CHAPTER III

EXPERIMENTAL

3.1 Instrumental and Apparatus

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.1.23 Graduated cylinders, 25mL and 100mL.

3.1.24 Spatulas.

3.1.25 Droppers.

3.1.26 Stirring rods.

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

3.2 Chemicals

3.2.1 Standard compounds

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

3.2.2 Organic solvents

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

3.2.3 Reagents

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

3.3 Preparation of standard solutions

3.3.1 Preparation of stock standard solutions

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

3.3.2 Preparation of mixture standard solutions

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

3.4 LC-MS/MS system

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

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

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

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

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

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

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

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

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

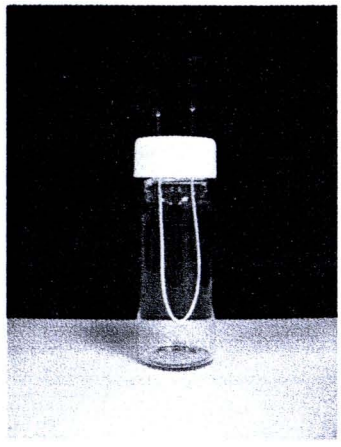

Figure 3.1 The studied HF-LPME configuration

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

3.5.1 The procedure of immersion time optimization

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

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

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

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

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

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

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

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

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

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

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

3.5.2 The procedure of organic solvent type optimization

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

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

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

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

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

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

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

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

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

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

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

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

3.5.3 The procedure of organic solvent composition optimization

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

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

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

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

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

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

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

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

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

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

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

3.5.4 The procedure of donor type optimization

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

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

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

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

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

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

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

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

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

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

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

3.5.5 The procedure of donor pH optimization

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

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

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

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

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

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

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

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

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

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

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

3.5.6 The procedure of acceptor type optimization

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

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

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

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

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

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

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

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

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

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

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

3.5.7 The procedure of acceptor pH optimization

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

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

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

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

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

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

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

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

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

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

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

3.5.8 The procedure of extraction time optimization

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

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

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

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

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

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

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

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

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

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

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

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

3.6 Method Validation

3.6.1 Standard calibration curve

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

3.6.2 Linearity

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

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

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

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

signal to noise ratio of S/N = 10. Both LODs and LOQs of method are shown in Table 4.12.

3.6.4 Enrichment factor

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

3.6.5 Accuracy

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

3.6.6 Precision

The precision is the closeness of agreement between independent test results obtained under same condition. The two categories of precision are intra-assay precision and intermediate precision. The intra-assay precision is the precision derived from repeated

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

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

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

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

where C is the concentration of the analyte in the sample

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

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

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

3.7.1 Water sample

The optimized HF-LPME condition was applied to preconcentrate four macrolides in water sample. Water samples were collected from Chaophaya River, Bangkok, Thailand. Four macrolides were spiked in water sample for determination because the water sample was not found macrolide antibiotic residues. The procedure for determining ERY, SPI, TIL, and TYL spiked in water sample was described as follows.

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

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

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

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

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

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

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

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

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

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

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

3.7.2 Poultry muscle sample

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

3.7.2.1 Method I: Donor solution

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.7.2.2 Method II: Meta-phosphoric acid-methanol

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

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

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

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

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

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

Composition I: 0.3% meta-phosphoric acid-methanol

Composition II: 0.5% meta-phosphoric acid-methanol

Composition III: 1% meta-phosphoric acid-methanol

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

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

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

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

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

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

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

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

3.7.2.3 Method III: McIlvaine buffer

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

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

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

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

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

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

Composition I: McIlvaine buffer

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

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

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

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

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

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

3.7.2.4 Method IV: Trichloroacetic acid (TCA)

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

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

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

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

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

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

Composition I: TCA

Composition II: TCA + McIlvaine buffer

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

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

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

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

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

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

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

3.7.2.5 Method V: KH_2PO_4-ACN

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

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

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

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

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

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

Composition I: KH_2PO_4-ACN

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

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

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

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

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

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

3.7.3 Method performance in water and poultry sample application

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

3.7.3.1 Water sample application

3.7.3.1.1 Recovery

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

3.7.3.1.2 Limit of detections (LODs)

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

3.7.3.2 Poultry sample application

3.7.3.2.1 Recovery

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

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

3.7.3.2.2 Limit of detections (LODs)

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