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HOLLOW FIBER SUPPORTED LIQUID PHASE MICROEXTRACTION FOR THE DETERMINATION OF QUATERNARY AMMONIUM HERBICIDES

Miss Wannakarn Nitayarerk

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

สาขาวิชา.....เคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

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477 24526 23: MAJOR CHEMISTRY
KEY WORD: SUPPORTED LIQUID MEMBRANE/ HOLLOW FIBER MEMBRANE/ QUATERNARY AMMONIUM HERBICIDES

WANNAKARN NITAYARERK: HOLLOW FIBER SUPPORTED LIQUID PHASE MICROEXTRACTION FOR THE DETERMINATION OF QUATERNARY AMMONIUM HERBICIDES. THESIS ADVISOR: ASST. PROF. NATCHANUN LEEPIPATPIBOON, Dr.,rer.nat, THESIS CO-ADVISOR: ASST. PROF. M.L. SIRIPASTR JAYANTA, 94 pp. ISBN 974-14-2976-2

A cheap and simple sample preparation technique, hollow fiber supported liquid phase microextraction, was used for the preconcentration of the quaternary ammonium herbicides from drinking water. The cationic herbicides were extracted through a hollow fiber membrane where the supported liquid membrane was 70% di-(2-ethylhexyl) phosphoric acid in di-n-hexyl ether. They were preconcentrated in a few microliters of an acidic acceptor. Liquid chromatography-mass spectrometry was used to analyze these herbicides in only 4-min run time without any ion-pairing agent. The enrichment factors were between 8.9 and 57.2 from a 60-minute extraction. The calibration curve after extraction of 2-10 μg/L spiking level presented the correlation coefficients in the range of 0.97 to 0.99. Relative standard deviations of the % recovery based on ten replicates of 5 and 50 μg/L spiked standard solutions were lower than the % R.S.D. calculated from the Horwitz equation. The method detection limits are 0.17, 0.39, 0.48 and 1.29 μg/L for chlormequat, mepiquat, diquat, and paraquat, respectively. This proposed method was validated and successfully applied for drinking water samples in Thailand with the acceptable % recovery values.

Department.....Chemistry..... Student's Signature Wannakarn Nitayarerk
Field of Study.....Chemistry..... Advisor's Signature Natchanun Leepipatpiboon
Academic Year.....2006..... Co-advisor's Signature

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CONTENTS

PAGE

PAGE

PAGE

LIST OF TABLES

TABLES **PAGE**

LIST OF FIGURES

FIGURES **PAGE**

LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
˚C	degree celsius
AOAC	Association of Official Analytical Chemists
APCI	atmospheric pressure chemical ionization
C_A	analyte concentration in acceptor phase
C_D	analyte concentration in donor phase
CE	capillary electrophoresis
CI	chemical ionization
cm	centimeter
CQ	chlormequat
Da	dalton
DC	direct-current
DEHPA	di-(2-ethylhexyl) phosphoric acid
DF	difenzoquat
DHE	di-n-hexyl ether
D_m	diffusion coefficient in membrane
DOA	Department of Agriculture
DQ	diquat
EE	extraction efficiency
EF	enrichment factor
ESI	electrospray ionization
FIA	flow injection analysis
GC	gas chromatography
HFBA	heptafluorobutyric acid
HILIC	hydrophilic interaction chromatography
h_m	thickness of membrane
HPLC	high-performance liquid chromatography
K	partition coefficient
K_a	acid dissociation constant
k_m	mass-transfer coefficient of membrane

K_{ow}	octanol-water partition coefficient
kV	kilovolt
L/min	liter per minute
LC-MS	liquid chromatography-mass spectrometry
LLE	liquid-liquid extraction
M	molar
m/z	mass per charge ratio
MDL	method detection limit
MESI	membrane extraction with a sorbent interface
mL/min	milliliter per minute
mm	millimeter
mM	millimolar
MMLLE	microporous membrane liquid-liquid extraction
MOAC	Ministry of Agriculture and Cooperatives
MQ	mepiquat
MQL	method quantitation limit
n_A	number of moles of the analyte in acceptor solution
n_D	number of moles of the analyte in donor solution
ng/L	nanogram per liter
PGRs	plant growth regulators
PME	polymeric membrane extraction
ppb	part per billion
PQ	paraquat
psi	pound per square inch
PTFE	polytetrafluoro ethylene
QAHs	quaternary ammonium herbicides
R.S.D.	relative standard deviation
R^2	correlation coefficient
RF	radio frequency
SD	standard deviation
SLME	supported liquid membrane extraction
SLMME	supported liquid membrane microextraction

SPE	solid phase extraction
TOPPS	1,2,3,4-tetrahydro-1-oxo-pyridyl 1,2-a-5-pyrazinium bromide
US EPA	United States Environmental Protection Agency
UV	ultraviolet
v/v	volume by volume
V_A	acceptor volume
V_D	donor volume
V_D/V_A	phase ratio
WHO	World Health Organization
α_A	fraction of analyte in acceptor phase
α_D	fraction of analyte in donor phase
ΔC	concentration difference
η	viscosity
µg/L	microgram per liter
µL	microliter
µm	micrometer
µs	microsecond
π	3.14

CHAPTER I

INTRODUCTION

1.1 Problem Definition

In agricultural areas, the main problem is weeds, the unwanted plants that prevent the growing of target agricultural plants. In recent years, the most popular way used for killing weeds is the use of herbicides due to their low cost while giving the best result in removing the weeds. Unfortunately, most of herbicides are quite harmful not only to plants but also to human and animals.

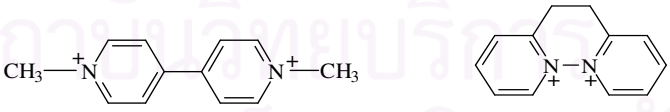
Quaternary ammonium herbicides, known as quats, are the one famous group of weeds killer. The popular compounds of this group consist of paraquat (PQ), diquat (DQ), difenzoquat (DF), chlormequat (CQ) and mepiquat (MQ), of which the structures were shown in Figure 1.1. PQ, DQ and DF are non-selective contact herbicides used to control weeds, grasses and aquatic weeds, while CQ and MQ are plant growth regulators used to reduce risk of lodging and increasing yields in barley, wheat, rye and oats. Because of their charged chemical structures, the five quats are highly soluble in water and their residues have high potential to be presented in the natural water or agricultural products. These contaminants can harm human and animals due to their toxicity.

In the 2004 WHO recommended guidelines, pesticides are classified by their hazard level. PQ, DQ and DF are classified as moderately hazardous pesticides (Class II) while CQ and MQ are classified as slightly hazardous pesticides (Class III) *(1)*. Their contaminant levels are limited in drinking water by the US Environmental Protection Agency (US EPA) who set allowable maximum contaminant levels at 3 and 20 µg/L for PQ and DQ, respectively *(2)*. The European Union has not specifically regulated the levels of these compounds in water but applies the value of 0.1 µg/L for individual pesticide and 0.5 µg/L for total pesticides in general *(3)*. The EU sets no regulation for CQ and MQ in water however the contaminant levels of CQ

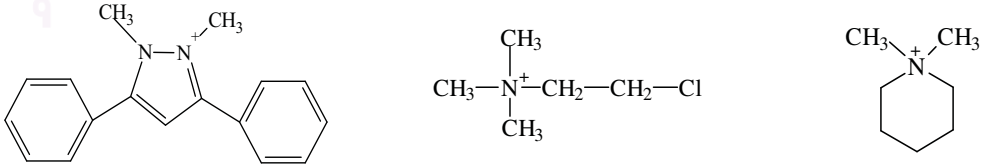
and MQ are limited in some agricultural products, e.g., the Codex Alimentarius Commission has set maximum residue limits for CQ in pears, wheat and oats at 3, 5 and 10 mg/kg, respectively *(4)*.

In Thailand, these herbicides are defined as dangerous substances and are under the control of the Department of Agriculture (DOA), Ministry of Agriculture and Cooperatives (MOAC) who control the approving and restricting the usage and allowable import volumes of pesticides *(5)*. In 2003, MOAC reported that the import volumes of PQ and MQ were 8,366,582 and 14,325 kilograms, respectively which corresponded to 10.5% of annual pesticide volume *(6)*. This demonstrated that the quaternary ammonium herbicides are favorite chemicals used for controlling the weeds in Thailand.

Because the amounts of quats presented as contaminants in water are very low due to dilution factor and adsorption in soil, sample preparation and enrichment are important for the success of their analysis. Due to their good solubility in water, they cannot be extracted by any organic solvent. The most common method is solid phase extraction (SPE), using solid sorbent with ion-pairing capability to retain the quats and eluting them by acidic-aqueous solution. Although the technique is quite successful to preconcentrate the quats, it still has some drawbacks such as breakthrough volume, high cost of the SPE sorbents, and the interferences of organic matters or surfactants in the water matrices. Therefore, a stable, selective and inexpensive sample preparation and enrichment technique is still in demand for quats.



Paraquat Diquat



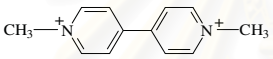
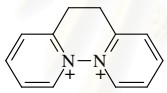
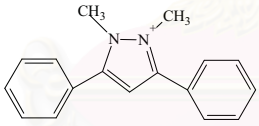
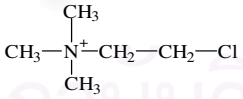
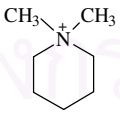
Difenzoquat Chlormequat Mepiquat

Figure 1.1 Structures of the quaternary ammonium herbicides

1.2 Quaternary Ammonium Herbicides

Quaternary ammonium herbicides (QAHs) are widely used in the agriculture. They are divided by their operation into two groups; the non-selective herbicides and the plant growth regulators. Some properties of QAHs are presented in Table 1.1.

Table 1.1 Properties of the quaternary ammonium herbicides *(7, 8)*

Name	Chemical Structure	IUPAC Name	Molecular Weight (g/mol)	Log K_{ow} (20 ºC)	Solubility in Water
Paraquat		1,1'-dimethyl-4,4-bipyridinium	186	-4.7	700 g/L
Diquat		1,1'-ethylene-2,2'-bipyridyldiylium	184	-4.6	700 g/L
Difenzoquat		1,2-dimethyl-3,5-diphenyl pyrazolium	249	0.2	765 g/L
Chlormequat		2-chloro ethyltrimethyl ammonium	122	-1.6	>1,000 g/kg
Mepiquat		1,1-dimethyl piperidinium	114	-2.8	>1,000 g/kg

1.2.1 Non-Selective Herbicides: Paraquat, Diquat and Difenzoquat

PQ, DQ and DF are classified as the non-selective herbicides which mean that they kill all plants in contact by influencing physiological processes of the vegetation, e.g., cell membrane disruption by PQ and DQ. Once inside, PQ and DQ move to the photosynthesis reaction centers in the chloroplasts and form the radicals and auto oxidized to produce hydrogen peroxides (H_2O_2). These superoxides can further react to form hydroxyl radicals which are quickly and effectively initiate lipid peroxidation. This process allows cellular components to leak into the intercellular space and causes cell membrane damage and cell death *(9)*. However, the mode of action of DF has not yet been found *(10)*.

1.2.1.1 The Decomposition in Sunlight and in Water

On the plant surface (and in solution), PQ is quickly broken down photochemically. Two degradation products were identified, 1-methyl-4-carboxypyridinium ion and methylamine hydrochloride, both of which have very low toxicities in mammals. The photolysis pathway of PQ is shown in Figure 1.2.

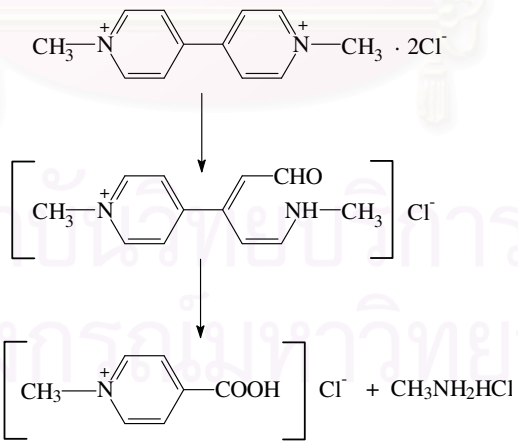


Figure 1.2 Photolysis pathway of paraquat *(11)*

It was found that both the rings and ethylene bridges of DQ breakdown into volatile products by photolysis. About 75% of DQ was lost after 96 hours of sunlight exposure. The photo-chemical breakdown resulted in 1,2,3,4-tetrahydro-1-oxo-pyridyl 1,2-a-5-pyrazinium bromide (TOPPS) as shown in Figure 1.3 as a major product. It was found that in water maintained in sunlight, 70% degradation of diquat occurred in 1-3 weeks and also produced TOPPS. This compound has low mammalian toxicity. All other degradation products are also presence in minor amount. The proposed scheme for the photochemical degradation of DQ in water is shown in Figure 1.4.

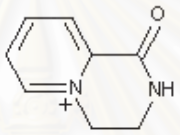


Figure 1.3 Structure of 1,2,3,4-tetrahydro-1-oxo-pyridyl 1,2-a-5-pyrazinium bromide (TOPPS) *(12)*

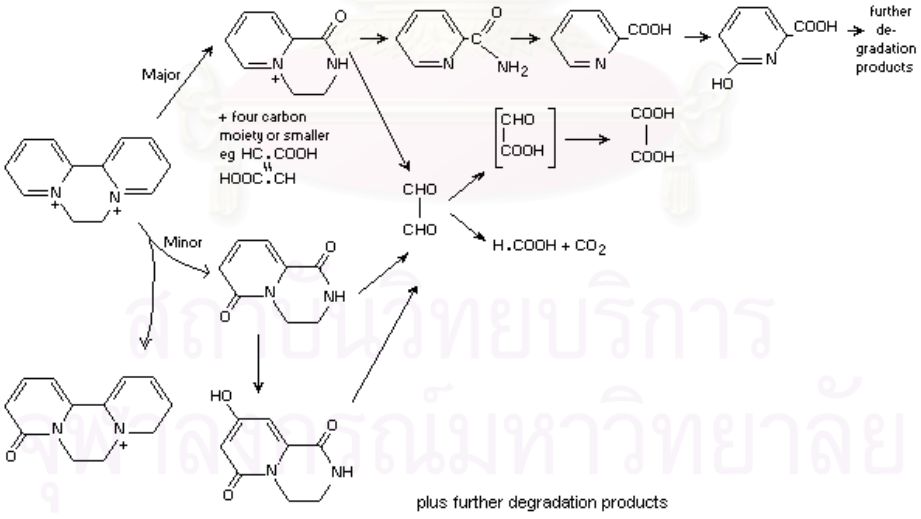


Figure 1.4 The proposed scheme for the photochemical degradation of diquat in water *(13)*

1.2.1.2 The Decomposition in Soil

PQ has been shown to be degraded by soil microorganisms to demethylated paraquat (1-methyl-4,4'-dipyridinium ion) and another compound characterized as the 1-methyl-4-carboxy-pyridinium ion (N-methylisonicotinic acid). Figure 1.5 shows the bacteria-demethylation degradation pathway of PQ and ring cleavage of one of the heterocyclic rings to eventually form the carboxylated N-methyl-pyridinium ion.

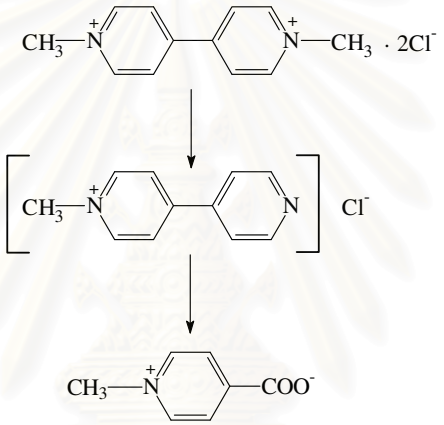


Figure 1.5 Degradation pathways by bacteria-demethylation of paraquat *(11)*

In soil, DQ can be inactivated rapidly and completely. This inactivation results from a reaction complex formed between the positively charged diquat cation and the negatively charged sites on the clay minerals present in soil *(12)*.

1.2.2 Plant Growth Regulators: Chlormequat and Mepiquat

Plant growth regulators (PGRs) are a class of natural and synthetic organic compounds that affect growth, development and maturation of vegetative and reproductive plant structures. CQ and MQ are categorized as PGRs. They inhibit the action of gibberellic acid, a plant growth substance involved in promotion of stem elongation, mobilization of food reserves in seeds and other processes, thus they

permits shortening and strengthening of stems in plants. They also reduce branching and leaf growth in certain species of shrubs and trees. Simultaneously, the product increases the formation of chlorophyll and the development of the root system, resulting in greater crop yield. CQ and MQ are most widely used for increasing the yield of barley, wheat, rye, oats, pears, cotton and grapes *(14, 15)*.

1.2.2.1 The Decomposition in Plants

CQ (chlorine choline chloride) can decompose into choline chloride by the enzymatic system and changed into betain by oxidation in a central position in plant metabolism. It is noticeable that CQ decomposes differently in leaf extracts of different plants. Plants sensitive to CQ seem to decompose the growth regulator more slowly than plants which are less sensitive. The decomposition of CQ into choline chloride is pH-dependent and thermostable. Figure 1.6 shows general decomposition of CQ in plants.

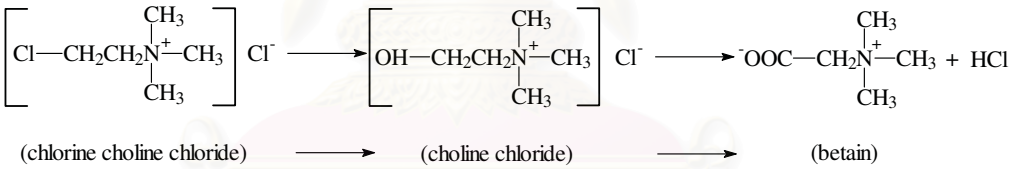


Figure 1.6 The decomposition of chlormequat in plants *(16)*

1.2.2.2 The Decomposition in Soil

CQ decomposition in soil takes place relatively fast. The persistency of choline chloride in soil was assessed as only three weeks. Four different soils were treated with 5 mg chlormequat in 0-6 weeks before sowing wheat seeds. After 4-6 weeks nearly complete inactivation were defected in all pots *(16)*.

1.3 Literature Reviews

In 1992, the US EPA developed standard method 549.1 for the determination of PQ and DQ in drinking water. Water sample pH was kept at alkalinized region and passed through C8 solid phase extraction (SPE) cartridge or disk using 1-hexanesulfonic acid sodium salt as the ion-pair reagent to retain both analytes. Elution was accomplished by acidic solution. The analysis is recommended using high-performance liquid chromatography with ultraviolet detection (HPLC-UV). The method detection limits are 0.80 and 0.44 µg/L for PQ and DQ, respectively *(17)*.

In 1996, M. Ibáñez, Y. Picó and J. Mañes studied the influence of organic matter and surfactants on solid phase extraction of PQ, DQ and DF from waters, using Silica Sep-Pak cartridge. This study proved that some compounds such as surfactants and humic acid contaminated in natural water have negative effect on the recoveries of the three cationic herbicides *(18)*. As a result, they tried to develop a better strategy for increasing the extraction efficiency of three quats using SPE technique in 1998. They found that anionic surfactants and humic acid in water samples bound to the herbicides. To prevent this undesirable effect, a cationic surfactant called cetrimide was added to the water samples. By this approach, the quat recoveries were improved from approximately 40 to 88% and the method detection limits were in the range of 0.05 to 0.08 µg/L *(19)*.

For fast and convenient analysis, on-line instrumental method was developed. In 1996, M. Ibáñez et al. applied on-line liquid chromatography with UV detection technique for the determination of PQ, DQ and DF. The preconcentration column, LiChrospher 60 silica cartridge, connected to an analytical column, Spherisorb SW3, was used. Although the on-line clean-up system was an advantage of this work, the complicated instrument was expensive and the performance of this system required an interval check every 2 months. The method detection limits of three quats for 200-mL water samples were about 20 pg/L *(20)*.

Ion chromatography method was proposed for the determination of CQ residue in pears by M.C. Peeters et al. in 2001. Pear samples were extracted with

hydrochloric acid prior to the analysis by ion chromatography. The method detection limit was 0.5 mg/kg and the recoveries were in the range of 92-95% *(21)*.

In 2002, S. Riediker et al. developed an on-line solid phase extraction coupled with liquid chromatography-electrospray ionization tandem mass spectrometry for the determination of CQ and MQ in pear, tomato and wheat flour. The analytes were extracted with a mixture of methanol-water (1:1 v/v) before passing through the SPE cartridge. However, the samples fortified with analytes above 0.78 mg/kg overloaded the SPE cartridge and caused the carry-over effects of the analytes. The method detection limits for CQ and MQ were below 6 µg/kg in all three matrices tested and the recoveries were between 90 and 96% *(22)*.

For simultaneous detection of PQ, DQ, DF, CQ and MQ, the liquid chromatography-mass spectrometry (LC-MS) technique was developed in 1999 by R. Castro, E. Moyano and M.T. Galceran. The tap water samples were still treated by SPE technique using the silica Sep-Pak cartridge. Heptafluorobutyric acid (HFBA) was used as the volatile ion-pair reagent for the LC separation. The method detection limits ranged from 0.05 to 4.70 µg/L for spiked water samples *(23)*. In the next year, these researchers employed on-line ion-pair SPE-LC-MS for the analysis of five quats in drinking water samples using HFBA as ion-pair reagent and C8 disk for the extraction. The method detection limits were between 6 and 85 ng/L *(24)*.

The application in surface water and groundwater was studied by J.L. Martínez Vidal et al. in 2004. They used a silica cartridge and ion-pair LC-MS for the preconcentration and analysis of PQ, DQ, CQ and MQ in natural water samples. Herbicides were detected in 24 of 40 samples from Andalusia (South of Spain) ranging from 1 - 42 µg/L for DQ and 2-12 µg/L for PQ. However, CQ and MQ could not be detected in all water samples. The method detection limits of four analytes were below 0.5 µg/L and the recoveries were between 88.9 and 99.5% *(25)*.

The membrane technique was introduced to enrich PQ and DQ firstly in 2004 by M. Mulugeta and N. Megersa. The porous-PTFE flat sheet membrane was used in the supported liquid membrane (SLM) extraction mode. Di-(2-ethylhexyl) phosphoric

acid (DEHPA) in dihexyl ether (DHE) was impregnated in the pores of membrane. The donor solution, the pH-adjusted water sample, was continuously flowed to contact with the membrane surface. Another side of membrane was touched with the acidic acceptor solution. DEHPA acts as a carrier transferring two quats from the donor solution into the acceptor solution which was analyzed by HPLC-UV. One piece of membrane was used for several samples with carry-over effect being observed. Therefore, regeneration of membrane was required in between runs. The method detection limits was 0.74 and 0.56 µg/L for PQ and DQ, respectively *(26)*.

1.4 Purpose of the Study

From literature reviews, the commonly chosen sample preparation technique for the quaternary ammonium herbicides is the SPE technique. Most of the literatures suggested using SPE sorbent with ion-pairing capability for retaining the quats on the sorbent and eluting them using acidic aqueous solution. This process is tedious and some contaminants in water samples still interfere in the extraction causing inconsistant results. Moreover, the SPE cartridges are expensive resulting in high cost per an extraction.

Although many on-line systems were proposed, some drawbacks such as the carry-over effect and the high-cost instrument were presented. On-line system of supported liquid membrane (SLM) was also used as a sample preparation technique for the quaternary ammonium herbicides. This membrane technique is less expensive than SPE technique due to lower cost of membrane than the SPE sorbent. However, the carry-over effect is still the main problem.

Because the US EPA regulated MRLs of QAHs in drinking water are very low, the analysis requires highly sensitive instrumentation which is limited to very few choices. Therefore, most research is concentrated in the sample preparation and enrichment step for the same reason, the study aimed to develop a simple, rapid, selective, and inexpensive sample preparation technique for matrix clean-up and enrichment of four QAHs (PQ, DQ, CQ, and MQ) in drinking water.

Off-line supported liquid membrane microextraction mode (off-line SLMME) was chosen for this work. The analytes in the donor solution was extracted through the porous hollow fiber membrane whose pores were filled with organic solvent which acted as the analytes carrier. A small volume of the acceptor solution contained in the membrane lumen provided hydrogen ions in exchange with the analytes. After extraction, the acceptor solution was analyzed by LC-MS. Because of a large difference between donor volume and acceptor volume, good preconcentration of analyte was obtained. This technique can be used to enrich the analytes and clean up the sample matrix simultaneously because the donor solution does not contact with the acceptor solution. Cost per extracting by hollow-fiber is lower than SPE technique due to cheaper price of hollow fiber membranes. Another important aspect is the minimum volume of organic solvent used in this technique making it suitable for routine analysis in laboratory of the future where environmental regulation seems to limit use of organic solvent for the sake of environmental protection.

In this study, we optimized the SLMME parameters for achieving the best enrichment of analytes. The optimized parameters are as follows: the donor pH, the acceptor pH, the donor volume, the acceptor volume, the organic carrier immersion time, the carrier concentration, and the extraction time. The optimized extraction method was validated and tested on the commercial drinking water samples in Thailand.

CHAPTER II

THEORY

Sample preparation technique is used for preconcentration and cleaning-up the analytes prior to the actual analysis by adjusting the analyte concentration in the appropriate range of the analytical method of choice. This may require removing of other interferences such as matrix to enhance method sensitivity. Liquid-liquid extraction (LLE), the most popular sample preparation technique, uses an organic solvent to extract organic analytes from aqueous sample solutions in separation funnels or other vessels. The basis is the partitioning of the dissolved analytes between the organic phase (extraction liquid) and the aqueous solution (sample solution) which depends on their partition coefficients. Unfortunately, this technique is less attractive because it is tedious and time consuming, not easy to automate, easily form emulsion that makes it difficult to separate the two phases, and environmentally unfriendly due to large volumes of organic solvents used.

Solid-phase extraction (SPE) is the main competing extraction technique. The aqueous sample passes the solid sorbent in SPE column and the analytes are first trapped and then eluted with suitable small volume of organic solvent. It deviate some drawbacks from LLE such as it uses little amounts of organic solvents, is less demanding with respect to manual work and is available for the automatic instruments. However, some disadvantages with SPE technique can be identified as limited selectivity, insufficient retention of very polar compounds and high costs of disposable sorbent materials.

Nowadays, the development of sample preparation technique aims at decreasing organic solvents due to their toxicities and environmentally-unfriendly properties. Membrane extraction is a recent sample preparation technique with high potential to solve this problem. One type of this extraction technique in particular is hollow-fiber supported liquid membrane microextraction (SLMME) which is simple

and cheap due to the low cost of membrane per extraction and requires only a small volume of organic solvent.

2.1 Membrane Extraction Techniques ***(27,28,29,30,31)***

Membrane extraction techniques can be classified by their characteristic as porous and non-porous membranes. In porous membrane techniques, the solution on each side of the membrane is physically contacted through the pores of a membrane. These techniques are commonly used to separate low-molecular-mass analytes from high-molecular-mass matrix components. The selectivity of these processes is based on pore size and pore-size distribution. Therefore, porous membranes are mainly use for size-exclusion application such as filtration, reverse osmosis and dialysis. On a contrary in non-porous membrane techniques, the membrane, a liquid-impregnating porous membrane or absolutely solid membrane, forms a separate phase between the two phases called the donor phase and the acceptor phase. Analytes move through membrane either by diffusion flux or by partitioning, therefore these techniques are very versatile and are common called membrane extraction.

Membrane extraction techniques can also be divided by their mechanism into 2 categories: two-phase and three-phase, which are described below.

2.1.1 Two-Phase System: Microporous Membrane Liquid-Liquid Extraction (MMLLE)

MMLLE is a system which one aqueous phase is separated from one organic phase by porous hydrophobic membrane. In this technique, the acceptor is an organic solvent and the same solvent is filled in the membrane pores while the donor is an aqueous solution contacts on the other side of the membrane. In this set-up, the phases are never mixed and all mass transfer between the phases take place at the membrane surface. The extraction mechanism of this technique can be explained in a similar way to liquid-liquid extraction (LLE) technique. MMLLE is easily interfaced to gas chromatography (GC) or to normal phase liquid chromatography because the final

extract is usually an organic phase. Therefore, this technique is often employed in a dynamic system.

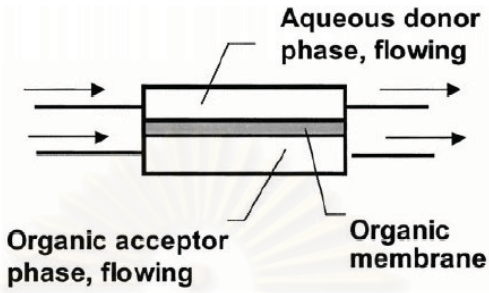


Figure 2.1 Schematic description of MMLLE *(28)*

As in classical LLE, the extraction efficiency of the MMLLE is controlled by partition coefficients. If the value is very high (i.e., the hydrophobicity of the analyte is high), it is possible to work with stagnant acceptor and still obtains a considerable enrichment of the analyte into a small extraction volume. If the partition coefficient is low, the acceptor phase might be circulated with a slow flow-rate in order to successively remove the analyte and to maintain sufficient diffusion through the membrane.

2.1.2 Three-Phase System: Supported Liquid Membrane Extraction (SLME)

In this technique, a porous hydrophobic polymer membrane is impregnated with an organic solvent, which is held in the pores of membrane by capillary forces. Typical solvents are long chain hydrocarbons like n-undecane, kerosene, di-hexyl ether, tri-octyl phosphate and several others. This immobilized organic solvent separates two aqueous solutions called the donor solution and the acceptor solution. The SLME is chemically similar to a LLE from an aqueous sample (donor) into an organic solvent, followed by a "back extraction" of the organic phase to a second aqueous phase (acceptor). The driving force is the difference of the analyte concentration between the donor and the acceptor phases.

In order to get the best extraction efficiency, the concentration gradient across the two phases must be maintained. At the donor side, the analyte must be in a non-ionic form for the ability to be extracted into the membrane. In contrast, it must be in an ionic form on the acceptor side for irreversible trapping. The most-simple way to achieve this condition is to adjust the pH of the two aqueous phases. Therefore, this technique is very suitable for ionizable compounds such as medium to weak acids and bases.

As shown in Figure 2.2, for example of SLME, if the acidic analytes are extracted, the donor pH must be adjusted to the value which is lower than the pK_a of analyte by at least 2-3 pH units. At this pH, the acidic analytes are in the non-ionic form and they can be extracted into the organic phase impregnated in the membrane pores. The basic compounds are in the ionic form at this low pH and cannot pass through the membrane. In the acceptor solution, the pH must be adjusted to the value which is higher than the pK_a of the analytes by at least 2-3 pH units for promoting good extraction of ionic analytes which cannot be back extracted into the membrane organic phase. Moreover, the concentration gradient of the diffusing species (non-ionic analytes) is largely unaffected by the total concentration of non-ionic analytes in the acceptor phase and the enrichment of the analytes is well achieved. For neutral compounds, they may be extracted but the concentration in the acceptor phase will never exceed that in the donor phase, so no enrichment is obtained. Macromolecule such as proteins may also be extracted but the extraction rate will be very low due to their low diffusions coefficients. In summary, SLME can provide very selective enrichment for small ionizable compounds.

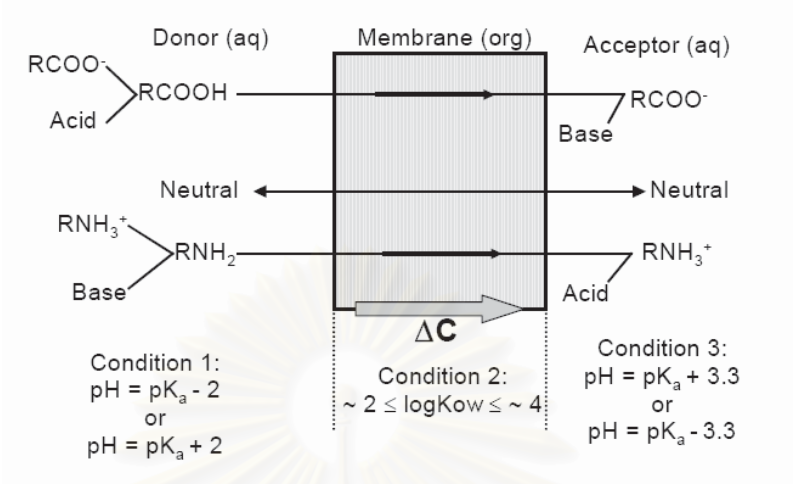


Figure 2.2 Schematic description of SLME *(27)*

In some case, extraction based on the difference of analyte solubility in the membrane and the acceptor solution may be difficult to perform. For example, the permanently charged compounds are too hydrophilic and cannot be changed into non-ionic form, so they cannot be extracted by SLME technique. The approach for this case is to incorporate a mobile carrier into the membrane that selectively binds the analytes. This is called the carrier facilitated transport or carrier-mediated SLME technique.

The carrier facilitated transports are divided into two types: the facilitated transport (or simple carrier transport) and the coupled transport illustrated in Figure 2.3 and Figure 2.4.

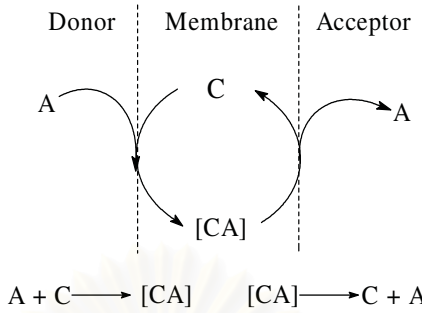


Figure 2.3 Facilitated transport (simple carrier transport) of carrier-mediated SLME

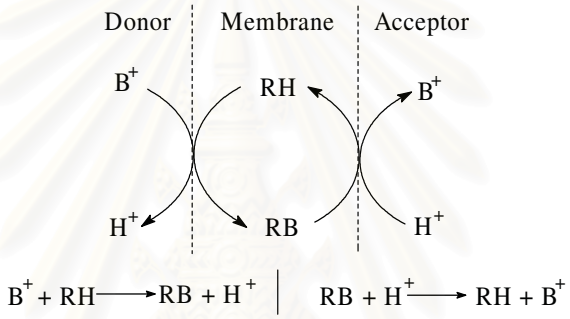


Figure 2.4 Coupled transport of carrier-mediated SLME

In facilitated transport (Figure 2.3), the liquid membrane phase contains a carrier agent (C) that forms a complex (CA) with the analyte (A) at interface. The complex is transported through the membrane and at membrane-acceptor interface, it is converted to a non-complex form in the acceptor solution (A).

In coupled transport (Figure 2.4), the charged carrier agent (RH) is incorporated in the membrane which has an ionic interaction with the charged analyte (B^+) to form an organic-soluble complex (RB) that can be transferred through the membrane organic phase. At the membrane-acceptor boundary, the reaction is reversed because of the higher concentration of hydrogen ion in the acceptor solution. The analyte ion is released to the acceptor solution and hydrogen ion is picked up. The re-formed carrier (RH) molecule diffuses back to the membrane-donor boundary. Hydrogen ion is the driving force of this system and transfers from the acceptor to the

donor solution, the opposite direction of how the analytes move. The carriers commonly used in this system are tri-n-octylphosphine oxide (TOPO), di-(2-ethylhexyl) phosphoric acid (DEHPA) and tricaprylmethylammonium chloride (Aliquat 336).

2.1.2.1 Theory of SLME

In SLME, extraction is usually evaluated as the extraction efficiency (EE), which can be expressed as:

$$EE = \frac{n_A}{n_D} = \frac{C_A V_A}{C_D V_D} \qquad \text{(Eq. 1)}$$

where n_D and n_A are the number of moles of analyte input to donor solution and collected in acceptor solution, respectively. EE is the fraction of analyte molecules that are transferred to the acceptor. This value is a function of many parameters including the magnitude of the partition coefficients of the analyte species between the aqueous phase and the organic (membrane) phase, the trapping conditions in the acceptor, and characteristics and dimension of the membrane.

The influence of the partition coefficient, K, on EE is rather complex. When the value of K is low, the membrane-controlled extraction condition is presented. Therefore, the analyte is insufficiently extracted into the organic membrane and the mass transfer is limited by the diffusion of the analyte compound through the membrane. The mass-transfer coefficient, k_m, is defined as:

$$k_m \propto \frac{K \cdot D_m}{h_m} \qquad \text{(Eq. 2)}$$

where D_m is the membrane diffusion coefficient and h_m is the membrane thickness

At intermediate values of K, the donor-controlled extraction condition is presented. Therefore, a considerably higher mass-transfer rate is normally obtained. Mass transfer is limited by the diffusion in the donor phase and thus depends on the

diffusion coefficient in the donor phase and on the donor flow conditions. In this region, the most efficient extraction is gained.

However, high hydrophobic species with too large values of K do not provide favorable extraction because significant amounts of analyte will be left in the membrane leading to low extraction efficiency. For this condition, the stripping of analyte into the acceptor phase becomes the limiting factor.

In conclusion, it was found that the most efficient extraction is obtained when the octanol-water partition coefficient (as a measure of polarity) of the diffusing species is around 10^3 ($\log K_{ow} = 3$).

The rate of mass transfer from donor to acceptor is proportional to the concentration difference, ΔC, over the membrane, which can be written:

$$\Delta C = \alpha_D C_D - \alpha_A C_A \qquad \text{(Eq. 3)}$$

where C_D and C_A are the analyte concentrations in the donor and acceptor phases, respectively and α_D and α_A are the fractions of analytes that are in extractable (uncharged) form in the indicated phase. Generally, the extraction conditions are set up such that α_D is close to 1 and α_A is a very small value. At the beginning of the extraction, the value C_A is zero and increases during the operation, usually to values well over C_D.

As long as α_A is sufficiently small, the term $\alpha_A C_A$ in Eq. 3 is negligible and EE will be constant during the course of the extraction, so the extracted amount will be directly proportional to the volume that is extracted and also to the concentration of analyte in the sample. This is usually the preferred situation and it is referred to as complete trapping.

If the trapping is not complete, EE will decrease with time, leading to less precise quantitation. In practical work, time is an important issue, and it is

therefore often more suitable to maximize the concentration enrichment factor rather than to maximize EE. The concentration enrichment factor (EF) and its relative with EE are expressed as:

$$EF = \frac{C_A}{C_D} \quad \text{(Eq. 4)}$$

$$EF = EE \cdot \frac{V_D}{V_A} \quad \text{(Eq. 5)}$$

where V_D and V_A are donor volume and acceptor volume, respectively. The term V_D/V_A is called phase ratio.

If the extraction is performed at longer time, the concentration in the acceptor, C_A, increases and eventually the second term in Eq. 3 will become significant and ΔC decreases. This leads to a lower rate of mass transfer and a decrease in EE. When ΔC has reached zero, all three phases are in equilibrium and the maximum concentration enrichment factor is attained. This is given by:

$$EF_{max} = \left(\frac{C_A}{C_D}\right)_{max} = \frac{\alpha_D}{\alpha_A} \quad \text{(Eq. 6)}$$

Note that this equation does not include the partition coefficients between the phases, in contrast to the conditions for classical LLE.

2.1.3 Other Membrane Based Extraction Techniques

2.1.3.1 Polymeric Membrane Extraction (PME)

In this technique, polymeric membrane (non-porous membrane) is used in place of porous membrane to separate the donor and the acceptor solutions. The most commonly used membrane material is silicone rubber because it is hydrophobic and has long lifetime. PME is possible for both aqueous-polymer-

aqueous extraction (similar to SLME) and also aqueous-polymer-organic extraction (like MMLLE).

Due to its solid nature, relative instability that is the drawback of liquid membrane is removed, allowing aqueous, organic and gaseous samples to be processed. Particularly, it is an ideal method for extracting analytes in complex samples with high amounts of organic materials such as lipids since the instability associated with liquid membranes does not exist. However, a fixed composition of the membrane reduces the scope of application for example the carrier incorporation, so the extraction of polar analytes is limited. Also, polymeric membranes lead to slower extraction because the diffusion coefficients in polymers are lower than in liquids.

The mass transfer of PME depends on the partition and diffusion of the analytes into the polymer and the partition into the receiving phase. The difference in the solubility and diffusion of various analytes into the polymer is the basis of selectivity.

2.1.3.2 Membrane Extraction with a Sorbent Interface (MESI)

In this technique, the hollow fiber non-porous membrane (usually silicone rubber) is used for the extraction of the analytes from the surrounding liquid or gaseous sample. Inside the fiber, a gaseous receiving phase (always a carrier gas of gas chromatography) flows and transports the analyte molecules from the membrane for trapping into a cooled solid-sorbent. The analytes are desorbed from this sorbent by heating and are transferred into a gas chromatographic system for the analysis. The technique is therefore suitable for volatile organic compounds in air or gaseous samples. The membrane probe and sorbent trap (sampling components) can be made either in-line or off-line for easy automation or field sampling, respectively. The differences in solubility and diffusion of various analytes into the membrane are the basis of the method selectivity.

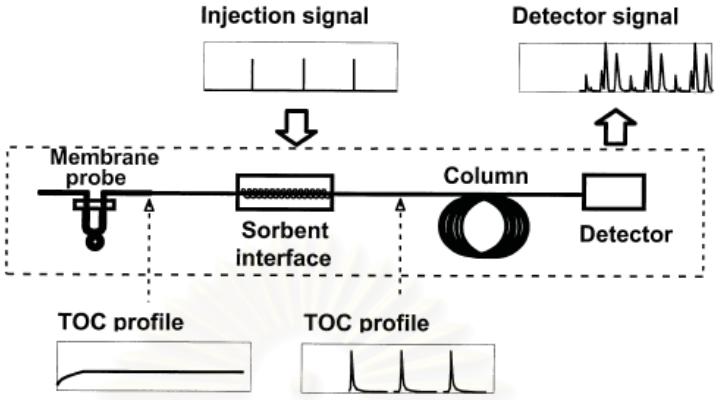


Figure 2.5 Components of membrane extraction with a sorbent interface (MESI) system *(28)*

2.2 Hollow Fiber Membrane Extraction ***(32,33,34)***

Hollow fiber membrane is one type of membrane using in membrane extraction. Generally, single, low-cost, disposable, porous, hydrophobic hollow fiber membranes is impregnated with an organic phase and can be used in either MMLLE and SLME modes. Similarly to planar membrane extraction, hollow fiber membrane extraction can be divided into two modes: two-phase extraction and three-phase extraction.

In two-phase system, the extraction mechanism is similar to the description of MMLLE (section 2.1.1). As shown in Figure 2.6, the donor solution is the aqueous sample, in which the analyte is extracted through the organic solvent immobilized in the membrane pores. The analyte then passes into the acceptor solution, which is the same as the solvent in the membrane pores. It should be mentioned that in two-phase extraction the final extract is usually an organic phase which is compatible with analytical techniques such as GC or normal-phase HPLC.

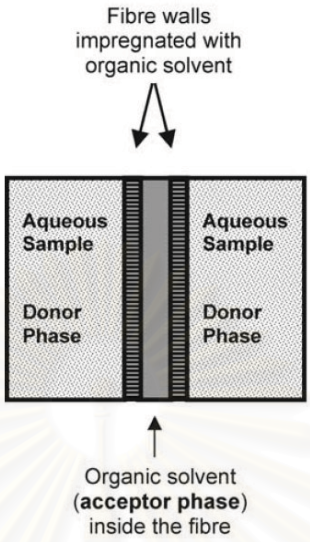


Figure 2.6 Cross-section of the hollow fiber in two-phase extraction system *(32)*

The extraction process of the two-phase system for analyte X is illustrated as follows:

$$X_D \longleftrightarrow X_A$$

where X_D and X_A are the analytes in aqueous donor solution and organic acceptor solution, respectively. This process depends on the partition coefficient between the acceptor solution and the donor solution ($K_{A/D}$), defined by:

$$K_{A/D} = \frac{C_{eq,A}}{C_{eq,D}} \qquad \text{(Eq. 7)}$$

where $C_{eq,A}$ and $C_{eq,D}$ are the analyte concentration at equilibrium in the organic acceptor solution and aqueous donor solution, respectively.

The extraction efficiency (EE) and the enrichment factor (EF) in the two-phase system may be calculated by Eq. 8 and Eq. 9:

$$EE = \frac{K_{A/D} \cdot V_A}{K_{A/D} \cdot V_A + V_D} = \frac{C_{eq,A} \cdot V_A}{C_D V_D} \quad \text{(Eq. 8)}$$

$$EF = EE \cdot \frac{V_D}{V_A} = \frac{C_{eq,A}}{C_D} \quad \text{(Eq. 9)}$$

where V_A and V_D are the acceptor volume and the donor volume (sample volume), respectively and C_D is the initial analyte concentration in aqueous donor solution.

In three-phase system, the extraction mechanism is similar to the description of SLME (section 2.1.2). SLME using hollow fiber membrane is called supported liquid membrane microextraction (SLMME). As shown in Figure 2.7, the analyte is extracted from an aqueous sample solution (donor solution) through the organic solvent immobilized in the membrane pores into another aqueous phase (acceptor solution) presented in the membrane lumen. The organic phase in this case acts as a barrier between the acceptor and the donor aqueous solution preventing the mixing of these two phases. Note that, this sampling mode is usually combined with a reverse-phase HPLC or a CE system, as the acceptor phase is aqueous.

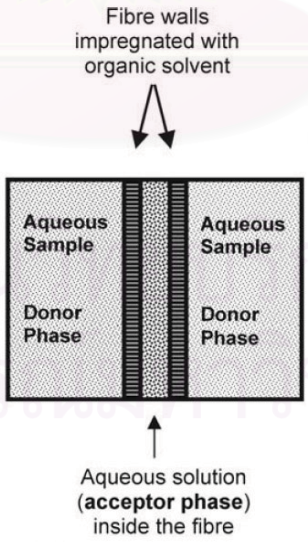


Figure 2.7 Cross-section of the hollow fiber in three-phase extraction system *(32)*

The extraction process of the three-phase system for analyte X is illustrated as follows:

$$X_D \longleftrightarrow X_M \longleftrightarrow X_A$$

where X_D, X_M and X_A are the analytes in aqueous donor solution, organic phase in the membrane pores and aqueous acceptor solution, respectively. The total extraction process is affected by both the partition coefficients between the organic phase and the aqueous donor solution ($K_{M/D}$) and that between the aqueous acceptor solution and the organic phase ($K_{A/M}$), defined by Eq. 10 and Eq. 11:

$$K_{M/D} = \frac{C_{eq,M}}{C_{eq,D}} \qquad \text{(Eq. 10)}$$

$$K_{A/M} = \frac{C_{eq,A}}{C_{eq,M}} \qquad \text{(Eq. 11)}$$

where $C_{eq,M}$, $C_{eq,D}$ and $C_{eq,A}$ are the analyte concentration at equilibrium in the organic phase, aqueous donor solution and aqueous acceptor solution, respectively. Therefore, the partition coefficient between the acceptor phase and the donor phase, $K_{A/D}$, can be considered as the overall driving force for the extraction and can be written as:

$$K_{A/D} = K_{M/D} \cdot K_{A/M} = \frac{C_{eq,A} V_A}{C_D V_D} \qquad \text{(Eq. 12)}$$

The extraction efficiency (EE) and the enrichment factor (EF) in the three-phase system may be calculated by Eq. 13 and Eq. 14:

$$EE = \frac{K_{A/D} V_A}{K_{A/D} V_A + K_{M/D} V_M + V_D} = \frac{C_{eq,A} V_A}{C_D V_D} \qquad \text{(Eq. 13)}$$

$$EF = EE \cdot \frac{V_D}{V_A} = \frac{C_{eq,A}}{C_D} \qquad \text{(Eq. 14)}$$

where V_D, V_M and V_A are the volume of aqueous donor solution (sample), organic phase and aqueous acceptor solution, respectively and C_D is the initial analyte concentration in aqueous donor solution.

2.2.1 Parameters and Practical Consideration Affecting Two-Phase and Three-Phase Hollow Fiber Membrane Extraction

2.2.1.1 Hollow Fiber Membrane

The hollow fiber membranes should be hydrophobic for the compatibility with the organic solvent being used. Polypropylene capillary membranes are used in almost all published reports of which the most common inner diameter is 600 μm that is compatible with the microliter volumes of the acceptor solution. In order to control high extraction speed, the membrane must have a large surface area that is in contact with the sample solution and a short diffusion distance between membrane and the fiber. Wall thickness is 200 μm because this seems to be the standard among commercial fibers. Wall thickness below 200 μm may result in poor mechanical stability, while values above 200 μm may result in extended extraction times and reduced recovery because of increases in the volume and the thickness of the organic phase. The pore sizes usually range from 0.2 and 0.64 μm for efficient microfiltration by allowing penetration of only small molecules (target analytes) through the pores of the hollow fiber. Figure 2.8 is illustrated the cross-section of a hollow fiber membrane.

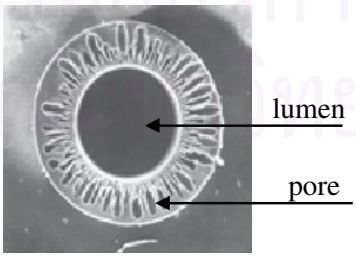


Figure 2.8 Cross-sectional of a hollow fiber membrane *(35)*

2.2.1.2 Organic Solvent

The selection of a suitable organic solvent is the key to the success in both two-phase and three-phase systems. Normally, various water-immiscible solvents differing in polarity and water solubility should be tested. The solvent should have low volatility for minimum loss during extraction, low water solubility compatible for avoiding dissolution into the aqueous phase, and with the polypropylene fiber for easy immobilized within the pores.

In two-phase system, the partition coefficient ($K_{A/D}$ in Eq. 7) is the key parameter for improving both the extraction efficiency and the enrichment factor. For non-ionizable analytes, $K_{A/D}$ is determined by the organic solvent selected as the acceptor solution which should provide high solubility for target analyte for obtaining high $C_{eq,A}$ value. Therefore, optimization of the organic solvent is valuable for this type of analytes.

In three-phase system, the selected solvent should ensure high values for both $K_{M/D}$ and $K_{A/M}$ in Eq. 12 to obtain large $K_{A/D}$. Therefore, the organic solvent should provide medium solubility for the analyte. If the solubility in the organic phase is too low, the analytes are poorly extracted from the donor solution. Whereas high analyte solubility within the organic phase may result in poor back extraction into the acceptor solution.

2.2.1.3 Agitation of the Sample

In both two-phase and three-phase system, the acceptor solution is restricted within the fiber, and it can tolerate very high agitation speed. Thus, the agitation of the donor solution can be applied to accelerate the extraction kinetic. This agitation facilitates the diffusion of analytes through the interfacial layer of the hollow fiber. Vibration and magnetic stirring are typically used for agitating the sample. Vibration had the advantage that it eliminated the possibility of sample contamination with the use of Teflon-coated magnetic stirrer. The use of magnetic stirrer was found to promote the formation of air bubbles adhere to the hollow fiber surface, thus accelerating solvent evaporation and introducing imprecision in the measurements.

2.2.1.4 Donor Volume and Acceptor Volume

From Eq. 9 and Eq. 14, the enrichment factors of both two-phase and three-phase system enhance when the ratio between donor volume and acceptor volume increase. However, adjusting the acceptor volume is related to the membrane length and also depends on the analytical technique coupled to the extraction device. For example, in contrast to GC and CE, acceptor volumes in the range 10-25 μL are easily injectable into a HPLC instrument, so the whole acceptor solution may be analyzed, potentially providing lower detection limits. The donor volume may also be adjusted according to the nature of sampl e.g., a few milliters for blood sample. On the other hand, the range of 50-250 mL of donor volumes can be used when natural water is applied. Eventhough the small donor volume is used, the donor-acceptor volume ratio is still high because the acceptor volume required in this technique is in microliter scale.

2.2.1.5 Adjustment of pH

Adjustment of pH can affect the extraction of acidic/basic target analytes. For two-phase system, pH adjustment in the donor solution is important to ensure that the analytes are present in their deionized state to enhance their extraction by organic acceptor solution. If charged compounds are very hydrophilic, two-phase system should not be chosen.

For three-phase extraction, both donor pH and acceptor pH are very importance. The pH adjustment of this system for the extraction of acidic analytes is described previously in section 2.1.2 (SLME technique). In addition, analogous observations can be made for basic analytes.

2.2.1.6 Extraction Time

Extraction efficiency of both two-phase and three-phase system is determined by extraction time because mass transfer of this technique is a time-dependent process. The rate of mass transfer is reduced when the system is near

equilibrium. The extraction eventually reaches equilibrium, after this point extended extraction times provide no more gain in extraction efficiency. In most cases, the extraction is carried out with extraction times close to equilibrium to maximize extraction efficiency. However, fast non-equilibrium extraction is possible and the extraction time should be one of the parameter studied and controlled for precise result.

2.2.2 Technical Set-Up

The first technical set-up is illustrated in Figure 2.9. The length of porous hollow fiber membrane can be adjusted depending on the acceptor volume (such as 8 cm for containing 25 μL of the acceptor solution). The fiber is first immersed in an organic solvent until saturation. Each end of the fiber is connected to a medical needle allowing a U-shape portion dipped into the sample vial (U-shape configuration). One steel needle is served to guide a microsyringe into the lumen of the fiber to deliver the acceptor solution, while the other steel needle serves as an exit tube for the acceptor solution after extraction. The fiber is placed in a sample vial with a silicone-septum screw cap containing the aqueous donor solution. During extraction, this vial might be extensively shaken or vibrated to speed up the process. After the extraction, a small head-pressure supplied by medical syringe is applied on the first steel needle, and the acceptor solution is collected from the exit tube in a HPLC vial insert for further analysis.

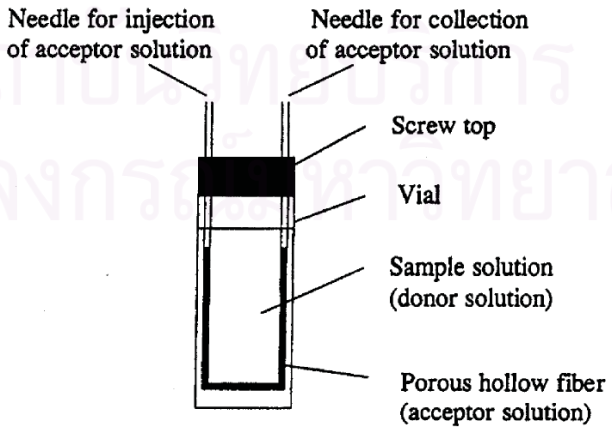


Figure 2.9 U-shape configuration of hollow fiber membrane technical set-up *(33)*

Figure 2.10 illustrated a rod-like configuration for the fiber. A microsyringe may be introduced down to the bottom of the fiber for delivery and removal of the acceptor solution. This concept is much more compatible with modern autosamplers. A conical guide is placed on the top of the fiber to ensure that the needle is effectively guided into the fiber. For this set-up, one end of the fiber is flame sealed and the length can be reduced to 1.5 cm for improving compatibility with small sample volumes, which are highly suitable for biomedical applications.

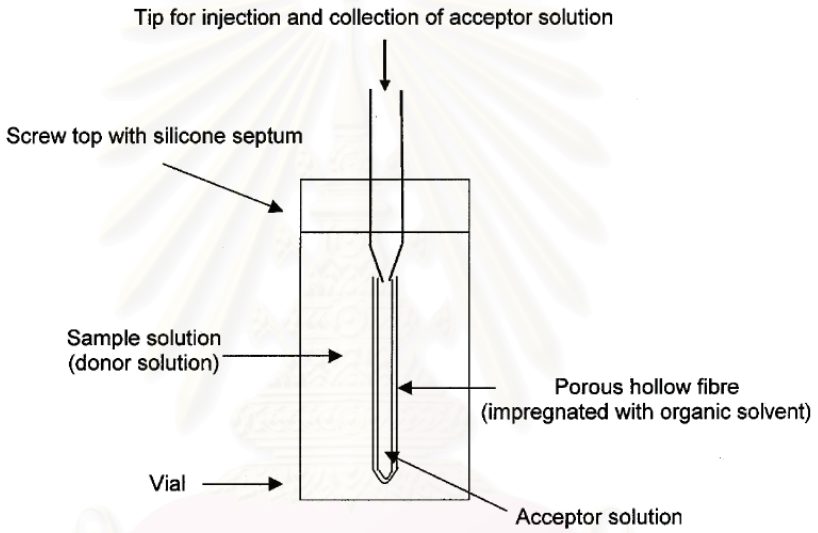


Figure 2.10 Rod-like configuration of hollow fiber membrane technical set-up *(33)*

Another interesting technical development is illustrated in Figure 2.11. In this concept, a microsyringe is used as a support for the hollow fiber. The acceptor solution is drawn into the microsyring and transferred into the hollow fiber. The fiber is then immersed in the organic solvent to fill the pores. After that, the fiber which is still attached to the microsyringe, is placed in the sample for extraction. At the end of the extraction, the acceptor solution is drawn into syringe again and injected into a chromatographic system. One end of the hollow fiber is flame sealed before using. However, without flame sealing, the same set-up is utilized for dynamic extractions, in which a programmable syringe pump automatically moves the plunger of the microsyringe.

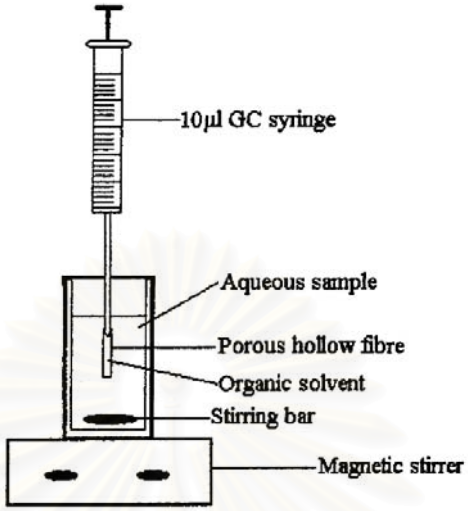


Figure 2.11 Technical set-up for static or dynamic hollow fiber membrane *(33)*

2.3 Liquid Chromatography-Mass Spectrometry (LC-MS) ***(36,37,38)***

Nowadays, high-performance liquid chromatography (HPLC) is the most widely used of all of the analytical separation techniques because of its widespread applicability to many substances. It is suitable for separating non-volatile species or thermal-unstable compounds. It is sensitive and readily adapted for accurate quantitative determinations. However, the main limitation of HPLC is its inability to provide an unequivocal identification of the mixture components even if they can be completely separated from each other.

Mass spectrometry (MS) is the identification technique for many compounds with a high degree of confidence. For the mixture of compounds, however, the mass spectrum obtained will contain ions from all of the compounds present. So, the combination of the separation capability of HPLC to introduce “pure” compounds into the mass spectrometer is the advantage, particularly for compounds with similar or identical retention characteristics usually have quite different mass spectra and can therefore be differentiated.

The combination of HPLC and MS has been investigated for over 30 years. These two techniques cannot be linked directly due to the incompatibilities between HPLC and MS. The reason is that the HPLC mobile phase is a liquid which is pumped at a flow rate of typically 1 mL/min, while the mass spectrometer operates at a pressure of around 10^{-6} torr. Therefore, it is impossible to pump the HPLC eluate directly into the source of a mass spectrometer and an interface system must be used to remove this problem. Moreover, the majority of analytes, which are separated by HPLC, are relatively involatile and/or thermally labile, so the suitable ionization methods have been developed.

2.3.1 Liquid Chromatography

Chromatographic system consists of five components: a mobile phase, a pump, an injector (sample introduction), a column (filled with a stationary phase) and a detector as shown in a block diagram in Figure 2.12. The following description of technique simply described the HPLC major components which are essential to the successful application of the LC-MS combination.

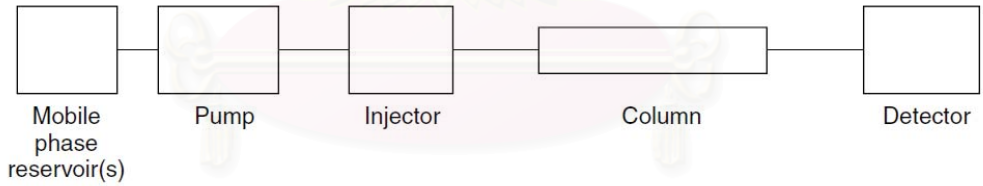


Figure 2.12 Block diagram of a typical HPLC system *(36)*

2.3.1.1 Pump

The pump is used for delivering the mobile phase into the system. It must provide stable flow rates from between 0.01 mL/min and 2 mL/min with the LC-MS requirement dependent on the interface being used and the diameter of the HPLC column. The most popular pump used today is the reciprocating pump.

2.3.1.2 Sample Introduction (Injector)

The loop injector is used almost exclusively in HPLC for introducing a liquid sample into a flowing liquid stream using a conventional syringe. While the loop is filled, mobile phase is pumped through the valve to the column. When the liquid sample in the loop is injected, a rotating switch is moved and the flow is diverted through the loop, thus flushing its contents into the column. It is important to ensure that air bubbles are not introduced into the injector because the liquid flow may be interrupted, so resulting in an imprecise retention time of analytes and an unstable response from the mass spectrometer.

2.3.1.3 Mobile Phase

For HPLC system, a mobile phase is a liquid in which the analytes are soluble. In reversed-phase chromatography, the majority of HPLC separations, the mobile phase is the more polar compound than the stationary phase and the more polar analytes elute more rapidly than the less polar ones.

The mixtures of solvents can be used as the mobile phase for improving the separation of analytes of widely different polarities. A separation using a constant composition of mobile phase is termed isocratic elution, while that in which the composition of the mobile phase is changed is termed gradient elution.

The use of buffers in mobile phase can control the degree of ionization of the analyte, reduce peak tailing and improve the reproducibility of retention. In LC-MS, the buffers must be volatile, such as ammonium acetate, because the mobile phase must be removed at LC-MS interface.

2.3.1.4 Stationary Phase

The majority of HPLC columns range in length from 10 to 30 cm and inner diameter for the columns used in LC-MS is between 1 and 4.6 mm. The common particle sizes of packings are 3, 5 and 10 μm. The most widely used columns

contain a chemically modified silica stationary phase, with the chemical modification determining the polarity of the column. For reverse-phase system, a very popular stationary phase is one in which a C18 alkyl group is bonded to the silica surface.

2.3.1.5 Detectors

Detectors for HPLC must have low dead volume to minimize extra-column band broadening. The detector should be small and compatible with liquid flow. The detector used will depend on the nature of the sample. However, the most widely used detectors for HPLC are based on absorption of ultraviolet or visible radiation because many organic molecules absorb UV radiation at 254 nm. In the case that analytes not absorb UV-radiation, the use of indirect UV detection, in which a UV-active compound is added to the mobile phase, may be employed.

Mass spectrometer is used as a detector in widely applications. The advantage is that it may allow differentiation of compounds with similar retention characteristics or may allow the identification and/or quantitative determination of components that are only partially resolved chromatographically, or even those that are totally unresolved. This may reduce the time required for method development.

2.3.2 Mass Spectrometry (MS)

A mass spectrometer can be divided into three main component parts, as follows:

- ion source
- mass analyzer
- ion detector

Figure 2.13 shown the block diagram of an LC/MS system and each MS components, which are the suitable types for LC, are described below.

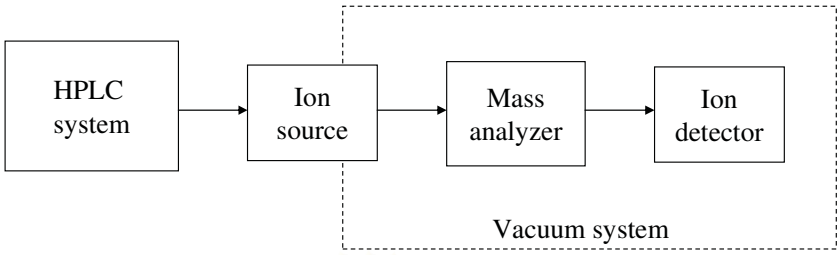


Figure 2.13 Block diagram of an LC/MS system

2.3.2.1 Ion Source

Ion source is used to ionize the analytes. In LC-MS, ionization of analyte occurs in the interface system which is the mass spectrometer inlet system for LC. The function of this system is to remove as much of the unwanted mobile phase as possible while still passing the maximum amount of analyte into the mass spectrometer and the analyte is ionized in this system simultaneously. The popular interface systems are the atmospheric pressure-electrospray ionization (AP-ESI) and the atmospheric pressure chemical ionization (APCI) described below.

Atmospheric Pressure-Electrospray Ionization (AP-ESI): Electrospray is the soft ionization technique. Ionization occurs at atmospheric pressure by the four steps:

- the formation of droplets from the HPLC eluate
- charging of these droplets
- desolvating of the droplets
- the formation of analyte ions

Electrospray spectra are produced by passing a liquid stream through a metal capillary maintained at high voltage (typically 3-4 kV for the production of positive ions; slightly less, and of opposite polarity, for the production of negative ions). This high voltage disperses the liquid stream, forming a mist of highly charged droplets that undergo desolvation during their passages across the source of the mass spectrometer. As the size of the droplet reduces, a point is reached (within 100 μs) at

which the repulsive forces between charges on the surface of the droplets are sufficient to overcome the cohesive forces of surface tension. A “Coulombic explosion” then occurs, producing a number of small droplets with a radius approximately 10% of that of the parent droplet.

A mobile phase with high surface tension and/or high viscosity should be avoided because it deters desolvation of the droplets. Ionization of the analytes is favored by the initial production of small droplets. The buffer concentration directly affects the size of droplets produced. Small droplets are gained when high buffer concentration is used. However, at buffer concentration over 10^{-3} M, the relationship between detector response and analyte concentration is not linear.

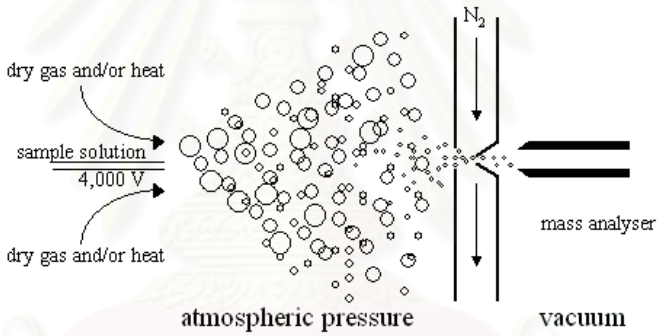


Figure 2.14 Schematic of electrospray ionization system *(36)*

The flow rate of HPLC mobile phase affects both the size and size distribution of the droplets formed during the electrospray process and, consequently, the number of charges on each droplet. This has an effect on the appearance of the mass spectrum which is generated.

The pH of the solution is also critical for the occurring of ionization and the appearance of the spectrum. The production of positive ions is favored at acidic pH but ions have been observed at pH at which a particular analyte would be expected to be fully deprotonated.

Electrospray ionization is applicable to a wide range of polar and thermally labile analytes of both low and high molecular weight. It produces predominantly multiple charged ions of the intact solute molecule. This effectively extends the mass range of the mass spectrometer and allows the study of molecules with molecular weights well outside its normal range. However, it has some drawback such as the observation of suppression effect and the sometime impossibility of direct analysis of mixtures. Structural information of analyte species is not usually available because electrospray is a soft-ionization method. Nevertheless, it is now the most widely used LC-MS interface and is compatible with a wide range of HPLC conditions.

Atmospheric-Pressure Chemical Ionization (APCI): APCI is another ionization techniques in which the stream of liquid emerging from an HPLC column is dispersed into small droplets by the combination of heat and a nebulizing gas, as shown in Figure 2.15. Two APCI systems are shown in Figure 2.16, with the major difference between them being the use of a heated capillary for desolvation and droplet transport in the second of these. Here, the HPLC effluent is passed through a pneumatic nebulizer where the droplets are both generated and desolvated. The spray so formed then passes through a heated region where the vapor is dried. The neutral species produced are then passed through a corona discharge. The latter occurs when the field at the tip of the electrode is sufficiently high to ionize the gas surrounding it but insufficiently high to cause a spark-where ionization of the analyte is effected by CI-type processes with the vaporized solvent acting as the reagent gas. The technique is capable of dealing with flow rates between 0.5 and 2 mL/min and is much more tolerant to a range of buffers.

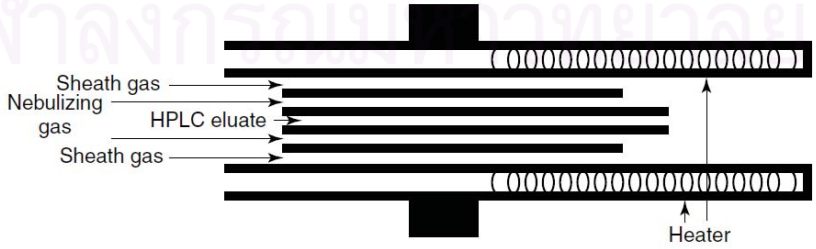


Figure 2.15 Schematic of an atmospheric-pressure-chemical-ionization probe *(36)*

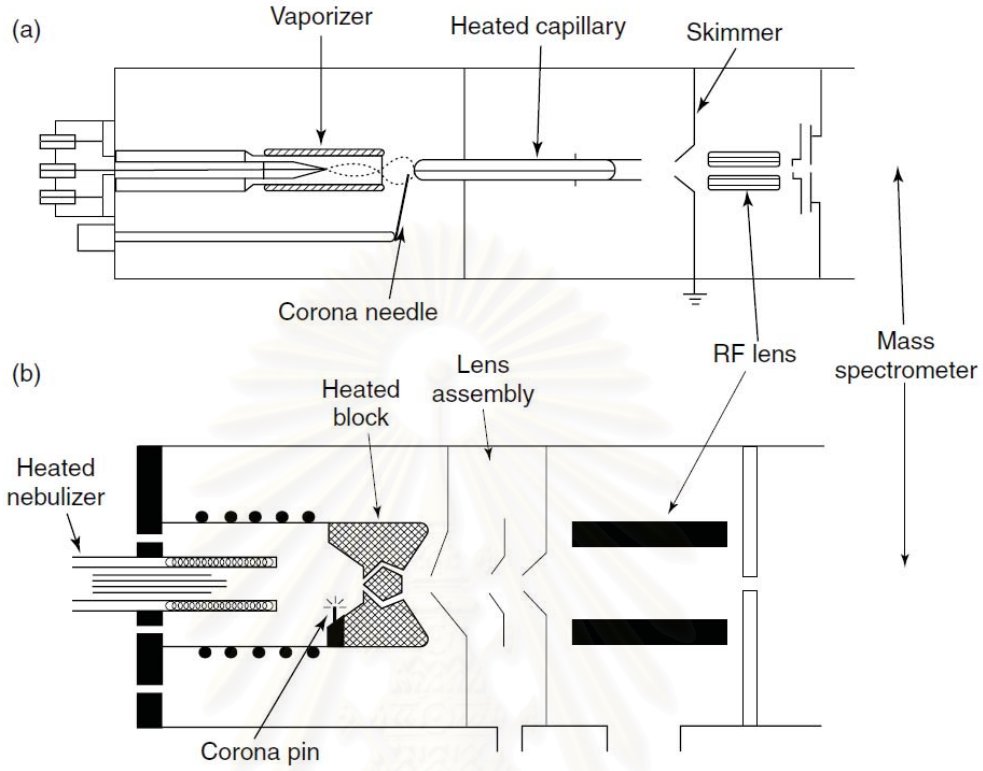


Figure 2.16 Schematics of two different designs of atmospheric-pressure-chemical-ionization LC-MS interfaces *(36)*

The ionization processes of the analyte are produced by the interaction of the electrons with the surrounding gas, undergo a number of reactions leading to the generation of reactive ions which interact with the analyte molecules. APCI leads to the formation of ion clusters involving solvent molecules, so these tend to make interpretation more difficult.

APCI is applicable to non-polar and slightly polar compounds. The mass limit for this technique is generally considered as below 2000 Da. It can be used for thermal instability compounds without their decomposition because it produces ions from solution (in contrast to ESI). The molecular weight of the analyte can be determined from this soft-ionization technique. This technique is more tolerant to the presence of buffers in the mobile phase stream than AP-ESI and able to use with flow

rates up to 2 mL/min, higher than that in AP-ESI. However, APCI spectra can contain ions from adducts of the analyte with the HPLC mobile phase or organic modifier and this technique is not suitable for analytes that are charged in solution.

2.3.2.2 Mass Analyzer

A mass spectrum may be considered to be a plot of the number of ions of each mass per charge ratio (m/z ratio) by an analyte upon ionization. Mass analyzer is used to separate the ions of different m/z ratios and determine these m/z values.

Quadrupole mass analyzer is the most widely used mass analyzer. It consists of four rods arranged as shown in Figure 2.17. The opposite pairs are connected electrically and a voltage, consisting of both radiofrequency (RF) and direct-current (DC) components, is applied, with the RF components on the two pairs of rods being 180° out-of-phase. At a specific value of these voltages, ions of a particular m/z follow a stable trajectory through the rods and reach the detector. A mass spectrum is therefore produced by varying the RF and DC voltages in a systematic way to bring ions of increasing or decreasing m/z ratios to the detector. This mass analyzer is classified as a low-resolution device (resolution about 500-4,000 at maximum). However, the instrument is cheap, small, robust and reliable.

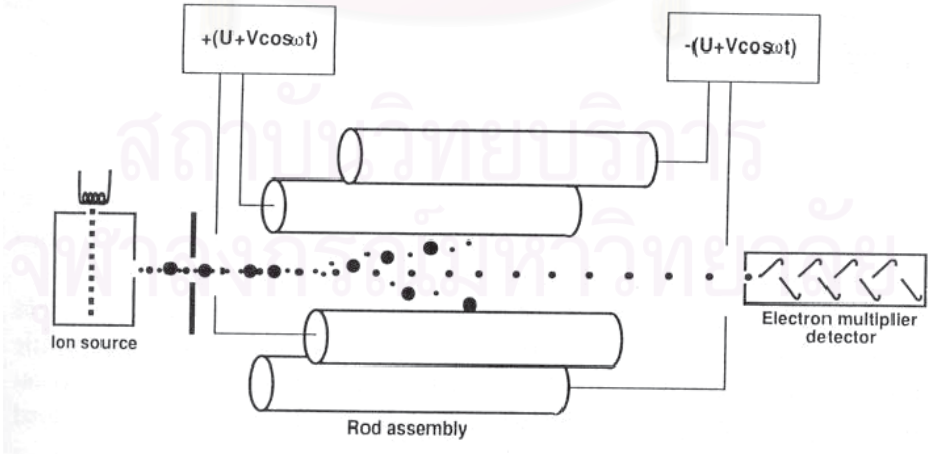


Figure 2.17 Schematic of the quadrupole mass analyzer *(39)*

2.3.2.3 Ion Detector

Ion detector is used for detecting the separated ions. The most popular is the electron multiplier which multiplies ion charges. Electron multiplier tube is made from glass or metal curved tube of which the inner surface is coated with electron-emissive material (such as PbO_2). It is biased at the entrance with large potential of opposite charge to the analyzed ion. When an ion hits the entrance of detector, it knocks electrons out of the surface then electron is accelerated to strike the next portion of tube by electrostatic force produced more and more electron. This process can amplifies the electrical current (electrons) about 10^6-10^8.

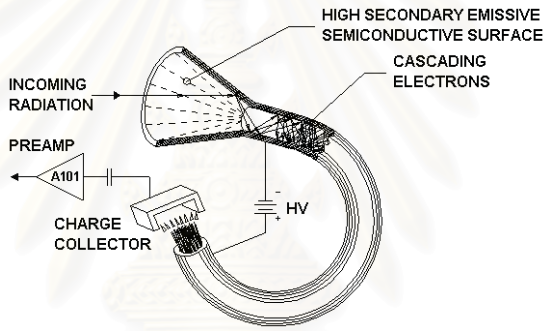


Figure 2.18 Schematic of the electron multiplier tube *(40)*

CHAPTER III

EXPERIMENTAL

3.1 Instrument and Apparatus

3.1.1 Liquid chromatograph module 1100^{TM} coupled to mass spectrometer module G1946D (LC/MS) equipped with an automatic degasser, an autosampler, a quaternary pump, a column thermostat and a mass spectrometer detector with an atmospheric pressure electrospray ionization (AP-ESI) interface, Agilent Technologies, USA.

3.1.2 HPLC column: Atlantis HILIC silica column, 150 x 2.1 mm I.D., 3.0 μm particle sizes, Waters, Switzerland.

3.1.3 Magnetic stirrer, model MUA/USEEP, Fisher Scientific, UK.

3.1.4 Vortex mixer, model G-560E, Scientific Industries, USA.

3.1.5 Milli-Q ultra-pure water system, model Millipore ZMQS5V00Y, Millipore, USA.

3.1.6 pH meter, model 744, Metrohm Ltd., Switzerland.

3.1.7 Vacuum pump, model DOA-V130-BN, Millipore, USA.

3.1.8 Porous polypropylene hollow fiber membranes, 600 μm I.D., 200 μm wall thickness and 0.2 μm pore size, Accurel PP Q3/2, Membrana, Germany.

3.1.9 Medical syringe needles, 800 μm O.D., Nipro Medical Corporation, Japan.

3.1.10 Medical syringes, 3 mL, Nipro Medical Corporation, Japan.

3.1.11 Microsyringe, 100 μL, Hamilton, Switzerland.

3.1.12 Nylon membrane filter, 0.45 μm pore size, 47 mm diameter, Alltech Associated Inc., USA.

3.1.13 PTFE membrane filter, 0.45 μm pore size, 47 mm diameter, Alltech Associated Inc., USA.

3.1.14 Micropipettes 10-100 μL and 100-1,000 μL.

3.1.15 Micropipette tips 100 μL, 1,000 μL.

3.1.16 Glass filter holder set (300 mL funnel, 1 L flask, glass base and tube cap, and 47 mm spring clamp) for HPLC mobile phase filtration, Millipore, USA.

3.1.17 Magnetic bars.

3.1.18 Solvent bottles 250, 500 and 1,000 mL.

3.1.19 Vials with silicone-septum screw caps 2 and 4 mL.

3.1.20 Bottles with silicone-septum screw caps 20, 50 and 120 mL.

3.1.21 Micro-insert vials, 200 μL.

3.1.22 Beakers 50, 100, 250 and 600 mL.

3.1.23 Stirring rods.

3.1.24 Spatulas.

3.1.25 Graduated cylinder 100 mL.

3.1.26 Volumetric flask 100 mL.

3.1.27 Nitrogen gas 99.99% purity, TIG, Thailand.

3.1.28 Liquid nitrogen 180 L, TIG, Thailand.

All glass apparatus were washed with detergent and rinsed with distilled water before used.

3.2 Chemical

3.2.1 Standard Compounds

Paraquat chloride tetrahydrate (99% purity) and diquat dibromide monohydrate (99% purity) were purchased from Chemservice, USA. chlormequat chloride (99.8% purity) was from Riedel-de Haën, Germany, and mepiquat chloride (98.7% purity) was from Dr.Ehrenstorfer GmbH, Germany.

3.2.2 Organic Solvents

Acetonitrile (J.T. Baker Chemical Company, Holland) was ultra-residue of analytical grade. Di-n-hexyl ether (Fluka, Switzerland) was analytical grade and di-(2-ethyl hexyl) phosphoric acid (Fluka, Switzerland) was technical grade.

3.2.3 Reagents

Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) and O-phosphoric acid 85% (H_3PO_4) were analytical grade purchased from Fluka, Switzerland. Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), hydrochloric acid fuming 37% (HCl) and ammonium formate (NH_4COOH) were analytical grade supplied by Merck, Germany. Formic acid 98% (HCOOH) was analytical grade purchased from Fisher Scientific, UK.

3.3 Preparation of Solution

3.3.1 Preparation of Standard Solutions

Paraquat, diquat, chlormequat, and mepiquat 1,000 mg/L stock standard solutions were individually prepared by dissolving each standard (0.1762 g, 0.1955 g, 0.1762 g, and 0.1342 g of paraquat chloride tetrahydrate, diquat dibromide monohydrate, chlormequat chloride, and mepiquat chloride, respectively) in Milli-Q water and diluting them to the 100.00-mL mark in volumetric flask. All of these stock solutions were kept in refrigerator at about 8˚C.

3.3.2 Preparation of Mobile Phase for LC-MS

Mobile phase A was prepared by dissolving 0.63 g of ammonium formate in 1,000 mL of Milli-Q water and 2 mL of formic acid was added into the solution. The mobile phase was filtered through a nylon membrane filter using a vacuum pump.

Mobile phase B is acetronitrile which was filtered through a PTFE membrane filter using a vacuum pump.

3.3.3 Preparation of 0.1 M Phosphate Buffer pH 3.0, 4.0, 5.0, 6.0 and 6.5

Solution A: Phosphoric acid 0.1 M was prepared by adding 6.7 mL of O-phosphoric acid (H_3PO_4) into Milli-Q water and adjusted the total volume to 1,000 mL.

Solution B: Sodium dihydrogen phosphate solution 0.1 M was prepared by dissolving 13.8 g of sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) in 1,000 mL of Milli-Q water.

Solution C: Disodium hydrogen phosphate solution 0.1 M was prepared by dissolving 35.9 g of disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) in 1,000 mL of Milli-Q water.

Phosphate buffer pH 3.0 and 4.0 were prepared by adding solution A into solution B until the pH of the solution detected from a pH meter equaled 3.0 and 4.0, respectively.

Phosphate buffer pH 5.0, 6.0 and 6.5 was prepared by adding solution C into solution B until the pH of the solution detected from a pH meter equaled 5.0, 6.0 and 6.5 respectively.

3.4 The Study of LC-MS Optimization

Flow injection analysis (FIA) in full scan mode was used to optimize the MS conditions which affected the sensitivity of analytes. The HPLC conditions used for FIA are as follows: the mobile phase was 50% B, the flow rate was 0.5 mL/min and the injection volume was 1 µg/L. Each individual quats at 10-µg/L concentration were injected. The mass ions and their relative abundances of all analytes were shown in Table 4.2. The results of optimized fragmentor voltage were illustrated in Figure 4.1 and the optimum MS conditions were shown in Table 4.1. The mass ions and their relative abundances of all analytes were shown in Table 4.2. The mass spectra of PQ, DQ, CQ and MQ were shown in Figure 4.2, 4.3, 4.4 and 4.5, respectively.

The HPLC conditions were developed by varying the flow rate and percentage of mobile phase in order to obtain the optimum separation for four quats. A 100 µg/L of the mixed standard solutions was injected into the LC-MS under optimum MS and HPLC conditions listed in Table 4.1 and 4.3, respectively. The extracted ion chromatograms of mixed standard solutions were shown in Figure 4.6.

3.5 The Study of SLMME Optimization

In this section, parameters that affected the extraction efficiency of SLMME were optimized as follows: the donor pH, the acceptor pH, the donor volume, the acceptor volume, the carrier concentration, the immersion time and the extraction time. For all parameters, the enrichment factor (EF) values were used to evaluate the extraction efficiency.

3.5.1 The Study of Donor pH

The procedure used to study effect of the donor pH on extraction can be described as follows:

3.5.1.1 A 12-cm length of hollow fiber membrane was immersed into 100% di-(2-ethyl hexyl) phosphoric acid (DEHPA) for 20 minutes. The excess solvent in the membrane lumen was flushed out by water and blow-dry.

3.5.1.2 Two medical syringe needles, inserted through a silicone septum of the screw cap were used to hold the two ends of the membrane.

3.5.1.3 The 30 µL of HCl pH 0.5 (acceptor solution) was injected into the membrane lumen by a microsyringe.

3.5.1.4 The donor solution was prepared by spiking 100 µg/L of PQ, DQ, CQ and MQ standard solutions in phosphate buffer pH 3.0. The membrane from 3.5.1.3 was placed in a 4-mL vial filled by 3.5 mL of the donor solution. This solution was vibrated using a vortex mixer for 30 minutes.

3.5.1.5 The acceptor solution was flushed into a 200-µL micro-insert by pressure from a medical syringe. It was then dried by blowing of nitrogen gas. The

residue was dissolved in mobile phase before injected into the LC-MS set at the optimum conditions (Table 4.1 and 4.3).

3.5.1.6 The procedures from 3.5.1.1 to 3.5.1.5 were repeated by changing the pH of phosphate buffer (in 3.5.1.4) to 4.0, 5.0, 6.0 and 6.5. Each pH condition was repeated in triplicate.

The EFs of the donor pH optimization were shown in Table 4.4. The averages and standard deviations of these values were graphed in Figure 4.7.

3.5.2 The Study of Acceptor pH

The procedure used to study effect of the acceptor pH on extraction can be described as follows:

3.5.2.1 A 12-cm length of hollow fiber membrane was immersed into 100% DEHPA for 20 minutes. The excess solvent in the membrane lumen was flushed out by water and blow-dry.

3.5.2.2 Two medical syringe needles, inserted through the silicone septum of the screw cap were used to hold the two ends of the membrane.

3.5.2.3 The 30 µL of HCl pH 0.0 (acceptor solution) was injected into the membrane lumen by a microsyringe.

3.5.2.4 The donor solution was prepared by spiking 100 µg/L of PQ, DQ, CQ and MQ standard solutions in phosphate buffer pH 3.0. The membrane from 3.5.2.3 was placed in a 4-mL vial filled by 3.5 mL of the donor solution. This solution was vibrated using a vortex mixer for 30 minutes.

3.5.2.5 The acceptor solution was flushed into a 200-µL micro-insert by pressure from a medical syringe. It was then dried by blowing of nitrogen gas. The residue was dissolved in mobile phase before injected into the LC-MS set at the optimum conditions (Table 4.1 and 4.3).

3.5.2.6 The procedures from 3.5.2.1 to 3.5.2.5 were repeated by changing the pH of HCl (in 3.5.2.3) to 0.5, 1.0, 1.5 and 2.0. Each pH condition was repeated in triplicate.

The EFs of the acceptor pH optimization were shown in Table 4.5. The averages and standard deviations of these values were graphed in Figure 4.8.

3.5.3 The Study of Donor Volume

The procedure used to study effect of the donor volume on extraction can be described as follows:

3.5.3.1 A 12-cm length of hollow fiber membrane was immersed into 100% DEHPA for 20 minutes. The excess solvent in the membrane lumen was flushed out by water and blow-dry.

3.5.3.2 Two medical syringe needles, inserted through the silicone septum of the screw cap were used to hold the two ends of the membrane.

3.5.3.3 The 30 μL of HCl pH 0.5 (acceptor solution) was injected into the membrane lumen by a microsyringe.

3.5.3.4 The donor solution was prepared by spiking 100 μg/L of PQ, DQ, CQ and MQ standard solutions in phosphate buffer pH 5.0. The membrane from 3.5.3.3 was placed in a 4-mL vial filled by 3.5 mL of the donor solution. This solution was vibrated using a vortex mixer for 45 minutes.

3.5.3.5 The acceptor solution was flushed into a 200-μL micro-insert by pressure from a medical syringe. It was then dried by blowing nitrogen gas. The residue was dissolved in mobile phase before injected into the LC-MS set at the optimum conditions (Table 4.1 and 4.3).

3.5.3.6 The processes from 3.5.3.1 to 3.5.3.5 were repeated by changing the volume of the phosphate buffer pH 5.0 (in 3.5.3.4) to 15.0, 25.0, 40.0 and 120.0 mL. Each donor volume was repeated in triplicate.

3.5.3.7 The processes from 3.5.3.1 to 3.5.3.5 were repeated by changing the volume of the phosphate buffer pH 5.0 (in 3.5.3.4) to 120.0 and 250.0 mL. The system agitation was also changed from vibration to stirring at 450 rpm for 45 minutes, using a magnetic stirrer. Each donor volume was repeated in triplicate.

The EFs of the donor volume optimization were shown in Table 4.6. The averages and standard deviations of these values were graphed in Figure 4.9.

3.5.4 The Study of Acceptor Volume

The procedure used to study the effect of the acceptor volume on extraction can be described as follows:

3.5.4.1 An 8-cm length of hollow fiber membrane was immersed into 100% DEHPA for 20 minutes. The excess solvent in the membrane lumen was flushed out by water and blow-dry.

3.5.4.2 Two medical syringe needles, inserted through the silicone septum of the screw cap were used to hold the two ends of the membrane.

3.5.4.3 The 20 μL of HCl pH 0.5 (acceptor solution) was injected into the membrane lumen by a microsyringe.

3.5.4.4 The donor solution was prepared by spiking 100 μg/L of PQ, DQ, CQ and MQ standard solutions in phosphate buffer pH 5.0. The membrane from 3.5.4.3 was placed in a 120-mL vial filled by 120 mL of the donor solution. This solution was stirred using a magnetic stirrer for 45 minutes.

3.5.4.5 The acceptor solution was flushed into a 200-μL micro-insert by pressure from a medical syringe. It was then dried by blowing nitrogen gas. The residue was dissolved in mobile phase before injected into the LC-MS set at the optimum conditions (Table 4.1 and 4.3).

3.5.4.6 The procedures from 3.5.4.1 to 3.5.4.5 were repeated by changing the membrane length (in 3.5.4.1) to 12, 25, and 37 cm and the volume of HCl (in 3.5.4.3) to 30, 60, and 100 μL, respectively. Each acceptor volume was repeated in triplicate.

The EFs of the acceptor volume optimization were shown in Table 4.7. The averages and standard deviations of these values were graphed in Figure 4.10.

3.5.5 The Study of Carrier Concentration

The procedure used to study the effect of the carrier concentration on extraction can be described as follows:

3.5.5.1 A 12-cm length of hollow fiber membrane was immersed into 30% v/v of DEHPA in di-n-hexyl ether (DHE) for 20 minutes. The excess solvent in the membrane lumen was flushed out by water and blow-dry.

3.5.5.2 Two medical syringe needles, inserted through the silicone septum of the screw cap were used to hold the two ends of the membrane.

3.5.5.3 The 30 μL of HCl pH 0.5 (acceptor solution) was injected into the membrane lumen by a microsyringe.

3.5.5.4 The donor solution was prepared by spiking 100 μg/L of PQ, DQ, CQ and MQ standard solutions in phosphate buffer pH 5.0. The membrane from 3.5.5.3 was placed in a 4-mL vial filled by 3.5 mL of the donor solution. This solution was vibrated using a vortex mixer for 45 minutes.

3.5.5.5 The acceptor solution was flushed into a 200-μL micro-insert by pressure from a medical syringe. It was then dried by blowing nitrogen gas. The residue was dissolved in mobile phase before injected into the LC-MS set at the optimum conditions (Table 4.1 and 4.3).

3.5.5.6 The procedures from 3.5.5.1 to 3.5.5.5 were repeated by changing the ratio (percentage) of DEHPA in DHE (in 3.5.5.1) to 40, 50, 60, 70, 80, 90 and 100 %v/v. Each carrier concentration was repeated in triplicate.

The EFs of the carrier concentration optimization were shown in Table 4.8. The averages and standard deviations of these values were graphed in Figure 4.11.

3.5.6 The Study of Immersion Time

The procedure used to study the effect of the immersion time on extraction can be described as follows:

3.5.6.1 A 12-cm length of hollow fiber membrane was immersed into 70% v/v of DEHPA in DHE for 5 minutes. The excess solvent in the membrane lumen was flushed out by water and blow-dry.

3.5.6.2 Two medical syringe needles, inserted through the silicone septum of the screw cap were used to hold the two ends of the membrane.

3.5.6.3 The 30 µL of HCl pH 0.5 (acceptor solution) was injected into the membrane lumen by a microsyringe.

3.5.6.4 The donor solution was prepared by spiking 100 µg/L of PQ, DQ, CQ and MQ standard solutions in phosphate buffer pH 5.0. The membrane from 3.5.6.3 was placed in a 4-mL vial filled by 3.5 mL of the donor solution. This solution was vibrated using a vortex mixer for 45 minutes.

3.5.6.5 The acceptor solution was flushed into a 200-µL micro-insert by pressure from a medical syringe. It was then dried by blowing nitrogen gas. The residue was dissolved in mobile phase before injected into the LC-MS set at the optimum conditions (Table 4.1 and 4.3).

3.5.6.6 The procedures from 3.5.6.1 to 3.5.6.5 were repeated by changing the immersion time (in 3.5.6.1) to 10, 20, 40, 90, 180 min and 1 day. Each immersion time was repeated in triplicate.

The EFs of the immersion time optimization were shown in Table 4.9. The averages and standard deviations of these values were graphed in Figure 4.12.

3.5.7 The Study of Extraction Time

The procedure used to study the effect of the extraction time on extraction can be described as follows:

3.5.7.1 A 12-cm length of hollow fiber membrane was immersed into 70% v/v of DEHPA in DHE for 20 minutes. The excess solvent in the membrane lumen was flushed out by water and blow-dry.

3.5.7.2 Two medical syringe needles, inserted through the silicone septum of the screw cap were used to hold the two ends of the membrane.

3.5.7.3 The 30 µL of HCl pH 0.5 (acceptor solution) was injected into the membrane lumen by a microsyringe.

3.5.7.4 The donor solution was prepared by spiking 100 µg/L of PQ, DQ, CQ and MQ standard solutions in phosphate buffer pH 5.0. The membrane from 3.5.7.3 was placed in a 4-mL vial filled by 3.5 mL of the donor solution. This solution was vibrated using a vortex mixer for 30 minutes.

3.5.7.5 The acceptor solution was flushed into a 200-µL micro-insert by pressure from a medical syringe. It was then dried by blowing nitrogen gas. The residue was dissolved in mobile phase before injected into the LC-MS set at the optimum conditions (Table 4.1 and 4.3).

3.5.7.6 The procedures from 3.5.7.1 to 3.5.7.5 were repeated by changing the extraction time (in 3.5.7.4) to 45, 60, 90 and 120 min. Each extraction time was repeated in triplicate.

The EFs of the extraction time optimization were shown in Table 4.10. The averages and standard deviations of these values were graphed in Figure 4.13.

The SLMME optimum conditions were summarized and in Table 4.11. To obtain the maximum EFs of all analytes, 10 replications of 5-µg/L spiked four quats in reagent water was extracted using SLMME conditions from Table 4.11.

3.6 The Method Validation

The purpose of method validation process is to confirm that the developed analytical procedure is suitable for its intended use. The parameters for method validation studied in this research are as follows: the selectivity, the calibration curve, the method quantitation limits (MQLs), the method detection limits (MDLs), the accuracy and the precision.

3.6.1 The Study of Selectivity

Selectivity refers to the extent to which a method can determine particular analytes in mixtures or matrices without interferences from other components. Selectivity in the chromatography technique was determined by monitoring the retention time of each analyte from each injection. For mass spectrometry technique, target ion and qualified ion were used to confirm the selectivity. In this study, the retention times of all analytes of the 5 µg/L spiked standard solutions ran in 10 replications were tested. The results are listed in Table 4.12.

3.6.2 The Study of Calibration Curve

In this study, donor solutions which are the 2, 4, 6 and 10-µg/L spiked standard solutions in phosphate buffer pH 5.0, were extracted using the optimum conditions shown in Table 4.10 in triplicate. The calibration curve of each analyte was obtained from the relationship between the analyte peak area and the spiked analyte concentration. The calibration curves of PQ, DQ, CQ and MQ were shown in Figure 4.14, 4.15, 4.16 and 4.17, respectively. The slope, intercept and correlation coefficient (R^2) values of each calibration curve were reported and shown in Table 4.13.

3.6.3 The Studies of Method Quantitation Limits (MQLs) and Method Detection Limits (MDLs)

The method quantitation limit is the analyte concentration of spiked standard solution that yields a peak after extraction at signal-to-noise ratios (S/N) equals to 10. The donor solution, 5-µg/L spiked standard solutions in phosphate buffer pH 5.0, was extracted using the optimum conditions shown in Table 4.11 in eight replications. A chromatogram of each standard solution was used to calculate the MQL value.

The method detection limit is the lowest analyte concentration of spiked standard solution that can be detected after the extraction. In this research, the method detection limit was obtained by calculation from the method quantitation limit by using the S/N equal to 3. The MQLs and MDLs of all analytes were shown in Table 4.14.

3.6.4 The Study of Accuracy

The accuracy of a method is the closeness of the measured values to the true value (concentration) of the sample. Accuracy was determined by analyzing the extraction of 10 replications of water samples spiked with 5 µg/L of the analytes using the optimum conditions shown in Table 4.11. The concentration after extraction was determined by the calibration curve obtained in Section 3.6.2. This concentration

is compared with the spiked concentration to obtain the % relative recovery as shown in Table 4.15.

3.6.5 The Study of Precision

Precision is the deviation of the results obtained from multiple analyses of homogeneous sample(s). In this research, within-day and between-day precisions were studied. Within-day precision was obtained from the repeated analysis of the same sample in one day. The study was performed by extracting the 5 and 50 µg/L spiked standard solutions in reagent water using the optimum conditions shown in Table 4.11. Each concentration was repeated in 10 replications. Within-day precision was reported as the standard deviation of the analyte enrichment factor for each concentration and shown in Table 4.16.

Between-day precision was obtained from the same analytical procedure as within-day precision and this procedure was performed repeatedly on three different days. The P-value calculated from ANOVA was used to determine between-day precision and the results were shown in Table 4.16.

3.7 The Application of Method in Drinking Water Samples

For the application of validated method, three commercial drinking waters, Crystal, Namthip, and Siam, were used as blank samples drinking waters. The procedure for determining PQ, DQ, CQ and MQ spiked in blank sample can be described as follows:

3.7.1 A 12-cm length of hollow fiber membrane was immersed into 70% v/v of DEHPA in DHE for 20 minutes. The excess solvent in the membrane lumen was flushed out by water and blow-dry.

3.7.2 Two medical syringe needles, inserted through the silicone septum of the screw cap were used to hold the two ends of the membrane.

3.7.3 The 30 µL of HCl pH 0.5 (acceptor solution) was injected into the membrane lumen by a microsyringe.

3.7.4 The donor solution was prepared by adding the crystal of 1.638 g $NaH_2PO_4.2H_2O$ and 0.0469 g $Na_2HPO_4.12H_2O$ into 120-mL of blank drinking water sample to form a buffer system at pH 5.0. After that, 5 µg/L of PQ, DQ, CQ and MQ standard solutions were spiked into the sample.

3.7.5 The membrane from 3.7.3 was placed in the donor solution. This solution was stirred using a magnetic stirrer for 60 minutes.

3.7.6 The acceptor solution was flushed into a 200-µL micro-insert by pressure from a medical syringe. It was then dried by blowing nitrogen gas. The residue was dissolved in mobile phase before injected into the LC-MS set at the optimum conditions (Table 4.1 and 4.3).

3.7.7 The procedures from 3.7.1 to 3.7.6 were repeated by changing the spiked standard concentration (in 3.7.4) to 20 µg/L. Each concentration was repeated in duplicate. The results were summarized in Table 4.17 and the chromatograms of spiked drinking water sample and blank drinking water sample after extraction were shown in Figure 4.18 and 4.19 respectively.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The Result of LC-MS Optimization

4.1.1 ESI Parameters Optimization

For MS detector, the sensitivity of analyte detection depends on the efficiency of analyte ionization. In this study, the analytes were ionized using ESI mode, thus ESI parameters were optimized in order to obtain the maximum sensitivity of analyte detection. Flow injection analysis (FIA) was used to optimize ESI parameters. The mobile phase in this study was 10mM ammonium formate:acetonitrile 50:50 v/v.

In ESI mode, the fragmentor voltage is an important parameter because it is the voltage that applied at the exit of the capillary for fragmenting the analyte ions. Typically, the optimum of fragmentor voltage depends on compound nature. In this study, the fragmentor voltage was varied between 30 and 200 V to select the optimum value for the ionization of PQ, DQ, CQ and MQ. The molecular radical ions $[M]^{+\bullet}$ of each compound were monitored in this observation. From Figure 4.1, the voltages ranged from 110 to 140 show a little difference of the peak areas. However, the fragmentor voltage at 120 V provided maximum sensitivities of CQ and PQ and gave the acceptable sensitivities for MQ and DQ. Thus, 120 V was selected as the optimum fragmentor voltage.

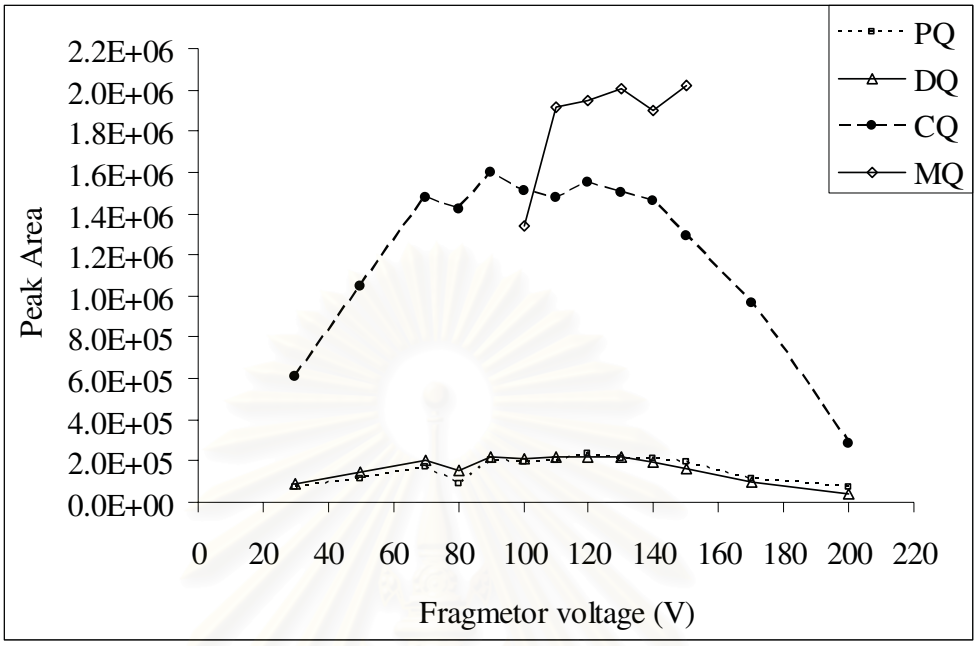


Figure 4.1 Comparison of the peak area of PQ, DQ, CQ and MQ by FIA with ESI mode at fragmentor voltage ranged from 30 to 200 V

Summary of the optimum MS conditions for the detection of PQ, DQ, CQ and MQ throughout this study were shown in Table 4.1.

Table 4.1 Mass spectrometer conditions

MS Parameter	Condition
Ionization mode	positive ESI
Drying gas flow rate	13.0 L/min
Drying gas temperature	350 ˚C
Nebulizer pressure	30 psi
Capillary voltage	3,000 V
Gain	0.2
Fragmentor voltage	120 V

4.1.2 Ion Characteristic

From the flow injection analysis of PQ, DQ, CQ and MQ, the mass spectra of each analyte were shown in Figure 4.2-4.5. The target ions and fragment ions, together with their relative abundance, are shown in Table 4.2. Because all of the analytes were cationic compounds, the molecular radical ions of them $[M]^{+\bullet}$ were detected. PQ gave the molecular radical ion $[M]^{+\bullet}$ at m/z 186.0 and the fragment ions at m/z 185.0 and 171.1 assigned to $[M-H]^+$ and $[M-CH_3]^+$, respectively. DQ gave the molecular radical ion $[M]^{+\bullet}$ at m/z 184.0 and the fragment ions at m/z 183.0 and 92.1 assigned to $[M-H]^+$ and $[M-C_6H_6N]^+$, respectively. The highest relative abundance of DQ mass spectra was obtained from m/z 183.0 but it was not selected as the target ion because this mass also showed in the spectrum of PQ and therefore to eliminate inconclusiveness of mass spectrum determination among the different derivatives, m/z of 184.0 was selected for DQ. For CQ, the molecular radical ion $[M]^{+\bullet}$ was observed at m/z 122.0. Because CQ has one chlorine atom in its structure, it gave the ion at m/z 124.0 corresponding to the ^{37}Cl isotopic contribution. The ratio of the relative abundance at m/z 122.0 to 124.0 is 3:1 which is the same as the isotope ratio of ^{35}Cl to ^{37}Cl. MQ gave the molecular radical ion $[M]^{+\bullet}$ at m/z 114.1 and the isotopic ion of ^{15}N at m/z 115.1. In this research, selected ion monitoring (SIM) mode was used and the signal of target ion of each analyte was monitored.

Table 4.2 Mass ion characteristic and relative abundance of PQ, DQ, CQ and MQ in positive ion detection mode, fragmentor 120 V

		Compound-specific ion, m/z *(relative abundance)*	
Analyte	MW	Target ion $[M]^+$	Fragment ion
PQ	186	186.0 *(59.3)*	185.0 *(39.9)*, 171.0 *(50.3)*
DQ	184	184.0 *(75.1)*	183.0 *(100.0)*, 92.1 *(27.0)*
CQ	122	122.0 *(100.0)*	124.0 *(32.8)*
MQ	114	114.1 *(100.0)*	115.1 *(8.1)*

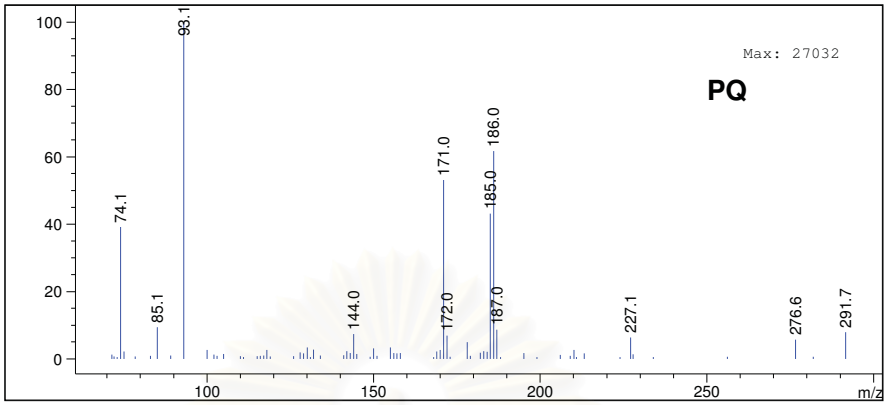


Figure 4.2 Mass spectra of PQ in positive ion detection mode, fragmentor 120 V

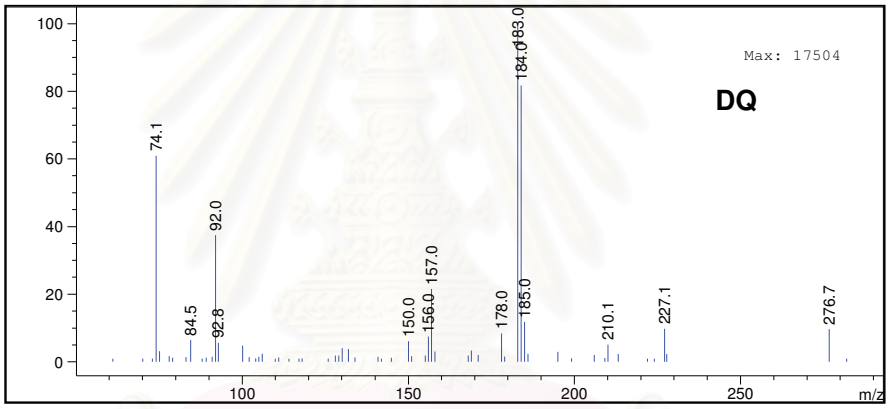


Figure 4.3 Mass spectra of DQ in positive ion detection mode, fragmentor 120 V

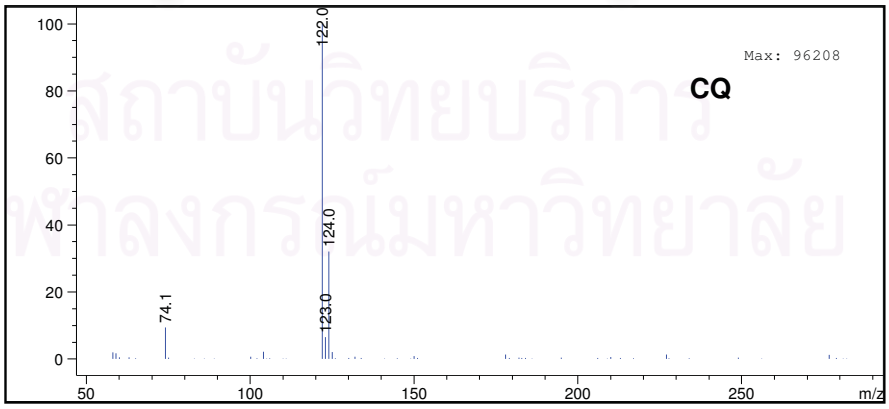


Figure 4.4 Mass spectra of CQ in positive ion detection mode, fragmentor 120 V

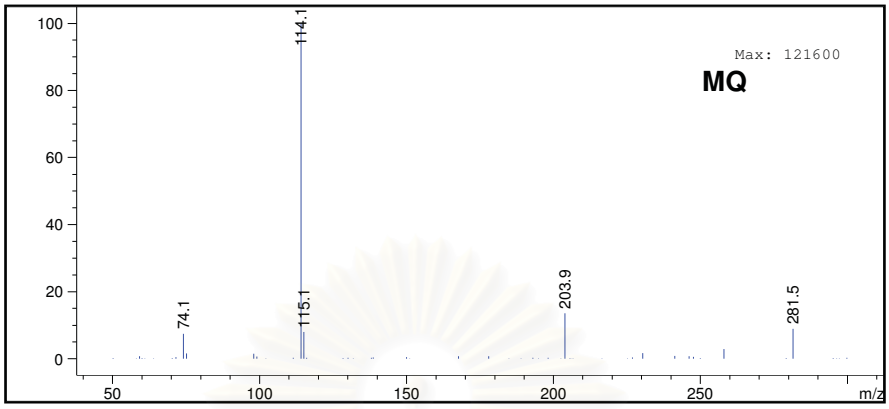


Figure 4.5 Mass spectra of MQ in positive ion detection mode, fragmentor 120 V

4.1.3 HPLC Optimum Conditions

Hydrophilic interaction chromatography (HILIC) was used to separate four quaternary ammonium herbicides. The HILIC silica column was suitable for the analysis of the charged compounds. The analysis was accomplished in simple and fast isocratic mode without the use of any ion-paring agent. The method required very low concentration of buffer (10 mM) which is suitable for MS by causing less ion-source problem. Table 4.3 shows the HPLC optimum conditions used in this research. As shown in Figure 4.6, the separation of four quats required only four minutes. However, band broadening of both PQ and DQ still occurred because of their strong cationic charges that react with silanol groups on the stationary phase.

Table 4.3 Optimum HPLC conditions

HPLC Parameter	Condition
Column	Atlantis HILIC silica (150 x 2.1 mm, 3.0 µm)
Mobile phase A	10 mM ammonium formate pH 3.0
Mobile phase B	Acetonitrile
Elution mode	Isocratic
%B	40%
Injection volume	1 µL
Flow rate	0.35 mL/min
Column temperature	35 ºC

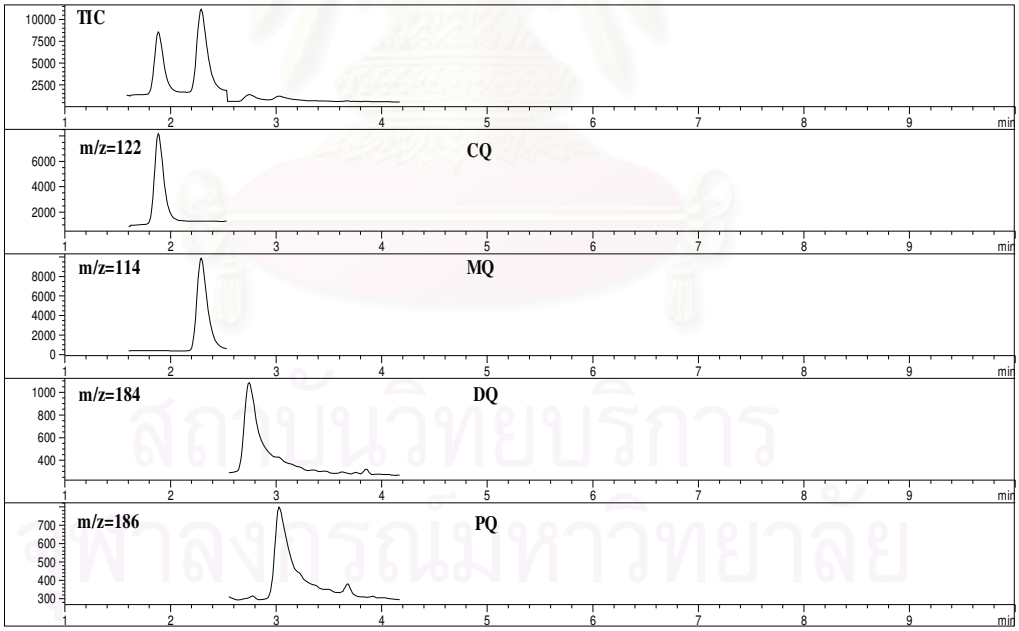


Figure 4.6 Extracted ion chromatograms of mixed standard PQ, DQ, CQ and MQ 100 µg/L

4.2 The Result of SLMME Optimization

Due to the ionizable and hydrophilic nature of PQ, DQ, CQ and MQ, the preconcentration and extraction from the sample matrix by conventional method such as LLE is very difficult. Three-phase SLMME is an alternative technique that can be used to preconcentrate and extract these analytes. The analytes are extracted from an aqueous donor solution through the organic solvent, immobilized in the pores of the membrane, and extracted out into another aqueous acceptor solution. Thus, the organic phase in this case acts as a barrier between two aqueous solutions and prevents the mixing of these solutions. Normally, this approach is used for the extraction of acidic or basic compounds which can be converted in the acceptor phase by protonation or complexation to species that cannot dissolve in the organic phase preventing the back extraction from the acceptor to the donor solution. The quaternary ammonium herbicides are charged compounds which cannot dissolve in the organic solvent. Therefore, a carrier is required to form a neutral analyte-carrier complex which can pass into the organic phase. This complex will release only the charged analyte into the aqueous acceptor solution. This process is called the coupled transport SLM. The extraction mechanism was illustrated in Figure 2.4.

As described above, the carrier substance plays an important role in transferring the analytes from the donor solution into the acceptor solution. Because the analytes are positively-charged compounds, the carrier selected is an anionic compound that can bind with the analytes. Di-(2-ethylhexyl) phosphoric acid (DEHPA; pKa = 3.24) was selected as the carrier because it is commonly used in SLM system for metal ion extraction. DEHPA is an acidic compound which can dissolve in the organic solution. In addition, M. Mulugeta and N. Megersa *(26)* also used DEHPA as a carrier in SLM extraction of PQ and DQ.

In this research, the enrichment factor (EF) was used to evaluate the extract as expressed in Eq. 4 and 5 of chapter 2 as showed below.

$$EF = \frac{C_A}{C_D} \qquad \text{(Eq. 4)}$$

$$EF = EE \cdot \frac{V_D}{V_A} \qquad \text{(Eq. 5)}$$

It can be extrapolated from Eq. 5 that EF can be improved by increasing the ratio of the donor volume to the acceptor volume (called phase ratio). Typically, hollow fiber base microextraction have high phase ratio therefore, it can provide high EF.

4.2.1 Effect of Donor pH

The donor pH should be higher than the pKa of DEHPA to promote maximum dissociation. Dissociation of DEHPA gave a hydrogen ion into the donor solution and the remained DEHPA anion at the membrane-donor interface would form the analyte-carrier complex. This complex can pass into the organic phase filled in the membrane pores. Therefore, the effect of different donor pH was studied.

The EFs of the four quats are shown in Figure 4.7. As the donor pH increased, higher proton concentration gradient existed across the membrane. However, solubility of DEHPA in aqueous solution also increased with pH, the SLM was destroyed and the acceptor solution could diffuse out of the membrane lumen. When the donor pH was higher than 6.5, the turbid donor solution was observed around the fiber due to increasing misciblility of DEHPA in the donor solution. Therefore, the pH was maintained below 6.5. For subsequent experiment, donor pH at 5.0 which provided maximum EF was selected. The average and standard deviation of EF values at each donor pH were shown in Table 4.4.

Table 4.4 Enrichment factor of PQ, DQ, CQ and MQ at different donor pH

Donor pH	Average EF ± SD (n=3)			
	PQ	DQ	CQ	MQ
3.0	1.61 ± 0.10	3.27 ± 0.07	1.37 ± 0.09	0.84 ± 0.07
4.0	5.64 ± 0.91	11.39 ± 1.14	3.11 ± 0.06	2.07 ± 0.04
5.0	7.55 ± 0.99	12.49 ± 0.87	3.18 ± 0.36	2.07 ± 0.22
6.0	4.52 ± 0.70	5.56 ± 0.60	1.63 ± 0.10	1.43 ± 0.10
6.5	6.29 ± 0.88	9.72 ± 1.43	2.46 ± 0.39	1.84 ± 0.29

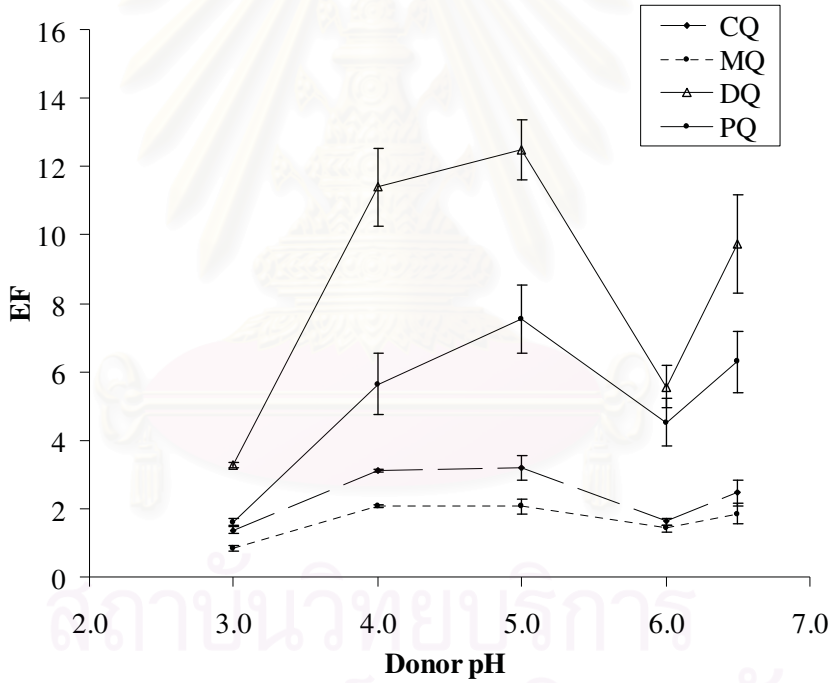


Figure 4.7 Enrichment factors as a function of donor pH

4.2.2 Effect of Acceptor pH

At the membrane-acceptor interface, the DEHPA anion in the analyte-carrier complex received a hydrogen ion to form the neutral DEHPA and released the cationic analyte into the acceptor solution. Therefore, the acceptor pH should be

lower than the pKa of DEHPA and the difference between the donor pH and the acceptor pH should be enough to produce the hydrogen ion gradient across the membrane. The effect of various acceptor pH was studied.

As illustrated in Figure 4.8, maximum EFs of all analytes were obtained at an acceptor pH of 0.5. The decreasing of EFs at higher acceptor pH was due to insufficient pH difference to force mass transfer between the donor pH and the acceptor pH. Decreasing value at lowest acceptor pH (0.0) occurred from protonated DEHPA which lacked the ability to transport the charged analyte. Eq. 15 shows the protonated of DEHPA at low pH. The average and standard deviation of EF values at each acceptor pH were shown in Table 4.5.

$$RH + H^{+} \longrightarrow RH_2^{+} \qquad \text{(Eq. 15)}$$

Table 4.5 Enrichment factor of PQ, DQ, CQ and MQ at different acceptor pH

Acceptor pH	Average EF $\pm$ SD (n=3)			
	PQ	DQ	CQ	MQ
0.0	4.12 $\pm$ 1.27	8.49 $\pm$ 2.04	3.17 $\pm$ 0.62	2.07 $\pm$ 0.39
0.5	7.55 $\pm$ 0.99	12.49 $\pm$ 0.87	3.18 $\pm$ 0.36	2.07 $\pm$ 0.22
1.0	7.46 $\pm$ 1.56	12.03 $\pm$ 2.04	2.51 $\pm$ 0.28	1.69 $\pm$ 0.18
1.5	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.37 $\pm$ 0.05	0.37 $\pm$ 0.04
2.0	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.21 $\pm$ 0.01	0.23 $\pm$ 0.00

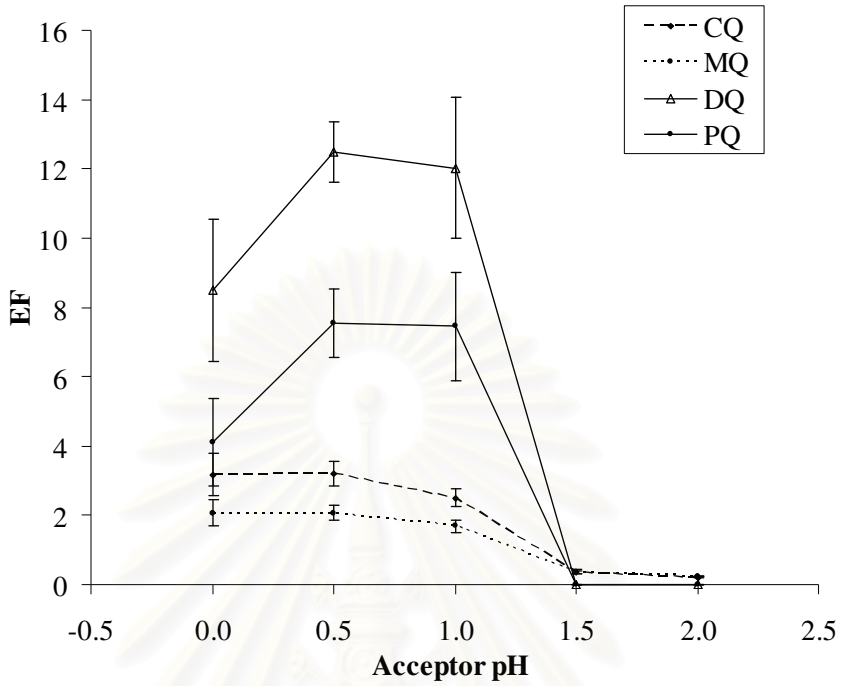


Figure 4.8 Enrichment factors as a function of acceptor pH

4.2.3 Effect of Donor Volume

In theory, when the donor volume increased, EF rose because large amount of the analyte was accessible. This can be observed when change the donor volume from 3.5 to 120 mL (Figure 4.9). However, when the donor volume was increased to 250 mL, EF did not rise due to reduced contact surface area between the membrane and the analyte at high donor volume. Therefore, at this high volume longer extraction time is required to get the system to equilibrium state. The extraction time at each donor volume was set to 60 minutes, the system had not yet reach the equilibrium state and more analyte can still be extracted out of the donor solution. The extraction at this non-equilibrium condition resulted in lower EE that is directly related to lower EF as can be described by Eq. 5 in chapter 2:

$$EF = EE \cdot \frac{V_D}{V_A} \qquad \text{(Eq. 5)}$$

At 120-mL donor volume, different agitations were compared by vibrating and stirring at 450 rpm and the results are shown in the insignificant differences of EF. Therefore, stirring using a magnetic stirrer was preferred over vibration using a Vortex Mixer for the former was easier to use. At donor volume of 250 mL, agitation by stirring was also investigated but the EFs of all analytes were almost equal with these using 120-mL donor volume due to incomplete extraction. Therefore, extended extraction time is required for large volume. A 120-mL donor volume was selected because the portion size is reasonable but large enough to minimize error. The average and standard deviation of EF values at each donor volume were shown in Table 4.6.

Table 4.6 Enrichment factor of PQ, DQ, CQ and MQ at different donor volume

Donor volume (mL)	Average EF $\pm$ SD (n=3)			
	PQ	DQ	CQ	MQ
3.5 [a]	15.33 $\pm$ 1.57	31.02 $\pm$ 3.49	6.86 $\pm$ 0.08	4.55 $\pm$ 0.18
15.0 [a]	17.13 $\pm$ 5.20	34.25 $\pm$ 6.83	7.65 $\pm$ 0.71	5.29 $\pm$ 0.49
25.0 [a]	27.76 $\pm$ 1.14	50.83 $\pm$ 1.22	8.93 $\pm$ 0.28	6.11 $\pm$ 0.22
40.0 [a]	24.95 $\pm$ 4.21	45.83 $\pm$ 4.98	9.61 $\pm$ 0.52	6.51 $\pm$ 0.37
120.0 [a]	36.41 $\pm$ 1.24	63.70 $\pm$ 1.83	9.28 $\pm$ 0.40	6.29 $\pm$ 0.15
120.0 [b]	33.92 $\pm$ 3.44	53.66 $\pm$ 4.52	10.96 $\pm$ 1.14	7.56 $\pm$ 0.76
250.0 [b]	33.49 $\pm$ 1.38	53.34 $\pm$ 4.50	11.33 $\pm$ 0.39	7.80 $\pm$ 0.15

[a] Agitation by vibration

[b] Agitation by stirring

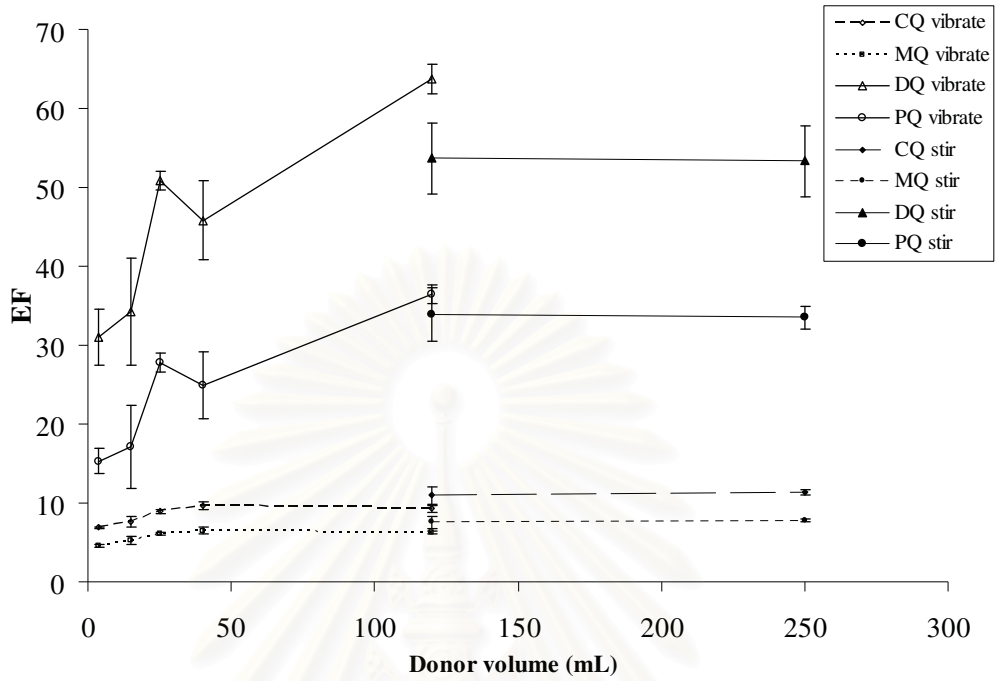


Figure 4.9 Enrichment factors as a function of donor volume

4.2.4 Effect of Acceptor Volume

To study the effect of acceptor volume, the length of membrane was varied. In this research, hollow fiber lengths of 8, 12, 24 and 37 cm corresponded to 20, 30, 60 and 100 μL of the acceptor volumes, respectively. Figure 4.10 shows the enrichment factors at each acceptor volume. For a 120-mL donor volume, the maximum EF was obtained at 30 μL of acceptor volume. Eq. 5 in chapter 2 as showed below stated that the lowest acceptor volume provides the highest EF due to the higher phase ratio when donor volume and EE are kept constant.

$$EF = EE \cdot \frac{V_D}{V_A} \qquad \text{(Eq. 5)}$$

However, a longer hollow fiber holds a larger volume of the carrier in its pores, and has a larger analyte-membrane contact area. From a comparison between a 20 and 30-μL acceptor volume, the increase in membrane length was more effective

than decreasing the phase ratio. For acceptor volumes higher than 30 μL, a decline in EF was observed because of lower phase ratio. The average and standard deviation of EF values at each acceptor volume were shown in Table 4.7.

Table 4.7 Enrichment factor of PQ, DQ, CQ and MQ at different acceptor volume

Acceptor volume (μL)	Average EF ± SD (n=3)			
	PQ	DQ	CQ	MQ
20.0	44.86 ± 2.29	61.08 ± 2.34	9.61 ± 0.66	6.12 ± 0.50
30.0	54.50 ± 0.82	71.07 ± 1.21	9.62 ± 0.41	6.11 ± 0.42
60.0	41.89 ± 2.65	48.07 ± 1.26	9.16 ± 0.70	5.88 ± 0.47
100.0	48.79 ± 1.85	50.25 ± 0.45	10.33 ± 0.27	6.95 ± 0.20

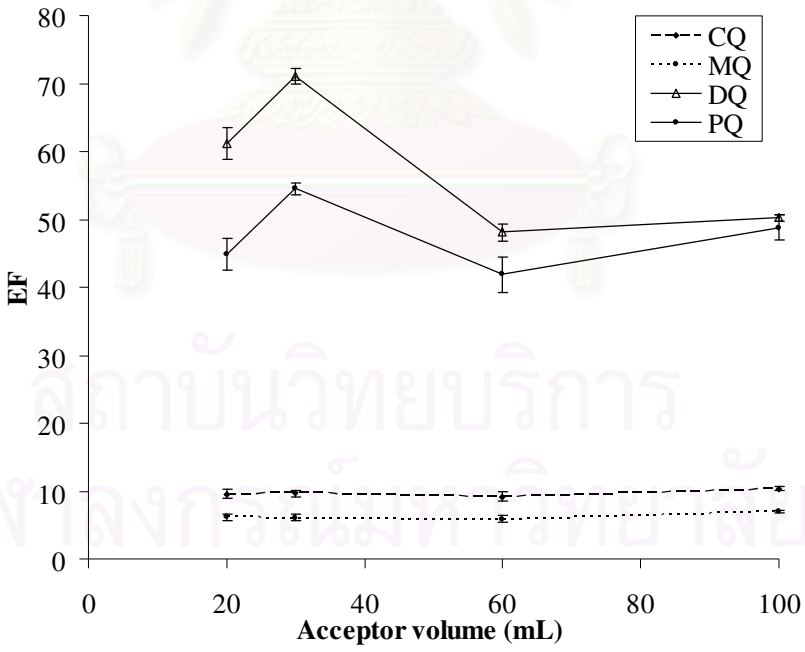


Figure 4.10 Enrichment factors as a function of acceptor volume

4.2.5 Effect of Carrier Concentration

The carrier (DEHPA) contained in the membrane pores is the essential component of the SLM. Typically, increasing of DEHPA in the SLM leads to higher EF. However, base on the Stokes-Einstein relationship, $D = kT/6\pi\eta r$, where D is the analyte diffusion coefficient, k is the Boltzman constant, T is the absolute temperature, r is the molecular radius of the species and η is the viscosity of the organic phase *(26)*, the analyte diffusion decreases with the increasing of the organic phase viscosity. In this study, the concentration of DEHPA was varied in the range of 30-100% v/v dissolved in DHE. The results are shown in Figure 4.11. The EFs of all analytes increased with the rising amount of DEHPA. However, above 70 %v/v of DEHPA in DHE, the EFs decreased because of increasing in organic phase viscosity. Typically, DEHPA is significantly more viscous than DHE. Thus the addition of DEHPA increased the organic phase viscosity, thereby reducing the analyte diffusion. Therefore, 70 % v/v of DEHPA in DHE was considered to the optimum carrier concentration. The average and standard deviation of EF values at each carrier concentration were shown in Table 4.8.

Table 4.8 Enrichment factor of PQ, DQ, CQ and MQ at different carrier concentration

DEHPA in DHE (%v/v)	Average EF ± SD (n=3)			
	PQ	DQ	CQ	MQ
30.0	1.63 ± 0.32	8.57 ± 2.06	5.86 ± 1.55	2.80 ± 0.75
40.0	6.67 ± 1.94	20.80 ± 4.58	8.24 ± 1.80	4.39 ± 0.95
50.0	10.83 ± 1.63	31.70 ± 3.69	13.78 ± 0.84	7.72 ± 0.39
60.0	13.84 ± 0.97	36.36 ± 1.70	12.99 ± 0.79	7.36 ± 0.50
70.0	24.15 ± 2.30	42.56 ± 1.63	14.06 ± 0.75	8.31 ± 0.44
80.0	16.24 ± 2.28	31.85 ± 1.18	11.76 ± 1.33	7.19 ± 0.83
90.0	16.32 ± 1.35	29.00 ± 0.42	9.28 ± 0.32	5.98 ± 0.13
100.0	17.99 ± 4.30	28.14 ± 3.70	7.69 ± 0.36	5.23 ± 0.23

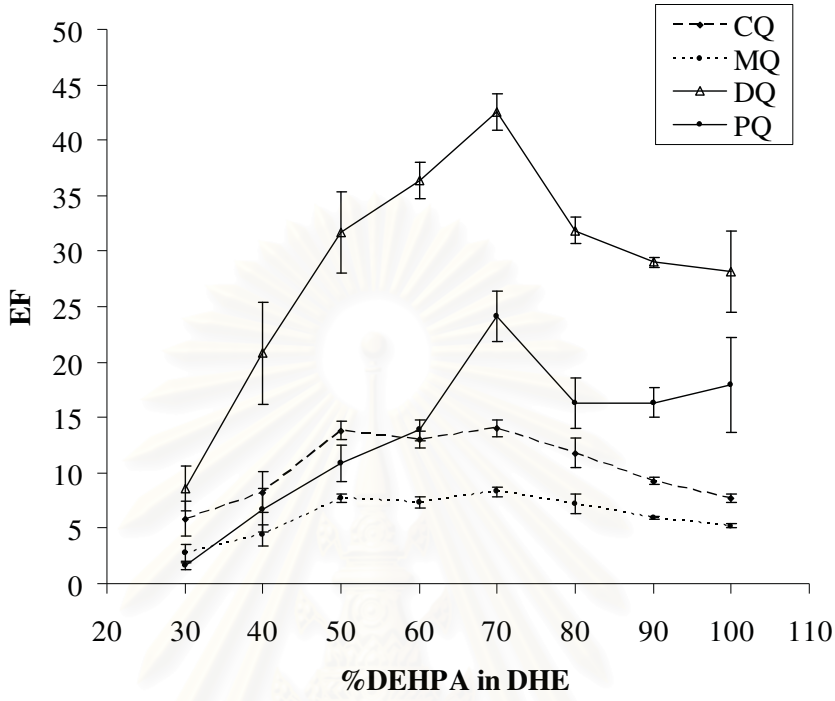


Figure 4.11 Enrichment factors as a function of carrier concentration

4.2.6 Effect of Immersion Time

Because the carrier is the key component in this research, the amount of carrier filled into the membrane pores was of great concerned. Because it takes time for the carrier to soak completely in the membrane pores, the immersion time was studied. The membranes were immersed for 5, 10, 20, 40, 90, 180 min and 1.5 days. The results in Figure 4.12 show insignificant difference of EF. However, the standard deviations (SD) of EF obtained from 180-min and 1.5-day immersion were lower than that from others due to the formation of a more stable membrane solvent in the membrane pores. Therefore, the membrane was immersed for at least 1 day in the organic solvent before the extraction. The average and standard deviation of EF values at each immersion time were shown in Table 4.9.

Table 4.9 Enrichment factor of PQ, DQ, CQ and MQ at different immersion time

Immersion time (min)	Average EF ± SD (n=3)			
	PQ	DQ	CQ	MQ
5.0	23.09 ± 3.23	48.98 ± 3.27	15.86 ± 0.73	9.22 ± 0.39
10.0	25.02 ± 1.32	44.48 ± 2.05	14.71 ± 1.20	8.62 ± 0.68
20.0	26.40 ± 4.79	46.78 ± 6.30	15.36 ± 2.52	8.91 ± 1.49
40.0	26.06 ± 1.70	45.66 ± 2.58	14.63 ± 0.95	8.45 ± 0.52
90.0	17.03 ± 3.22	31.86 ± 5.43	10.31 ± 2.20	5.95 ± 1.23
180.0	21.93 ± 0.51	40.39 ± 0.70	13.28 ± 0.56	7.79 ± 0.36
1.5 days	27.35 ± 0.75	46.56 ± 1.93	15.60 ± 0.57	9.05 ± 0.26

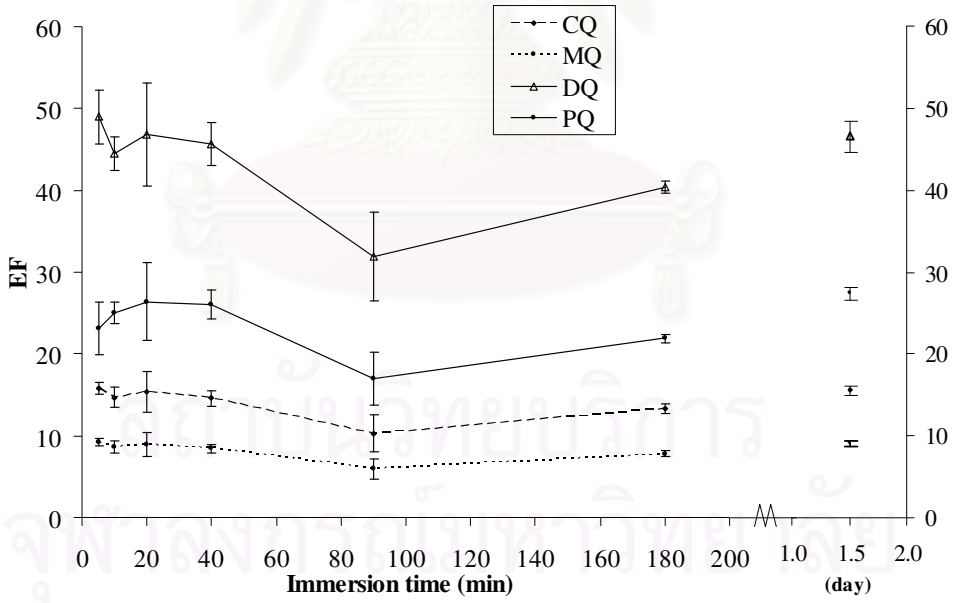


Figure 4.12 Enrichment factors as a function of immersion time

4.2.7 Effect of Extraction Time

SLMME is not an exhaustive extraction technique and it is based on the analyte's partitioning between the aqueous sample and the organic solvent. Therefore, equilibration plays an important role. As a result, increasing of extraction time can improve extraction efficiency until equilibrium is reached. In this study, the extraction time was varied from 15 to 120 min. Figure 4.13 shows that EFs of all analytes increased with extraction time. However beyond 60-minute, the EFs decreased because the longer extraction time led to the loss of the organic solvents from the membrane pores and in turn resulted in loss of a barrier between the donor solution and the acceptor solution destroying the preconcentration process. Thus, 60 minutes was selected as the optimum extraction time. The average and standard deviation of EF values at each extraction time were shown in Table 4.10.

Table 4.10 Enrichment factor of PQ, DQ, CQ and MQ at different extraction time

Extraction time (min)	Average EF $\pm$ SD (n=3)			
	PQ	DQ	CQ	MQ
15.0	10.66 $\pm$ 1.82	18.31 $\pm$ 2.29	5.83 $\pm$ 0.30	3.22 $\pm$ 0.14
30.0	18.51 $\pm$ 1.77	32.72 $\pm$ 3.31	10.68 $\pm$ 0.43	6.17 $\pm$ 0.19
45.0	26.40 $\pm$ 2.40	46.78 $\pm$ 4.35	15.36 $\pm$ 0.73	8.91 $\pm$ 0.42
60.0	29.22 $\pm$ 1.95	50.77 $\pm$ 2.62	18.02 $\pm$ 0.83	10.81 $\pm$ 0.29
90.0	31.62 $\pm$ 0.74	39.60 $\pm$ 2.10	9.36 $\pm$ 0.57	8.05 $\pm$ 0.28
120.0	22.20 $\pm$ 1.08	24.80 $\pm$ 2.22	6.58 $\pm$ 0.14	6.38 $\pm$ 0.12

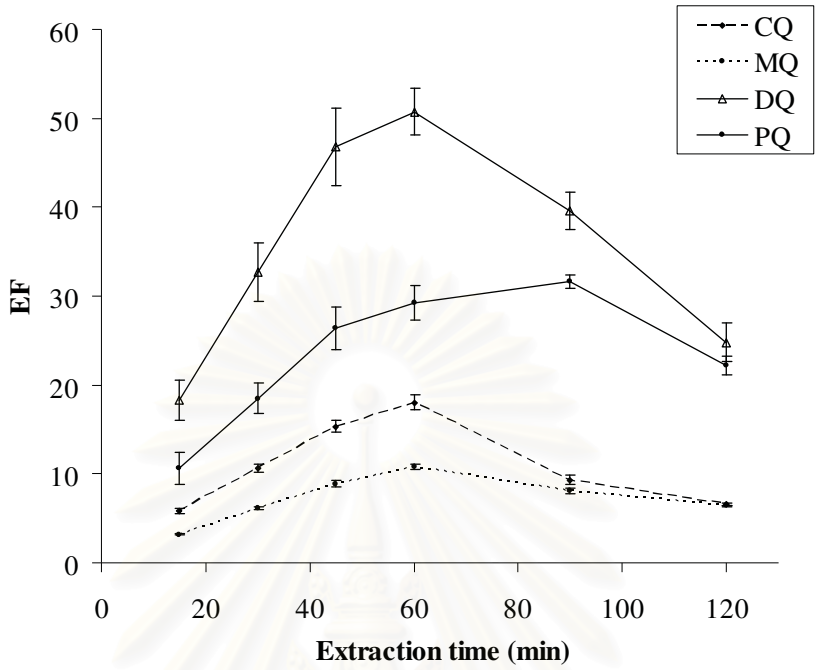


Figure 4.13 Enrichment factors as a function of extraction time

The optimum values of all SLMME conditions obtained is summarized in Table 4.11. The procedure was used for 10 replicated extraction of 5-µg/L spiked standards in reagent water gave the maximum EFs as follows: 57.19 for PQ, 55.11 for DQ, 11.33 for CQ and 8.85 for MQ. The relatively lower values for four quats were due to the fact that all analytes are still ionized and fairly hydrophilic at this condition. Even though DEHPA, an organic compound, was used as the carrier for the formation of ion-pair complex with the analytes, the quats preferred to partition in the aqueous donor.

Table 4.11 Summary of SLMME optimum conditions

SLMME parameter	Condition
Donor type	Phosphate buffer pH 5.0
Donor volume	120.0 mL
Membrane length	12 cm
Carrier type	70% v/v DEHPA in DHE
Immerging time	> 1 day
Acceptor type	Hydrochloric acid pH 0.5
Acceptor volume	30.0 µL
Agitation type	Stir
Extraction time	60 minutes

4.3 The Method Validation

4.3.1 Method Selectivity

Selectivity refers to the extent to which a method can determine particular analytes in mixtures or matrices without interferences from other components. For HPLC method, selectivity can be defined as the retention time of each analyte in repeated extractions. For MS method, target ion and qualified ion were used to confirm the selectivity. The selectivity results were shown in Table 4.12.

Table 4.12 Average retention time and %R.S.D. of retention time of PQ, DQ, CQ and MQ at 5 μg/L spiking level (n=10) and their target ions and qualified ions

	PQ	DQ	CQ	MQ
Retention time (min)	3.148 ± 0.017	2.810 ± 0.006	1.878 ± 0.003	2.304 ± 0.006
% R.S.D.	0.548	0.200	0.156	0.242
Target ion [M^+]	186.0	184.0	122.0	114.1
Qualified ion	185.0	183.0	124.0	115.1

4.3.2 Calibration Curve

Since SLMME is a non-exhaustive extraction, calibration curves of the four quats were obtained from the relationship of the peak area of all spiked analytes after SLMME extraction and their spiked concentrations. These calibration curves, shown in Figure 4.14-4.17, were obtained from triplicate analyses of each analyte from 2 to 10 μg/L. The correlation coefficients (R^2) were in the range of 0.97-0.99. Slopes, intercepts and correlation coefficients of the analytes were summarized in Table 4.13.

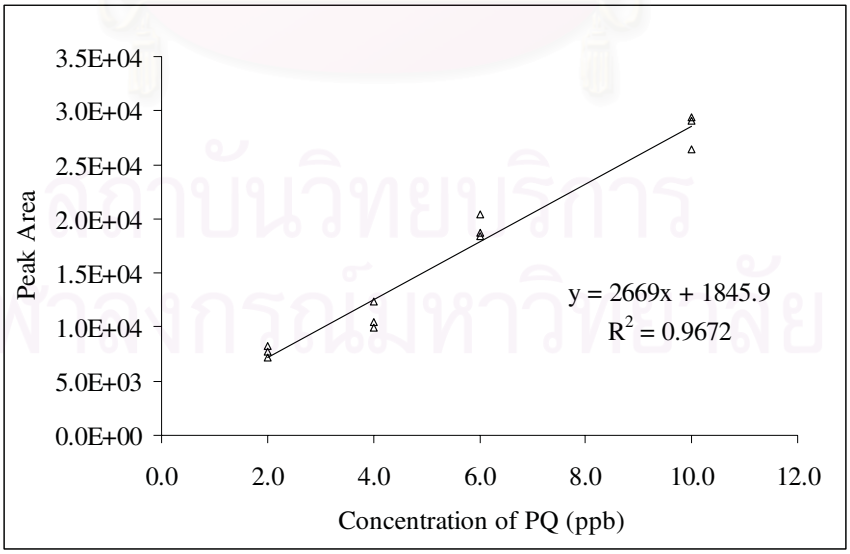


Figure 4.14 Calibration curve of PQ after extraction by SLMME-LC-MS

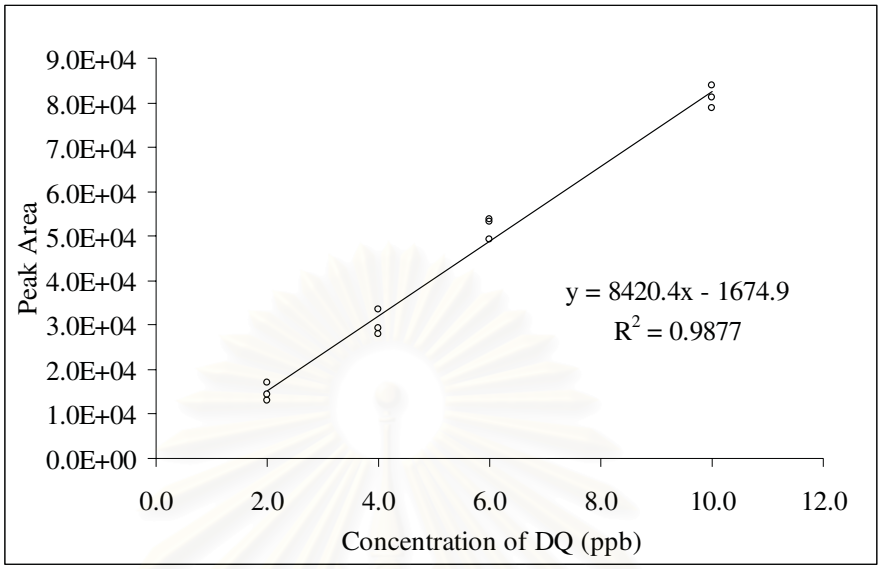


Figure 4.15 Calibration curve of DQ after extraction by SLMME-LC-MS

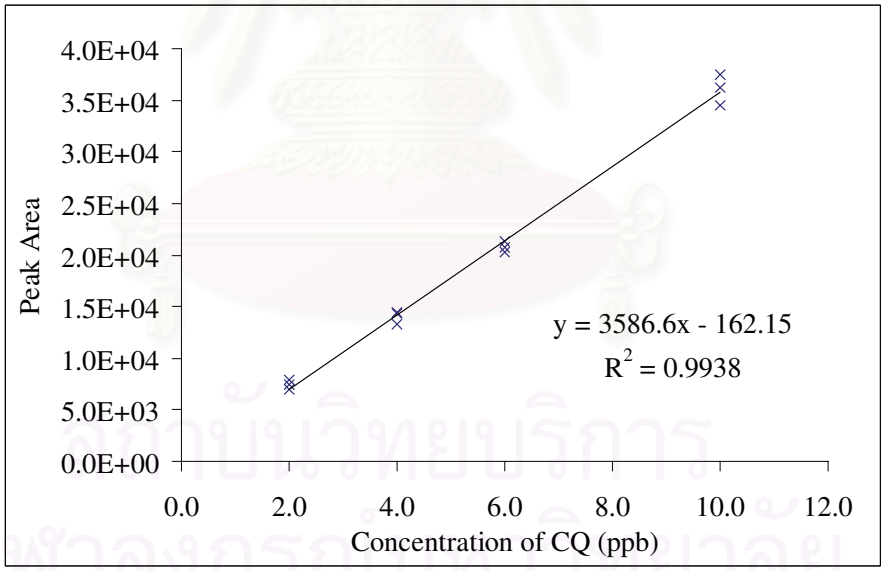


Figure 4.16 Calibration curve of CQ after extraction by SLMME-LC-MS

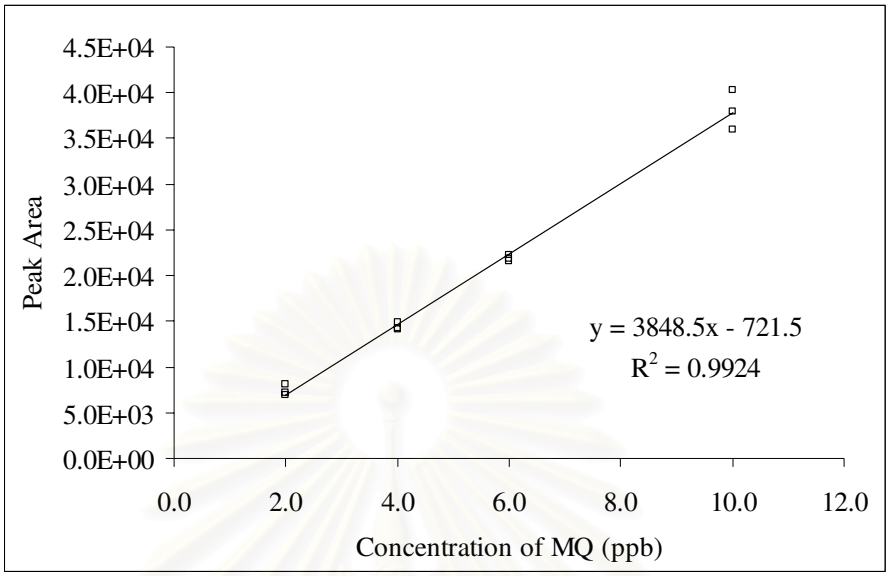


Figure 4.17 Calibration curve of MQ after extraction by SLMME-LC-MS

Table 4.13 Slopes, intercepts and correlation coefficients (R^2) of PQ, DQ, CQ and MQ

	PQ	DQ	CQ	MQ
Slope	2,669.0	8,420.4	3,586.6	3,848.5
Intercept	+1,845.9	-1,674.9	-162.2	-721.5
Correlation coefficient	0.9672	0.9877	0.9938	0.9924

4.3.3 Method Quantitation Limits (MQLs) and Method Detection Limits (MDLs)

The method detection limits (MDLs) for DQ and PQ were comparable to those recommended by the EPA method 549.1. CQ and MQ are not regulated for drinking water and there are no standard methods for their determination in water. The MDLs of CQ and MQ were at low to sub-ppb levels. Overall, the MDLs achieved by this method are better or comparable to that from others published

methods for quaternary ammonium herbicides in water *(23-25, 41-45).* Table 4.14 shows the summary of MDLs and MQLs of all analytes done in eight replications.

Table 4.14 Method detection limits (MDLs) and method quantitation limits (MQLs) of PQ, DQ, CQ and MQ (n=8)

	PQ	DQ	CQ	MQ
MDL (μg/L)	1.29 ± 0.17	0.48 ± 0.07	0.17 ± 0.03	0.39 ± 0.06
MQL (μg/L)	4.30 ± 0.57	1.60 ± 0.22	0.57 ± 0.11	1.30 ± 0.19
MDL (μg/L) from EPA method 549.1	0.80	0.44	-	-

4.3.4 Method Accuracy

Due to the non-exhaustive extraction property of SLMME, the accuracy of this technique is reported as relative recovery determined by the comparison between the concentration after extraction obtained from calibration curve (in Section 4.3.2) and the spiked concentration. Percent Relative recoveries at 5 μg/L spiking levels were between 71.64 and 78.74 % as shown in Table 4.15. From the AOAC manual for the Peer Verified Methods program, the estimated recoveries at 5 μg/L analyte concentrations is in the range of 50-117% *(42)*. Thus, the values obtained from the experiment were acceptable.

Table 4.15 % Relative recoveries of PQ, DQ, CQ and MQ at 5 μg/L spiking levels (n=10)

	PQ	DQ	CQ	MQ
% Recovery	74.34 ± 5.10	71.64 ± 4.31	74.76 ± 5.90	78.74 ± 2.94
% R.S.D.	6.86	6.02	7.89	3.74

4.3.5 Method Precision

Normally, the precision of a method was expressed as relative standard deviation (R.S.D.) of repeated analysis. In this research, the R.S.D. of 10 replications obtained from SLMME on the same day at 5 and 50 µg/L spiked levels were compared for within-day precision. The procedure was repeated for three consecutive days to evaluate the between-day precision. The precision data are shown in Table 4.16. The R.S.D values used as the standard for comparing with the experimental R.S.D. is calculated from the Horwitz equation *(41)*.

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

Where R.S.D._r is the relative standard deviation calculated from the within-day result and C is the mass fraction of analyte in sample (g/g). The R.S.D. values calculated from the Horwitz equation at 5 and 50 µg/L are 23.4 and 16.6%, respectively. For the within-day precision, the R.S.D. values obtained from the experiment were acceptable because they were not larger than the calculated values. However, the R.S.D. values obtained from 50-µg/L concentration were not better than that from 5-µg/L concentration. The reason is that at 50-µg/L concentration the analyte concentration in the acceptor solution is high and overloaded the analytical column therefore, the acceptor solution was diluted before injecting into the LC-MS. This additional procedure contributed to a scatter deviation of the R.S.D. From this observation, SLMME technique is suitable for the extraction of low analyte concentration. Overall, R.S.D. values were satisfactory and demonstrated that SLMME technique is comparable to other sample preparation techniques of QAHs reported in published papers *(17-19, 23-25)*.

To evaluate the between-day precision, the comparison between % recovery of the first (n=10), the second (n=10) and the third (n=10) was determined by ANOVA. P-values at 95% confidence limit of PQ, DQ, CQ and MQ at 5 and 50 µg/L were shown in Table 4.16. All of the analytes presented a P-value greater than 0.05, therefore these four herbicides showed insignificant differences at both concentration.

Table 4.16 % Relative standard deviations (R.S.D.) and P-value from ANOVA single factor of PQ, DQ, CQ and MQ at 5 and 50 µg/L spiking levels (n=10)

Concentration		Analysis Day	PQ	DQ	CQ	MQ
5 µg/L	%R.S.D.	1	6.86	6.02	7.89	3.74
		2	5.62	7.07	8.32	7.37
		3	1.71	6.36	7.88	6.31
	P-value		0.1664	0.0870	0.6054	0.5230
50 µg/L	%R.S.D.	1	9.23	2.96	12.06	9.26
		2	10.84	6.70	4.83	3.87
		3	8.97	11.03	5.32	4.61
	P-value		0.0801	0.1122	0.0723	0.1249

4.4 The Analysis of Drinking Water Samples

Because the international organizations such as the US EPA set the maximum residual limits of PQ and DQ in drinking water, this developed SLMME-LC-MS method was tested for their capability with commercial drinking water samples in Thailand. Three brands of drinking water tested were Crystal, Siam and Namthip.

To determine the recovery, three commercial drinking water samples were spiked with 5 and 20 µg/L of the four quats and extracted. Table 4.17 shows the average recoveries of all samples. The relative recoveries of the four quats at 5 µg/L spiked level ranged between 56.1 and 105.4% and that for the 20 µg/L spiked level ranged between 62.2 and 106.9%. From the AOAC manual for the Peer Verified Methods program, the estimated recoveries should be between 50-117% for 5 µg/L analyte concentrations and between 62-115% for 20 µg/L *(42)*. Therefore, the recoveries obtained from the experiment were acceptable because the values fall within the range of AOAC. The chromatograms of the spiked drinking water samples

with and without extraction were shown in Figure 4.18 and 4.19, respectively. After extraction, greater improvement in term of noise and peak shape was observed in all chromatograms especially for PQ and DQ. This observation indicated that SLMME is very effective in the simultaneous clean-up of interferences and analyte enrichment. As a result, greater sensitivity was obtained. Therefore, the SLMME-LC-MS can be used effectively for the determination of residual PQ, DQ, CQ and MQ in drinking water.

Table 4.17 % Recovery PQ, DQ, CQ and MQ in Crystal, Namthip and Siam drinking water samples at 5 and 20 µg/L spiking levels (n=2)

		Average %Recovery (n=2)			
		PQ	DQ	CQ	MQ
5 µg/L	Crystal	105.4 ± 3.4	86.5 ± 7.2	88.5 ± 3.1	85.8 ± 7.6
	Namthip	64.4 ± 4.4	59.4 ± 1.2	84.4 ± 6.7	74.1 ± 5.6
	Siam	61.5 ± 2.2	56.1 ± 3.7	78.5 ± 3.6	71.2 ± 4.6
20 µg/L	Crystal	65.6 ± 3.2	94.9 ± 7.2	106.9 ± 1.2	96.5 ± 0.7
	Namthip	62.4 ± 1.7	62.2 ± 2.7	70.9 ± 1.4	71.8 ± 0.6
	Siam	96.2 ± 3.9	84.0 ± 7.4	82.9 ± 2.9	82.7 ± 3.5

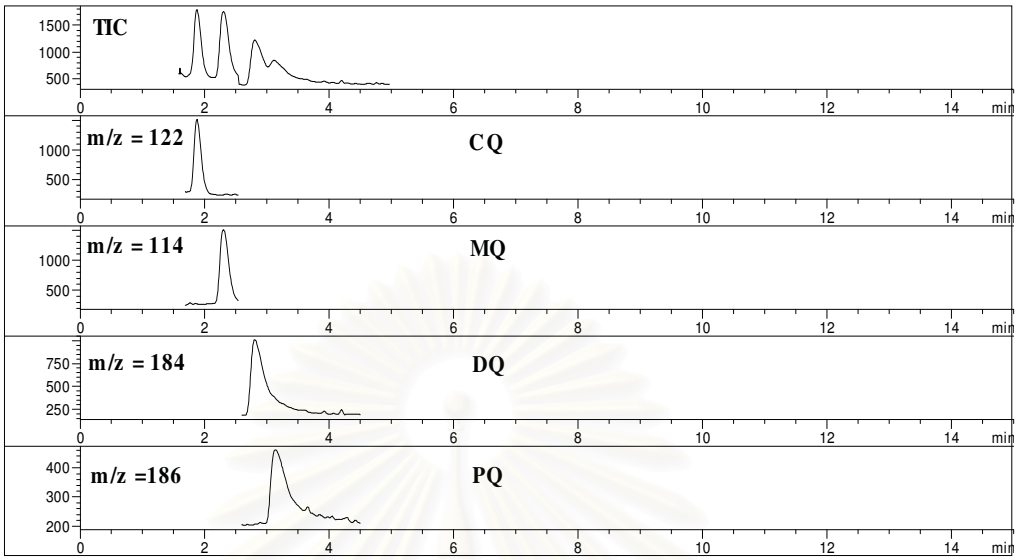


Figure 4.18 Extracted ion chromatogram of PQ, DQ, CQ and MQ after SLMME-LC-MS of spiked standard solutions 5 µg/L in drinking water sample

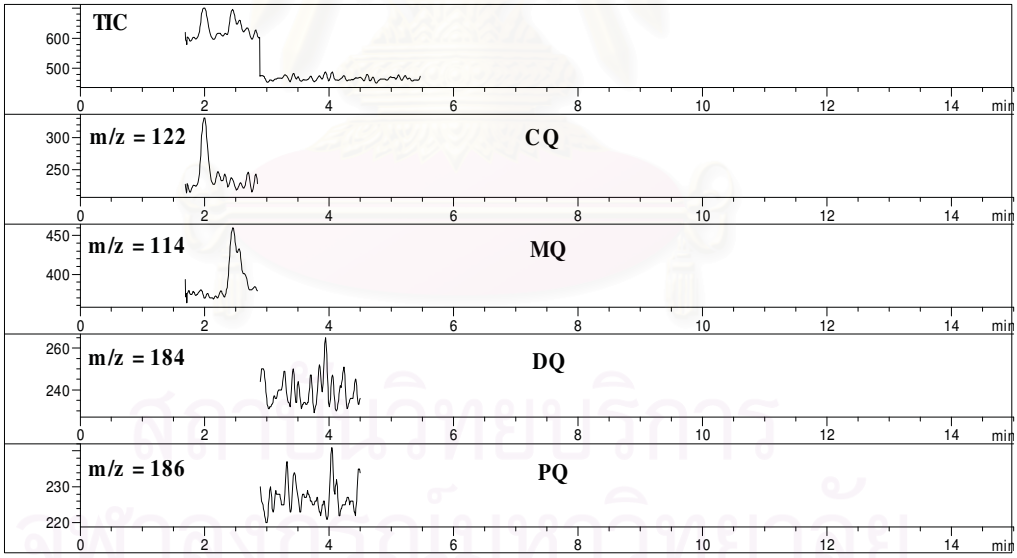


Figure 4.19 Extracted ion chromatogram of PQ, DQ, CQ and MQ of spiked standard solutions 5 µg/L in drinking water sample, direct injection without SLMME

CHAPTER V

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDY

In this study, a new method for preconcentration and determination of quaternary ammonium herbicides (QAHs): paraquat (PQ), diquat (DQ), chlormequat (CQ), and mepiquat (MQ), was developed. The analysis was performed by liquid chromatography-mass spectrometry (LC-MS). Mass spectrometer equipped with a single quadrupole was used and the MS conditions were shown in Table 5.1.

Table 5.1 Mass spectrometer conditions for the analysis of PQ, DQ, CQ and MQ

MS Parameter	Condition
Ionization mode	positive ESI
Drying gas flow	Nitrogen, 13.0 L/min
Drying gas temperature	350 ˚C
Nebulizer gas pressure	Nitrogen, 30 psi
Capillary voltage	3,000 V
Gain	0.2
Fragmentor voltage	120 V

For quantitative analysis, mass spectrometer was operated using selected ion monitoring (SIM) of the molecular radical ion [$M^{+\bullet}$] of each analyte as shown in Table 5.2. The retention time windows in Table 5.2 are the timing program for SIM of each m/z. Peak retention times obtained from the extraction were matched with that from the standard peaks for the analyte qualitative confirmation.

Table 5.2 Time schedule and m/z of SIM for detecting PQ, DQ, CQ and MQ

Compound	Retention time window (min)	Selected ion monitoring (m/z) *(Relative abundance)*
CQ	1.60-2.56	122.0 *(100.0)*
MQ		114.1 *(100.0)*
DQ	2.56-4.20	184.0 *(75.1)*
PQ		186.0 *(59.3)*

A good separation with short analysis time was succeeded by using an Atlantis hydrophilic interaction (HILIC) silica column (150 x 2.1 mm, 3.0 μm). This column is suitable for hydrophilic compounds which, normally unretained by reverse phase chromatography. HILIC silica column has water layer on its modified-silica stationary phase and hydrophilic analytes can retain in this column by their partition between the water-rich stationary phase and the water-poor mobile phase. Thus, this column is suitable for the separation of QAHs without using any ion-pairing reagents. The mobile phase was 40:60 v/v of acetonitrile:10 mM ammonium formate pH 3.0 and the flow rate was 0.35 mL/min. The four QAHs were successfully separated and eluted in only 4 minutes. CQ and MQ gave good peak shapes, however band broadening were observed for PQ and DQ due to their strong cationic properties.

For the preconcentration of QAHs, supported liquid membrane microextraction (SLMME) procedure was developed. The simple and cheap SLMME device was set up as shown in Figure 2.8. To avoid carry-over effect and membrane lifetime problem, new membrane was used in each extraction. However, the cost of hollow fiber membrane is very low, making this an inexpensive method. The developed extraction method is summarized in Figure 5.1.

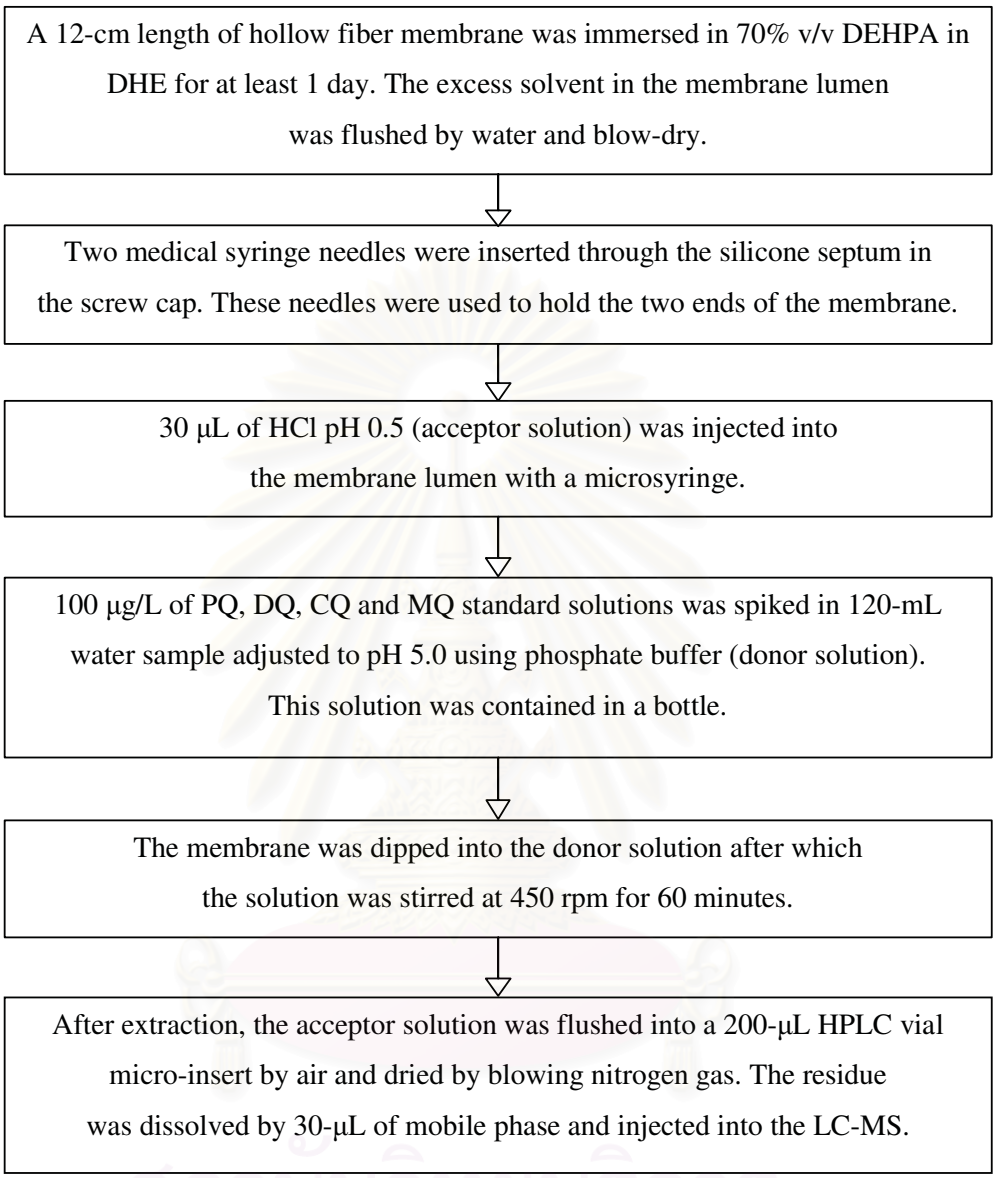


Figure 5.1 Schematic of the SLMME procedure for the extraction of QAHs

Due to the high hydrophilic and ionic properties of QAHs, the enrichment factors from this procedure were not high and ranged from 8.85 to 57.19. The method detection limits (MDLs) ranged from 0.17 to 1.29 μg/L and the method quantitation limits (MQLs) ranged from 0.57 to 4.30 μg/L (Table 5.3). These MDLs and MQLs values show the method capability to enrich QAHs for successive analysis at concentration below the maximum residue limits (MRLs) regulated by U.S. EPA. The

correlation coefficients (R^2) of the 2 to 10 μg/L calibration curves of all analytes were over 0.97 representing good linear dynamic range of the method.

The method's within-day precision was reported as the % relative standard deviation (%R.S.D.). The %R.S.D. values at 5 μg/L were in the range of 1.71 to 8.32% and the %R.S.D. values at 50 μg/L were in the range of 3.87 to 12.06%. All experimental %R.S.D. values were lower than %R.S.D. calculated from the Horwitz equation indicating satisfactory within-day precision. The method's between-day precision was reported as the P-value at 95% confidence of the extraction results obtained from three days (n=10). All P-values were higher than 0.05 indicating insignificant difference of the results from different-day experiment. For the application of this method in drinking water samples, % relative recoveries ranged from 59.4-105.4% were obtained from 5 μg/L spiking level and 62.6-106.9% from 50 μg/L spiking level. All values were acceptable according to the AOAC standard at each concentration level. These recovery values proved that the developed SLMME-LC-MS method can be successfully applied for the determination of QAHs in drinking water sample.

Table 5.3 Method validation data of SLMME-LC-MS for the determination of QAHs consists of retention time, correlation coefficience (R^2), method detection limit (MDL), and method quantitation limit (MQL)

	PQ	DQ	CQ	MQ
Retention time (min)	3.148±0.017	2.810±0.006	1.878±0.003	2.304±0.006
R^2	0.9672	0.9877	0.9938	0.9924
MDL (μg/L)	1.29 ± 0.17	0.48 ± 0.07	0.17 ± 0.03	0.39 ± 0.06
MQL (μg/L)	4.30 ± 0.57	1.60 ± 0.22	0.57 ± 0.11	1.30 ± 0.19

Overall, SLMME can be an alternative technique for clean-up and enrichment of QAHs in water. The advantages are: low extraction cost, device simplicity, and uncomplicated extraction procedure. However, due to a small the miniature size of the fiber membrane and the extraction device, the performing analyst must be trained for proper skills in SLMME technique to gain precise result. To improve method reproducibility and increasing through-put, further study should be conducted for on-line SLMME. The developed SLMME couple to LC-MS using hydrophilic interaction column is suitable for the analysis of QAHs.

The developed SLMME-LC-MS method was directly applied to drinking water because we were interested to find a simple and reliable method that meets the regulations at the time. And only the regulations to control QAHs in drinking water were regulated by many control authorities. This technique can be applied for natural water samples as well. However, interference from other positively charged species such as metal ions is expected because these positively charged species can be extracted into the acceptor solution and may result in the lower EF of QAHs. Therefore the method should be fine-tuned before using for natural water samples such as surface water and ground water. This procedure also has good potential for testing of QAH residues in agricultural products such as rice, wheat, pear, etc. Interfering food matrix can be simply eliminated by this technique making sample preparation very simple, fast, and inexpensive. However, the procedure must be fine-tuned for proper extraction parameters of each different matrix in a similar way that we described in this work.

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