CHAPTER V

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDY

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

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

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

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

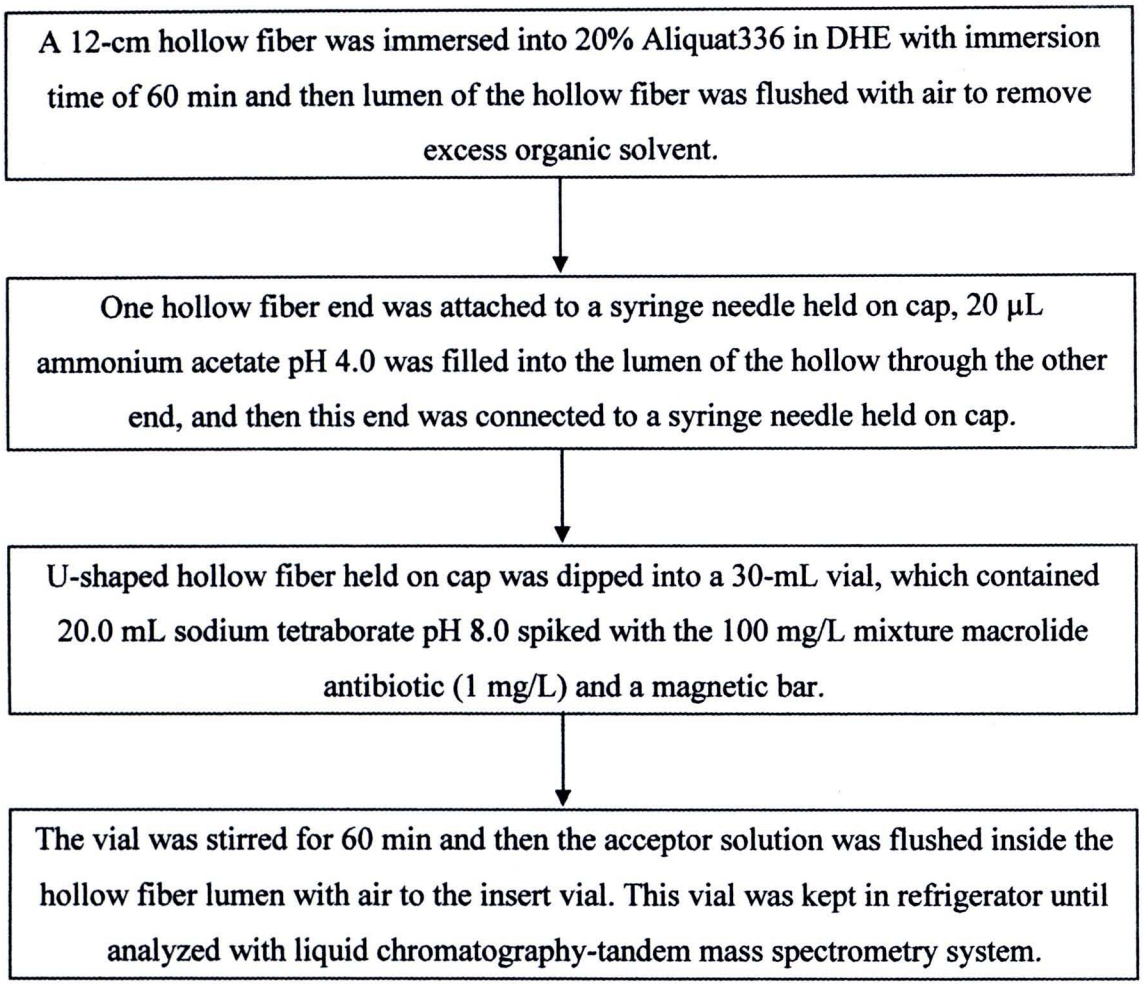

Figure 5.1 Schematic diagram of HF-LPME procedure with optimized condition.

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

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

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

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

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

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

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

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

The LOD of this proposed method are promising when compared with other works. The method recovery representing accuracy ranged from 89.09 to 102.99 % at 50 μg/L spiking level. The intra-assay precision was reported as relative standard deviation (%R.S.D.) and the value of %R.S.D. for within-day precision ranged from 7.68 to 10.23%. The %R.S.D. values obtained from the experiments were lower than %R.S.D calculated from Horwitz equation, which indicates the satisfactory of method capability. For intermediate precision, the %R.S.D. was calculated from the results on two analytical days and two-tailed F-test were used to evaluate the significance of different %R.S.D. between two days. The values of %R.S.D did not significantly differ

on two working days because the calculated F values were lower than the critical F values (P=0.05). Both intra-assay and intermediate precisions were in acceptable ranges.

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

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

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

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

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