiber lumen. After extraction, the acceptor solution was directly injected to LC-ESI-MS/MS. Because of the difference between volume of donor and acceptor solution, analytes were preconcentrated with good performance. HF-LPME can simultaneous enrich and clean-up analytes from sample matrix. The related parameters were optimized such as the donor pH, the acceptor pH, type of donor and acceptor, organic solvent type, organic solvent composition, immersion time of hollow fiber in organic solvent and extraction time. The optimized HF-LPME method was applied with various extraction methods for extract macrolide antibiotics in poultry muscle obtained from a local market and water sample collected from the river.

Table 1.3 The studied macrolide antibiotics properties *(19,20)*

Analyte	Chemical structure	Chemical formula	Molecular mass (g/mol)	pKa	Log K_{ow}
Erythromycin		$C_{37}H_{67}NO_{13}$	733.93	8.9	3.06
Spiramycin		$C_{43}H_{74}N_2O_{14}$	843.05	7.9	2.49
Tilmicosin		$C_{46}H_{80}N_2O_{13}$	869.13	7.4 8.5	2.60
Tylosin		$C_{46}H_{77}NO_{17}$	916.10	7.1	2.50

CHAPTER II

THEORY

Sample preparation procedure is one of the most important parts of the analysis to influence the analytical results. The objective of sample preparation process is to isolate target analytes from various matrices and convert the analytes into a more suitable form for separation and detection. Matrix effects are considered as a major problem in extracting analytes as they may lead to low recovery. These effects depend on sample properties and the concentration of analytes in the sample. Several studies have attempted to develop sample preparation procedures to remove interferences, increase the concentration of analytes, and provide a simple, inexpensive, robust, and reproducible method. The traditional sample preparation method, liquid-liquid extraction (LLE) is still in use because of its simplicity. LLE uses an organic solvent to extract analytes from aqueous sample in the principle of phase partition. With LLE, it is possible to achieve both analyte enrichment and sample clean-up, but the main disadvantage is the consumption of large quantities of organic solvent, which may result in environmental impacts and potential health hazards.

Solid-phase extraction (SPE) has gradually replaced classical LLE and become the common sample preparation technique. SPE utilized a solid sorbent or bonded organic phase material to preconcentrate and clean-up analytes from sample. Analytes are extracted and partitioned between a solid stationary phase and a liquid sample phase. These analytes must have greater affinity for the solid phase than the sample matrix. Choice of sorbents and elution solvents are the essential parameters affecting recovery and LOD of target compounds. SPE is used for extraction, preconcentration, and clean-up purposes. Compared with LLE, this technique offers high recovery, specificity, automation possibility, less organic solvent usage, and SPE sorbents are commercially available in form of disposable cartridges. However, SPE technique is time-consuming, expensive, labor-intensive, has limited selectivity, and low preconcentration ability.

An optional technique, which provides some distinctive advantages over LLE and SPE, especially in case of selectivity, enrichment power, and automation potential, is membrane extraction, where the attempt is to use LLE advantages by avoiding its disadvantages.

2.1 Membrane extraction *(21,22,23,24,25)*

Membrane extraction was introduced in 1999 by Jönsson et al.. *(21)* A membrane was applied as a selective barrier between two aqueous phases. The phase in which the transfer of analytes occurrs is called feeding or donor phase, and the phase into which the analytes are extracted is called stripping or acceptor phase. The membrane is a synthetic product of different chemical natures and displays different properties. Common membrane characterization is based on the porosity of the membrane, which can be porous or non-porous. In porous membranes, two liquid phases are in contacted through the membrane pores and only particles smaller than the pore size can pass through the membrane. These membranes are used for the separation of analytes from matrix with particle size selection, which leads to an efficient clean-up without enrichment of analytes. Thus, the separation by porous membranes is a function of analyte molecular size and pore size distribution of the membrane. Porous membranes are often applied in filtration, reverse osmosis, and dialysis process. On the other hand, non-porous membranes have been widely used for extraction. Analytes are transported from donor to acceptor phase by diffusion under the driving force of pressure, concentration, or electrical potential gradient. Non-porous membranes, which act as interface between two liquid solutions, can consist of a liquid or a solid phase. It can be a liquid-impregnating porous membrane or an absolute solid membrane. The extraction from non-porous membrane provides efficient clean-up with high enrichment factors. Non-porous membranes offer a powerful membrane extraction technique without significance use of organic solvents.

One remarkable non-porous membrane extraction is supported liquid membrane (SLM). This technique employs a polymeric pore membrane as support for an organic solvent and creates three-phase system. The organic solvent within the membrane is a barrier between the aqueous donor and the acceptor solution. If the acceptor solution is

also organic solvent, it is called two-phase system, which is also named microporous membrane liquid-liquid extraction (MMLLE). Both SLM and MMLLE principles are frequently applied rather for on-line membrane system than off-line. However, the off-line membrane configuration, which is a versatile non-porous membrane extraction technique, was developed by Rasmussen et al. and is termed liquid-phase microextraction (LPME). *(22)* Nowadays, LPME has been widely used for many applications not only its simplicity, low-cost, and elimination of carry-over effects, but also for its fast and almost solvent-free use.

2.2 Liquid-Phase Microextraction (LPME) *(26,27)*

LPME, a miniaturized LLE, was firstly based on the extraction of analytes from aqueous sample into a small droplet of organic solvent hanging at the end of microsyringe needle. The organic solvent droplet was placed into aqueous solution and analytes were extracted into the organic hanging droplet by passive diffusion. After extraction, the droplet was withdrawn into the syringe and transferred to inject into gas chromatography (GC). Afterwards, this technique has been separately termed from LPME as single-drop microextraction (SDME). Due to the instability of organic droplet in SDME, LPME was improved to a more robust configuration, which utilized disposable low-cost hollow fiber membranes to stabilize the extracting phase. This technique is called hollow-fiber liquid-phase microextraction (HF-LPME).

2.2.1 Hollow-Fiber Liquid-Phase Microextraction (HF-LPME) *(28,29,30,31)*

HF-LPME utilizes porous, hydrophobic, hollow fibers impregnated with an organic phase to perform both SLM and MMLLE systems. A hollow fiber membrane employed in HF-LPME is shown in Figure 2.1. The basic principle of HF-LPME is to fill the aqueous sample into vial and then a piece of hollow fiber impregnated with organic solvent in the pores is placed into the sample solution. The solvent must be immiscible with water to remain in the fiber pores. Analytes are extracted from the aqueous sample through the organic solvent in pores of the hollow fiber and further into an acceptor

solution. Figure 2.2 illustrates the basic diagram of extraction in HF-LPME. Similar to the principle of MMLLE and SLM, membrane extraction technique can be divided into two modes of extraction depending on the extracting or acceptor phase type. In HF-LPME, there are called two-phase and three-phase systems.

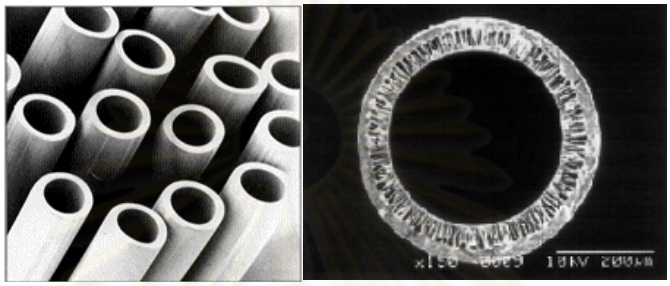


Figure 2.1 Hollow fiber membrane. *(32)*

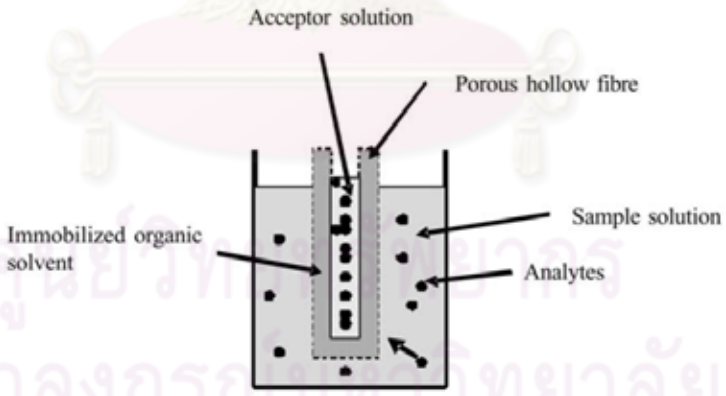


Figure 2.2 Diagram of basic HF-LPME principle. *(28)*

.

2.2.1.1 Two-phase HF-LPME

In two-phase LPME, the extraction principle is similar to MMLLE in membrane extraction. The analytes are extracted from aqueous sample (donor) phase into organic solvent presented in both the porous wall and lumen of the hollow fiber. A two-phase cross-section diagram of hollow fiber inside the aqueous sample is shown in Figure 2.3. In this case, the acceptor solution is the same organic solvent as impregnated in the fiber porous wall. Two-phase systems are applied for analytes with a high solubility in non-polar organic solvents.

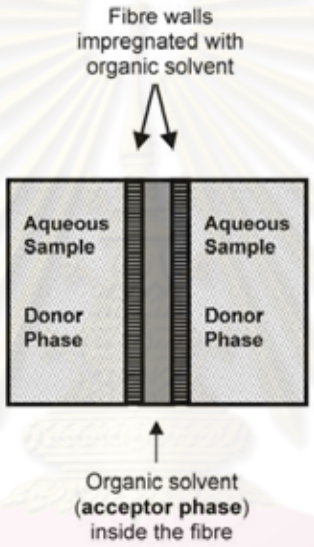


Figure 2.3 Two-phase cross-section diagram of HF-LPME in the aqueous sample. *(26)*

The extraction process of two-phase extraction is shown in Eq. 1.

$$A_{donor} \longleftrightarrow A_{organic\ acceptor} \qquad \text{(Eq. 1)}$$

where A refers to target analyte in sample (donor) and in organic acceptor. The partition coefficient (K) of analytes between acceptor and donor phase is defined in Eq. 2.

$$K_{acceptor/donor} = \frac{C_{eq,acceptor}}{C_{eq,donor}} \quad \text{(Eq. 2)}$$

where $C_{eq,acceptor}$ is the concentration of analytes in the acceptor solution (organic phase) at equilibrium and $C_{eq,donor}$ is the concentration of analytes in the sample (donor phase) at equilibrium.

From Eq. 1 and Eq. 2, the two-phase recovery of analytes at equilibrium (R), the extraction efficiency (EE), and enrichment factor (EF) are calculated by the following equations (Eq. 3-5).

$$R\ (\%) = \frac{K_{acceptor/donor} V_{acceptor}}{K_{acceptor/donor} V_{acceptor} + V_{donor}} \times 100 \quad \text{(Eq. 3)}$$

$$EE = \frac{R}{100} = \frac{K_{acceptor/donor} V_{acceptor}}{K_{acceptor/donor} V_{acceptor} + V_{donor}} = \frac{C_{eq,acceptor} V_{acceptor}}{C_{donor} V_{donor}} \quad \text{(Eq. 4)}$$

$$EF = EE \frac{V_{donor}}{V_{acceptor}} = \frac{C_{eq,acceptor}}{C_{donor}} \quad \text{(Eq. 5)}$$

where $V_{acceptor}$ is the volume of acceptor solution, V_{donor} is the volume of sample (donor) solution, and C_{donor} is the initial analyte concentration in the aqueous donor solution.

It can be predicted that the recovery is related to the partition coefficient, the volume of organic solvent in acceptor phase, and the volume of sample (donor). To obtain high

recovery, sample volume should be low and the partition coefficient ($K_{acceptor/donor}$) should be high. The value of partition coefficient depends on the selection of organic solvent and the selection of pH in aqueous solution for acidic or basic analytes in order to obtain non-ionic species. In two-phase systems, the extracted analytes must be more miscible with the organic solvent than aqueous medium to obtain high partition coefficient (K) values for analytes between organic acceptor and aqueous donor phase ($K_{acceptor/donor}$). High $K_{acceptor/donor}$ for analytes are obtained for moderately or highly hydrophobic compounds containing acidic or basic groups, and neutral compounds with hydrophobic properties.

The enrichment factor provided by two-phase extraction is noticeable high because the ratio ($V_{donor}/V_{acceptor}$) is frequently high. While the donor volume is in mL-level, the volume of acceptor is in μL-level. This is the main advantage of HF-LPME because high enrichment of analyte is achieved.

After extraction with two-phase system, the organic acceptor solution is compatible with GC and normal-phase HPLC detection. For reversed-phase HPLC analysis, the solvent should be evaporated and the analyte dissolved in aqueous medium prior to injection.

2.2.1.2 Three-phase HF-LPME

In three-phase LPME, the extraction principle is similar to SLM in membrane extraction technique. It differs from two-phase system in the type of acceptor solution used. The analytes are extracted from the aqueous sample (donor) phase, through organic solvent immobilized in hollow fiber pores, which acts as a barrier between the two phases. Analytes are further extracted into aqueous acceptor solution inside the lumen of hollow fiber. A three-phase cross-section diagram of hollow fiber inside the aqueous sample is shown in Figure 2.4. In this case, the acceptor solution is another aqueous solution.

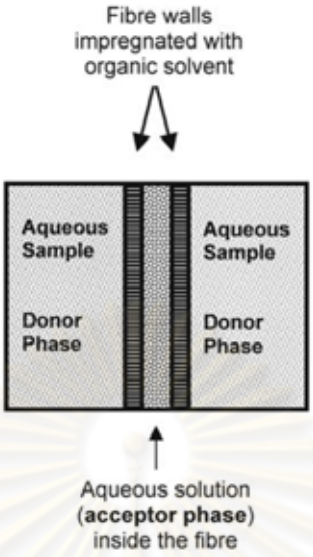


Figure 2.4 Three-phase cross-section diagram of HF-LPME in the aqueous sample *(26)*

Three-phase extraction is suitable for acidic or basic analytes with ionizable functionalities and its process is described in Eq. 6.

$$A_{donor} \longleftrightarrow A_{organic} \longleftrightarrow A_{aqueous\ acceptor} \qquad \text{(Eq. 6)}$$

where A refers to target analyte in the sample (donor), the organic, and in the aqueous acceptor phase. Among the partitioning of analytes in the three phases, the partition coefficient of analytes between the acceptor and donor phase ($K_{acceptor/donor}$) is considered as the overall driving force of three-phase extraction and defined in Eq. 7.

$$K_{acceptor/donor} = K_{organic/donor} \times K_{acceptor/organic} = \frac{C_{eq,acceptor}}{C_{eq,donor}} \qquad \text{(Eq. 7)}$$

where $K_{organic/donor} = \frac{C_{eq,organic}}{C_{eq,donor}}$

and $K_{acceptor/organic} = \frac{C_{eq,acceptor}}{C_{eq,organic}}$

where $C_{eq,donor}$, $C_{eq,organic}$, and $C_{eq,acceptor}$ refer to the concentration of analytes in the sample (donor phase), organic phase, and acceptor solution (organic phase) at equilibrium, respectively.

From Eq. 6 and Eq. 7, the three-phase recovery of analytes at equilibrium (R), the extraction efficiency (EE), and enrichment factor (EF) are calculated by the following equations (Eq. 8-10).

$$R\ (\%) = \frac{K_{acceptor/donor} V_{acceptor}}{K_{acceptor/donor} V_{acceptor} + K_{organic/donor} V_{organic} + V_{donor}} \times 100 \qquad \text{(Eq. 8)}$$

$$EE = \frac{R}{100} = \frac{K_{acceptor/donor} V_{acceptor}}{K_{acceptor/donor} V_{acceptor} + K_{organic/donor} V_{organic} + V_{donor}}$$

$$= \frac{C_{eq,acceptor} V_{acceptor}}{C_{donor} V_{donor}} \qquad \text{(Eq. 9)}$$

$$EF = EE \frac{V_{donor}}{V_{acceptor}} = \frac{C_{eq,acceptor}}{C_{donor}} \qquad \text{(Eq. 10)}$$

where $V_{donor,}$ $V_{organic}$, and $V_{acceptor}$ are the volume of sample (donor) solution, organic phase, and acceptor solution, respectively, and C_{donor} is the initial analyte concentration in aqueous donor solution.

The extraction mechanism of three-phase system is shown in Figure 2.5, where pH is the critical driving force to promote the extraction. For basic analyte compounds, the pH of donor solution should be adjusted to alkaline to promote only basic analyte in deionized form partitioning into organic phase, while other acidic compounds which is ionized in the donor solution cannot partition into organic phase. Meanwhile, pH of acceptor solution should be acidic to promote high extraction efficiency from organic phase into acceptor phase. On the other hand, for acidic analytes, the donor pH should adjust to be acidic to allow analyte in deionized form extraction into organic phase, and the acceptor pH is adjusted to alkaline in order to prevent analyte back-extraction into organic phase.

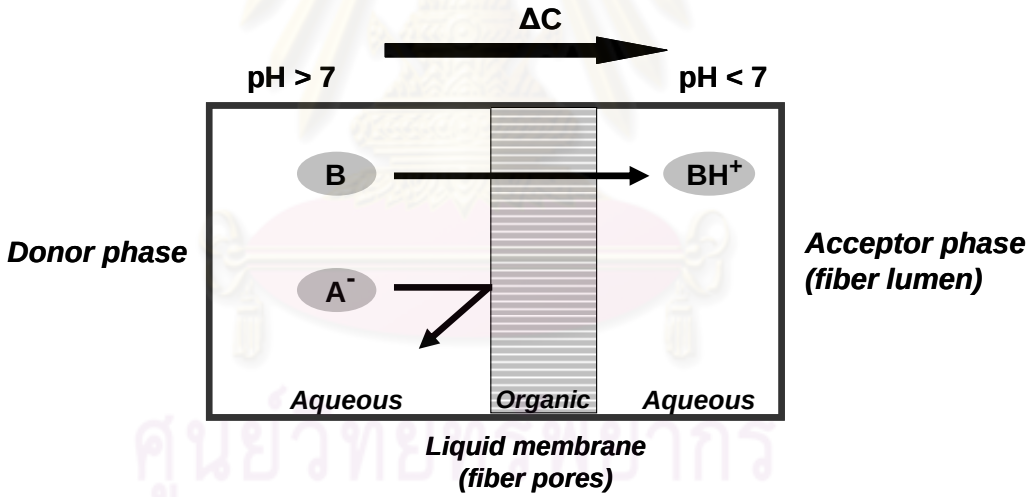


Figure 2.5 Three-phase extraction mechanism in HF-LPME for basic analyte.

(B = basic species , A = acidic species) (adapted from *(25)*)

The recovery of analyte in three-phase system is controlled by two individual partition coefficients ($K_{organic/donor}$ and $K_{acceptor/organic}$) to reach high recoveries. High partition coefficients are performed by proper selection of organic solvent to create SLM and

proper selection of pH conditions in both aqueous solutions (donor and acceptor). In addition, another parameter affecting the recovery is the volume of sample and organic, which should be low to increase recovery.

Similar way to two-phase systems, the enrichment of three-phase systems depends on the volume ratio of donor and acceptor ($V_{donor}/V_{acceptor}$), which should be low and then the enrichment factor is normally found to be high.

Following three-phase extraction, the acceptor solution can directly be injected into HPLC or capillary electrophoresis without prior treatment.

Besides high analyte enrichment ability, both two-phase and three-phase provide efficient clean-up from matrix components by excluding the acceptor phase from macromolecules and other compounds in the sample that could interfere with analysis.

Two-phase and three-phase extractions are based on diffusion, which means that the extraction can be promoted by high partition coefficients. However, for very hydrophilic compounds that have low partition coefficients, the extraction is not possible with both two- or three-phase modes. Low partition coefficients indicate that analytes cannot be extracted based on diffusion alone. To solve this problem, HF-LPME has further been developed into carrier-mediated HF-LPME.

2.2.1.3 Carrier-mediated HF-LPME *(33,34)*

Carrier-mediated HF-LPME utilizes ion-pairing agents to transfer analytes from donor solution to acceptor solution. This method was developed by Ho et al. *(34),* for the attempt to extract high hydrophilic or polar analytes with HF-LPME. This ion-pair HF-LPME is well-known as carrier-mediated membrane transport or carrier-mediated HF-LPME.

The carriers employed in carrier-mediated HF-LPME mode are various types of compounds and can be cationic, neutral, and anionic carrier. Some carriers are illustrated in Figure 2.6.

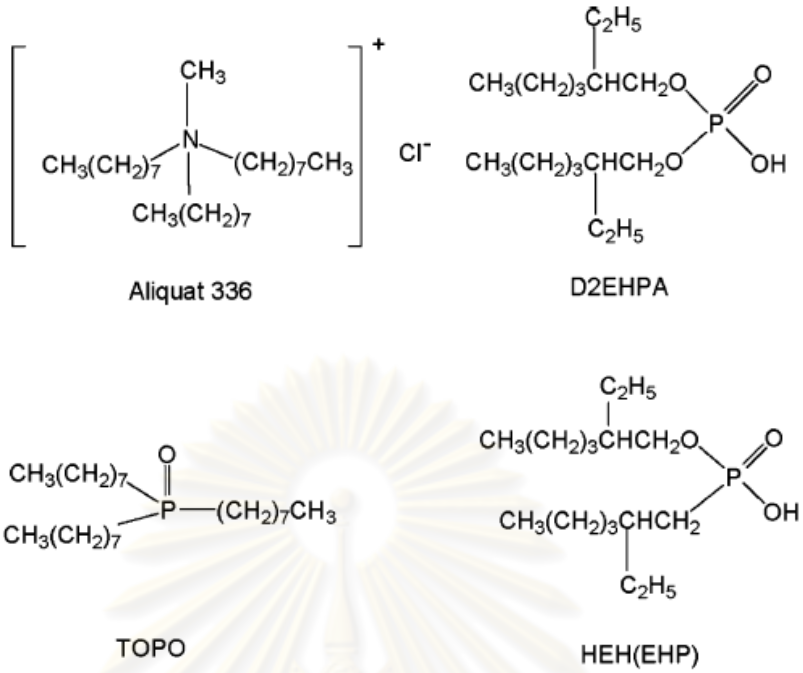


Figure 2.6 Some carriers in carrier-mediated HF-LPME. *(35)*

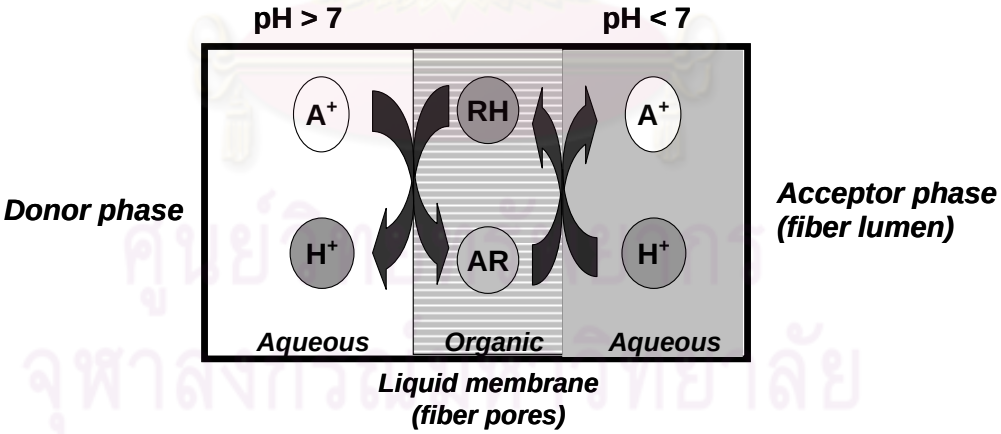


Figure 2.7 Carrier-mediated HF-LPME mechanism.

(A^+ = hydrophilic analyte species , RH = carrier , AR = ion-pairs between analyte and carrier) (adapted from*(31)*)

The principle of carrier-mediated HF-LPME is to add a carrier, an ion-pairing agent into sample solution or organic solvent in order to form ion-pair complex with hydrophilic analytes. The ion-pairs between carrier and target analyte offer a higher partition possibility into organic solvent than the native analyte; therefore, target analytes can be transfered from sample solution through organic solvent, and subsequently extracted into acceptor solution in their native forms. Carrier-mediated HF-LPME is usually applied in three-phase extraction and the analytes in the acceptor solution should be performed in a condition suitable for detection with analytical methods such as HPLC or CE.

In three-phase systems, the carrier-mediated process is controlled by two individual partition coefficients ($K_{organic/donor}$ and $K_{acceptor/organic}$). Besides diffusion and ion-pairing effects, the counter-ions present in acceptor solution and pH gradient between donor and acceptor phase are the essential driving forces to promote extraction. As seen in the carrier-mediated mechanism from Figure 2.7, the counter-ions in acceptor solution should be in sufficient quantity to form ion-pair complexes with carrier in the contact area and then these counter-ions are back-extracted into sample solution to allow the carrier to form new ion-pair complex with analyte, and the carrier-mediated extraction process of analyte is repeated again. For basic analytes, the adjustment of sample pH is to ensure target analytes are in their ionized state, whereas the acceptor pH should be adjusted to acidic to have sufficient protons, which behaved as counter-ions and to release the carrier within acceptor phase.

In present, carrier-mediated HF-LPME has been efficiently applied for the extraction and determination of polar analytes from complex matrices such as environmental and biological samples with high enrichment characteristics and remarkable clean-up efficiency.

2.2.1.4 Parameters affecting HF-LPME procedure

2.2.1.4.1 Hollow fiber membrane

Hollow fiber is a synthetic membrane classified based on the geometry. HF-LPME hollow fiber membranes are porous and mainly made of polypropylene polymer. Nowadays, hollow fibers are commercially available with inner diameter of 600 μm, wall thickness of 200 μm, and average pore size of 0.2 μm. The character of hollow fiber membrane for HF-LPME should be hydrophobic and inert. For extraction, the hollow fiber should be compatible and resist in the choice of organic solvent. Because the hollow fiber size affects the mechanical stability, the inner diameter size is important to have a proper volume to contain the acceptor solution in the fiber lumen. Additionally, the wall thickness should be convenient to create a thin layer of SLM and provide a short diffusion distance. Besides all, the fiber porosity should be high enough to promote extraction speed by providing large surface area of impregnated organic solvent and to be in contact with the sample solution. Pore sizes of 0.2 μm are suitable for the penetration of small molecules of target analytes through the fiber pores. Compared with the structure and ability of flat sheet membranes, hollow fibers allow low-cost extraction with reducing carry-over effects, provide higher surface area per unit volume, and have lower solvent usage. For their advantages, hollow fibers are more extensively used for LPME than flat sheet membranes.

2.2.1.4.2 Organic solvent

The selection of organic solvent in HF-LPME is an essential step for two-phase and three-phase systems. The organic solvent chosen for extraction must be immiscible with water, strongly immobilized within the fiber pores to prevent leakage of analytes into sample solution, and provide appropriate extraction selectivity related to extraction recoveries. Due to the fact that the partition coefficients of analyte in aqueous and organic phase control the extraction efficiency in HF-LPME process, the organic solvent type and composition are necessarily optimized parameter.

In two-phase extraction, the organic solvent should be selected to provide high solubility of hydrophobic analytes in organic phase (high $K_{organic/donor}$) and should have suitable properties to be compatible with GC analysis. 1-octanol is the most popular organic solvent used in two-phase HF-LPME, but some organic solvents used in three-phase system can also be applied in many two-phase systems.

In three-phase extraction, organic solvent immobilized within fiber pores serve as a barrier between the two aqueous phases (donor and acceptor). Therefore, selected solvent should offer high $K_{organic/donor}$ and high $K_{acceptor/organic}$ for target analytes together with proper polarity when combined with polypropylene hollow fibers. The volume of organic solvent is related to recovery, extraction efficiency, and enrichment factor in three-phase system as seen in Eq. 8-10. However, the volume of organic solvent employment depends on hollow fiber porosity, which is difficult to optimize. For three-phase mode, 1-octanol and dihexyl ether are extensively used as organic solvent.

The composition of organic solvent is another choice to improve extraction of target analytes. In case that one organic solvent cannot extract a large group of analytes with different polarity, mixed solvent systems are applied to cover the dissimilar properties. In carrier-mediated HF-LPME, the addition of ion-pairing agent into organic solvent has been proven to effectively enhance extraction of very hydrophilic analytes. Therefore, carrier type and its composition in organic solvent are alternative parameters to increase the extraction efficiency.

2.2.1.4.3 Extraction kinetics

To obtain high recovery and enrichment, high extraction speed is required in HF-LPME and agitation or stirring are effective ways to achieve that. These techniques are related the increase extraction kinetics. Agitation or stirring of sample solution cause faster diffusion of analytes into organic or acceptor solution. With enhanced extraction kinetics, the extraction time is reduced and the repeatability of extraction method is improved. However, improper agitation can affect the organic solvent immobilized in fiber pores. Hence, magnetic stirring can properly promote the diffusion of analytes. To

date, there are multi-stirrer devices that are convenient for extraction. Many sample solutions can be extracted simultaneously and help to decrease time and labor for extraction.

2.2.1.4.4 Donor solutions

Donor or aqueous sample solution has three main parameters to be optimized such as pH, volume, and composition. For donor volume, it directly related with acceptor volume to create the volume ratio ($V_{donor}/V_{acceptor}$) affecting the enrichment factor and recovery of analytes. From Eq. 5 and Eq. 10, the donor volume should be relatively high to provide large volume ratio in both two-phase and three-phase systems corresponding with mL-level of the sample volume. The pH of donor solution is associated with the extraction enhancement and donor pH changes may lead to higher analyte preconcentration. pH in the donor solution should be adjusted as such deionized analytes are obtained in order to reduce their solubility in the sample solution and to promote their transport to organic phase. In addition, a carrier can be added to the sample solution instead of organic solvent to efficiently transfer analytes. Besides carrier which is the one additive in sample solution, the solution filled to adjust donor pH is another consideration. The pH adjustment solutions should not react with analytes or carrier and must not interfere the extraction process.

2.2.1.4.5 Acceptor solutions

Acceptor solutions in two-phase and three-phase extraction are different. It can be defined that the acceptor phase is a parameter that separates the two modes of extraction. While in two-phase systems the acceptor solution is an organic solvent, the acceptor solution in a three-phase system is aqueous. Two-phase systems properly extract hydrophobic analytes into organic acceptor phases, whereas hydrophilic analytes are to be extracted with three-phase systems. Like the donor solution, the three considerations of acceptor solution are volume, type, and composition. The acceptor volume is relatively low in μL-level to be easily directly injected into HPLC analysis.

Low amounts of acceptor combined with the large donor volume in solution are also inducing high volume ratio of donor and acceptor solution ($V_{donor}/V_{acceptor}$). Besides high recovery and enrichment factor obtained, the sensitivity of method is increased by a high volume ratio. In three-phase mode, pH of the aqueous acceptor solution be adjusted to ensure efficient extraction of analytes from organic phase and to prevent analytes to be trapped in the organic phase. Hence, acceptor pH should be adjusted to obtain analytes in their ionized form. The composition of acceptor phase is determined by the analytical method chosen. For two-phase mode, organic acceptor should match with GC behavior, while aqueous acceptor in three-phase mode should be appropriate for HPLC or CE detection.

2.2.1.4.6 Extraction time

In HF-LPME, mass transfer is based on time-dependent equilibrium process. When the extraction system is close to equilibrium, the mass transfer rate is reduced. In other words, HF-LPME is defined as a non-exhaustive method. It, therefore, may consume long time for the system to reach equilibrium. Even the longer extraction times result in increased extraction efficiencies; short time is strongly required in practical analysis. During the experiment, consistent and precise timing is necessary for good precision in simultaneous extraction of a large number of samples. High sample throughput capacities compensate long extraction time.

2.2.1.4.7 HF-LPME configuration

There are several configuration utilized in HF-LPME. The U-shaped configuration seems to extensively used compared to other configurations. The technical set-up of this configuration is illustrated in Figure 2.8.

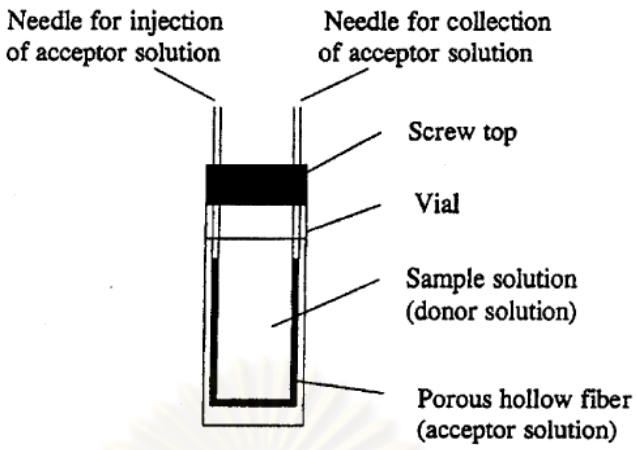


Figure 2.8 HF-LPME technical set-up in U-shaped configuration. *(28)*

In U-shaped configuration, the two porous hollow fiber ends are connected to syringe needles to hold the fiber in U-shape within the sample solution. One fiber end is used to fill acceptor solution into fiber lumen, while the other end is employed to collect acceptor solution after extraction. This configuration provides excellent extractions, but it has some drawbacks in transferring the acceptor solution into the instrument, which leads to difficulties in automation.

There is another U-shaped configuration, where one end of the fiber is connected to a funnel-shaped injection guide, while the other end is held by a small dent in the injection guide. This configuration is shown in Figure 2.9. The set-up decreases air-bubble formation in the acceptor solution and the device can directly be transferred to an autosampler for further analysis.

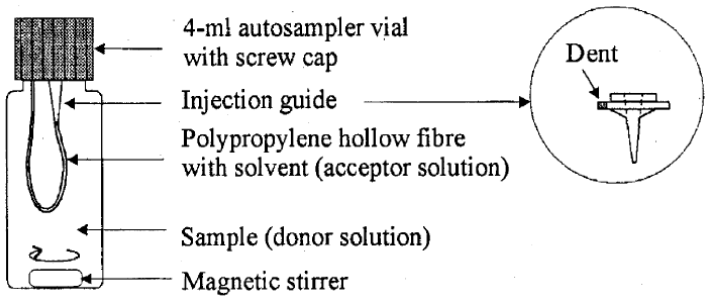


Figure 2.9 Alternative HF-LPME technical set-up in U-shaped configuration. *(28)*

Besides U-shaped, HF-LPME has rod-like configuration as shown in Figure 2.10. This configuration has resolved the U-shaped problem with the application of one tip for both addition and removal of acceptor solution and lead to a more convenient automated system.

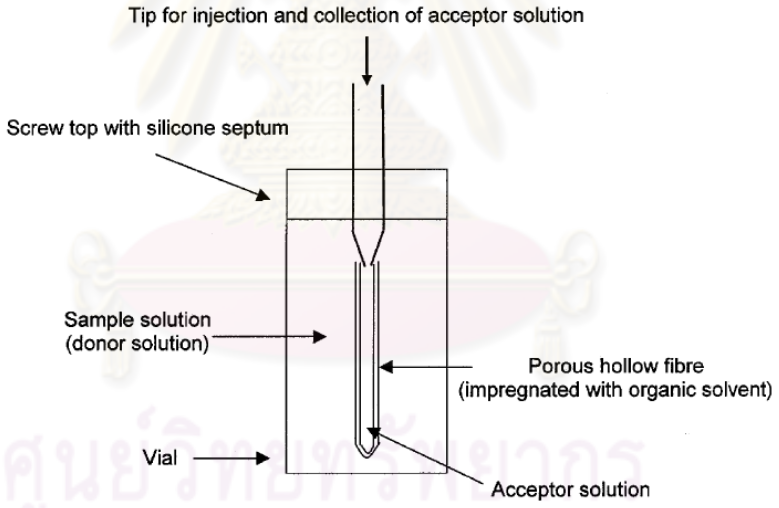


Figure 2.10 HF-LPME technical set-up in rod-like configuration. *(28)*

However, the hollow fiber configuration is crucial to extraction efficiency and enrichment factor of HF-LPME. The selected configuration may have to be additionally optimized (e.g., length of hollow fiber, the volume of acceptor, and suitable supplementary devices).

2.3 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) *(36, 37, 38, 39, 40)*

Currently, high-performance liquid-chromatography (HPLC) has been widely applied as determination step for residual analysis. HPLC accurately and precisely provides capabilities in separation and quantification of polar, non-volatile, and thermally unstable analytes. However, HPLC cannot provide enough information regarding the identity of compounds. Mass spectrometry (MS) is the detection system that can overcome this limitation and also offers high sensitivity and selectivity of analysis. MS can provide absolute identification by providing information about the molecular weight, structure, identity, and quantity of specific sample components. As a result of the resolving power of LC and the detection specificity of MS, LC is coupled with MS and has become the method of choice for routine qualitative and quantitative analysis.

2.3.1 High Performance Liquid Chromatography (HPLC)

Chromatography is a technique to separate mixtures into their individual components, so they can be identified and measured. In liquid chromatography (LC), the separation principle is based on the interaction of a solute with a stationary and a mobile phase. These interactions can be controlled through different choices of both stationary and mobile phases. A schematic diagram of a typical HPLC instrument is shown in Figure 2.11.

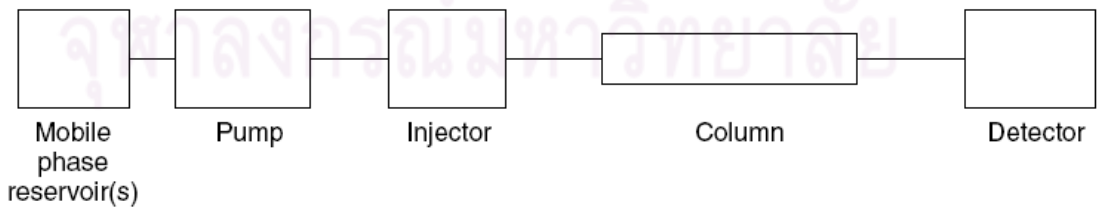


Figure 2.11 Schematic diagram of a typical HPLC instrument. *(36)*

In HPLC, the chromatographic process begins when the solute is injected into the injector, then the mobile phase, which is forced by a pumping system, carries the solute and flows through a chromatographic column. In the column, the mixture is separated into its components by the individual interaction of each component with mobile and stationary phase; and then the components are determined at the detector. The result of separation is shown in forms of chromatogram. From the HPLC diagram, the instrument consists of five parts (i.e., mobile phase, pump, injector, column, and detector).

2.3.1.1 Mobile phase and mobile phase reservoir

The most common type of mobile phase reservoir is a glass bottle. Most of the manufacturers supply these bottles with special caps, Teflon tubing, and filters to connect the bottles to the pumping system. The mobile phase reservoir should be placed away from sunlight and temperature gradients should be avoided.

Mobile phases in HPLC are usually mixtures of two or more individual solvents with or without additional additives or modifiers. The solvents chosen affects the elution of solute. In column HPLC there are two elution types such as isocratic and gradient mode. The selection of elution mode depends on the polarities of analytes. In isocratic elution, the mobile phase is employed at constant composition, while change in mobile phase compositions during the separation is called gradient elution. Gradient elution mode reduces analysis time and increases resolution for complex mixtures.

Solvents used must be high purity, most often HPLC grade because impurities in solvents or reagents can react with solute. Besides purity, there are other considerations to be made in solvent selection such as viscosity, polarity, toxicity, boiling point, and detector compatibility. Mobile phases must be filtered and degassed before used because the dissolved gases in solvents can be collected in the columns, pumps, and detectors and, therefore, affect the reproducibility of the volume delivered. Additionally, large bubbles may stop the pump from working.

2.3.1.2 Pump

The mobile-phase solvents are delivered from their reservoirs by toa pump. High pressure pumps are needed to force solvents flow through column with a controlled flow rate because the particles in column are packed with high density. For HPLC, typical flow rates of 0.5-5.0 mL/min are produced by pumps operating at 300-6000 psi. The two major categories of pumps applied are constant flow or volume and constant pressure. Constant flow pumps generate a certain flow rate of mobile phase, while constant pressure pumps apply a constant pressure to the mobile phase flowing through column. Most HPLC instruments use a reciprocating pump for both maintaining a constant flow rate up to several milliliters per minute and obtaining high output pressure to push the mobile phase through the chromatographic column. Reciprocating pump results in a pulsed flow that induces noise to the chromatogram. To eliminate this problem a pulse damper is placed at the outlet of the pump.

2.3.1.3 Injector

The purpose of the injection system is to apply the sample extract onto the column in a narrow band. The three available techniques of injection are direct syringe injection, stop flow syringe injection, and injection valve. The sample injected should be in solution, so solid samples need to be dissolve in an appropriate solvent, which must not be the same type as mobile phase prior to injection. The injection valve is widely used as injection device for reproducibly introducing sample extracts into pressurized columns without flow interruption. After the valve is loaded with sample, it switches mode sample and mobile phase flow to the column.

2.3.1.4 Column

In HPLC, the two columns typically utilized are an analytical and a guard column. An analytical column is used to separate the sample, while the guard column is placed before the analytical column to protect the analytical column from contamination.

Typical analytical columns are 10, 15, or 25 cm in length and are fitted with extremely small diameter particles (3, 5, or 10 μm). The internal diameter of the columns is usually between 1 and 4.6 mm. The major advantages of these shorter columns are faster separations and improved sensitivity of detection. The most widely used columns contain chemically modified silica stationary phase with the chemical modification determining the polarity of the column. The stationary phase selection is based on the surface interactions and the adsorption sites. Modern HPLC adsorbents are small rigid porous particles with high surface area. A very popular stationary phase is C18 alkyl group, which is bonded to the silica surface.

The guard column is employed to eliminate two threats to the analytical column. Firstly, solutes binding irreversibly to the stationary phase will degrade the analytical column's performance by decreasing the available of the stationary phase. Secondly, particulate material injected with the sample may clog the analytical column. Guard columns usually contain the same particulate packing material and stationary phase as the analytical column but are significantly shorter and less expensive.

2.3.1.5 Detector

The function of an HPLC detector is to continuously and instantaneously monitor the components emerging from the column. The most popular HPLC detectors based on spectroscopic measurements are UV/Visible and fluorescence detectors. The analytical wavelength is selected in a modified spectrophotometer equipped with a flow cell. When using a UV/Visible detector, the resulting chromatogram is a plot of absorbance as a function of elution time. An instrument utilizing a diode array detector (DAD) is giving a three-dimensional chromatogram showing absorbance as a function of wavelength and elution time. One limitation in using absorbance is that the mobile

phases must not have absorbance at the chosen wavelength. Fluorescence detectors provide additional selectivity when solutes can fluorescence. The resulting chromatogram is a plot of fluorescence intensity as a function of time. Another common group of HPLC detectors are those based on electrochemical measurements such as amperometry, voltammetry, coulometry, and conductivity. 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.

2.3.2 Ultra Performance Liquid Chromatography (UPLC)

UPLC has been developed with the same practicality and principles as HPLC. With smaller size of packing materials from HPLC, UPLC offers greater resolution, speed, and selectivity. Owing to the efficiency of HPLC increased as particle sizes of the column packing decreased, the UPLC, which has particle size of 1.7 µm, provides efficiency three times greater when compared with 5 µm particle sizes of typical HPLC. In addition, the resolution can be increased up to 70%. Because of the small particle packing, the UPLC column length can be reduced by three times and the flow rate can grow up three times compared to HPLC. 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 2.12.

The Acquity system from Waters is the only UPLC system that is commercially available. UPLC is operated at high pressure of around 8000 psi due to the small size particle packing in column. Therefore, UPLC application requires a better pumping system than HPLC and the detector for UPLC must have a high sampling rate for sensitive detection and reliable quantification of the narrow peaks produced.

Figure 2.12 Acquity™ UPLC column *(41)*

2.3.3 Mass Spectrometry (MS)

Mass spectrometry is one of the most important analytical tools, in order to obtain information about the chemical composition and abundance of isotopes. A mass spectrometer produces ions from the substance, separates them according to their mass to charge ratio (m/z), and records the relative abundance of each ionic species present. The three major components of a MS instrument are ion source, mass analyzer, and detector. Figure 2.13 shows a schematic diagram of the mass spectrometry process.

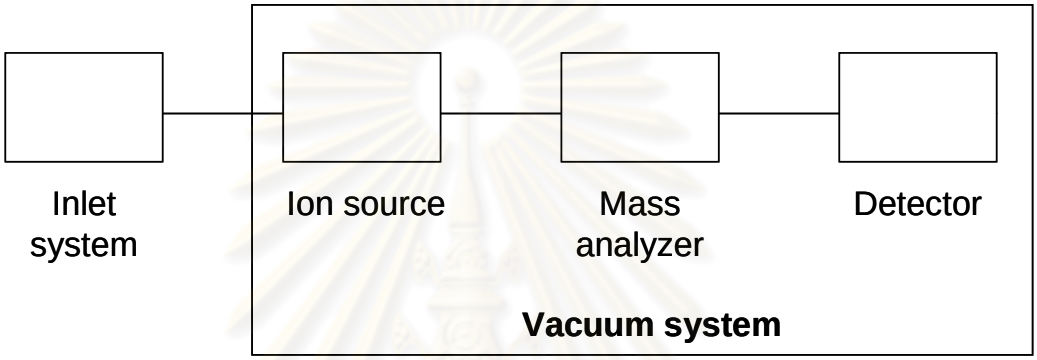


Figure 2.13 Schematic diagram of MS system.

In MS, samples are transferred through the introduction system into the vacuum area of the mass spectrometer. In the ion source region, sample molecules are ionized to gas phase ions and accelerated into mass analyzer, where all ions are separated according to their mass to charge ratio. Finally, separated ions are determined with a detector and signals are delivered to data system analysis. All MS instruments have a high vacuum system to minimize the collision between ions, prevent the loss of ions, and increase the mean free path of ions.

2.3.3.1 Ion source

Ion source is the region, where ionization of analytes occurs. In hyphenated systems of LC and MS, the ionization appears on the interface area of LC and MS, where the separated components from LC are introduced. The LC-MS interface is utilized to eliminate the mobile phase from LC and produce gas phase ions of analytes for further separation and detection in the MS system. Extensive ionization techniques in LC-MS

are atmospheric pressure electrospray ionization (AP-ESI) and atmospheric pressure chemical ionization (APCI). The ionization technique is selected based on analyte properties. Most mass spectrometers use positive ions, which are easily created. However, sometimes negative ions are required.

2.3.3.1.1 Atmospheric pressure electrospray ionization (AP-ESI)

AP-ESI is a useful ionization technique to analyze samples that become single or multiple charged depending on their molecular structures. It can be used to create either positive or negative ions, and it also ionizes high molecular weight components. AP-ESI ionization process is followed by evaporation. The three basic steps of AP-ESI are nebulization and charging, desolvation, and ion evaporation. These steps are shown in Figure 2.14.

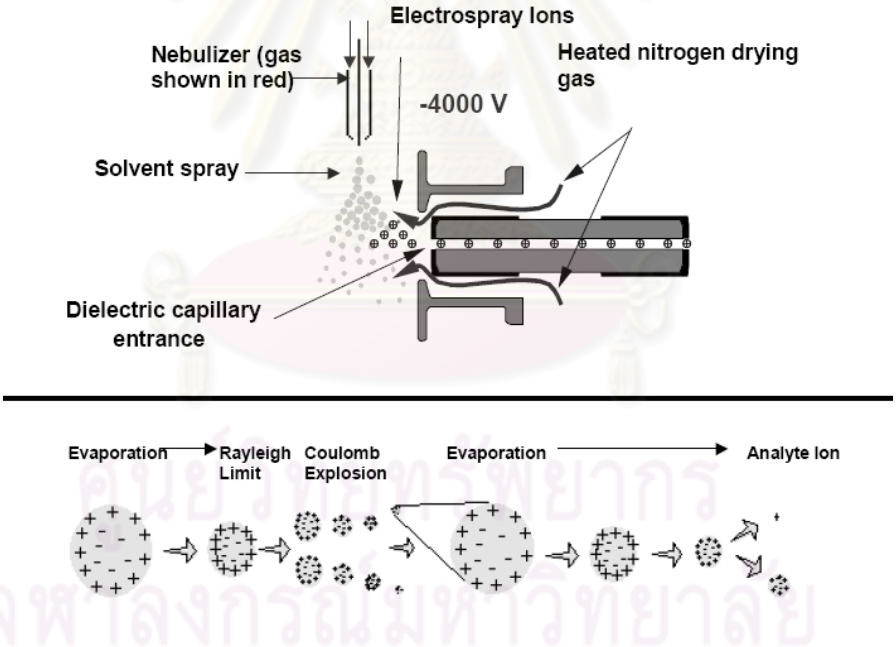


Figure 2.14 Atmospheric pressure electrospray ionization process. *(38)*

Firstly, the HPLC effluent is pumped through a nebulizing needle, which is set at ground potential. The spray passes an electrode, which is held at high potential. The potential difference between the needle and the electrode produces a strong electrical

field. This field charges the surface of the liquid and forms a spray of charged droplets. During the desolvation step, the droplets are attracted to the capillary and dried with a heated nitrogen gas flow and uncharged species are eliminated. After the charged droplet size is reduced, the repulsive force within charges overcomes the cohesive force of surface tension and creates coulombic explosion. This process is repeated until the analyte ions are desorbed into the gas phase. These gas-phase ions are then continuously passed to the mass analyzer.

AP-ESI is a concentration dependent technique and has many advantages such as high sensitivity to polar compounds, it produces multiply charged ions, and is suitable to reverse phase solvents.

2.3.3.1.2 Atmospheric pressure chemical ionization (APCI)

APCI is an ionization technique that is applicable to a wide range of polar and nonpolar analytes of moderate molecular weight. APCI differs from AP-ESI as evaporation process occurs and is followed by ionization. APCI also has three basic steps; nebulization, desolvation, and ionization. These steps are shown in Figure 2.15.

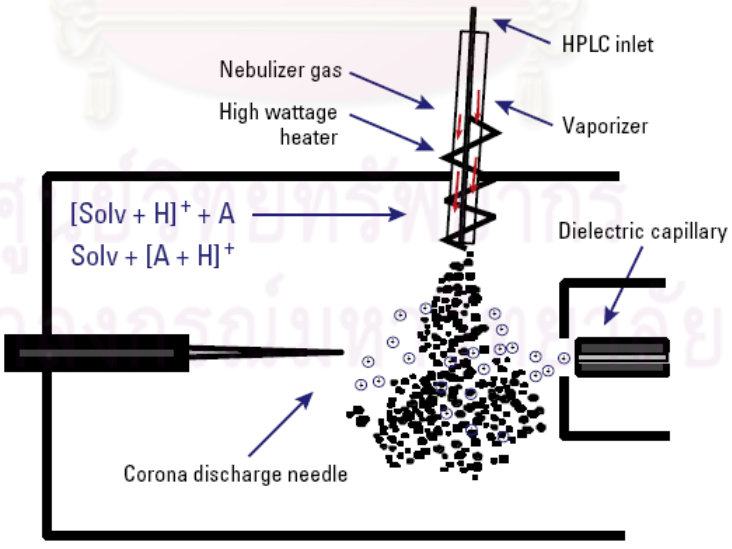


Figure 2.15 Atmospheric pressure chemical ionization process. *(38)*

APCI nebulization is similar to API-ES, but APCI nebulization occurs in a hot vaporizer chamber (typically 250°C–400°C). The effluents from HPLC are evaporated to spray droplets of solvent and analytes in gas phase. The gas-phase solvent molecules are ionized by a corona needle discharge. Then, the charge is transfered from the ionized solvent species to the analyte molecules, and the charged analytes are delivered to the mass analyzer.

APCI can handle HPLC flow rates up to 2 mL/min, efficiently works with many compounds, especially non-polar, and produces only single charged ions. Nevertheless, possible thermal degradation is of concern in APCI; furthermore, compounds require a certain vapor pressure.

2.3.3.2 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 have some ion focusing device to prevent the spread of ions from ion source. The selection of mass analyzer depends on the resolution, mass range, scan rate, and detection limits required for the application. Each analyzer has different operating characteristics, and an additional instrument. In hyphenated LC-MS, quadrupole and time-of-flight (TOF) are widely used mass analyzers. Both techniques are considered as ion transmission system.

2.3.3.2.1 Quadrupole mass analyzer

The quadrupole mass spectrometer is the most common mass analyzer because of its compact size, fast scan rate, high transmission efficiency, and moderate vacuum requirements. In the mass spectrometer, the quadrupole analyzer consists of four parallel metal rods or electrodes. Two parallel rods are connected to direct current (DC), while the others are connected to radio frequency (RF). Both DC and RF are chosen to filtered ions. When the ions travel through the quadrupole, they are selected

by DC and RF according to their m/z, only ion of selected m/z or resonance ion pass through quadrupole analyzer. A quadrupole mass analyzer is schematically shown in Figure 2.16.

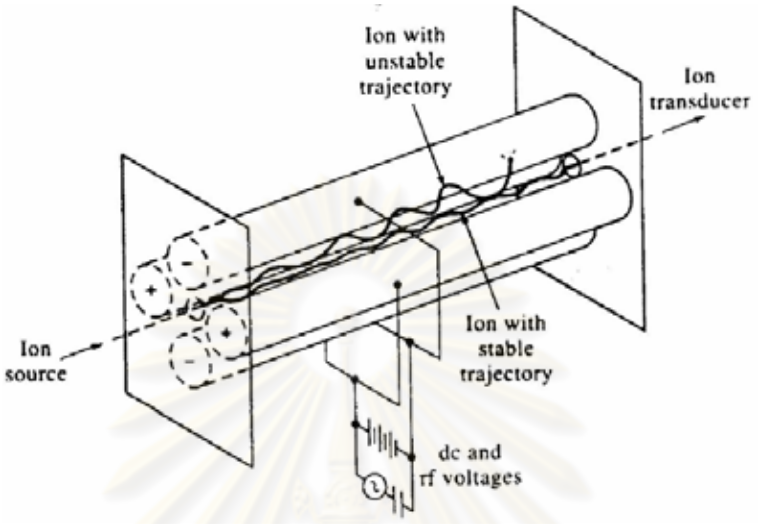


Figure 2.16 Quadrupole mass analyzer. *(39)*

2.3.3.2.2 Time-of-flight mass analyzer (TOF)

The time-of-flight mass analyzer (TOF) is the simplest configuration of the mass separation devices. The selection of ions is based on the movement of ion through the flight tube (Figure 2.17). TOF is usually applied to separate macromolecules with large m/z. The separation is based on the principle that ion of different masses experience individual velocities in the flight tube, and, in conclusion, have different flying time to the end of the tube, where transferred to the detector.

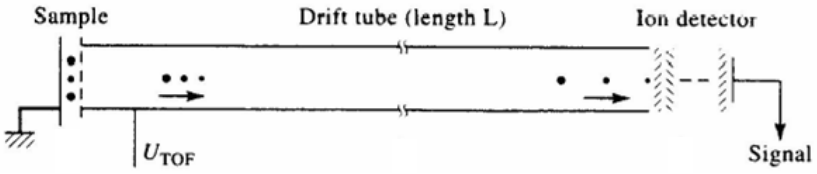


Figure 2.17 Time-of-flight mass analyzer. *(39)*

2.3.3.3 Detector

The detector is used to measure the ions leaving from the mass analyzer by converting ions into an electrical current or other forms of signal, processing and recording into 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 2.18) and photo multiplier tube (Figure 2.18). Electron multiplier tube offers electron from surface of tube for analyte ions. The entrance of tube is held with potential charge opposite from the analyte ions. Analyte ions are attracted to the entrance of tube and collide with tube surface, then the inner surface coated with electron-emissive material releases electrons. These electrons are accelerated to hit another portion of tube by electrostatic force and the surface loses more electrons in every collision. 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 photon 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 measured at the anode.

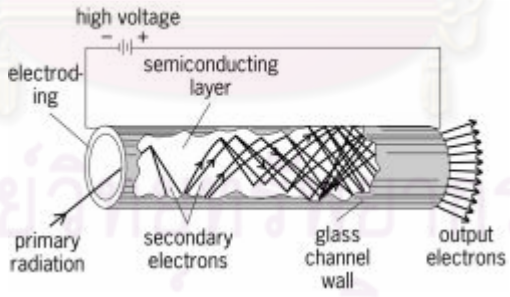


Figure 2.18 Electron multiplier tube. *(42)*

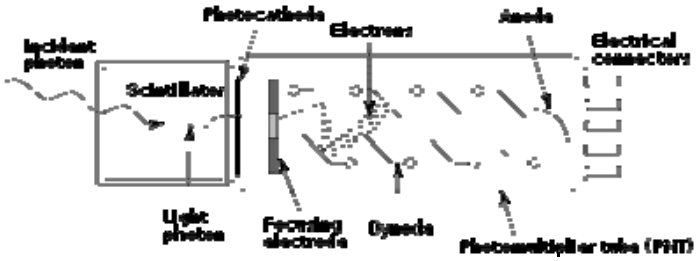


Figure 2.19 Photo multiplier tube. *(43)*

2.3.4 Tandem Mass spectrometry (MS/MS)

Tandem mass spectrometry uses two or more sequential mass spectrometers. MS/MS is a powerful technique that provides both the molecular weight of an analyte and information about the structure of the molecule involved. Therefore, MS/MS has been applied for many qualitative and quantitative applications. MS/MS is used to isolate an ion of interest in first mass analyzer (MS1) and then chemically or energetically modifies these ions with second mass analyzer (MS2). MS/MS process involves the determination of mass relationship between a precursor or parent ion in MS1 and a product or fragmented ion in MS2. The most commonly used tandem mass spectrometer is the triple quadrupole (QqQ). The configuration of QqQ consists of three sets of quadrupole rods in a series (Figure 2.19). Both the first and third sets of quadrupoles are used for mass separation, while the second set acts as a collision cell. The selected precursor ions pass from first quadrupole, are then fragmented and focused in the second quadrupole before transmitted into third quadrupole, where the fragmented ions of analytes are separated and subsequently detected. With this mechanism, MS/MS separates components of same molecular masses but different product ions with high specificity.

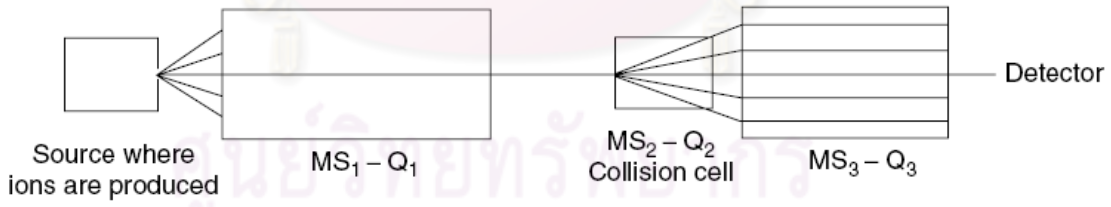


Figure 2.20 Triple quadrupole mass analyzer (QqQ). *(36)*

CHAPTER III

EXPERIMENTAL

3.1 Instrumental and Apparatus

3.1.1 Liquid chromatography-tandem mass spectrometer (LC-MS/MS): Waters Acquity UPLC system with an autosampler, a binary pump and a column thermostat compartment coupled to a Micromass Quattro Premier™ XE benchtop tandem quadrupole mass spectrometer using an atmospheric pressure electrospray (AP-ESI) interface and Masslynx 4.1 software processing, Water Corporation, MA, USA.

3.1.2 HPLC column: C_{18} Acquity UPLC BEH (100mm x 2.1mm I.D., 1.7μm) Water Corporation, MA, USA.

3.1.3 Multi-station magnetic stirrer: model RCT basic IKAMAG®, IKA® Werke GmbH & Co. KG, Staufen, Germany.

3.1.4 Milli-Q ultra-pure water system: model Millipore ZMQS5V00, Millipore, USA.

3.1.5 Ultrasonicate: model crest575d, Crest Ultrasonic corporation, NY, USA.

3.1.6 Balance: model XS, Mettler-Toledo, Inc., OH, USA.

3.1.7 pH meter: model 744, Metrohm Ltd., Herisau, Switzerland.

3.1.8 Blender: model HGBTWTQ4, Waring Commercial, CT, USA.

3.1.9 Centrifuge: model sorvall biofuge stratos, Utech Products,Inc., NY, USA.

3.1.10 Micro-porous polypropylene hollow fiber membrane: Accurel® PP Q3/2 with 600 μm i.d., 200 μm wall thickness, and 0.2 μm pore size, Membrana GmbH, Wuppertal, Germany.

3.1.11 Microsyringes, 100-μL, Hamilton, Bonaduz, Switzerland.

3.1.12 Medical syringes, 3 mL, Nipro Medical Corporation, Osaka, Japan.

3.1.13 Medical syringe needles, 500 µm O.D., Nipro Medical Corporation, Osaka, Japan.

3.1.14 Micropipettes, 2-20 µL, 50-200 µL, and 200-1000 µL, Gilson, Inc., Middleton, USA.

3.1.15 Micropipette tips, 200 µL and 1000 µL, Gilson Inc., Middleton, USA.

3.1.16 HPLC amber vials, 2 ml with PTFE cap, Agilent Technologies, CA, USA.

3.1.17 HPLC insert vials, 200 µL, Agilent Technologies, CA, USA.

3.1.18 Vials, 30 mL with silicone-septum screw caps, N.K. Supply, Bangkok, Thailand.

3.1.19 Magnetic bars, Lab systems Co., LTD., Bangkok, Thailand.

3.1.20 Volumetric flasks, 5.00 mL, 10.00 mL, 25.00 mL, 50.00 mL, 100.00 mL, 250.00 mL, and 500.00 mL.

3.1.21 Solvent bottles, 25 mL, 100 mL, 250 mL, and 1000 mL.

3.1.22 Beakers, 10 mL, 50 mL, 100 mL, 250 mL, and 1000 mL.

3.1.23 Graduated cylinders, 25mL and 100mL.

3.1.24 Spatulas.

3.1.25 Droppers.

3.1.26 Stirring rods.

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

3.2 Chemicals

3.2.1 Standard compounds

Erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), and tylosin (TYL) were all purchased from Dr.Ehrenstorfer (Augsburg, Germany) with purity of 93.5%, 98.5%, 98.5%, and 95.0 %, respectively.

3.2.2 Organic solvents

HPLC gradient grade acetonitrile was supplied by Merck (Darmstadt, Germany) and di-n-hexyl ether (DHE) was obtained from Fluka (Buchs, Switzerland). Methanol in HPLC gradient grade and analytical grade acetone were purchased from J.T. Baker (Deventer, The Netherlands). Analytical grade 1-octanol, 1-decanol, undecane, and dodecane were supplied by Aldrich (WI, USA) and toluene was purchased from Merck (Darmstadt, Germany)

3.2.3 Reagents

Tricaprylmethylammonium chloride (Aliquat 336), di(2-ethylhexyl)phosphoric acid (D2EHPA), ammonium acetate (CH_3COONH_4), ammonium formate ($HCOONH_4$), succinic acid, ethylenediaminetetraacetic acid disodium salt dihydrate and ($Na_2EDTA{\cdot}2H_2O$) were purchased from Fluka (Buchs, Switzerland) and 2-hydroxy-5-nonylacetophenone oxime (LIX 84) was obtained from Henkel (Tucson, AZ). Di-sodium tetraborate decahydrate ($Na_2B_4O_7{\cdot}10H_2O$) and sodium carbonate (Na_2CO_3) were supplied by BDH (Poole, England) and J.T. Baker (Deventer, The Netherlands), respectively. Disodium hydrogenphosphate dehydrate ($Na_2HPO_4{\cdot}12H_2O$), potassium dihydrogen phosphate (KH_2PO_4), m-phosphoric acid, glacial acetic acid, and sodium hydroxide pellets were purchased from Merck (Darmstadt, Germany) and trichloroacetic acid was supplied by Riedel de Häen (Seelze, Germany). 37% hydrochrolic acid fuming was obtained from Fisher Scientific (Loughborough, LE, UK).

3.3 Preparation of standard solutions

3.3.1 Preparation of stock standard solutions

1000 mg/L macrolide standard solutions of erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), and tylosin (TYL) were individually prepared by dissolving 0.0107 g of ERY, 0.0102 g of SPI, 0.0102 g of TIL, and 0.0105 g of TYL in 10.00 mL volumetric flasks with acetonitrile. All stock standard solutions were stored in closed vials with Teflon screw cap at 4 ºC in a refrigerator until use.

3.3.2 Preparation of mixture standard solutions

A 100 mg/L mixture standard solution was prepared by pipetting 1 mL of 1000 mg/L ERY, SPI, TIL, and TYL stock solution into a 10.00 mL volumetric flask and diluting with acetonitrile. The mixture standard solution was kept in closed vials with Teflon screw cap and prepared daily.

3.4 LC-MS/MS system

A Waters Acquity Ultra Performance Liquid Chromatography was connected to a Micromass Quattro Premier^{TM} XE benchtop tandem quadrupole mass spectrometer (Milford, MA, USA). Electrospray ionization (ESI) was used as ionization source in positive mode.

In the LC system, chromatographic separation was performed in an UPLC column C_{18} Acquity BEH (100mm x 2.1mm I.D., 1.7μm) with binary mobile phase in a gradient elution mode. Mobile phase A was an aqueous solution of 10 mM Ammonium acetate and 0.3% (v/v) acetic acid, while mobile phase B was methanol:acetonitrile (50:50, v/v) with 0.3% (v/v) acetic acid. The flow rate was set at 0.2 mL/min and column temperature was 40°C. The injection volume was 10 μL. The separation of four macrolides antibiotics was achieved within 5.5 min in the following gradient program: the mobile phase ratio of A:B was 95:5 at 0.0 min and maintained for 1.5 min, 35:65 at

3.0 min and maintained for 2 min. Then, 100% B was held at 5.5 min for 4.5 min with a return to 5% B at 10.5 min.

The tandem mass spectrometer parameters are 1 kV capillary voltage, 3 V extractor voltage, 120 °C source temperature, 50 L/h cone gas (nitrogen) flow, 1000 L/h desolvation gas (nitrogen) flow, 350 °C desolvation temperature, 0.22 mL/min collision gas (argon) flow, and 0.35 Pa cell pressure. Multiple reactions monitoring mode (MRM) with the most two sensitive transition used for both quantification and confirmation purposes of ERY, SPI, TIL, and TYL. The quantification and confirmation information of four macrolides is shown in Table 3.1. Instrument control and data acquisition and evaluation were performed with MassLynx 4.1 software package provided by Micromass^{TM}.

These proposed LC-MS/MS conditions were entirely employed in this work in order to determine the optimization of sample preparation step in Chapter IV because of the clarification and significance in the separation of four macrolide antibiotics with LC-ESI-MS/MS.

Table 3.1 Multiple Reaction Monitoring (MRM) used in MS/MS analysis

	ERY	**SPI**	**TIL**	**TYL**
Retention time (min)	4.76	4.22	4.49	4.76
Cone voltage (V)	40	30	55	57
Quantification transition	734.45 > 158.28	843.51 > 174.10	869.53 > 696.51	916.48 > 174.19
Collision energy (eV)	30	45	55	40
Confirmation transition	734.45 > 576.26	843.51 > 101.07	869.53 >174.39	916.48 > 772.94
Collision energy (eV)	30	58	50	35

3.5 Hollow-Fiber Liquid-Phase microextraction (HF-LPME) optimization

Parameters affecting HF-LPME procedure such as organic solvent type, organic solvent composition, donor type, donor pH, acceptor type, acceptor pH, immersion time, and extraction time were investigated with U-shaped configuration of HF-LPME as seen in Figure 3.1. The results are displayed as enrichment factors in order to evaluate the method efficiency.

Figure 3.1 The studied HF-LPME configuration

In every optimization processes, the 12-cm hollow fiber was first sonicated with acetone to remove any contaminants and allowed to completely dry in air. Each piece of hollow fiber was single used to prevent carry-over effect.

3.5.1 The procedure of immersion time optimization

The process of immersion time optimization in HF-LPME was performed as follows:

3.5.1.1. A 12-cm hollow fiber was immersed into 30% Aliquat336 in DHE with one immersion time to the fill organic solvent into hollow fiber pores. Immersion times of 5, 15, 30, 60, 120, and 180 min were investigated in three replicates.

3.5.1.2. The lumen of hollow fiber was flushed with air few times by a syringe needle connected with a 3-mL medical syringe to remove excess organic solvent.

3.5.1.3. One end of hollow fiber was attached to a syringe needle held with silicone septum on cap.

3.5.1.4. 20 µL of ammonium acetate pH 4.0 were filled into the lumen of hollow through the free end of the hollow fiber by a 100- µL microsyringe.

3.5.1.5. The free end of the hollow fiber was connected to another syringe needle held with silicone septum on cap.

3.5.1.6. The U-shaped hollow fiber holding on cap was dipped into a 30-mL vial, which contained 20 mL sodium tetraborate pH 9.0 spiked with the 100 mg/L mixture macrolide antibiotics (1 mg/L), and a magnetic bar and then the vial was closed.

3.5.1.7. The 30-mL vial was placed on a multi-station magnetic stirrer and was extracted for 60 min.

3.5.1.8. After extraction, one end of the hollow fiber was induced to the insert vial placed in 2-mL HPLC vial and then the acceptor solution was flushed inside the lumen of the hollow fiber with air through the syringe needle on cap by a 3-mL medical syringe.

3.5.1.9. The acceptor solution was collected and the vial was kept in refrigerator under 4°C until analyzed with liquid chromatography-tandem mass spectrometry system.

The results of immersion time optimization are shown in Table 4.1 and Figure 4.1.

3.5.2 The procedure of organic solvent type optimization

The process of organic solvent type optimization in HF-LPME was performed as follows:

3.5.2.1. A 12-cm hollow fiber was immersed into organic solvent with immersion time of 60 min to fill the organic solvent into hollow fiber pores. 1-octanol, 1-decanol,

dihexyl ether, undecane, dodecane, and toluene were investigated as organic solvents in two replicates.

3.5.2.2. The lumen of the hollow fiber was flushed with air few times by a syringe needle connected with a 3-mL medical syringe to remove excess organic solvent.

3.5.2.3. One end of the hollow fiber was attached to a syringe needle held with silicone septum on cap.

3.5.2.4. 20 µL of ammonium acetate pH 4.0 were filled into the lumen through the free end of the hollow fiber by a 100- µL microsyringe.

3.5.2.5. The free end of hollow fiber was connected to another syringe needle held with silicone septum on cap.

3.5.2.6. The U-shaped hollow fiber holding on cap was dipped into a 30-mL vial, which contained 20 mL sodium tetraborate pH 9.0 spiked with the 100 mg/L mixture macrolide antibiotics (1 mg/L), and a magnetic bar and then the vial was closed.

3.5.2.7. The 30-mL vial was placed on a multi-station magnetic stirrer and was extracted for 60 min.

3.5.2.8. After extraction, one end of the hollow fiber was induced to the insert vial placed in 2-mL HPLC vial and then the acceptor solution was flushed inside the lumen of the hollow fiber with air through the syringe needle on cap by a 3-mL medical syringe.

3.5.2.9. The acceptor solution was collected and the vial was kept in refrigerator under 4°C until analyzed with liquid chromatography-tandem mass spectrometry system.

The results of organic solvent type optimization are shown in Table 4.2 and Figure 4.2.

3.5.3 The procedure of organic solvent composition optimization

The process of organic solvent composition in HF-LPME was performed as follows:

3.5.3.1. A 12-cm hollow fiber was immersed into dihexyl ether adding carrier in various contents of 5%, 10%, 20%, 30% and 40% with immersion time of 60 min to fill the organic solvent into hollow fiber pores. The three carriers studied were Aliquat 336, D2EHPA, and LIX 84. Each carrier was studied with two replicates and each composition was investigated with optimized carrier in three replicates.

3.5.3.2. The lumen of the hollow fiber was flushed with air few times by a syringe needle connected with a 3-mL medical syringe to remove excess organic solvent.

3.5.3.3. One end of the hollow fiber was attached to a syringe needle held with silicone septum on cap.

3.5.3.4. 20 µL of ammonium acetate pH 4.0 were filled into the lumen through the free end of the hollow fiber by a 100- µL microsyringe.

3.5.3.5. The free end of hollow fiber was connected to another syringe needle held with silicone septum on cap.

3.5.3.6. The U-shaped hollow fiber holding on cap was dipped into a 30-mL vial, which contained 20 mL sodium tetraborate pH 9.0 spiked with the 100 mg/L mixture macrolide antibiotics (1 mg/L), and a magnetic bar and then the vial was closed.

3.5.3.7. The 30-mL vial was placed on a multi-station magnetic stirrer and was extracted for 60 min.

3.5.3.8. After extraction, one end of hollow fiber was induced to the insert vial placed in 2-mL HPLC vial and then the acceptor solution was flushed inside the lumen of the hollow fiber with air through the syringe needle on cap by a 3-mL medical syringe.

3.5.3.9. The acceptor solution was collected and the vial was kept in refrigerator under 4°C until analyzed with liquid chromatography-tandem mass spectrometry system.

The results of organic solvent composition optimization are shown in Table 4.3, Table 4.4, Figure 4.3, and Figure 4.5.

3.5.4 The procedure of donor type optimization

The process of donor type optimization in HF-LPME was performed as follows:

3.5.4.1. A 12-cm hollow fiber was immersed into 20% Aliquat336 in DHE with immersion time of 60 min to fill the organic solvent into hollow fiber pores.

3.5.4.2. The lumen of the hollow fiber was flushed with air few times by a syringe needle connected with a 3-mL medical syringe to remove excess organic solvent.

3.5.4.3. One end of the hollow fiber was attached to a syringe needle held with silicone septum on cap.

3.5.4.4. 20 µL of ammonium acetate pH 4.0 were filled into the lumen through the free end of the hollow fiber by a 100- µL microsyringe.

3.5.4.5. The free end of the hollow fiber was connected to another syringe needle held with silicone septum on cap.

3.5.4.6. The U-shaped hollow fiber holding on cap was dipped into a 30-mL vial, which contained 20 mL donor solution pH 9.0 spiked with the 100 mg/L mixture macrolide antibiotics (1 mg/L), and a magnetic bar and the vial was closed. Sodium tetraborate, sodium hydrogen phosphate, and sodium carbonate were investigated as donor types in two replicates.

3.5.4.7. The 30-mL vial was placed on a multi-station magnetic stirrer and was extracted for 60 min.

3.5.4.8. After extraction, one end of the hollow fiber was induced to the insert vial placed in 2-mL HPLC vial and then the acceptor solution was flushed inside the lumen of the hollow fiber with air through the syringe needle on cap by a 3-mL medical syringe.

3.5.4.9. The acceptor solution was collected and the vial was kept in refrigerator under 4°C until analyzed with liquid chromatography-tandem mass spectrometry system.

The results of donor type optimization are shown in Table 4.5 and Figure 4.6.

3.5.5 The procedure of donor pH optimization

The process of donor pH optimization in HF-LPME was performed as follows:

3.5.5.1. A 12-cm hollow fiber was immersed into 20% Aliquat336 in DHE with immersion time of 60 min to fill the organic solvent into hollow fiber pores.

3.5.5.2. The lumen of the hollow fiber was flushed with air few times by a syringe needle connected with a 3-mL medical syringe to remove excess organic solvent.

3.5.5.3. One end of the hollow fiber was attached to a syringe needle held with silicone septum on cap.

3.5.5.4. 20 µL of ammonium acetate pH 4.0 were filled into the lumen through the free end of the hollow fiber by a 100- µL microsyringe.

3.5.5.5. The free end of the hollow fiber was connected to another syringe needle held with silicone septum on cap.

3.5.5.6. The U-shaped hollow fiber holding on cap was dipped into a 30-mL vial, which contained 20 mL sodium tetraborate spiked with the 100 mg/L mixture macrolide antibiotics (1 mg/L), and a magnetic bar and then the vial was closed. Donor pH of 7.0, 8.0, 9.0, 10.0, and 11.0 were investigated in two replicates.

3.5.5.7. The 30-mL vial was placed on a multi-station magnetic stirrer and was extracted for 60 min.

3.5.5.8. After extraction, one end of the hollow fiber was induced to the insert vial placed in 2-mL HPLC vial and then the acceptor solution was flushed inside the lumen of the hollow fiber with air through the syringe needle on cap by a 3-mL medical syringe.

3.5.5.9. The acceptor solution was collected and the vial was kept in refrigerator under 4°C until analyzed with liquid chromatography-tandem mass spectrometry system.

The results of donor pH optimization are shown in Table 4.6 and Figure 4.7.

3.5.6 The procedure of acceptor type optimization

The process of acceptor type optimization in HF-LPME was performed as follows:

3.5.6.1. A 12-cm hollow fiber was immersed into 20% Aliquat336 in DHE with immersion time of 60 min to fill the organic solvent into hollow fiber pores.

3.5.6.2. The lumen of the hollow fiber was flushed with air few times by a syringe needle connected with a 3-mL medical syringe to remove excess organic solvent.

3.5.6.3. One end of the hollow fiber was attached to a syringe needle held with silicone septum on cap.

3.5.6.4. 20 µL of acceptor solution pH 4.0 was filled into the lumen through the free end of the hollow fiber by a 100- µL microsyringe. Ammonium acetate, ammonium formate, succinic acid, and trichloroacetic acid were investigated as acceptor types in two replicates.

3.5.6.5. The free end of the hollow fiber was connected to another syringe needle held with silicone septum on cap.

3.5.6.6. The U-shaped hollow fiber holding on cap was dipped into a 30-mL vial, which contained 20 mL sodium tetraborate pH 8.0 spiked with the 100 mg/L mixture macrolide antibiotics (1 mg/L), and a magnetic bar and then the vial was closed.

3.5.6.7. The 30-mL vial was placed on a multi-station magnetic stirrer and was extracted for 60 min.

3.5.6.8. After extraction, one end of the hollow fiber was induced to the insert vial placed in 2-mL HPLC vial and the acceptor solution was flushed inside the lumen of the hollow fiber with air through the syringe needle on cap by a 3-mL medical syringe.

3.5.6.9. The acceptor solution was collected and the vial was kept in refrigerator under 4°C until analyzed with liquid chromatography-tandem mass spectrometry system.

The results of acceptor type optimization are shown in Table 4.7 and Figure 4.8.

3.5.7 The procedure of acceptor pH optimization

The process of acceptor pH optimization in HF-LPME was carried out as follows:

3.5.7.1. A 12-cm hollow fiber was immersed into 20% Aliquat336 in DHE with immersion time of 60 min to fill the organic solvent into hollow fiber pores.

3.5.7.2. The lumen of the hollow fiber was flushed with air few times by a syringe needle connected with a 3-mL medical syringe to remove excess organic solvent.

3.5.7.3. One end of the hollow fiber was attached to a syringe needle held with silicone septum on cap.

3.5.7.4. 20 µL of ammonium acetate was filled into the lumen through the free end of the hollow fiber by a 100- µL microsyringe. Acceptor pH of 3.0, 4.0, 5.0, and 6.0 were investigated in two replicates.

3.5.7.5. The free end of the hollow fiber was connected to another syringe needle held with silicone septum on cap.

3.5.7.6. The U-shaped hollow fiber holding on cap was dipped into 30-mL vial, which contained 20 mL sodium tetraborate pH 8.0 spiked with the 100 mg/L mixture macrolide antibiotics (1 mg/L), and a magnetic bar and then the vial was closed.

3.5.7.7. The 30-mL vial was placed on a multi-station magnetic stirrer and was extracted for 60 min.

3.5.7.8. After extraction, one end of the hollow fiber was induced to the insert vial placed in 2-mL HPLC vial and then the acceptor solution was flushed inside the lumen of the hollow fiber with air through the syringe needle on cap by a 3-mL medical syringe.

3.5.7.9. The acceptor solution was collected and the vial was kept in refrigerator under 4°C until analyzed with liquid chromatography-tandem mass spectrometry system.

The results of acceptor pH optimization are shown in Table 4.8 and Figure 4.9.

3.5.8 The procedure of extraction time optimization

The process of extraction time optimization in HF-LPME was performed as follows:

3.5.8.1. A 12-cm hollow fiber was immersed into 20% Aliquat336 in DHE with immersion time of 60 min to fill the organic solvent into hollow fiber pores.

3.5.8.2. The lumen of the hollow fiber was flushed with air few times by a syringe needle connected with a 3-mL medical syringe to remove excess organic solvent.

3.5.8.3. One end of the hollow fiber was attached to a syringe needle held with silicone septum on cap.

3.5.8.4. 20 µL of ammonium acetate pH 4.0 were filled into the lumen through the free end of the hollow fiber of hollow by a 100- µL microsyringe.

3.5.8.5. The free end of the hollow fiber was connected to another syringe needle held with silicone septum on cap.

3.5.8.6. The U-shaped hollow fiber holding on cap was dipped into a 30-mL vial, which contained 20 mL sodium tetraborate pH 8.0 spiked with the 100 mg/L mixture macrolide antibiotics (1 mg/L), and a magnetic bar and then the vial was closed.

3.5.8.7. The 30-mL vial was placed on a multi-station magnetic stirrer and was extracted. The extraction times of 5, 15, 30, 45, 60, 90, and 120 minutes were investigated in two replicates.

3.5.8.8. After extraction, one end of the hollow fiber was induced to the insert vial placed in 2-mL HPLC vial and then the acceptor solution was flushed inside the lumen of the hollow fiber with air through the syringe needle on cap by a 3-mL medical syringe.

3.5.8.9. The acceptor solution was collected and the vial was kept in refrigerator under 4°C until analyzed with liquid chromatography-tandem mass spectrometry system.

The results of extraction time optimization are shown in Table 4.9 and Figure 4.10.

All optimized parameters for HF-LPME are summarized in Table 4.10.

3.6 Method Validation

3.6.1 Standard calibration curve

Standard calibration curves were prepared with spiked standard solution at various concentrations in donor solution and extracted in HF-LPME under optimized parameters. The spiked concentrations were in the range of 0.5-50.0 µg/L. Each concentration was studied in three replicates. The calibration curves were plotted as concentration over peak area of each analyte. The calibration curves of ERY, SPI, TIL and TYL are shown in Figure 4.11, 4.12, 4.13 and 4.14, respectively.

3.6.2 Linearity

Linearity of method was obtained from standard calibration curve of four analytes. Correlation coefficient (R^2) represents the linearity of the proposed method. Under optimized HF-LPME conditions, the linearity was performed over a concentration ranged of 0.5-50.0 µg/L with three replicates of each level. The slope, y-intercept, and correlation coefficient (R^2) of four macrolide antibiotics are shown in Table 4.11.

3.6.3 Limit of detections (LODs) and limit of quantifications (LOQs)

LOD and LOQ are important in the determination process and refer to the efficiency of the method in terms of detection and quantification. While LOD refers to the method lowest concentration of analyte detected, LOQ is the lowest concentration of analyte that can be quantitatively determined.

From chromatogram, the limits of detection were calculated as chromatographic signal (peak height) being three times higher than background noise (S/N = 3). The chromatographic signal was observed from extraction of the lowest spiked concentration of each standard (0.5 µg/L) under optimized HF-LPME condition in eight replicates. The limits of quantification were calculated similar to LOD, but with a signal to noise ratio of S/N = 10. Both LODs and LOQs of method are shown in Table 4.12.

3.6.4 Enrichment factor

Enrichment capability of the method was obtained from extraction of four spiked macrolide antibiotics with optimized HF-LPME condition at two spiked concentration levels of 25 and 50 µg/L and each concentration was studied in eight replicates. The enrichment factor was calculated from observed concentration and spiked concentration as seen in Eq. 10. The results of method enrichment factor at two spiked levels are shown in Table 4.13.

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. Accuracy was derived from the extraction of analyte spiked in donor solution under optimized HF-LPME parameters. In this work, two concentration levels of 25 and 50 µg/L were studied and each concentration was investigated in eight replicates. The observed concentration was determined from the calculation of obtained peak area in the regression equation from standard calibration curve and the average value of eight calculated concentrations was used to represent the observed concentration. The comparison between observed concentration and spiked concentration lead to the recovery of analytes. The recoveries (%) of four analytes at two spiked concentrations are presented in Table 4.14.

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 intra-assay precision and intermediate precision. The intra-assay precision is the precision derived from repeated tests on the same method with single analytical runs, while the intermediate precision is the precision acquired from repeated tests on the same method with different analytical runs or different times. In this work, precision was determined with four analytes spiked at 30 µg/L with the optimized HF-LPME conditions in eight replicates. The extractions were performed in eight replicates in both two analytical days.

The peak area obtained was calculated in the regression equation from standard calibration curve and resulted in concentration of analyte from the method. The percent of relative standard deviations (%R.S.D) were calculated from concentration obtained in eight replicates. The %R.S.D. obtained from the results of one analytical day refers to intra-assay precision, whereas intermediate precision was reported as the %R.S.D from the results of two analytical days.

The acceptable value for %R.S.D within day was calculated from Horwitz equation *(49)*:

$$R.S.D._r = 0.67 \times 2^{(1-0.5\log C)} \quad \text{(Eq.11)}$$

where C is the concentration of the analyte in the sample

To evaluate the intermediate precision, the two-tailed F test was employed to determine the significant difference of results obtained. The results of both intra-assay precision and intermediate precision were presented in Table 4.15.

3.7 The application of optimized HF-LPME method in water and poultry sample

After method validation, the optimized HF-LPME method was proved the effectiveness of procedure by the application in real sample confronted the macrolide antibiotics residue problem; water and poultry sample. The two samples have different matrices so they have different sample preparation process before preconcentration with HF-LPME method. After real sample analysis, the recovery and detection limits of two applications were defined to show the capability of HF-LPME method in real sample application.

3.7.1 Water sample

The optimized HF-LPME condition was applied to preconcentrate four macrolides in water sample. Water samples were collected from Chaophaya River, Bangkok, Thailand. Four macrolides were spiked in water sample for determination because the

water sample was not found macrolide antibiotic residues. The procedure for determining ERY, SPI, TIL, and TYL spiked in water sample was described as follows.

3.7.1.1. The water sample was stand overnight to precipitate sediment and then filtered and spiked with four macrolide antibiotics at 2, 8 and 20 µg/L and pH was adjusted to 8.0 with sodium tetraborate. 20 mL of prepared sample solution were filled into sample vial. Each concentration was done in three replicates.

3.7.1.2. A 12-cm hollow fiber was immersed into 20% Aliquat336 in DHE with immersion time of 60 minutes to fill organic solvent in hollow fiber pores.

3.7.1.3. The lumen of hollow fiber was flushed with air few times by syringe needle connected with 3-mL medical syringe to remove excess organic solvent.

3.7.1.4. One end of the hollow fiber was attached to syringe needle held with silicone septum on cap.

3.7.1.5. 20 µL of ammonium acetate pH 4.0 was filled into the lumen through the free end of the hollow fiber of hollow by a 100- µL microsyringe.

3.7.1.6. The free end of hollow fiber was connected to another syringe needle held with silicone septum on cap.

3.7.1.7. The U-shaped hollow fiber holding on cap was dipped into the prepared sample solution pH 8.0, a magnetic bar was added, and closed the vial.

3.7.1.8. The 30-mL vial was placed on multi-station magnetic stirrer and extracted for 60 minutes.

3.7.1.9. After extraction, the one end of hollow fiber was induced to the insert vial placed in 2-mL HPLC vial and then the acceptor solution flushed inside the lumen of the hollow fiber with air through the syringe needle on cap by 3-mL medical syringe.

3.7.1.10. The acceptor solution was collected and the vial was kept in refrigerator under 4°C until analyzed with liquid chromatography-tandem mass spectrometry system.

The results of the application of HF-LPME in water sample are shown in Table 4.16 and Figure 4.15.

3.7.2 Poultry muscle sample

After the successful application of the optimized HF-LPME condition in water sample, the method was also employed in poultry sample. The chicken sample was bought from a Lotus department store, Thailand. The muscle part was chosen because it is the main position of the injection of antibiotics into poultry. The sample was not detecting macrolide antibiotics. Four macrolides were spiked in chicken sample before sample preparation process. Before preconcentration with HF-LPME method, the analysis process is needed the extraction of analytes from chicken because of its complicated sample. Various extraction methods were studied to extract the four macrolide antibiotics from poultry sample. In addition, the purpose of extraction method is to be suitable to be combined with the HF-LPME process. The four analytes were extracted from the samples and preconcentrated with optimized HF-LPME conditions followed by analysis with LC-MS/MS. The extraction procedures were divided into five methods by the applied extracting solution. The extracting solutions were optimized donor solution, meta-phosphoric acid-methanol, McIlvaine buffer, trichloroacetic acid, and KH_2PO_4-ACN. The last four extracting solutions were adapted from methods for the extraction of various antibiotics from animal products [Meta-phosphosphoric acid-methanol *(15),(44),(45)*, McIlvaine buffer *(16),(46)*, trichloroacetic acid *(47),(48)* , KH_2PO_4-ACN *(11)*].

3.7.2.1 Method I: Donor solution

This method employed optimized donor solution from HF-LPME experiment as extracting solution in the extraction of four macrolides from chicken sample. The procedure of the extraction by this method was carried out as follows.

3.7.2.1.1. Blended: A chicken sample was sliced into smaller pieces, grinded, and blended with blender

3.7.2.1.2. Weighed: 5 g of minced sample were weight in a vial.

3.7.2.1.3. Spiked standard: Sample was spiked at 1 µg/L of analytes with 100 µg/L mixture solution of standard macrolides.

3.7.2.1.4. Kept in dark: The spiked sample was kept in dark for 30 minutes.

3.7.2.1.5. Added extracting solution: 20 mL donor solution were added into the spiked sample.

3.7.2.1.6. Agitation: The solution was shaked for 10 minutes to extract analytes from sample.

3.7.2.1.7. Extra process: After shaking, the extracted solution was studied in three pathways. Each pathway was studied with two replicates.

Pathway I: The extracted solution was forwarded to preconcentration step.

Pathway II: The extracted solution was left to stand for 30 min before forwarded to preconcentration step.

Pathway III: The extracted solution was centrifuged for 10 minutes at 8000 rpm and 18 ml of supernatant were taken to preconcentration step.

3.7.2.1.8. Preconcentration: pH of the extracts was adjusted to 8.0 and the solutions were used as donor for preconcentration with the proposed HF-LPME procedure using optimized conditions displayed in Table 4.10.

3.7.2.1.9. Analysis: The preconcentrated solutions were analyzed with LC-MS/MS.

The results of extraction with method I were determined as seen in Table 4.17.

3.7.2.2 Method II: Meta-phosphoric acid-methanol

This method employed meta-phosphoric acid-methanol as extracting solution in the extraction of four macrolides from chicken sample. The extraction procedure of this method was performed as follows.

3.7.2.2.1. Blended: A chicken sample was sliced into smaller pieces, grinded, and blended with blender

3.7.2.2.2. Weighed: 5 g of minced sample were weight in a vial.

3.7.2.2.3. Spiked standard: Sample was spiked at 1 µg/L of analytes with 100 µg/L mixture solution of standard macrolides.

3.7.2.2.4. Kept in dark: The spiked sample was kept in dark for 30 minutes.

3.7.2.2.5. Added extracting solution: 20 mL meta-phosphoric acid-methanol extracting solution was studied in six compositions. Each composition was studied with two replicates.

Composition I: 0.3% meta-phosphoric acid-methanol

Composition II: 0.5% meta-phosphoric acid-methanol

Composition III: 1% meta-phosphoric acid-methanol

Composition IV: 0.3% meta-phosphoric acid-methanol + optimized donor solution from HF-LPME experiment

Composition V: 0.5% meta-phosphoric acid-methanol + optimized donor solution from HF-LPME experiment

Composition VI: 1.0% meta-phosphoric acid-methanol + optimized donor solution from HF-LPME experiment

3.7.2.2.6. Agitation: The solutions of each extracting solution composition were shaked for 10 minutes to extract analytes from sample.

3.7.2.2.7. Extra process: After shaking, the extracted solutions were centrifuged for 10 minutes at 8000 rpm and 18 ml of supernatant were taken to preconcentration step.

3.7.2.2.8. Preconcentration: pH of the extracts was adjusted to 8.0 and the solutions were used as donor for preconcentration with the proposed HF-LPME procedure using optimized conditions displayed in Table 4.10.

3.7.2.2.9. Analysis: The preconcentrated solutions were analyzed with LC-MS/MS.

The results of extraction with method II were determined as seen in Table 4.18.

3.7.2.3 Method III: McIlvaine buffer

This method employed McIlvaine buffer (citric acid monohydrate + Na_2HPO_4 + Na_2EDTA) as extracting solution in the extraction of four macrolides from chicken sample. The procedure of the extraction by this method was performed as follows.

3.7.2.3.1. Blended: A chicken sample was sliced into smaller pieces, grinded, and blended with blender.

3.7.2.3.2. Weighed: 5 g of minced sample were weight in a vial.

3.7.2.3.3. Spiked standard: Sample was spiked at 1 µg/L of analytes with 100 µg/L mixture solution of standard macrolides.

3.7.2.3.4. Kept in dark: The spiked sample was kept in dark for 30 minutes.

3.7.2.3.5. Added extracting solution: 20 mL McIlvaine buffer extracting solution were studied in two compositions. Each composition was studied with two replicates.

Composition I: McIlvaine buffer

Composition II: McIlvaine buffer + optimized donor solution from HF-LPME experiment

3.7.2.3.6. Agitation: The solutions of each extracting solution composition were shaked for 10 minutes to extract analytes from sample.

3.7.2.3.7. Extra process: After shaking, the extracted solutions were centrifuged for 10 minutes at 8000 rpm and then 18 ml of supernatant were taken to preconcentration step.

3.7.2.3.8. Preconcentration: pH of the extracts was adjusted to 8.0 and the solutions were used as donor for preconcentration with the proposed HF-LPME procedure using optimized conditions displayed in Table 4.10.

3.7.2.3.9. Analysis: The preconcentrated solutions were analyzed with LC-MS/MS.

The results of extraction with method III were determined as seen in Table 4.19.

3.7.2.4 Method IV: Trichloroacetic acid (TCA)

This method employed TCA as extracting solution in the extraction of four macrolides from chicken sample. The procedure of the extraction by this method was carried out as follows.

3.7.2.4.1. Blended: A chicken sample was sliced into smaller pieces, grinded, and blended with blender.

3.7.2.4.2. Weighed: 5 g of minced sample were weight in a vial.

3.7.2.4.3. Spiked standard: Sample was spiked at 1 µg/L of analytes with 100 µg/L mixture solution of standard macrolides.

3.7.2.4.4. Kept in dark: The spiked sample was kept in dark for 30 minutes.

3.7.2.4.5. Added extracting solution: 20 mL TCA extracting solution were studied in four compositions. Each composition was studied with two replicates.

Composition I: TCA

Composition II: TCA + McIlvaine buffer

Composition III: TCA + optimized donor solution from HF-LPME experiment

Composition IV: TCA + McIlvaine buffer+ optimized donor solution from HF-LPME experiment

3.7.2.4.6. Agitation: The solutions of each extracting solution composition were shaked for 10 minutes to extract analytes from sample.

3.7.2.4.7. Extra process: After shaking, the extracted solutions were centrifuged for 10 minutes at 8000 rpm and then 18 ml of supernatant were taken to preconcentration step.

3.7.2.4.8. Preconcentration: pH of the extracts was adjusted to 8.0 and the solutions were used donor for preconcentration with the proposed HF-LPME procedure using optimized conditions displayed in Table 4.10.

3.7.2.4.9. Analysis: The preconcentrated solutions were analyzed with LC-MS/MS.

The results of extraction with method IV were determined as seen in Table 4.20.

3.7.2.5 Method V: KH_2PO_4-ACN

This method employed KH_2PO_4-ACN as extracting solution in the extraction of four macrolides from chicken sample. The procedure of the extraction by this method was performed as follows.

3.7.2.5.1. Blended: A chicken sample was sliced into smaller pieces, grinded, and blended with blender.

3.7.2.5.2. Weighed: 5 g of minced sample were weight in a vial.

3.7.2.5.3. Spiked standard: Sample was spiked at 1 µg/L of analytes with 100 µg/L mixture solution of standard macrolides.

3.7.2.5.4. Kept in dark: The spiked sample was kept in dark for 30 minutes.

3.7.2.5.5. Added extracting solution: 20 mL of KH_2PO_4-ACN extracting solution were studied in two compositions. Each composition was studied with two replicates.

Composition I: KH_2PO_4-ACN

Composition II: KH_2PO_4-ACN + optimized donor solution from HF-LPME experiment

3.7.2.5.6. Agitation: The solutions of each extracting solution composition were shaked for 10 minutes to extract analytes from sample.

3.7.2.5.7. Extra process: After shaking, the extracted solutions were centrifuged for 10 minutes at 8000 rpm and then 18 ml of supernatant were taken to preconcentration step.

3.7.2.5.8. Preconcentration: pH of the extracts was adjusted to 8.0 and the solutions were used as donor for preconcentration with the proposed HF-LPME procedure using optimized conditions displayed in Table 4.10.

3.7.2.5.9. Analysis: The preconcentrated solutions were analyzed with LC-MS/MS.

The results of extraction with method V were determined as seen in 4.21.

3.7.3 Method performance in water and poultry sample application

The recovery and limit of detection were studied to observe the ability of HF-LPME method in the application with real sample.

3.7.3.1 Water sample application

3.7.3.1.1 Recovery

Four macrolide antibiotics were spiked 20 µg/L in water sample and investigated in eight replicates under optimized HF-LPME parameters. The comparison between observed concentration and spiked concentration lead to the recovery of analytes. The observed concentration was determined from the calculation of obtained peak area in the regression equation from standard calibration curve and the average value of eight calculated concentrations was used to represent the observed concentration. The recoveries (%) of spiked four analytes in water sample are presented in Table 4.22.

3.7.3.1.2 Limit of detections (LODs)

LODs refers to the method lowest concentration of analyte detected. From chromatogram, the limits of detection were calculated as chromatographic signal (peak height) being three times higher than background noise (S/N = 3). The chromatographic signal was observed from extraction of the spiked concentration of each standard (20 µg/L) in water sample under optimized HF-LPME condition in eight replicates The LODs of four macrolides in the application of HF-LPME method in water sample are shown in Table 4.22.

3.7.3.2 Poultry sample application

3.7.3.2.1 Recovery

Four macrolide antibiotics were spiked 20 µg/L in poultry muscle sample and investigated in eight replicates under optimized HF-LPME parameters. The comparison between observed concentration and spiked concentration lead to the recovery of analytes. The observed concentration was determined from the calculation of obtained peak area in the regression equation from standard calibration curve and the average value of eight calculated concentrations was used to represent the observed

concentration. The recoveries (%) of spiked four analytes in poultry muscle sample are presented in Table 4.23.

3.7.3.2.2 Limit of detections (LODs)

LODs refers to the method lowest concentration of analyte detected. From chromatogram, the limits of detection were calculated as chromatographic signal (peak height) being three times higher than background noise (S/N = 3). The chromatographic signal was observed from extraction of the spiked concentration of each standard (20 µg/L) in poultry muscle sample under optimized HF-LPME condition in eight replicates The LODs of four macrolides in the application of HF-LPME method in poultry sample are shown in Table 4.23.

CHAPTER IV

RESULTS AND DISCUSSION

Macrolide antibiotics (ERY, SPI, TIL and TYL) are weak basic compounds with ionizable functionalities as seen in their chemical structures and pKa values from Table 1.3. Their low-level residues in food-producing animal may pose health risks to human. A HF-LPME method was developed to be an alternative technique for extraction, preconcentration, and clean-up purposes. Compared with traditional techniques such as LLE and SPE, HF-LPME can overcome some drawbacks in both techniques especially in terms of enrichment ability and organic solvent consumption. In this work, three-phase HF-LPME was investigated in the determination of ERY, SPI, TIL, and TYL in poultry muscle.

4.1 HF-LPME optimization

HF-LPME method was studied to define optimal preconcentration condition. With three-phase mode, ERY, SPI, TIL, and TYL spiked in aqueous donor solution were extracted through immobilized organic solvent in hollow fiber pore and back-extract to aqueous acceptor solution. Parameters affecting the HF-LPME ability, such as immersion time, organic solvent type, organic solvent composition, donor type and pH, the acceptor type and pH, and extraction time were optimized.

The enrichment factor (EF) was utilized to evaluate the experimental results from each parameter optimization. EF was calculated from Eq. 10 in Chapter II.

4.1.1 The optimization of immersion time

Immersion time is the time used for impregnation of organic solvent in the hollow fiber pores before extraction. The organic solvent immobilized with capillary force in the fiber pores is performed as a thin layer of organic phase. Therefore, the amount of immobilized organic solvent is necessary to provide sufficient extraction of analytes. The immersion time was optimized to completely impregnate the organic solvent in the pores. This work investigated immersion times of 5, 15, 30, 60, 120, and 180 min and the results are shown in Table 4.1 and Figure 4.1. The enrichment factor increased with longer immersion time until 60 min then decrease gradually owing to organic solvent leak out from fiber wall after saturation of organic solvent. For short immersion time, the low EF resulted from the incomplete addition of organic solvent in the porous hollow fiber, and this effect lead to a higher standard deviation range than for extended immersion time. The highest enrichment factor of four macrolide antibiotics was obtained at 60 minutes of immersion time with acceptable range of standard deviations. For different extraction methods, optimal immersion time was varied because of organic solvent chemistry and hollow fiber geometry.

Table 4.1 Effect of different immersion time on the enrichment factor of ERY, SPI, TIL, and TYL.

Immersion time (min)	Average EF ± S.D. (n=3)			
	ERY	SPI	TIL	TYL
5	0.33 ± 0.14	0.47 ± 0.40	0.21 ± 0.09	0.54 ± 0.12
15	0.58 ± 0.28	2.68 ± 1.01	1.11 ± 0.23	1.15 ± 1.04
30	0.47 ± 0.39	3.35 ± 0.84	2.84 ± 1.72	2.18 ± 0.27
60	1.89 ± 0.47	6.04 ± 0.77	3.32 ± 0.48	3.74 ± 0.66
120	1.21 ± 1.24	5.49 ± 0.36	3.19 ± 0.56	3.97 ± 0.23
180	0.98 ± 0.38	4.18 ± 0.93	2.62 ± 1.34	3.02 ± 0.42

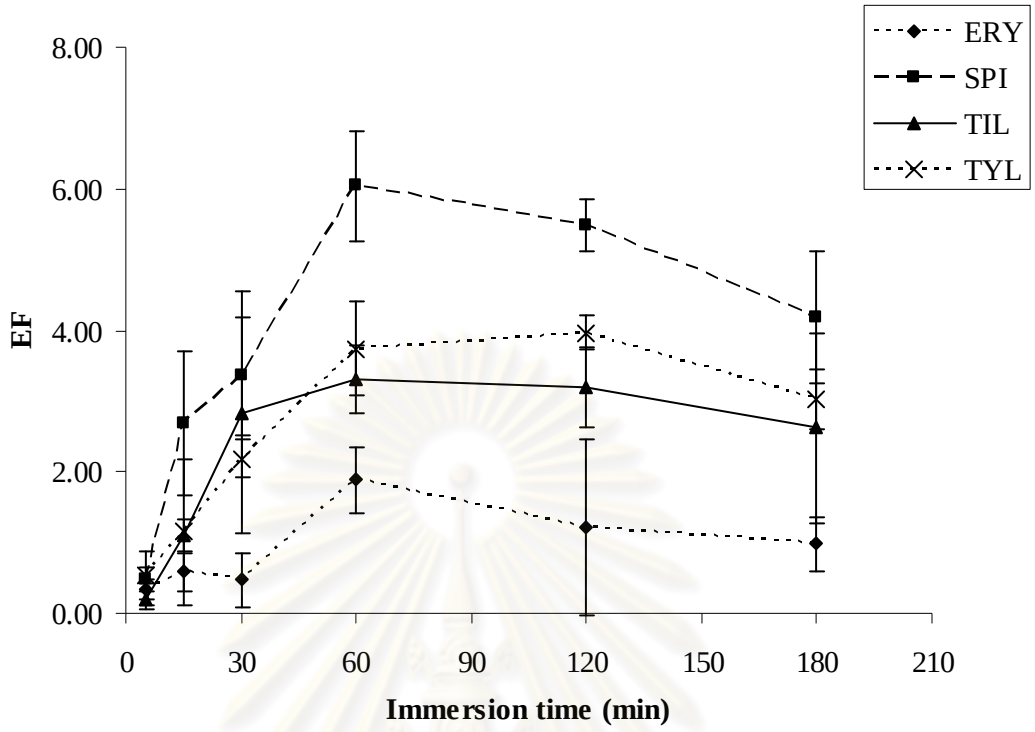


Figure 4.1 The influence of immersion time on enrichment factor.

4.1.2 The optimization of organic solvent type

In three-phase HF-LPME, the organic solvent impregnated in fiber pores is the extracting phase for the analyte in the donor phase and is used to promote the diffusion of analyte from donor to acceptor solution. The type of organic solvent influences the method extraction efficiency. The solvent chosen must be immiscible with water, compatible with the used type of hollow fiber, and highly stable in the pores. Polar and non-polar solvents were optimized including 1-octanol, 1-decanol, dihexyl ether, undecane, dodecane, and toluene. The results are shown in Table 4.2 and Figure 4.2. The four macrolides were almost not enriched in non-polar solvents, and the highest enrichment was obtained with dihexyl ether, which may be due to corresponding analytes solubility and solvent polarity. From macrolide properties in Table 1.3, most analytes are hydrophilic compounds with ionizable functionalities. This may lead to the extractability of macrolides with polar organic solvents. In addition, not optimized experimental parameters may result in low enrichment factor and the wide range of

analyte solubility combined with the complex structure of four macrolides led to a difficult extraction with a single solvent. Other parameters of HF-LMPE were optimized as discussed later. The selected organic solvent was dihexyl ether.

Table 4.2 Effect of different organic solvents on enrichment factor of ERY, SPI, TIL, and TYL.

Organic solvent	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
1-octanol	1.98 ± 0.23	3.76 ± 0.45	3.33 ±1.09	1.67 ± 0.14
1-decanol	2.89 ± 0.10	5.89 ± 1.22	6.05 ± 0.22	3.76 ± 0.27
dihexyl ether	5.96 ± 0.41	8.57 ± 0.51	8.49 ± 0.57	7.17 ± 0.51
undecane	0.80 ± 0.14	0.00 ± 0.00	0.07 ± 0.01	0.00 ± 0.00
dodecane	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
toluene	0.00 ± 0.00	0.14 ± 0.08	0.64 ± 0.13	0.16 ± 0.06

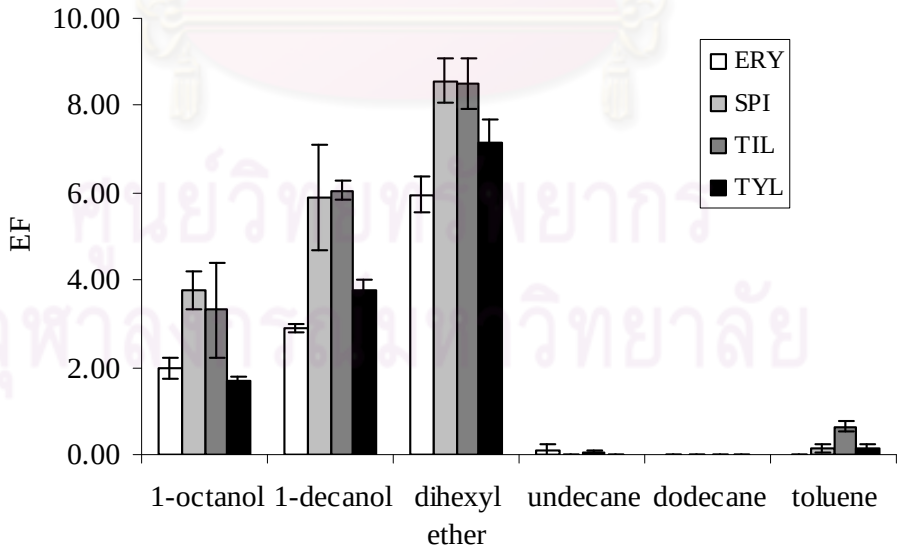


Figure 4.2 The influence of organic solvent type on enrichment factor.

4.1.3 The optimization of organic solvent composition

To improve the extraction of analytes in three-phase system, carrier-mediate mode of HF-LPME was applied. In this work, carrier was added to the organic solvent for the formation of ion-pairs between carrier and analytes at the sample-organic interface. The basic principle of carrier-mediated HF-LPME was described in Chapter II. The three carriers studied (Aliquat 336, D2EHPA, LIX 84) were filled into dihexyl ether (DHE) to increase the extraction efficiency. The effect of different carriers at 10 % carrier in DHE is shown in Table 4.3 and Figure 4.3.

In the experiment, basic donor solution was used to obtain macrolide antibiotics in their neutral and negative charged form as considered from their pK_a values (7.4-8.8) and their basic dimethylamine structure [$-N(CH_3)_2$] as seen in Table 1.3. Among the three studied carriers, Aliquat 336 effectively formed ion pairs with neutral and negative charged analytes compared to D2EHPA and LIX 84. This highest enrichment tendency of Aliquat 336 is related to its cationic characteristic and its permanent positive charge $(R_3NCH_3)^+$ in all pH ranges. Aliquat 336 easily formed ion pairs with negative charged macrolides and efficiently transferred the four macrolides to the acceptor phase. The mechanism of the carrier-mediate in this work is proposed in Figure 4.4.

Table 4.3 Effect of different carriers on enrichment factor of ERY, SPI, TIL, and TYL.

Carrier	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
Aliquat 336	9.66 ± 0.22	15.70 ± 0.67	12.21 ± 1.28	11.98 ± 0.88
D2EHPA	4.13 ± 0.34	7.24 ± 1.26	6.70 ± 0.19	2.44 ± 1.47
LIX 84	7.73 ± 1.21	9.98 ± 0.25	8.55 ± 2.07	5.13 ± 0.29

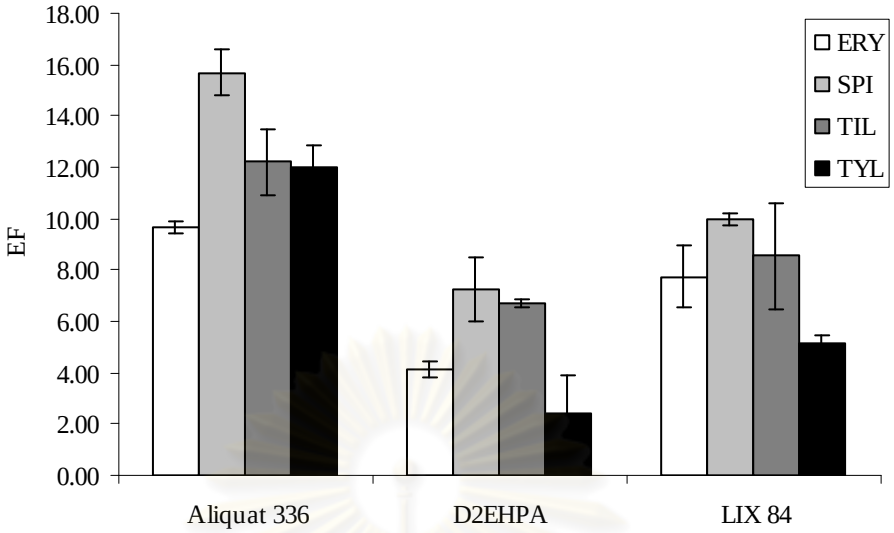


Figure 4.3 The influence of carrier type on enrichment factor.

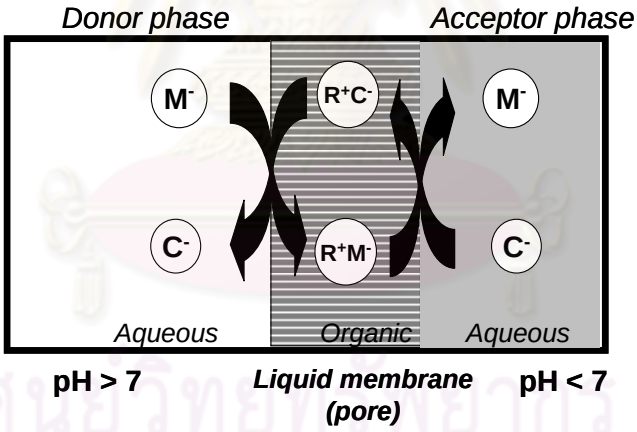


Figure 4.4 Mechanism of carrier-mediated mode in this work

(M^- = Macrolide, R^+ = Aliquat 336, C^- = acetate ion from acceptor)

Due to Aliquat 336 yielded the highest enrichment factor, the content of Aliquat 336 was optimized at 5%, 10%, 20%, 30%, and 40% in DHE and the results are presented in Table 4.4 and Figure 4.5. The enrichment factor from low content of Aliquat 336 in DHE led to low extraction efficiency because the amount of carrier was insufficient to

form ion-pair complex with analytes. On the other hand, high content of carrier resulted in high viscosity of organic phase inside hollow fiber, which decreased the flux of the compound through the membrane. Therefore, 20% Aliquat 336 in DHE was chosen for organic phase.

Table 4.4 Effect of Aliquat 336 content on enrichment factor of ERY, SPI, TIL, and TYL.

% Carrier in DHE	Average EF ± S.D. (n=3)			
	ERY	SPI	TIL	TYL
5	2.03 ± 1.37	5.98 ± 1.51	3.57 ± 0.26	2.86 ± 1.28
10	8.41 ± 0.54	14.11 ± 0.46	12.08 ± 2.97	10.25 ± 1.25
20	12.59 ± 2.32	22.28 ± 3.39	20.43 ± 1.05	18.22 ± 2.15
30	7.26 ± 1.28	15.34 ± 1.82	13.51 ± 1.12	9.87 ± 3.53
40	4.96 ± 0.77	9.18 ± 2.69	7.22 ± 3.45	5.41 ± 2.34

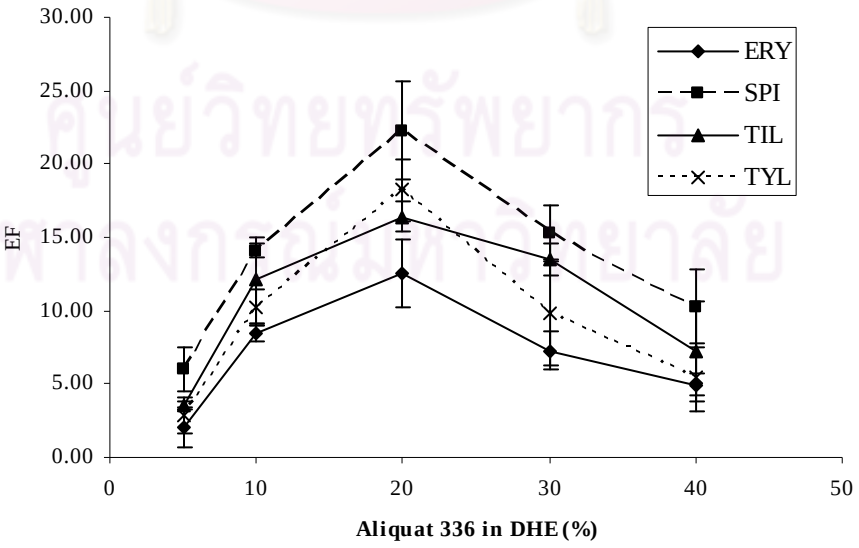


Figure 4.5 The influence of Aliquat 336 content on enrichment factor.

4.1.4 The optimization of donor type

After organic solvent optimization, donor or sample solution pH was adjusted with various solutions. The selected donor solution should not react with analytes. Three solutions (sodium tetraborate, sodium hydrogen phosphate, and sodium carbonate) were studied as donor at pH 9.0. The results are shown in Table 4.5 and Figure 4.6. The enrichment obtained was not significancly different between the three solutions; therefore, the solutions did not affect the enrichment or extraction efficiency. The solution was only used for pH adjustment solution. Sodium tetraborate was selected because of its small variation.

Table 4.5 Effect of donor type on enrichment factor of ERY, SPI, TIL, and TYL.

Donor	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
Sodium tetraborate	8.12 ± 0.48	21.59 ± 0.85	22.18 ± 1.34	18.75 ± 0.97
Sodium hydrogen phosphate	7.99 ± 0.65	19.74 ± 1.23	23.20 ± 1.61	14.92 ± 1.48
Sodium carbonate	6.82 ± 1.59	21.38 ± 0.56	20.93 ± 2.07	17.59 ± 1.34

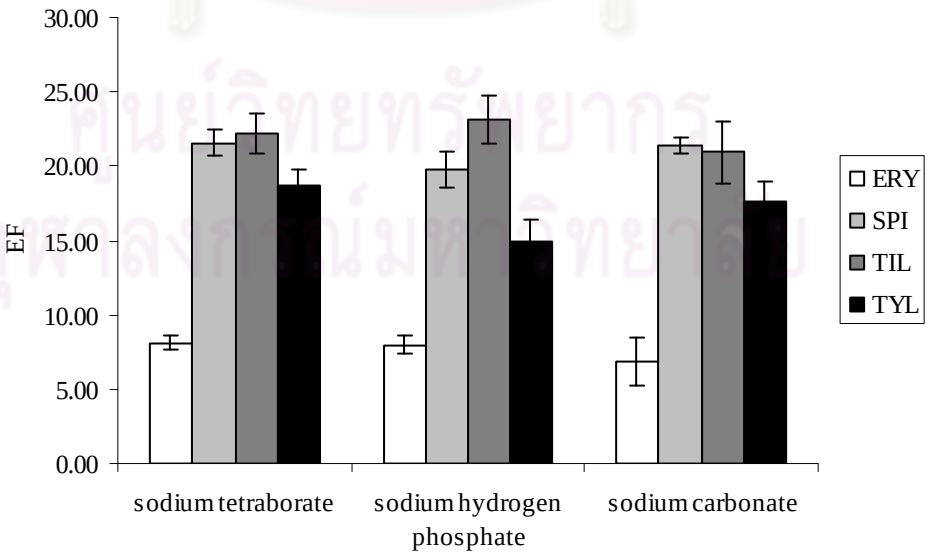


Figure 4.6 The influence of donor type on enrichment factor.

4.1.5 The optimization of donor pH

Donor pH is an essential parameter affecting the method extraction efficency. The pH of donor or sample solution should be higher than analyte's pKa in order to promote analytes in their appropriate species for the extraction into organic phase. From preliminary test, basic donor solution was suitable in case of macrolide antibiotics. Consequently, pH of donor solution was investigated at 7.0, 8.0, 9.0, 10.0, and 11.0. Table 4.6 and Figure 4.1 show the enrichment results. pH 8.0 was found to be most suitable to extract macrolides as considered from the results and pKa of the four analytes. At pH below 8.0, most analyte were in charged form, which results in a difficult transfer to the hydrophobic (organic) phase. However, if analytes were in neutral form at donor pH higher than 8.0, the carrier could not form ion-pair complex with neutral macrolides. Therefore, the optimized donor pH was 8.0.

Table 4.6 Effect of donor pH on enrichment factor of ERY, SPI, TIL, and TYL.

Donor pH	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
7.0	3.89 ± 0.58	12.87 ± 1.68	15.63 ± 2.39	9.95 ± 2.88
8.0	12.35 ± 2.04	32.18 ± 2.46	30.71 ± 1.54	26.27 ± 4.56
9.0	9.14 ± 2.98	25.97 ± 1.87	24.46 ± 0.97	20.55 ± 2.16
10.0	5.28 ± 1.33	17.88 ± 3.96	14.52 ± 1.62	15.43 ± 1.77
11.0	2.97 ± 1.02	10.23 ± 2.24	12.34 ± 2.59	6.26 ± 1.58

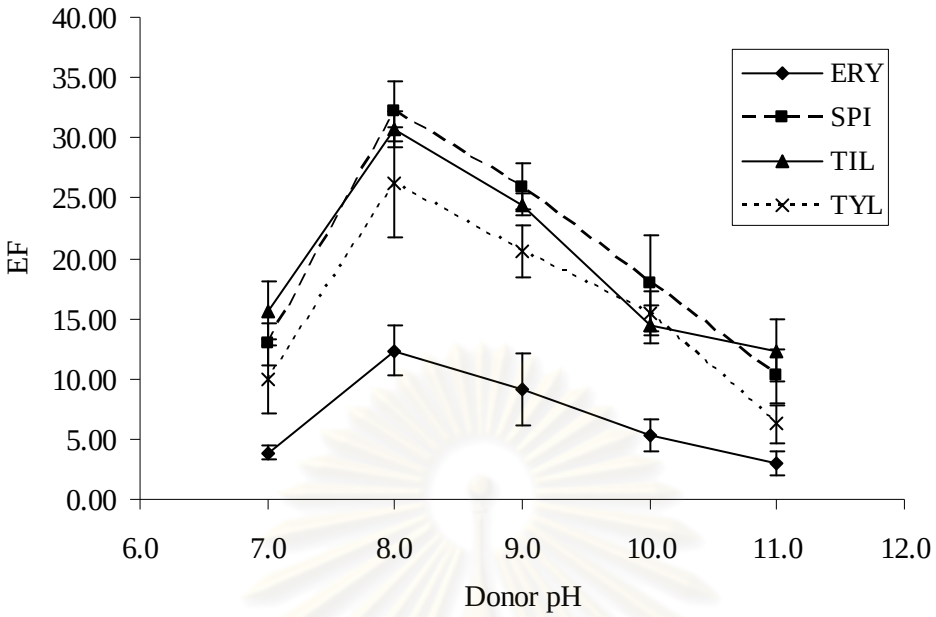


Figure 4.7 The influence of donor pH on enrichment factor.

4.1.6 The optimization of acceptor type

The selection of acceptor type was based on the compatibility with the analytical instrument. In this work, LC-ESI-MS/MS was employed, so the acceptor solution should be a volatile compound with no extremely low pH to protect the instrument and prevent clogging in the LC-MS interface. Acceptors studied were ammonium acetate, ammonium formate, succinic acid, and trichloroacetic acid. Weak acidic acceptors were selected over strong acidic ones to avoid a dilution step, which would have been necessary for strong acidic acceptors. The optimization results are presented in Table 4.7 and Figure 4.8. The enrichment factors of ammonium acetate and ammonium formate as acceptor solution gave satisfactory results. This may result from the fact that these two compounds were prepared in form of buffer solution and, therefore, could keep a constant pH in the acceptor solution. Therefore, analytes were efficiently trapped in ammonium acetate as acceptor solution at constant pH.

Table 4.7 Effect of acceptor type on enrichment factor of ERY, SPI, TIL, and TYL.

Acceptor type	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
ammonium acetate	11.97 ± 2.05	34.88 ± 2.48	32.46 ± 1.13	30.56 ± 4.74
ammonium formate	9.85 ± 3.84	30.27 ± 1.89	21.35 ± 3.95	17.83 ± 2.71
succinic acid	1.56 ± 0.69	4.65 ± 1.44	2.64 ± 1.84	1.85 ± 0.63
trichloroacetic acid	1.42 ± 0.23	1.82 ± 0.26	1.90 ± 1.14	1.13 ± 1.02

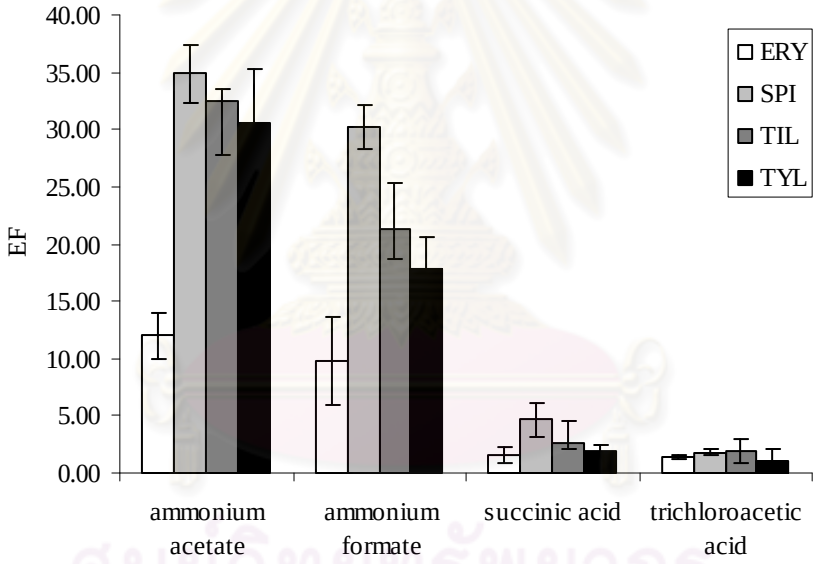


Figure 4.8 The influence of acceptor type on enrichment factor.

4.1.7 The optimization of acceptor pH

The ion-pair complexes between carrier and analyte were separated and released the analytes into acceptor phase. The pH of acceptor is an important parameter because it influences the potential to trap the analyte in the acceptor solution. The adjustment of acceptor pH ensures the extraction of analytes from organic phase and prevents analyte

to be trapped in the organic phase by changing the analyte to a charged form. The acceptor pH should be lower than pKa of analytes. The results of acceptor pH varied from 3.0 to 6.0 are shown in Table 4.8 and Figure 4.9. The acceptor pH higher than 4.0 did not provide a sufficient gradient concentration of counter-ion to create diffusion between two aqueous phases, while acceptor pH below 4.0 provided low enrichment because a high amount of protons may interfere ion-pairs formation. In addition, low pH is not suitable for LC-MS/MS system. An acceptor pH of 4.0 was chosen for the extraction of the four macrolide antibiotics..

Table 4.8 Effect of acceptor pH on enrichment factor of ERY, SPI, TIL, and TYL.

Acceptor pH	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
3.0	2.84 ± 0.26	4.55 ± 1.02	4.13 ± 0.48	3.74 ± 2.87
4.0	12.21 ± 1.74	27.46 ± 2.47	29.83 ± 1.24	23.62 ± 3.52
5.0	10.72 ± 1.08	24.91 ± 2.98	27.18 ± 1.57	19.94 ± 1.88
6.0	10.11 ± 2.51	21.27 ± 1.54	18.22 ± 3.23	17.31 ± 1.92

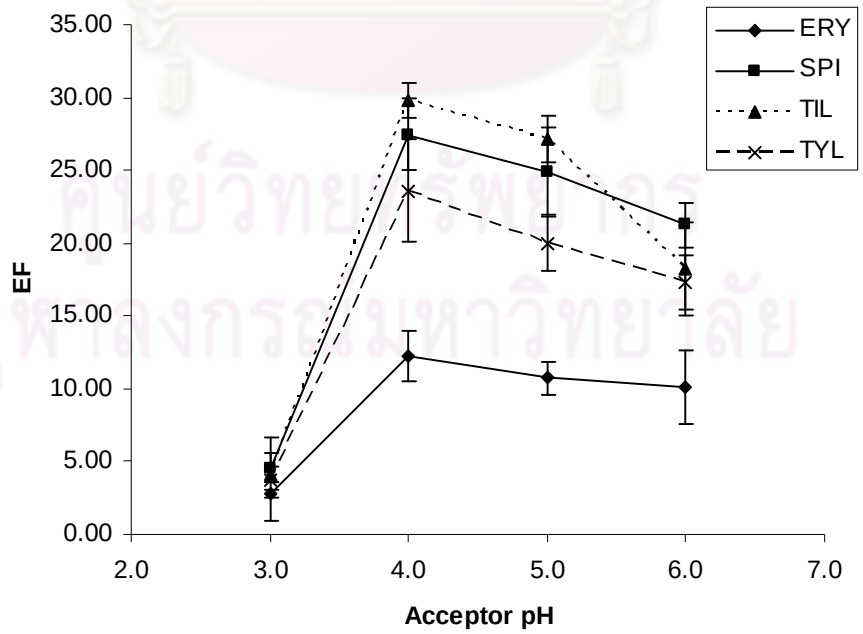


Figure 4.9 The influence of acceptor pH on enrichment factor.

4.1.8 The optimization of extraction time

HF-LPME is a non-exhaustive method and is based on time-dependent equilibrium process. At equilibrium, the enrichment factor reaches a maximum because then the highest partition coefficient of analytes between the three phases is obtained. Therefore, the extraction is optimized when the partitioning process reaches equilibrium and the highest enrichment is obtained. Extraction times of 5, 15, 30, 45, 60, 90, and 120 minutes were investigated and the results are showed in Table 4.9 and Figure 4.10. The enrichment factor increased when the extraction time extended. When reaching the equilibrium point, the enrichment factors were stable. The obtained results indicated that the equilibrium time for this method is 60 min.

Table 4.9 Effect of extraction time on enrichment factor of ERY, SPI, TIL, and TYL.

Extraction time (min)	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
5	1.06 ± 0.19	2.16 ± 1.04	1.25 ± 0.48	0.86 ± 0.20
15	3.22 ± 0.52	9.33 ± 3.37	4.12 ± 0.87	2.34 ± 1.13
30	9.78 ± 2.01	21.14 ± 1.59	19.51 ± 3.57	15.61 ± 1.54
45	13.02 ± 1.24	30.48 ± 2.78	24.48 ± 1.44	26.78 ± 3.67
60	12.85 ± 2.48	37.15 ± 1.64	28.97 ± 2.48	31.04 ± 2.52
90	10.51 ± 4.95	36.54 ± 2.17	26.18 ± 2.97	30.17 ± 3.10
120	11.65 ± 3.74	36.21 ± 1.09	28.41 ± 1.83	32.49 ± 1.86

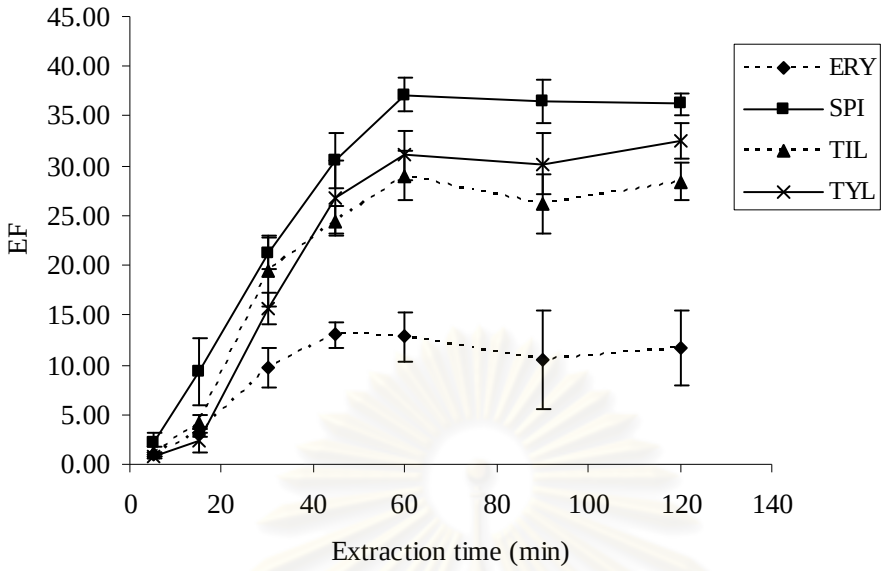


Figure 4.10 The influence of extraction time on enrichment factor.

Various parameters affecting the efficiency of extracting ERY, SPI, TIL, and TYL by HF-LPME were optimized and a summary of the optimal conditions is given in Table 4.10.

Table 4.10 The optimum conditions of HF-LPME.

HF-LPME parameter	Condition
Hollow fiber length	12 cm
Immersion time	60 minutes
Organic phase	20% Aliquat 336 in DHE
Donor solution	Sodium tetraborate pH 8.0
Donor volume	20.0 mL
Acceptor solution	Ammonium acetate pH 4.0
Acceptor volume	20.0 µL
Extraction time	60 minutes

4.2 Method Validation

The HF-LPME method was validated to prove the effectiveness in the application. The method validation was done with optimized condition of HF-LPME in water.

4.2.1 Standard calibration curve

Standard calibration curve was used to determine analytes concentration in sample solution because HF-LPME is a non-exhaustive method and is based on equilibrium process. Calibration curves of standard ERY, SPI, TIL, and TYL at various spiked concentrations in the range of 0.5-50.0 µg/L with three replicate analysis in HF-LPME method are displayed in Figure 4.11 (ERY), Figure 4.12 (SPI), Figure 4.13 (TIL), and Figure 4.14 (TYL). In the calibration curves peak area was plotted over the spiked standard concentration. Table 4.11 indicates the summarized information of calibration curves for ERY, SPI, TIL, and TYL.

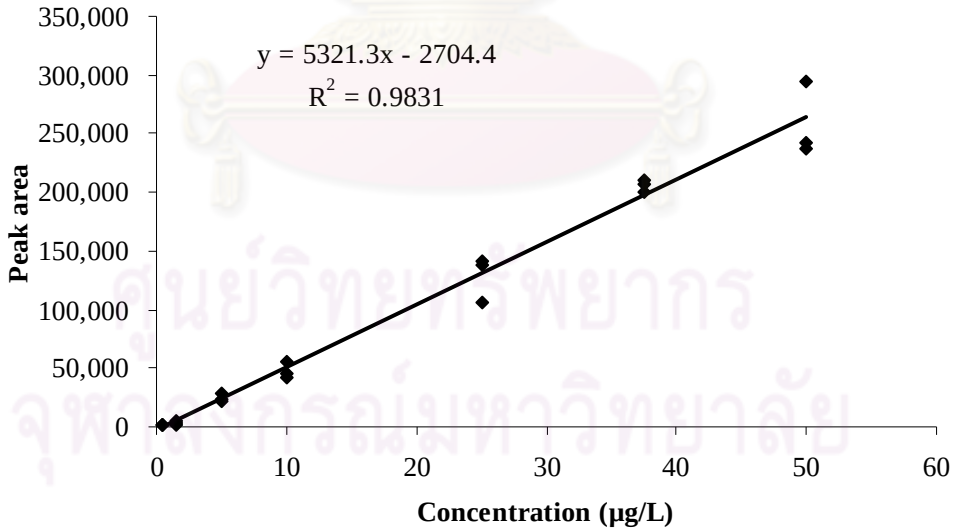


Figure 4.11 Standard calibration curve of erythromycin (ERY) in HF-LPME analysis.

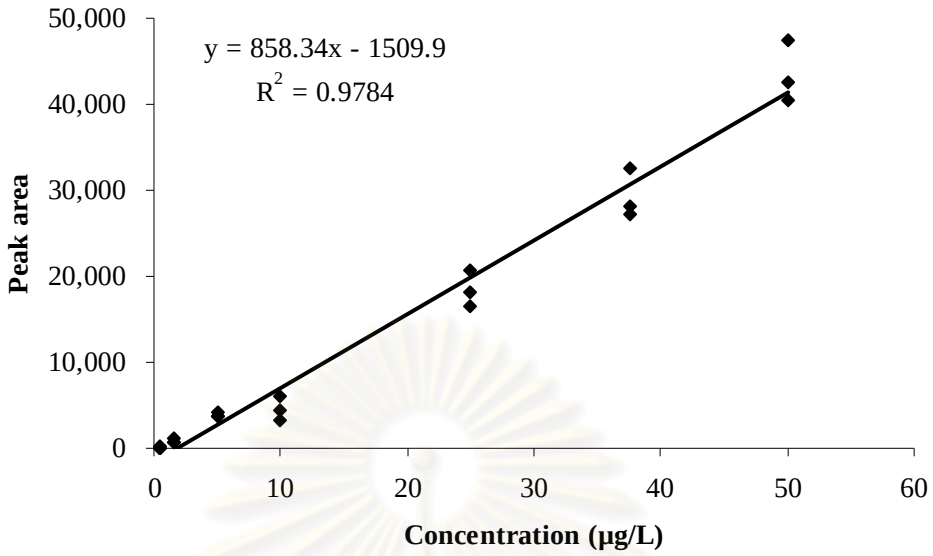


Figure 4.12 Standard calibration curve of spiramycin (SPI) in HF-LPME analysis.

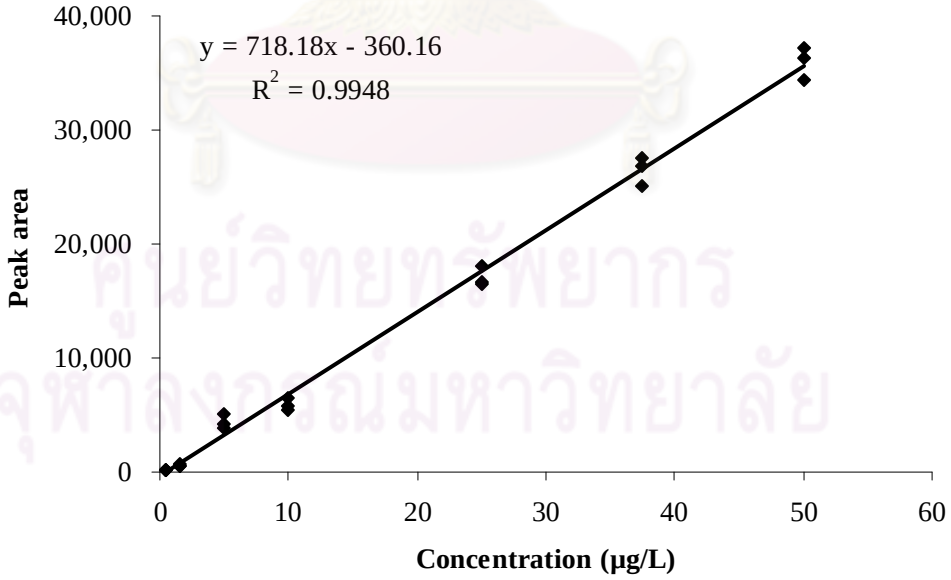


Figure 4.13 Standard calibration curve of tilmicosin (TIL) in HF-LPME analysis.

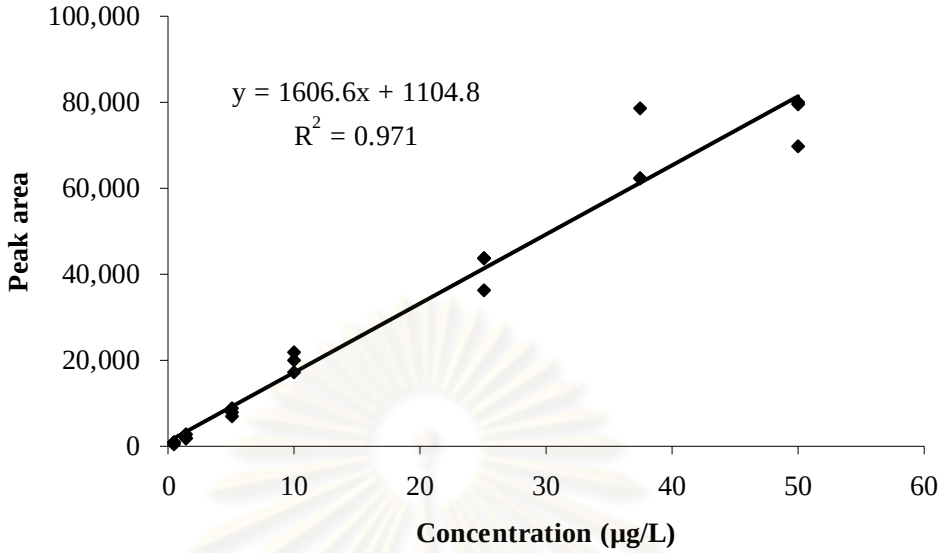


Figure 4.14 Standard calibration curve of tylosin (TYL) in HF-LPME analysis.

Table 4.11 Slope, y-intercept, and correlation coefficient from standard calibration curve of ERY, SPI, TIL and TYL in HF-LPME.

	ERY	SPI	TIL	TYL
Slope	5321.3	858.34	718.18	1606.6
y-intercept	-2704.4	-1509.9	-360.16	1104.8
Correlation coefficient (R^2)	0.9831	0.9784	0.9948	0.9710

4.2.2 Linearity

The linearity of method was derived from standard calibration curves of ERY, SPI, TIL, and TYL in HF-LPME with the concentration ranges of 0.5-50.0 µg/L and the correlation coefficients (R^2) represent the method linearity. The slope, y-intercept, and correlation coefficient (R^2) are listed in Table 4.11. The correlation coefficients (R^2)

ranged from 0.9710 to 0.9948. This method provided good linearity of four macrolide antibiotics in water with HF-LPME in low concentration ranges.

4.2.3 Limit of detections (LODs) and limit of quantifications (LOQs)

The method limits of detection were calculated from chromatographic signal (peak height) at three times higher than background noise (S/N=3). The lowest spiked concentration of each standard (0.5 µg/L) under optimized HF-LPME condition was employed to calculate LOD and the study was done in eight replicates. In the same way, the method limits of quantification were also calculated from chromatographic signal (peak height) but estimated at ten times higher than background noise (S/N=10). Eight replicates of LOQ were studied. The method LOD and LOQ are expressed in Table 4.12. There are regulations for macrolide antibiotic residue in water. Hence, the LOD and LOQ values in low µg/L level indicate the promise of method because macrolide antibiotics usually found in aquatic environment in ng/L to µg/L level.

Table 4.12 The limit of detections and limit of quantifications of ERY, SPI, TIL, and TYL in HF-LPME (n=8).

	Average value ± S.D. (n=8)			
	ERY	SPI	TIL	TYL
LODs (µg/L)	0.07 ± 0.05	0.14 ± 0.09	0.17 ± 0.06	2.28 ± 0.31
LOQs (µg/L)	0.40 ± 0.16	0.67 ± 0.22	0.43 ± 0.12	8.10 ± 0.84

4.2.4 Enrichment factor

The enrichment ability of this method under optimized HF-LPME conditions was determined in eight replicates and each analyte was spiked in water at 25 and 50 µg/L. After HF-LPME analysis, the enrichment factor from two spiking level was determined

as seen in Table 4.13. The results indicated the highest enrichment factors of the four macrolides with HF-LPME from two spiking levels are 13.10 (ERY), 38.54 (SPI), 30.24 (TIL), and 31.51 (TYL).

Table 4.13 The enrichment ability of ERY, SPI, TIL and TYL in HF-LPME (n=8).

Spiking level	Average EF ± S.D. (n=8)			
	ERY	SPI	TIL	TYL
25 µg/L	11.96 ± 4.59	34.03 ± 2.98	27.19 ± 5.23	27.43 ± 4.41
50 µg/L	12.38 ± 3.86	36.14 ± 6.54	30.57 ± 8.22	29.33 ± 2.35

4.2.5 Accuracy

The accuracy was reported in forms of recovery because HF-LPME is non-exhaustive method. The recovery is calculated from observed concentration and spiked concentration of analytes. The observed concentration derived from calculation of signal in regression equation from each standard calibration curve. The estimated recoveries were performed at 25 and 50 µg/L with eight replicates and the result was shown in Table 4.14.

Table 4.14 Recovery (%) of ERY, SPI, TIL, and TYL in HF-LPME (n=8).

Spiking level	% recovery ± S.D. (n=8)			
	ERY	SPI	TIL	TYL
25 µg/L	96.99 ± 4.13	112.68 ± 3.57	105.39 ± 3.72	91.58 ± 5.38
50 µg/L	89.09 ± 6.32	98.72 ± 5.32	102.99 ± 6.29	93.05 ± 3.07

4.2.6 Precision

The studied precision of this method was determined as intra-assay and intermediate precision. The intra-assay precision (within-day precision) was investigated in one day with eight replicates and the intermediate precision (between-day precision) was estimated from the results within two analytical days in eight replicates per day. In this work, precision was determined at 30 µg/L-spiked level of the four analytes under optimized HF-LPME conditions. The percent of relative standard deviations (%R.S.D) represented the intra-assay and the intermediate precision. The results are reported in Table 4.15. The % R.S.D of intra-assay precision were compared with calculated acceptable value of %R.S.D by Horwitz equation, which was 18.17% (at 30 µg/L). The % R.S.D. obtained in each day were in the range of 7.68 to 10.23 (day1) and 5.67 to 11.85 (day2). The intra-assay precision of this method was acceptable because the % R.S.D. values were not larger than the calculated value from Horwitz equation and overall R.S.D. values were also satisfactory. The % R.S.D of intermediate precision (n=2) determined the significant difference of result in two days by two-tailed F test. The calculated F value and critical F value (P=0.05) were shown in Table 4.15. Due to the calculated F values of four analytes were less than critical F value, the results (%R.S.D.) from two days are acceptable with no significance in difference.

Table 4.15 Relative standard deviation (%R.S.D.) and F-value of ERY, SPI, TIL, and TYL in HF-LPME (n=8).

	% R.S.D.			F-value	
	Day 1	Day 2	Overall	Calculated	Critical (P=0.05)
ERY	8.63	11.29	9.90	1.35	4.995
SPI	7.68	5.67	9.27	1.19	
TIL	9.29	7.53	8.22	1.25	
TYL	10.23	11.85	12.08	1.29	

4.3 The application of optimized HF-LPME method in water and poultry sample

From the validation, the performance of method was satisfactory and the optimized HF-LPME method was proved the effectiveness by the application of this method in real sample confronted the macrolide antibiotics residue problem; water and poultry sample.

After real sample analysis, the recovery and detection limits of two applications were investigated to show the capability of HF-LPME method in real sample application.

4.3.1 Water sample

Water sample is collected from Chaophaya River, Bangkok, Thailand. The sample is quite turbid and has many types of sediment which can act as matrix interference. Therefore, samples are allowed to stand overnight for sediment precipitation before analysis. Optimized HF-LPME was applied with real water sample analysis. The river water sample was filtered, spiked with four macrolides, and pH was adjusted with sodium tetraborate to pH 8.0 (optimized donor solution). Analytes were spiked 2, 8, and 20 µg/L in water sample. Each concentration was studied in three replicates. The enrichment factor and example LC-MS/MS chromatogram of these experiments are shown and illustrated in Table 4.16 and Figure 4.15. At the three low spiked concentration levels, the results indicated high enrichment factor with wide deviation ranges because of low concentration in the investigation. From the results obtained, the HF-LPME can efficiently enrich ERY, SPI, TIL, and TYL in water sample with a similar trend of highest enrichment factor from HF-LPME optimization condition and method validation results, even when spiking analytes at low concentrations which prove the effectiveness of HF-LPME method in the determination of four macrolides in real water sample.

Table 4.16 Enrichment factors of HF-LPME application in real water analysis.

Spiking level	Average EF ± S.D. (n=3)			
	ERY	SPI	TIL	TYL
2 µg/L	11.35 ± 4.52	31.30 ± 3.45	23.81 ± 5.33	31.34 ± 2.87
8 µg/L	14.15 ± 2.03	35.81 ± 4.85	19.78 ± 6.24	33.28 ± 3.07
20 µg/L	12.40 ± 1.68	33.14 ± 5.35	24.34 ± 2.69	31.92 ± 4.68

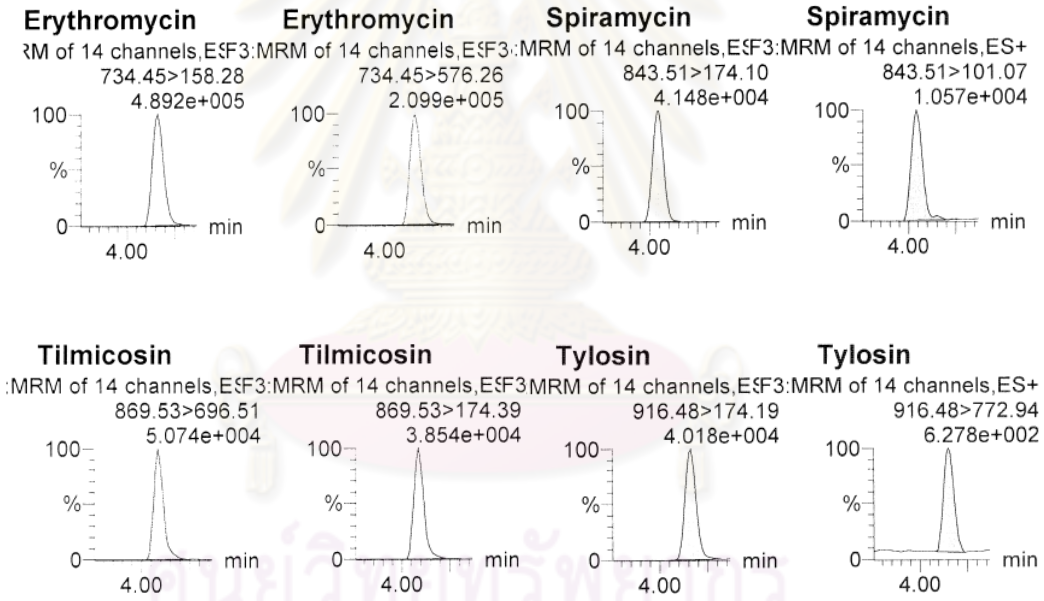


Figure 4.15 Example LC-ESI-MS/MS chromatogram of ERY, SPI, TIL and TYL spiking 20 µg/L in water sample after HF-LPME method at MS quantification and conformation transition.

4.3.2 Poultry sample

After optimum preconcentration conditions for HF-LPME of four macrolides were obtained, the method was validated. The application of HF-LPME method in real sample was successful in less complicated matrices such as river water sample. Additionally, the poultry sample was investigated to observe the ability of HF-LPME method in the complex matrices. Before preconcentration with optimized HF-LPME condition, the extraction is necessary step to separate analytes from poultry matrix. Therefore, the extraction method of analytes from poultry muscle was optimized. When analytes are extracted from real sample into extracting solution, this solution is further preconcentrated by HF-LPME method at optimum condition prior to LC-ESI-MS/MS analysis. Consequently, the extracting solution and extraction method should be simple and suitable to be combined with donor solution in HF-LPME. The extracting solution may be used as donor solution by itself or in solution modified with donor solution. The chicken sample was spiked with four analytes and extracted with five extraction methods classified by the method extracting solution as described in Chapter III. The results of each extraction methods are explained as follows.

4.3.2.1 Extraction Method I

The extracting solution of Method I was based on the donor solution from HF-LPME. The reason was the simplification of using the same extracting solution as donor phase with no modification. Sodium tetraborate pH 8.0 as HF-LPME donor solution was studied for extraction of macrolides from poultry. This donor solution was used as extracting solvent with three pathways of extraction. Firstly, after extracting the analytes into donor solution, this solution was immediately preconcentrated with HF-LPME. Secondly, after extracting the analytes into donor solution, this solution was left standing for 30 min before preconcentrated with HF-LPME. Thirdly, after extracting the analytes into donor solution, this solution was centrifuged and the supernatant was taken for preconcentration with HF-LPME procedure. The results were evaluated based on the enrichment factor as shown in Table 4.17.

From the results, the four analytes spiked at 1 mg/L could not be enriched or extracted with directly using donor solution as extracting solution with three pathways. Due to high protein and fat content in chicken sample, analytes were blocked to be enriched or extracted. However, the enrichment tendency could suggest that centrifugation is necessary to precipitate interferences or sample matrices and is better than the other pathways. A centrifugation step was used for further optimization.

Table 4.17 Effect of Method I extraction on enrichment factor of ERY, SPI, TIL, and TYL.

Method I	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
Pathway I	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
Pathway I	0.39 ± 0.02	0.49 ± 0.02	0.18 ± 0.11	0.29 ± 0.03
Pathway III	0.51 ± 0.14	0.76 ± 0.08	0.19 ± 0.05	0.96 ± 0.31

4.3.2.2 Extraction Method II

The extracting solution of Method II was based on meta-phosphoric acid-methanol. From various references, the utilization of meta-phosphoric acid-methanol as protein precipitating agent is well-known for extraction in animal products matrices. Due to extraction with the method I cannot extract or enrich analytes because of complex matrix, Method II was applied meta-phosphoric acid-methanol in extraction of macrolides from chicken sample. The various proportions of meta-phosphoric acid in methanol were modified with donor solution and optimized in six compositions. The meta-phosphoric acid proportions of 0.3%, 0.5% and 1.0% in methanol were investigated. Each proportion with donor solution (sodium tetraborate) modification was also studied. In six compositions, the experiment performed in two replicates. The results were evaluated with enrichment factor as shown in Table 4.18. Even the protein precipitating agent were applied, the enrichment factors are still low when compared with the value obtained from HF-LPME optimization part. The low proportion of meta-phosphoric acid in methanol cause higher enrichment factor than high proportion. The

reason for this tendency resulted from the nature of macrolide antibiotics, the four analytes are unstable in acidic solution. If high acidicity performed, analytes are degraded and cannot extract or enrich with HF-LPME. When compared enrichment factor between donor solution modification and not, the results showed that the modification with donor in meta-phosphoric acid-methanol give a better results because the existence of donor solution can comfortably transfer or extract analytes in the similar way with HF-LPME process.

Table 4.18 Effect of Method II extraction on enrichment factor of ERY, SPI, TIL, and TYL.

Extracting solvent	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
0.3% m-phosphoric acid in methanol	1.02 ± 0.23	2.34 ± 1.99	2.12 ± 1.13	1.85 ± 1.30
0.5% m-phosphoric acid in methanol	0.98 ± 0.45	2.10 ± 0.95	1.05 ± 0.51	1.13 ± 0.95
1.0% m-phosphoric acid in methanol	0.55 ± 0.16	1.31 ± 0.84	0.48 ± 0.32	0.28 ± 0.10
0.3% m-phosphoric acid in methanol + donor solution	3.94 ± 0.84	7.31 ± 1.99	4.87 ± 2.53	5.68 ± 2.30
0.5% m-phosphoric acid in methanol + donor solution	2.01 ± 1.59	3.22 ± 0.84	1.84 ± 0.22	2.41 ± 1.54
1.0% m-phosphoric acid in methanol + donor solution	0.54 ± 0.40	1.05 ± 0.76	0.55 ± 0.13	0.78 ± 0.35

4.3.2.3 Extraction Method III

The extracting solution of Method III was based on McIlvaine buffer. McIlvaine buffer consists of citric acid monohydrate, disodium hydrogenphosphate (Na_2HPO_4), and ethylenediaminetetraacetic acid disodium salt (Na_2EDTA). It is one of the most popular protein precipitating agents in the extraction from biomatrices. The presence of EDTA

and citric acid prevents the adsorbing sites of analytes from chelating with cations that can affect to extraction efficiency. Hence, McIlvaine buffer is an alternative way to precipitate protein in matrix. The extraction with McIlvaine buffer was investigated with two compositions, which are donor modification and no adjustment with donor in McIlvaine solution. Each composition was done in two replicates. The results in Table 4.19 display the low enrichment factor obtained from both compositions of McIlvaine buffer. It can be presumed that proteins from sample may have precipitated but not completely because McIlvaine buffer can only bind with cations from sample matrix. More clean-up of matrix should result in higher extraction efficiency. When comparing the results of donor and no donor added, the existence of donor in the extracting solution caused higher enrichment than if no donor was present.

Table 4.19 Effect of Method III extraction on enrichment factor of ERY, SPI, TIL, and TYL.

Extracting solvent	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
McIlvaine buffer	0.92 ± 0.24	1.53 ± 0.60	1.67 ± 0.15	0.28 ± 0.12
McIlvaine buffer + donor solution	1.72 ± 0.14	3.91 ± 0.08	1.95 ± 0.05	2.67 ± 0.55

4.3.2.4 Extraction Method IV

The extracting solution of Method IV was based on trichloroacetic acid (TCA). Few works *(45),(46)* have reported that TCA can also be used single or coupled with McIlvaine buffer for protein precipitation prior to analysis of food samples. In this method, TCA was used to precipitate protein from chicken sample with four compositions. Single TCA, TCA with McIlvaine buffer, TCA and donor modification, and mixing solution of TCA, McIlvaine buffer, and donor were studied. Each composition was optimized in two replicates and the results are shown in Table 4.20. The donor solution added in various TCA compositions indicated the same tendency of

enrichment factor as the other extracting solvents. The presence of donor in the extracting solvent improved the extraction of analytes in HF-LPME.

In the experiment, after the extraction of analytes with single TCA, the extracted solution was turbid compared with mixed McIlvaine buffer. In other words, when combined, the two extracting solvents precipitate more protein than if used single.

From four methods utilizing protein precipitating agent, all enrichment factors gained indicated to the enrichment of macrolides with HF-LPME method.

Table 4.20 Effect of Method IV extraction on enrichment factor of ERY, SPI, TIL, and TYL.

Extracting solvent	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
TCA	0.84 ± 0.20	1.23 ± 0.65	0.45 ± 0.13	1.67 ± 0.42
TCA+ McIlvaine buffer	2.35 ± 0.51	3.12 ± 0.46	3.01 ± 0.51	2.45 ± 0.74
TCA + donor solution	1.12 ± 0.34	2.03 ± 0.13	0.99 ± 0.05	2.11 ± 1.32
TCA + McIlvaine buffer + donor solution	3.01 ± 0.68	4.98 ± 0.57	3.42 ± 0.43	2.88 ± 0.97

4.3.2.5 Extraction Method V

The extracting solution of Method V was based on KH_2PO_4-ACN. This solution with organic solvent and the concept were adapted from *(47)*. The modification of organic solvent in extracting solution may induce transport of analytes through organic phase in hollow fiber membrane pores. The organic solvent used in modification must be more miscible with water than the organic phase to protect leakage of organic phase from hollow fiber during the preconcentration process. Acetonitrile was added to KH_2PO_4 to perform as extracting solution. This type of extracting solution was studied with two

compositions. Each composition was tested in two replicates and the results were determined as seen in Table 4.21. The enrichment factors obtained indicated that the addition of organic solvent in extracting solution did not significantly improved the extraction or preconcentration; even if in donor modification the extraction efficiency could be increased.

Table 4.21 Effect of Method V extraction on enrichment factor of ERY, SPI, TIL, and TYL.

Extracting solvent	Average EF ± S.D. (n=2)			
	ERY	SPI	TIL	TYL
KH_2PO_4-ACN	0.78 ± 0.12	0.66 ± 0.23	0.45 ± 0.20	0.83 ± 0.37
KH_2PO_4-ACN + donor solution	1.32 ± 0.54	3.85 ± 0.96	2.18 ± 0.33	2.46 ± 0.56

From the five methods extracting ERY, SPI, TIL, and TYL from chicken sample with various extracting solution, meta-phosphoric acid-methanol as extracting solution in Method II provided highest enrichment factors of ERY, SPI, TIL, and TYL of 3.94, 7.31, 4.87, and 5.68, respectively. Four macrolides are proved to extract and preconcentrate in complex sample such as poultry sample with method II and HF-LPME process.

4.3.3 Method performance in water and poultry sample application

4.3.3.1 Water sample application

In real sample analysis, the recovery represents the method accuracy and the limit of detections refers to the method lowest concentration detection. Both parameters were defined in eight replicates at spiking level of four macrolides 20 µg/L in water sample followed by preconcentrated with optimized HF-LPME method and detection with LC-ESI-MS/MS to evaluate the method effectiveness. The result of recovery and limits of

detections was shown in Table 4.22 All values of recovery and LODs of four macrolides gained are in the same tendency and range as the method validation results.

Table 4.22 Method performance of ERY, SPI, TIL, and TYL after HF-LPME application in water sample. (n=8)

	ERY	SPI	TIL	TYL
% recovery	82.93 ± 5.79	97.20 ± 3.47	94.16 ± 5.29	89.81 ± 4.02
LODs (µg/L)	0.09 ± 0.03	0.14 ± 0.05	0.57 ± 0.22	3.52 ± 0.78

4.3.3.2 Poultry sample application

The recovery and limit of detections also utilized to evaluate the effectiveness of HF-LPME method in the determination of macrolide antibiotics in complex matrices like poultry sample. Both parameters were defined in eight replicates at spiking level of four macrolides 20 µg/L in poultry muscle sample followed by preconcentrated with optimized HF-LPME method and detection with LC-ESI-MS/MS. The result of recovery and limits of detections was shown in Table 4.23. All ranges of recovery and LODs of four macrolides gained are less than the application in water sample and the method validation results. This tendency resulted from the complexity of poultry sample matrix, the analyte structure, and the hollow fiber geometry. Four macrolides can preconcentrated with low efficiency because the residue of sample matrices can block hollow fiber pores combined with the fact that the large structure of macrolides reduce the capability in transportation through very small pore size of hollow fiber. However, it can be concluded that the HF-LPME method can valuably be applied for real sample analysis such as water and poultry sample.

Table 4.23 Method performance of ERY, SPI, TIL, and TYL after HF-LPME application in poultry sample. (n=8)

	ERY	SPI	TIL	TYL
% recovery	71.78 ± 4.23	90.23 ± 6.48	87.44 ± 3.89	79.21 ± 5.61
LODs (µg/L)	5.47 ± 0.84	6.92 ± 2.46	12.33 ± 3.57	18.05 ± 6.12

CHAPTER V

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDY

A method for the preconcentration and determination of the macrolide antibiotics erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), and tylosin (TYL) was developed. Hollow-fiber liquid-phase microextraction (HF-LPME) was used to enrich the four analytes before detection with liquid chromatography-tandem mass spectrometry (LC-ESI-MS/MS). In this work, the optimization was particularly focused on HF-LPME and the extraction from poultry sample. The conditions of LC-MS/MS detection for ERY, SPI, TIL, and TYL were derived from the conditions of routine analysis and are described in Table 5.1. MS/MS was operated in multiple reactions monitoring mode (MRM) with the most two sensitive transitions used for both quantification and confirmation. The MRM transitions of ERY, SPI, TIL, and TYL were previously shown in Table 3.1.

Table 5.1 LC-MS/MS condition for the analysis of ERY, SPI, TIL, and TYL.

	Parameter	Condition
LC	Column type	UPLC C_{18} Acquity BEH
	Column size	100mm x 2.1mm I.D., 1.7µm
	Column temperature	40 °C
	Mobile phase	Binary with gradient elution
	Flow rate	0.2 mL/min
	Injection volume	10 µL
MS/MS	Ionization mode	Positive-ESI
	Capillary voltage	1 kV
	Extractor voltage	3 V
	Source temperature	120 °C
	Cone gas flow (Nitrogen)	50 L/h
	Desolvation gas flow (Nitrogen)	1000 L/h
	Desolvation temperature	350 °C
	Collision gas flow (Argon)	0.22 mL/min
	Cell pressure	0.35 Pa

For the preconcentration of macrolide antibiotics, HF-LPME was employed with simple configuration set-up as illustrated in Figure 3.1. Low-cost hollow fiber membrane was utilized once per experiment to reduce carry-over effect and this type of membrane also provide a low consumption of organic solvent, which results in an environmentally friendly technique. Due to the high efficiency in preconcentration, HF-LPME was investigated in this work to enrich the four macrolide antibiotics with optimization of related parameters. Immersion time, organic solvent type and composition, donor type and pH, acceptor type and pH, and extraction were considered. The HF-LPME procedure with optimized parameters is summarized in Figure 5.1.

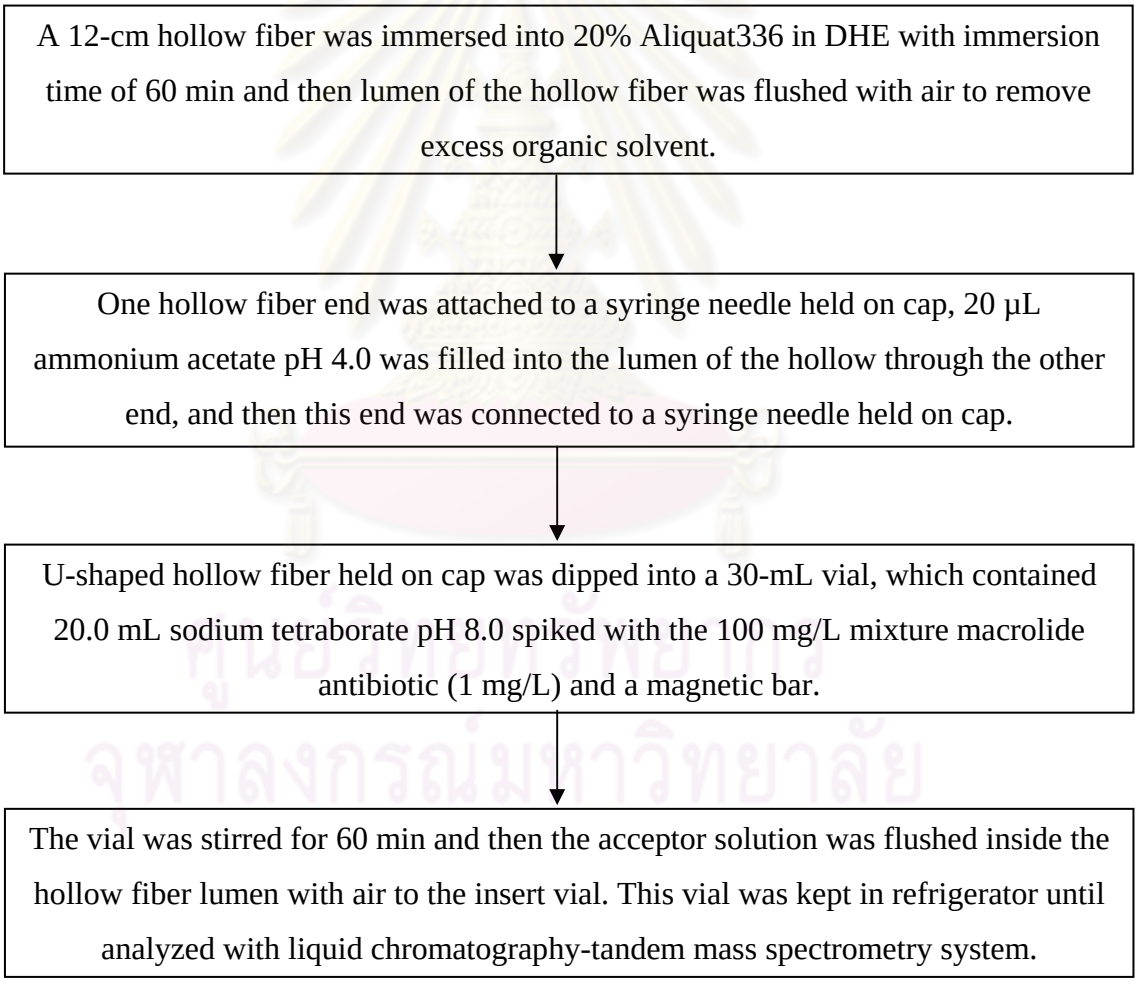


Figure 5.1 Schematic diagram of HF-LPME procedure with optimized condition.

All optimized parameters in HF-LPME were previously summarized in Table 4.10. For the four macrolide antibiotics, a wide range of solubility combined with the complex structure of the four macrolides led to difficulties in the extraction; therefore, a carrier ion-pairing agent was added into the organic solvent and carrier-mediate HF-LPME was performed to improve the extraction. The mechanism of carrier-mediate HF-LPME in this study is illustrated in Figure 4.4.

After the utilization of carrier-mediated HF-LPME in the determination of four macrolides, this optimized condition of HF-LPME method was validated to observe the performance of method before study in application with real sample. The summary of HF-LPME method validation is reported in Table 5.2.

Table 5.2 Method performance of HF-LPME with LC-MS/MS detection for ERY, SPI, TIL, and TYL.

	ERY	SPI	TIL	TYL
Linear range (µg/L)	0.5-50.0	0.5-50.0	0.5-50.0	0.5-50.0
Correlation coefficient (R^2)	0.9831	0.9784	0.9948	0.9710
LODs (µg/L)	0.07 ± 0.05	0.14 ± 0.09	0.17 ± 0.06	2.28 ± 0.31
LOQs (µg/L)	0.40 ± 0.16	0.67 ± 0.22	0.43 ± 0.12	8.10 ± 0.84
Enrichment factor	12.38 ± 3.86	36.14 ± 6.54	30.57 ± 8.22	29.33 ± 2.35
% Recovery	89.09 ± 6.32	98.72 ± 5.32	102.99 ± 6.29	93.05 ± 3.07
Intra-assay precision (% R.S.D.)	8.63	7.68	9.29	10.23
Intra-assay precision (% R.S.D.)	9.90	9.27	8.22	12.08

The linearity from standard calibration curve of ERY, SPI, TIL, and TYL revealed correlation coefficient value (R^2) of over 0.97 representing good linear dynamic range

of the method. The enrichment factors of ERY, SPI, TIL, and TYL derived from optimized HF-LPME condition ranged from 12.38 to 36.14. As a result of the large structure of four analytes, the enrichment process may be inconvenient and lead to low enrichment factors. Even though, low enrichment factors were obtained, the method limits of detection compensate this effect. The LOD are in low range of 0.07 to 2.28 µg/L, which can be considered as effective concentration detected when compared with LOD from other methods. Owing to no regulations about the concentration of macrolide antibiotic residues in water, the comparison with many publications is used to evaluate the efficiency of this method. The LOD of other methods in the determination of antibiotics in water are listed in Table 5.3.

Table 5.3 Comparison of limits of detection of this work and other publications determining antibiotics in water.

Method	LODs (µg/L)
Abuin *(50)*	0.01-1.90
Yang *(51)*	0.03-0.07
Batt *(52)*	0.03-0.19
Hao *(53)*	0.02-1.40
Rao *(54)*	0.60-8.10
This work	0.07-2.28

The LOD of this proposed method are promising when compared with other works. The method recovery representing accuracy ranged from 89.09 to 102.99 % at 50 µg/L spiking level. The intra-assay precision was reported as relative standard deviation (%R.S.D.) and the value of %R.S.D. for within-day precision ranged from 7.68 to 10.23%. The %R.S.D. values obtained from the experiments were lower than %R.S.D calculated from Horwitz equation, which indicates the satisfactory of method capability. For intermediate precision, the %R.S.D. was calculated from the results on

two analytical days and two-tailed F-test were used to evaluate the significance of different %R.S.D. between two days. The values of %R.S.D did not significantly differ on two working days because the calculated F values were lower than the critical F values (P=0.05). Both intra-assay and intermediate precisions were in acceptable ranges.

In real sample analysis, water and poultry samples are chosen to study with HF-LPME method because macrolide antibiotics were found to create residual problem in both types of sample. For water sample analysis, the river water was collected and filtered before preconcentration with optimized HF-LPME condition and detected by LC-ESI-MS/MS. The river water was not founded macrolide antibiotic residues so four macrolides were spiked at 2, 8 and 20 µg/L in sample to study the capability of HF-LPME application in water sample. The enrichment factor results are in range of 11.35-31.34 at 2 µg/L, 14.15-35.81 at 8 µg/L, and 12.40-33.14 at 20 µg/L. This range and tendency of enrichment factor from the application in water sample are the same as the results from method validation. This HF-LPME method was proved to efficiently apply in real water sample. For poultry sample analysis, the chicken was bought from local department store and the analysis need extraction step to separate four macrolides from sample matrices prior preconcentration with optimized HF-LPME method. The extraction process is necessary step for the determination in poultry samples. Therefore, the various extraction methods were developed to extract analytes from sample and transfered to preconcentrate with optimized HF-LPME. Five extraction methods with various types and compositions of extracting solvent were tested and were tried to be coupled with the preconcentration step. The studied extraction methods were quite efficient. The enrichment factor of four macrolides after extraction with Method II and preconcentration with HF-LPME method are in range of 3.94 to 7.31 which is the highest value when compared with other extraction methods. However, the enrichment factor from the application of HF-LPME in poultry sample is less than the results from the application in water sample and method validation because the matrices residue from poultry sample can obstruct the pore of hollow fiber that reduce the enrichment efficiency of method. Additionally, the large structures of four macrolide antibiotics are suffered from the transportation through very small pore sizes of hollow fiber. The enrichment factor obtained from water and poultry application depends on the

complexity of sample matrices and related to method limit of detections from each sample application.

The method recovery and limit of detections of the application in both water and poultry sample were defined to evaluate the method effectiveness. In water sample, the recovery and the LODs ranged from 82.93 to 97.20 % and 0.09-3.52 µg/L, respectively. For poultry sample, the method recoveries are in range of 71.78-90.23 % and the LODs ranged from 5.47-18.05 µg/L. From both recovery and LODs value from the application in water and poultry samples, the HF-LPME method was proved to successfully applied in real sample, even determination in complicated sample matrices such as animal products.

HF-LPME is an alternative technique to preconcentrate macrolide antibiotic residues in various types of sample because of it is easy to operate, inexpensive, and uses little organic solvent. The enrichment ability, low-level detection limit, and good linearity of this method provide benefits and overcome some sample preparation methods. On the other hand, this technique confronted problems from the miniature scale of extraction. The analysis required proficient skills in HF-LPME to reduce the variation of result.

The less complex matrix and analyte compounds of smaller structure were recommended for this HF-LPME method because the nature of hollow fiber membrane limited the determination of analytes in high matrix solution.. Macrolide antibiotics in poultry muscle should be determined with other sample preparation methods with high clean-up efficiency to reduce matrix effects.

The developed method proved its effectiveness in preconcentration and determination of macrolide antibiotics in both water and poultry sample. The extended HF-LPME could further be studied with other analytes, are of critical concern with residues in water sample such as pesticides. If high-level enrichment factors are obtained with this method, analytes in trace level residue could be detected with a less expensive and less complicated system than LC-MS/MS.

REFERENCES

(1) Mellon, M. Estimates of Antimicrobial Abuse in Livestock[Online]. (n.d.) Available from:www.ucsusa.org/food_and_environment/antibiotic_resistance /page.cfm?page ID=264 [2009, July 20]

(2) European Union. EC No. 96/23/EC On measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decision 89/187/EEC and 91/664/EEC., Brussels, 1996.

(3) Europian Union. EEC No. 2377/90 Laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin., Brussels, 1990.

(4) Europian Union. EEC No. 2002/657/EC Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results., Brussels, 2002.

(5) The Food Marketing Institute. Low-Level Use of Antibiotics In Livestock and Poultry[Online]. (n.d.) Available from: www.fmi.org/docs/media/bg/ antibiotics.pdf [2009, May 5]

(6) Kanfer, I.; Skinner, M. F.; and Walker, R. B. Analysis of macrolide antibiotics. Journal of Chromatography A 812 (1998): 255–286.

(7) Gaynor, M.; and Mankin, A. S. Macrolide Antibiotics: Binding Site, Mechanism of Action, Resistance. Frontiers in Medicinal Chemistry 2 (2005): 21-35.

(8) Chung, W.O.; Young, K.; Leng, Z.; and Roberts, M.C. Mobile element carrying ermF and tetQ in gram-positive and gram-negative bacteria. Journal of Antimicrobial Chemotherapy 44 (1999): 329-335.

(9) Horie, M.; Saito, K.; Ishii, R.; Yoshida T.; Haramaki Y.; and Nakazawa, H. Simultaneous determination of five macrolide antibiotics in meat by high-performance liquid chromatography Journal of Chromatography A 812 (1998): 295–302.

(10) Leal, C.; Codony, R.; Companñó, R.; Granados, M.; and Prat, M. D. Determination of macrolide antibiotics by liquid chromatography. Journal of Chromatography A 910 (2001): 285–290.

(11) Draisci, R.; Palleschi, L.; Ferretti, E.; Achene, L.; Cecilia, A. Confirmatory method for macrolide residues in bovine tissues by micro-liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 926 (2001): 97–104.

(12) Wang, J. Determination of Five Macrolide Antibiotic Residues in Honey by LC-ESI-MS and LC-ESI-MS/MS. Journal of Agricultural and Food Chemistry 52 (2004): 171-181.

(13) Mcardell, C.; Molnar, E.; Suter, M.J.F.; and Giger, W. Occurrence and Fate of Macrolide Antibiotics in Wastewater Treatment Plants and in the Glatt Valley Watershed, Switzerland. Environmental Science & Technology 37 (2003): 5479-5486.

(14) Abuin, S.; Codony, R.; Companñó, R.; Granados, M.; and Prat, M. D. Analysis of macrolide antibiotics in river water by solid-phase extraction and liquid chromatography–mass spectrometry. Journal of Chromatography A 1114 (2006): 73–81.

(15) Horie, M.; Takegami, H.; Toya, K.; and Nakazawa, H. Determination of macrolide antibiotics in meat and fish by liquid chromatography–electrospray mass spectrometry. Analytica Chimica Acta 492 (2003): 187–197.

(16) Berrada, H.; Borrull, F.; Font, G.; Moltó, J. C.; and Marcé, R. M. Validation of a confirmatory method for the determination of macrolides in liver and kidney animal tissues in accordance with the European Union regulation 2002/657/EC. Journal of Chromatography A 1157 (2007): 281–288.

(17) Berrada, H.; Borrull, F.; Font, G.; and Marcé, R. M. Determination of macrolide antibiotics in meat and fish using pressurized liquid extraction and liquid chromatography–mass spectrometry. Journal of Chromatography A 1208 (2008): 83–89.

(18) Msagati, T. A. M.; and Nindi, M. M. The Use of Supported Liquid Membranes in the Extraction of Macrolides in Biomatrices. Microchimica Acta 148 (2004): 199–214.

(19) Qiang, Z.; and Adams, C. Potentiometric determination of acid dissociation constants (pKa) for human and veterinary antibiotics. Water Research 38 (2004): 2874–2890.

(20) Pavlović, D. M.; Babić, S.; Horvat, A. J. M.; and Kaštelan-Macan, M. Trends in Analytical Chemistry 26 (2007): 1062-1075.

(21) Jönsson, J. A.; and Mathiasson, L. Liquid membrane extraction in analytical sample preparation. Trends in Analytical Chemistry 18 (1999): 318-325.

(22) Pedersen-Bjergaard, S.; and Rasmussen, K.E. Liquid−Liquid−Liquid Micro extraction for Sample Preparation of Biological Fluids Prior to Capillary Electrophoresis. Analytical Chemistry 71 (1999): 2650-2656.

(23) Jönsson, J. A.; and Mathiasson, L. Membrane-based techniques for sample enrichment. Journal of Chromatography A 902 (2000): 205–225.

(24) Jönsson, J. Å.; and Mathiasson, L. Membrane extraction in analytical chemistry. Journal of Separation Science 24 (2001): 495-507.

(25) Hylton, K.; and Mitra, S. Automated, on-line membrane extraction. Journal of Chromatography A 1152 (2007): 199–214.

(26) Psillakis, E.; and Kalogerakis, N. Developments in liquid-phase microextraction. Trends in Analytical Chemistry 22 (2003): 565-574.

(27) Lambropoulou, D. A.; and Albanis, T. A. Liquid-phase micro-extraction techniques in pesticide residue analysis. Journal of Biochemical and Biophysical Methods 70 (2007): 195–228.

(28) Rasmussen, K. E.; and Pedersen-Bjergaard, S. Developments in hollow fibre-based, liquid-phase microextraction. Trends in Analytical Chemistry 23 (2004): 1-10.

(29) Pedersen-Bjergaard, S.; and Rasmussen, K. E. Liquid-phase microextraction with porous hollow fibers, a miniaturized and highly flexible format for liquid–liquid extraction. Journal of Chromatography A 1184 (2008): 132–142.

(30) Lee, J.; Lee, H. K.; Rasmussen, K. E.; and Pedersen-Bjergaard, S. Environ mental and bioanalytical applications of hollow fiber membrane liquid-phase microextraction: A review. Analytica Chimica Acta 624 (2008): 253–268.

(31) Pedersen-Bjergaard, S.; and Rasmussen, K. E. Bioanalysis of drugs by liquid-phase microextraction coupled to separation techniques. Journal of Chromatography B 817 (2005): 3–12.

(32) Chang, Y. Membrane Applications[Online]. (2006). Available from: http://www.mnawwa.org/about/councils/training/research/Membrane%20Applications%20Presentation.ppt [2007, October 9]

(33) Xu, L.; Basheer, C.; and Lee, H. K. Chemical reactions in liquid-phase microextraction. Journal of Chromatography A 1216 (2009): 701–707.

(34) Ho, T. S.; Halvorsen, T. G.; Pedersen-Bjergaard, S.; and Rasmussen, K. E. Liquid-phase microextraction of hydrophilic drugs by carrier-mediated transport. Journal of Chromatography A 998 (2003): 61–72.

(35) Zhang, Z.; Zhang, C.; Su, X.; Ma, M.; Chen, B.; and Yao, S. Carrier-mediated liquid phase microextraction coupled with high performance liquid chromatography for determination of illicit drugs in human urine. Analytica Chimica Acta 621 (2008): 185–192.

(36) Ardrey, R. E. Liquid Chromatography –Mass Spectrometry: An Introduction. England: John Wiley & Sons, 2003.

(37) Skoog, D. A.; and Leary, J. J. Principles of instrumental analysis. 4th ed. United States of America: Saunders college publishing, 1992.

(38) Harvey, D. Modern Analytical Chemistry. 1st ed. United States of America: The McGraw-Hill Companies, Inc., 2000.

(39) Agilent Technologies, Inc. Agilent Technologies 1100 Series LC/MSD Techniques and Software Operation: student manual. Alpharetta: GA, 2001.

(40) Bramer, S. E. V. An Introduction to Mass Spectrometry[Online] (1998). Available from: http://science.widener.edu/~svanbram [2008, June 29]

(41) Selectscience. Acquity UPLC HSS T3 columns[Online] (2009). Available from:www.selectscience.net/images/products/7233_WatersChem_ProdMar07_HPLCcols2.jpg

(42) NASA. Mass Spectrometer[Online]. (n.d.) Available from: http://ael.gsfc.nasa.gov/saturnGCMSMass.shtml [2009, August 5]

(43) Wikipedia. Photomultiplier[Online].(n.d.) Available from: http://en.wikipedia. org/wiki/Photomultiplier [2009, August 5]

(44) Leal, C.; Codony, R.; Companñó, R.; Granados, M.; and Prat, M. D. Determination of macrolide antibiotics by liquid chromatography. Journal of Chromatography A 910 (2001): 285-290.

(45) Codony, R.; Compañó, R.; Granados, M.; García-Regueiro, J. A.; and Prat, M. D. Residue analysis of macrolides in poultry muscle by liquid chromatography–electrospray mass spectrometry. Journal of Chromatography A 959 (2002): 131-141.

(46) Oka, H.; Ikai, Y.; Ito, Y.; Hayakawa, J.; Harada, K.; Suzuki, M.; Odani, H.; and Maeda, K. Improvement of chemical analysis of antibiotics XXIII[1]. Identification of residual tetracyclines in bovine tissues by electrospray-high performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography B 693 (1997): 337-344.

(47) Cinquina, A. L.; Longo, F.; Anastasi,G.; Giannetti, L.; and Cozzani, R. Validation of a high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlortetracycline and doxycycline in bovine milk and muscle. Journal of Chromatography A 987 (2003): 227–233.

(48) Kaufmann, A.; and Maden, K. Determination of 11 Aminoglycosides in Meat and Liver by Liquid Chromatography with TandemMass Spectrometry. Journal of AOAC International 88 (2005): 1118-1125.

(49) Taverniers, I.; Loose, M. D.; and Bockstaele, E. V. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. Trends in Analytical Chemistry 23 (2004): 535-552.

(50) Abuin, S.; Codony, R.; Compañó, R.; Granados, M.; and Prat, M. D. Analysis of macrolide antibiotics in river water by solid-phase extraction and liquid chromatography–mass spectrometry. Journal of Chromatography A 1114 (2006): 73–81.

(51) Yang, S.; and Carlson, K.H. Solid-phase extraction–high-performance liquid chromatography–ion trap mass spectrometry for analysis of trace concentrations of macrolide antibiotics in natural and waste water matrices. Journal of Chromatography A 1038 (2004): 141–155.

(52) Batt, A. L.; and Aga, D. S. Simultaneous Analysis of Multiple Classes of Antibiotics by Ion Trap LC/MS/MS for Assessing Surface Water and Groundwater Contamination. Analytical Chemistry 77 (2005): 2940-2947.

(53) Hao, C.; Lissemore, L.; Nguyen, B.; Kleywegt, S.; Yang, P.; and Solomon, K. Determination of pharmaceuticals in environmental waters by liquid chromatography/electrospray ionization/tandem mass spectrometry. Analytical and Bioanalytical Chemistry 384 (2006): 505–513.

(54) Rao R. N.; Venkateswarlu, N.; and Narsimha, R. Determination of antibiotics in aquatic environment by solid-phase extraction followed by liquid chromatography–electrospray ionization mass spectrometry. Journal of Chromatography A 1187 (2008): 151–164.

VITA

Miss Soparat Yudthavorasit was born on June 25, 1985, in Bangkok, Thailand. She had graduated a Bachelor's degree of Science in Chemistry from Mahidol University in 2007. Afterwards, she continued her academic education for Master degree at Department of Chemistry, Faculty of Science, Chulalongkorn University. She had completed the program in September 2009 and received her Master's degree of Science in Analytical Chemistry in July 2010.