CHAPTER III

EXPERIMENTAL

3.1 Instrument and Apparatus

3.1.1 High Performance Liquid Chromatography (HPLC): A module 1100 consists of automatic degasser, binary pump, autosampler, column thermostat, diode-array detector, and fluorescence detector. Agilent Technologies, Palo Alto, CA, USA.

3.1.2 Post-column derivatization instrument: A module PCX5200 consists of two post-column pumps, column thermostat, heated reactor, and ambient reactor. Pickering Laboratories, Inc., Mountain View, CA, USA.

3.1.3 Milli-Q, ultrapure water systems, with Millipak 40 filter unit 0.22 µm, model Millipore ZMQS5VOOY, Millipore, Billerica, MA, USA.

3.1.4 Vortex mixer, model G-5605, Scientific Industries, Bohemia, NY, USA.

3.1.5 Ion-exchange column: Glyphosate column, 4.0 mm ID × 150 mm, Pickering Laboratories, Inc., Mountain View, CA, USA.

3.1.6 Guard column: glyphosate guard column, 3.0 mm ID × 20 mm, Pickering Laboratories, Inc., Mountain View, CA, USA.

3.1.7 A water vacuum pump, model DOA-V130-BN, with Pressure Regulator, Millipore, Billerica, MA, USA.

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

3.1.9 Polypropylene hollow fiber membranes, Accurel PP Q3/2, Membrana, Wuppertal, Germany.

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

3.1.11 Medical needles, 0.8 mm O.D., Nipro Medical Corporation, Osaka, Japan.

3.1.10 Nitrogen Gas 99.99% purity, TIG, Samutprakan, Thailand.

3.1.11 Universal indicator pH 0-14, MERCK, Whitehouse Station, NJ, USA.

3.1.12 Teflon filter membranes 47 mm, 0.45 µm, Altech, Deerfield, IL, USA.

3.1.13 Nylon filter membranes 47 mm, 0.45 µm, Osmonics Inc., Trevose, PA, USA.

3.1.14 Graduated pipette 10.00 mL.

3.1.15 Micro-pipettes 100-1,000 µL and tips, Eppendorf, Hamburg, Germany.

3.1.16 Volumetric flasks 10.00 and 100.00 mL.

3.1.17 Beakers 25, 50, 250, 600, and 1,000 mL.

3.1.18 Graduated cylinders 10.0, 100.0 and 1,000.0 mL.

3.1.19 HPLC vials 2 mL with caps and insert, Agilent Technologies, Palo Alto, CA, USA.

3.1.20 Bottles with screw caps and septums 4, 10, 20, 40, 60, 120 mL.

3.1.21 Microsyringe 100.0 µL, Gastight, Renu, NV, USA.

3.1.22 Solvent bottles 500 and 1,000 mL, Schott, Elmsford, NY, USA.

3.1.23 Plastic Bottles 100 mL.

3.1.24 Glass Bottles 20 mL.

All glass apparatus was washed thoroughly in detergent, rinsed with deionised water and then rinsed with eluting solvent before used.

3.2 Chemicals

3.2.1 The Standard Compounds

Glyphosate (CAS No. 1071-83-6) was supplied by Riedel-de Haen (Seelze, Germany) and its metabolite aminomethylphosphonic acid (AMPA) (CAS No. 1066-51-9) was supplied by Fluka (Steinheim, Germany). The percent purity of glyphosate is 99.2 and AMPA is 99.0.

3.2.2 Chemicals and Solvent

Analytical grade potassium tetraborate tetrahydrate, methyltrioctylammonium chloride and di-n-hexyl ether were purchased from Fluka (Steinheim, Germany). Sodium hydroxide, potassium dihydrogen phosphate, sodium chloride and *o*-phosphoric acid (80%) were purchased from Merck (Darmstadt, Germany). Analytical grade hydrochloric acid (37%) was from Fisher

Scientific (Loughborough, LE, UK). Thiofluor and *o*-opthalaldehyde (OPA) (chromatographic grade) were from Pickering Laboratories (Mountain View, CA, USA). The HPLC-grade methanol and 5% sodium hypochlorite solution were from J.T.Baker (Phillipsburg, NJ, USA.). High purity water was obtained from a Milli-Q water system.

3.3 Preparation of the Standard Solutions, HPLC-Mobile Phases and Post-Column Derivatization Reagents

3.3.1 The Single Standard Stock Solutions

Each standard stock solution of 1,000 mg/L was prepared by weighing 0.1003 g of glyphosate standard and 0.1014 g of AMPA standard and dissolving them to the mark with Milli-Q water in 100.00-mL volumetric flasks. The standard stock solutions were transferred into 100-mL plastic bottles and stored in a refrigerator at 4 °C.

3.3.2 The Diluted Standard Solutions

Each standard solution of 100 mg/L was prepared by pipetting 1,000.0 µL of standard stock solutions (section 3.3.1) and diluted them to the mark with Milli-Q water in 10.00-mL volumetric flasks. These standards were freshly prepared daily.

3.3.3 HPLC-Mobile Phases

There are two kinds of mobile phase used in the HPLC system. HPLC-mobile phase A is potassium dihydrogenphosphate buffer to pH 2 and HPLC-mobile phase B is potassium hydroxide solution. Preparations of the two mobile phases are as followed:

3.3.3.1 Potassium Dihydrogen Phosphate Buffer, pH 2 (Mobile phase A)

HPLC-mobile phase A was prepared by the following procedure:

- Weighed 1.00 g of potassium dihydrogen phosphate, dissolved with 1,000.0 mL Milli-Q water and stored in a 1,000-mL solvent bottle.
- Pipetted 4.70 mL of *o*-phosphoric acid (80%) into the reservoir by a 10.00- mL graduated pipette.
- Swirled the solvent bottle to complete the mixing.

3.3.3.2 Potassium Hydroxide Solution (Mobile phase B)

HPLC-mobile phase B was prepared by weighing 1.50 g of potassium hydroxide, dissolving with 500.0 mL Mill-Q water and stored in a 500-mL solvent bottle.

The two HPLC-mobile phases were filtered by a glass filter holder set and nylon filter membrane before use.

3.3.4 Post-Column Derivatization Reagents

There are two kinds of post-column derivatization reagents. One is an oxidizing reagent and another is an OPA reagent. Preparations of the two reagents are as followed:

3.3.4.1 Oxidizing Reagent

Oxidizing reagent was prepared by:

- Weighed 0.950 g of potassium dihydrogen phosphate, 9.50 g of sodium chloride and 0.950 g of sodium hydroxide.
- Dissolved all of weighed chemical in 950.0 mL of Milli-Q water and stored in a 1,000-mL solvent bottle. This solution is called hypochlorite diluent.
- Added 100.0 µL of 5% sodium hypochlorite solution to the hypochlorite diluent and swirled the bottle.

3.3.4.2 OPA Reagent

OPA reagent was prepared by:

- Weighed 67.10 g of potassium tetraborate tetrahydrate, dissolved in 950.0 mL of Milli-Q water and stored in a 1,000-mL solvent bottle. This solution is called OPA diluent.
- Sparged the OPA diluent with nitrogen gas for 10 minutes.
- Stored 5.0 mL of the OPA diluent in a 10.0-mL graduated cylinder for the following step.
- Weighed 0.1000 g of OPA, dissolved in 10.0 mL of methanol and stored in a 20-mL glass bottle.
- Added the OPA solution to the deoxygenated OPA diluent.
- Weighed 2.00 g of Thiofluor and dissolved in 5.0 mL of the OPA diluent in a 20-mL glass bottle and transferred into the OPA diluent.
- Continued sparging for 5 minutes.
- Swirled the reagent.

Methanol and the two post-column derivatization reagents were filtered by nylon membrane before use.

3.4 Analytical Method

In this work, the analysis was performed by HPLC with post-column derivatization instrument. The method which was used in this experiment was developed by Pickering Laboratories, Inc. (36). Method parameters/conditions are:

Analytical column:	glyphosate column, K^+ form, 4.0 mm. ID × 150 mm.
Mobile phase:	A:p dihydrogen phosphate buffer, pH2.0
	B: 0.3% wt/v potassium hydroxide in water
Flow rate:	0.4 mL/min
Injection volume:	5 µL

HPLC method:

Time (minutes)	%A	%B
0	100	0
15.0	100	0
15.1-17	0	100
17.1-25	100	0

Post-column conditions	Reagent 1:	oxidizing reagent
	Pump 1:	0.3 mL/min
	Reactor 1:	36 °C
	Reagent 2:	OPA reagent
	Pump 2:	0.3 mL/min
	Reactor 2:	ambient temperature

3.5 Hollow Fiber Liquid Phase Microextraction (HF-LPME)

A hollow fiber membrane (HFM) used in the experiment is Accurel PP Q3/2. It has an inner diameter of 0.6 mm, wall thickness of 0.2 mm and pore size of 0.2 μm. At the beginning of the extraction, a HFM was cut into different length segments depended on the required acceptor volume (the original length of a HFM is 54 cm). A segment of HFM was immersed overnight in a membrane solvent. Membrane solvent was a mixture of Aliquat 336 and di-n-hexyl ether. Then excess membrane solvent in the lumen of HFM was removed by forcing air through the lumen from a 3-mL medical syringe. To set up and extraction device, two medical needles were pierced through a septum that fit the screw cap of a glass bottle. The fiber was bent to a u-shape and one end of it was suspended by a medical needle. The acceptor solution was filled into the lumen by a 100.0-μL syringe. Another medical needle was attached to another end of the HFM and this module was submerged in the donor solution filled in the extraction bottle. The donor solution was prepared by a 100-mL volumetric flask. It was the Milli-Q water spiked with standard solutions of glyphosate and AMPA and adjusted pH by adding hydrochloric acid or sodium hydroxide to the desired values. The configuration of HF-LPME is illustrated in Figure 3.1.

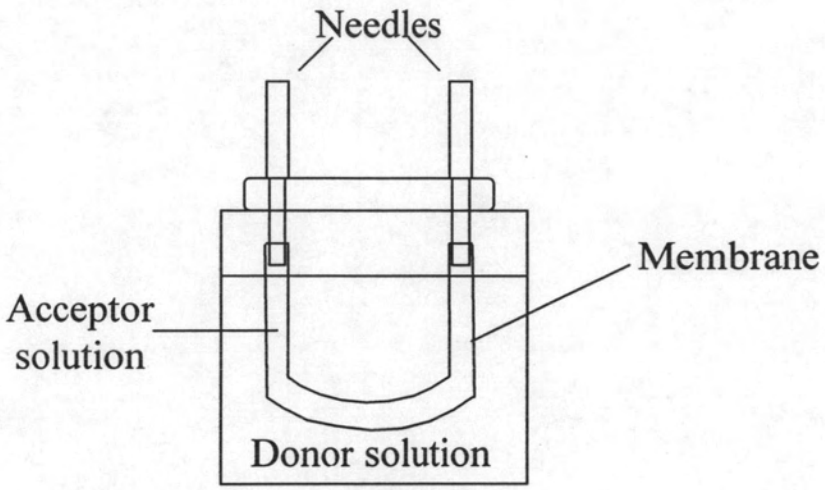

Figure 3.1 The schematic diagram of HF-LPME.

To prevent interference and cross contamination, magnetic stir bar was not used. The system was agitated by vortex during the extraction process at ambient temperature. After extraction, the acceptor solution was flushed out by forcing air through the lumen from a 3-mL medical syringe into a 200-µL HPLC insert vial. This solution was ready for analysis by HPLC with post-column derivatization. The HFM was discarded after use.

3.6 HF-LPME Optimization

To optimize HF-LPME method, the following parameters were studied in three replications.

3.6.1 Immersion Time

The duration that HFMs were soaked in membrane solvent was varied at 5, 30, 60, 120 minutes and overnight. Other fixed parameters were shown in Table 3.1.

The optimization results are shown in Table 4.1.

Table 3.1 HF-LPME conditions for the optimization process.

Optimized parameter	Extraction condition								
	Immersion time (min)	Donor pH	Aliquat 336 concentration (M)	Acceptor type	Acceptor solution concentration (M)	Acceptor solution volume (μL)	Donor solution volume (mL)	Agitation (unit)	Extraction time (min)
Immersion time	*	11.0	0.20	NaCl	1.0	30.0	20.0	Vortex 5	45
Donor pH	Overnight	*	0.20	NaCl	1.0	30.0	20.0	Vortex 5	45
Aliquat 336 concentration	Overnight	9.0	*	NaCl	1.0	30.0	20.0	Vortex 5	45
Acceptor type	Overnight	9.0	0.20	*	1.0	30.0	20.0	Vortex 5	45
Acceptor solution concentration	Overnight	9.0	0.20	KCl	*	30.0	20.0	Vortex 5	45
Acceptor solution volume	Overnight	9.0	0.20	KCl	1.0	*	20.0	Vortex 5	45
Donor solution volume	Overnight	9.0	0.20	KCl	1.0	20.0	*	Vortex 5	45
Agitation	Overnight	9.0	0.20	KCl	1.0	20.0	20.0	*	45
Extraction time	Overnight	9.0	0.20	KCl	1.0	20.0	20.0	Vortex 3	*

* varied parameters.

3.6.2 Donor Solution pH

The effect of donor solution pH was studied at pH 1.0, 3.0, 6.0, 8.0, 9.0, 10.0, 11.0 and 12.0. The donor solutions were prepared directly in 100-mL volumetric flasks. Milli-Q water was spiked with standard solutions of glyphosate and AMPA (10 μg/L) and adjusted to the desired value by adding hydrochloric acid or sodium hydroxide. The parameters in Table 3.1 shows controlled parameters in the extraction procedure.

The optimization results are shown in Table 4.2.

3.6.3 Concentration of Aliquat 336

Membrane solvent was prepared by mixing Aliquat 336 and di-n-hexyl ether in 2-mL HPLC vial. Ratios of the two chemicals were optimized as shows in Table 3.2. Hollow fiber membranes were immersed into these solutions overnight.

Table 3.2 Composition of Aliquat 336 and di-n-hexyl ether mixing in membrane solvent.

Aliquat 336 concentration (M)	Weight of Aliquat 336 (g)	Volume of di-n-hexyl ether (mL)
0.05	0.0202	0.9770
0.10	0.0404	0.9540
0.15	0.0606	0.9310
0.20	0.0808	0.9200
0.25	0.1010	0.8850
0.30	0.1212	0.8620

Other fixed extraction parameters are shown in Table 3.1.

The optimization data are shown in Table 4.3.

3.6.4 Types of Acceptor Solution

Formic acid, hydrochloric acid, sodium chloride, potassium chloride, and ammonium chloride of the same concentration (1.0 M) were tested. HF-LPME condition was controlled as in Table 3.1.

The optimization data are shown in Table 4.4.

3.6.5 Acceptor Solution Concentration

Potassium chloride was selected as the best acceptor solution. To further fine-tuning, its concentration was also evaluated at concentration of 0.25, 0.50, 0.75, 1.0, 1.50, and 2.0 M, respectively. Table 3.1 showed the extraction condition in this procedure.

The optimization results are shown in Table 4.5.

3.6.6 Acceptor Solution Volume

Suitable acceptor volume was evaluated in 10.0, 20.0, 30.0, 50.0, 80.0 and 100.0 μL and HFM length was shown in Table 3.3.

Table 3.3 Varied HFM lengths and acceptor volumes.

Acceptor solution volume (μL)	HFM length (cm)
10.0	4.0
20.0	8.0
30.0	12.0
50.0	20.0
80.0	31.0
100.0	37.0

The optimization results are shown in Table 4.6.

3.6.7 Donor Solution Volume

Optimum donor volumes were evaluated at 3.5, 5.0, 10.0, 20.0, 40.0, 60.0 and 120.0 mL. Other parameters were displayed in Table 3.1.

The optimization data are shown in Table 4.7.

3.6.8 Agitation

Vortex mixer was adjusted in arbitrary unit from 1 to 8. No agitation was studied and the fixed HF-LPME conditions were shown in Table 3.1.

The optimization results are shown in Table 4.8.

3.6.9 Extraction Time

Extraction time was studied last and was varied at 10, 20, 30, 45, 60, and 90 minute. Table 3.1 showed extraction condition at this step.

The optimization data are shown in Table 4.9.

The optimum HF-LPME conditions are concluded in Table 4.10.

3.7 Method Validation

Because the developed HF-LPME procedure will be used as an analytical sample preparation step, it is necessary to test the quality of the procedure especially how each parameter can affect the analytical data. Method validation is a process of proving that an analytical method is acceptable for its intended purpose in analytical chemistry. The developed HF-LPME method was fully tested for its analytical performance following a full method validation protocol. Linear dynamic ranges, method detection limits, method quantitation limits, precisions and accuracies are studied.

3.7.1 The Study of Linear Dynamic Range

The donor solutions which were spiked with standard solutions at 1, 20, 40, 60, 80, 100, 120, 140, 200, 300, 400, 600, 800, 1,000 and 3,000 μg/L for glyphosate and 5, 25, 45, 65, 85, 105, 125, 145, 205, 305, 405, 605, 805, 1,005 and 3,005 μg/L for AMPA were twice extracted by the optimum condition (Table 4.10). The acceptor solutions were injected into the HPLC with post-column derivatization instrument and the peak areas were measured. The relationships between concentrations and peak areas were plotted. The intercepts, slopes and correlation coefficients were obtained.

The linearity curves are shown in Figure 4.16 and Figure 4.17. The intercepts, slopes and correlation coefficients are shown in Table 4.11.

3.7.2 The Study of Method Detection Limits (MDLs) and Quantitation Limits (MQLs)

MDLs and MQLs were determined from the concentrations that give the peak signal as high as 3 and 10 times of the baseline signal, respectively. In the experiment, the spiked donor solutions (1 μg/L for glyphosate and 5 μg/L for AMPA) were extracted by HF-LPME by the optimum condition (Table 4.10) in 10 replications. MDLs and MQLs were calculated from the chromatograms attained.

The data of MDLs and MQLs are shown in Table 4.12.

3.7.3 The Study of Method Precision

In this thesis, precision was performed by extraction of spiked standard solutions at method quantification limits (MQL) and 5-fold MQL concentration levels. The optimum HF-LPME condition (Table 4.10) was used in this studied. Each level of concentration was repeated 10 times to measure accurate peak area and evaluates percent relative standard deviation.

The percent relative standard deviations obtained are shown in Table 4.13.

3.7.4 The Study of Accuracy

Accuracy of this method was determined by the analysis of 10 replications of standard spiked solutions. The standard glyphosate of 3 μg/L and standard AMPA of 8 μg/L were spiked and extracted by the optimum condition (Table 4.10). The accuracy was calculated in term of percent recovery.

The % recoveries data are shown in Table 4.14.

3.8 Application of the Developed HF-LPME Procedure for the Determination of Glyphosate and AMPA in Ground Water Sample

The developed HF-LPME procedure was tested on real water samples. Groundwater sample used as a blank collected from several sites around Thailand.

First site — Groundwater sample from Tombon Philom, Amphur Bangkratum, Phitsanulok Province.

Second site — Groundwater sample from Tombon Bangkratum, Amphur Bangkratum, Phitsanulok Province.

Third site — Groundwater sample from Tombon Wangchaoon, Amphor Boungsamokkee, Kamphaengphet Province.

Fourth site — Groundwater sample from Tombom Salokbath, Amphor Kanuworaluksaburee, Kamphaengphet Province.

Duplicate extractions of standard spiked underground water samples by the optimum HF-LPME procedure were injected into the HPLC with post-column derivatization instrument. The MQL concentrations of the two analytes were spiked (1.0 μg/L for glyphosate and 5.0 μg/L for AMPA). The final concentrations were determined by external standard calibration method and % recovery was calculated.

The % recoveries are shown in Table 4.15.