CHAPTER IV

RESULTS AND DISCUSSION

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

4.1 HF-LPME optimization

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

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

4.1.1 The optimization of immersion time

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

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

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

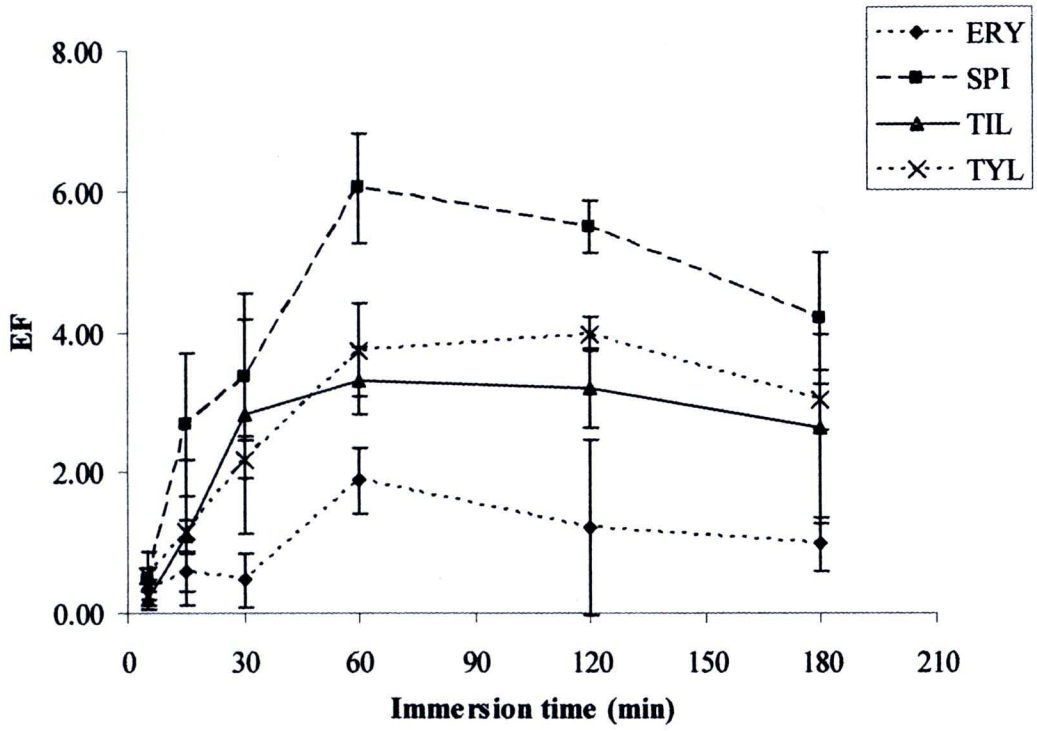

Figure 4.1 The influence of immersion time on enrichment factor.

4.1.2 The optimization of organic solvent type

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

difficult extraction with a single solvent. Other parameters of HF-LMPE were optimized as discussed later. The selected organic solvent was dihexyl ether.

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

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

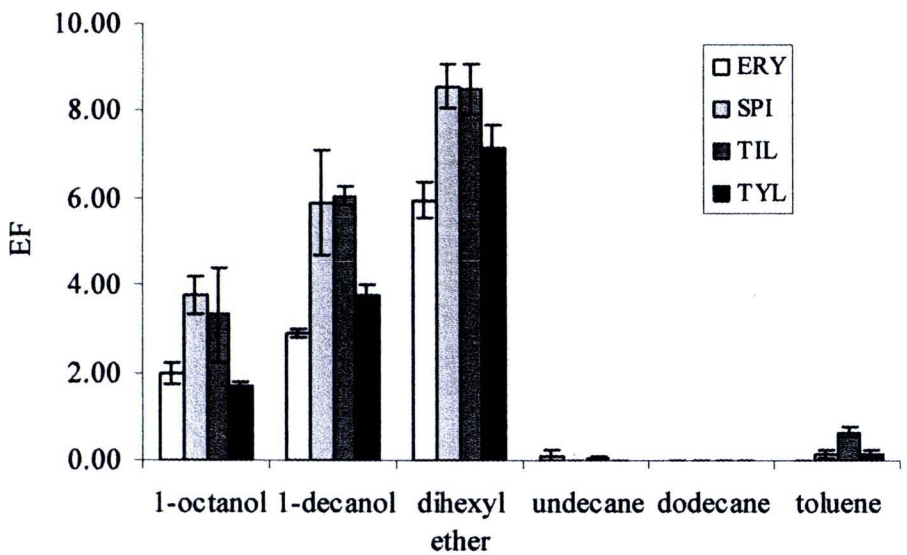

Figure 4.2 The influence of organic solvent type on enrichment factor.

4.1.3 The optimization of organic solvent composition

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

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

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

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

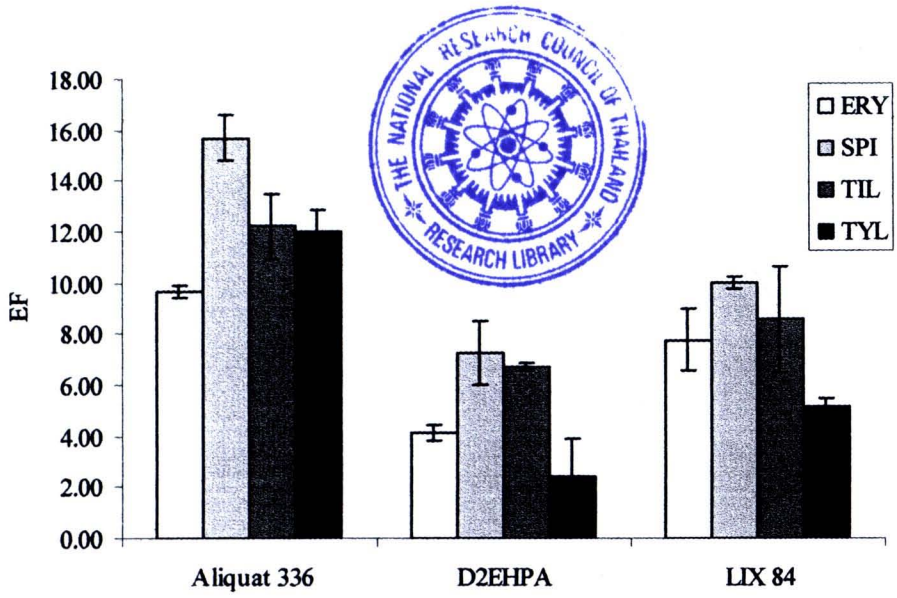

Figure 4.3 The influence of carrier type on enrichment factor.

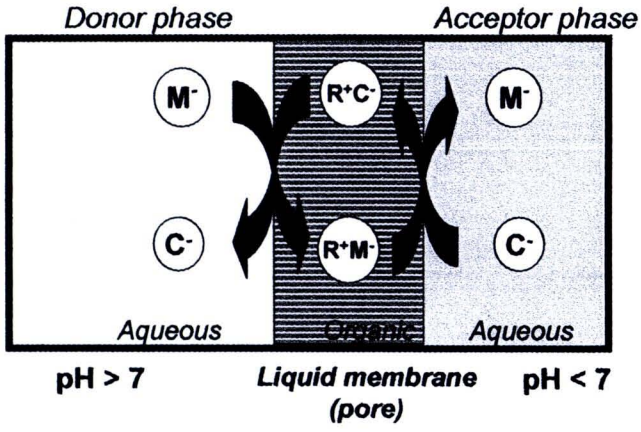

Figure 4.4 Mechanism of carrier-mediated mode in this work

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

Due to Aliquat 336 yielded the highest enrichment factor, the content of Aliquat 336 was optimized at 5%, 10%, 20%, 30%, and 40% in DHE and the results are presented in Table 4.4 and Figure 4.5. The enrichment factor from low content of Aliquat 336 in DHE led to low extraction efficiency because the amount of carrier was insufficient to form ion-pair complex with analytes. On the other hand, high content of carrier resulted

in high viscosity of organic phase inside hollow fiber, which decreased the flux of the compound through the membrane. Therefore, 20% Aliquat 336 in DHE was chosen for organic phase.

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

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

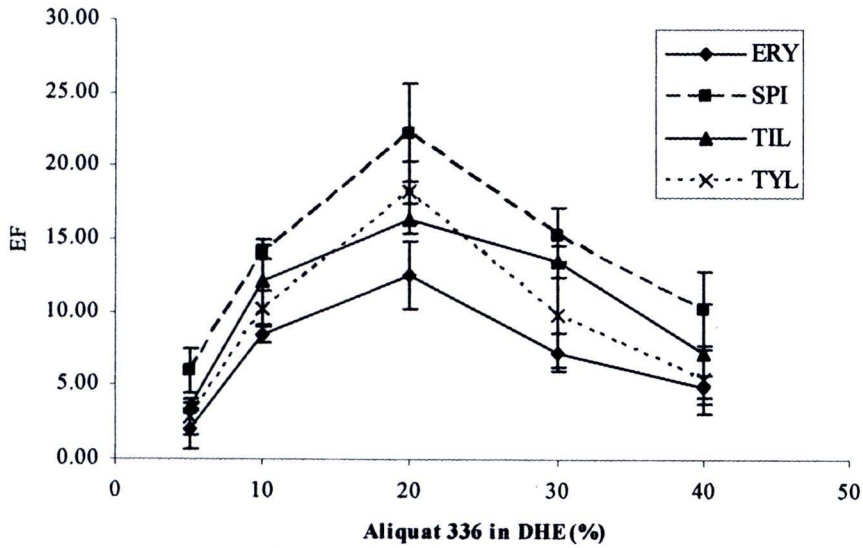

Figure 4.5 The influence of Aliquat 336 content on enrichment factor.

4.1.4 The optimization of donor type

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

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

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

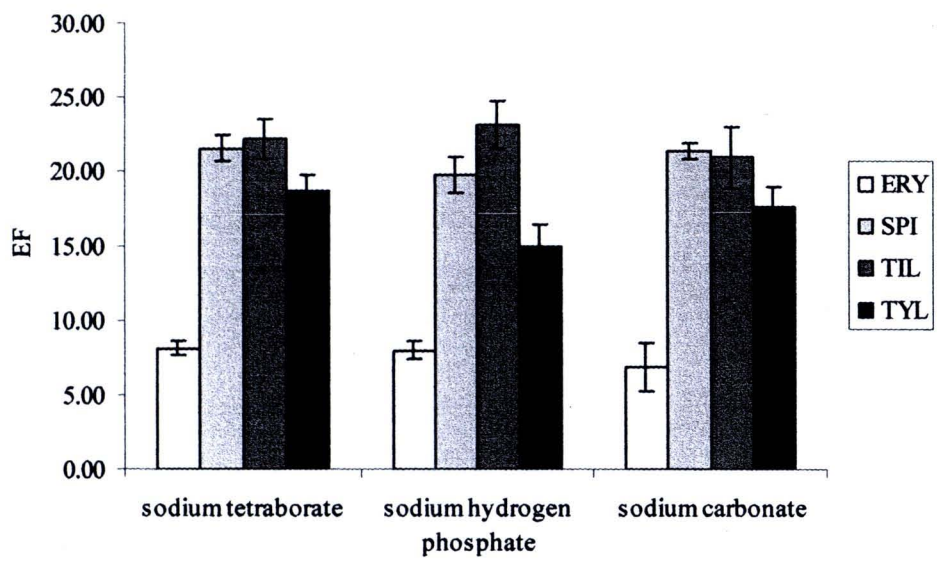

Figure 4.6 The influence of donor type on enrichment factor.

4.1.5 The optimization of donor pH

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

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

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

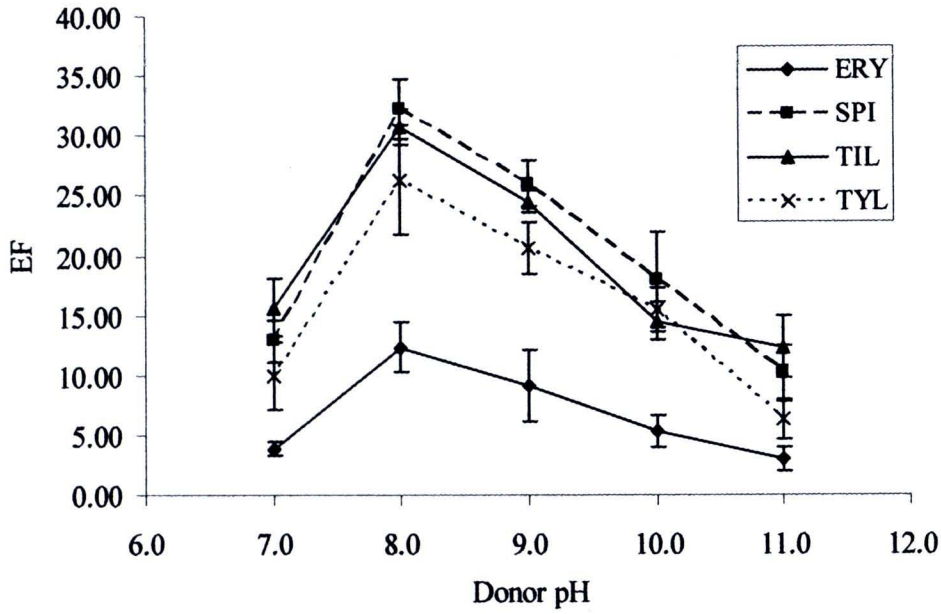

Figure 4.7 The influence of donor pH on enrichment factor.

4.1.6 The optimization of acceptor type

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

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

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

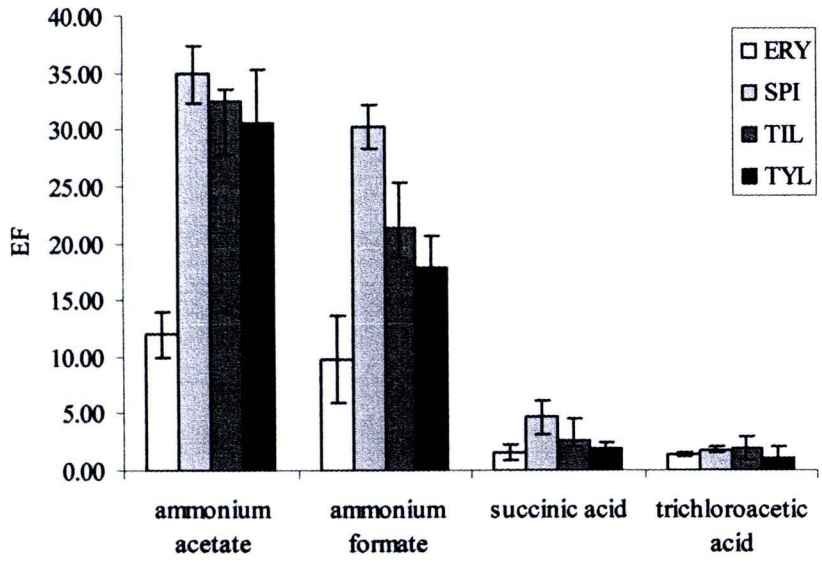

Figure 4.8 The influence of acceptor type on enrichment factor.

4.1.7 The optimization of acceptor pH

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

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

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

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

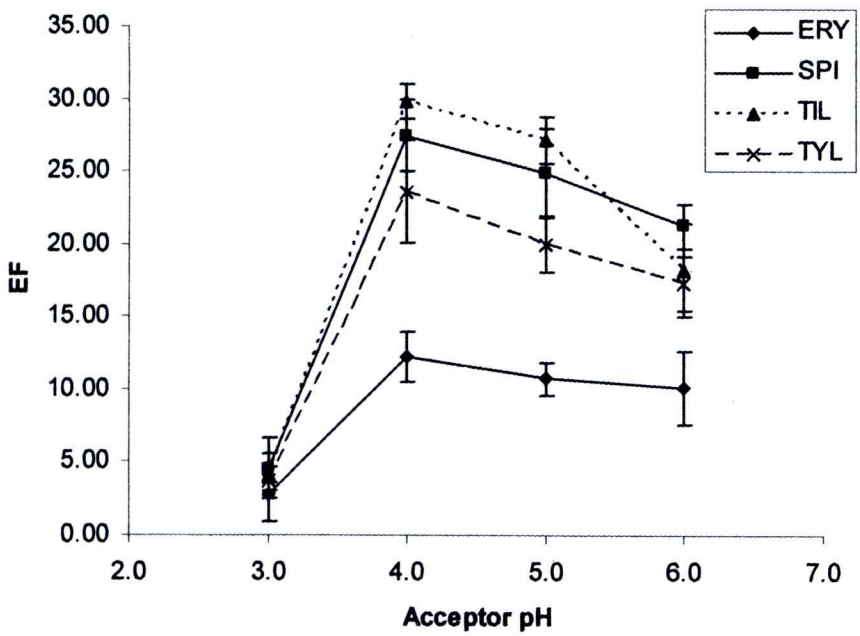

Figure 4.9 The influence of acceptor pH on enrichment factor.

4.1.8 The optimization of extraction time

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

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

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

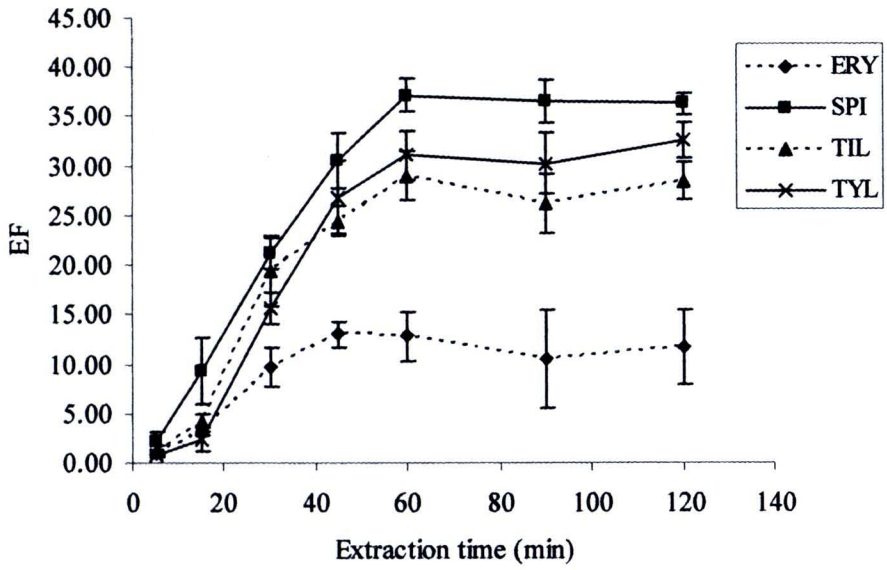

Figure 4.10 The influence of extraction time on enrichment factor.

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

Table 4.10 The optimum conditions of HF-LPME.

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

4.2 Method Validation

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

4.2.1 Standard calibration curve

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

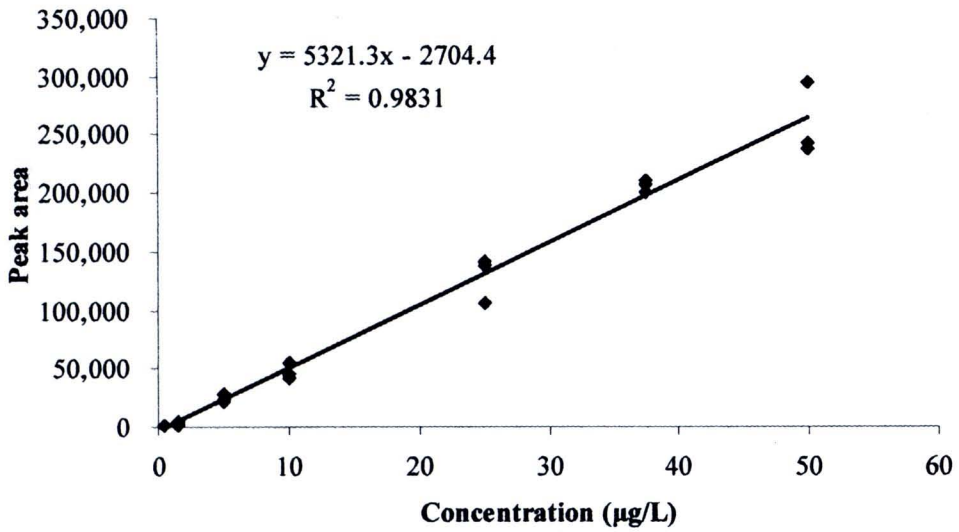

Figure 4.11 Standard calibration curve of erythromycin (ERY) in HF-LPME analysis.

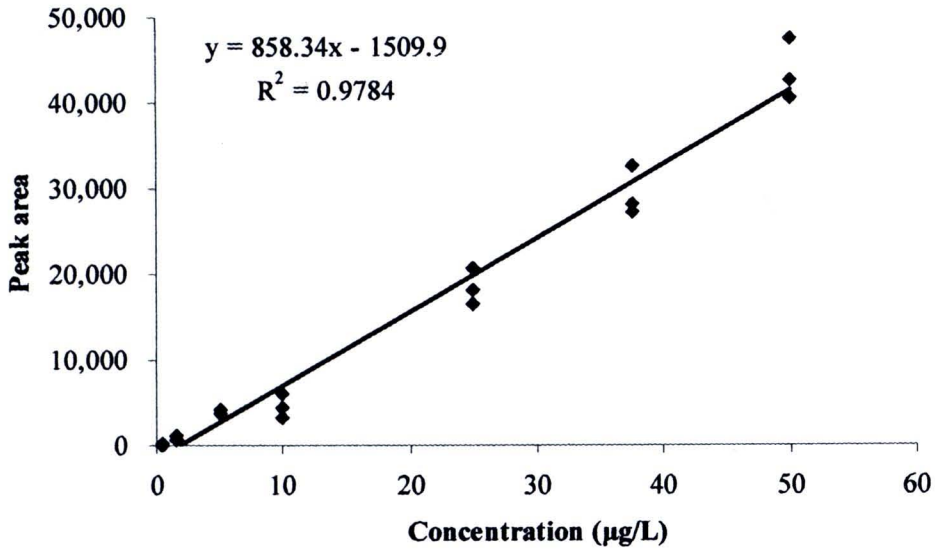

Figure 4.12 Standard calibration curve of spiramycin (SPI) in HF-LPME analysis.

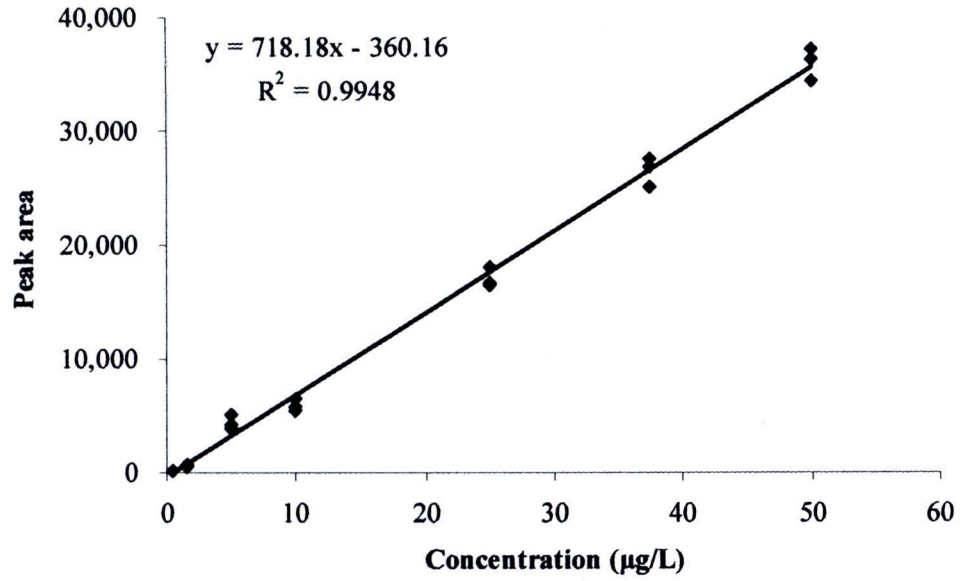

Figure 4.13 Standard calibration curve of tilmicosin (TIL) in HF-LPME analysis.

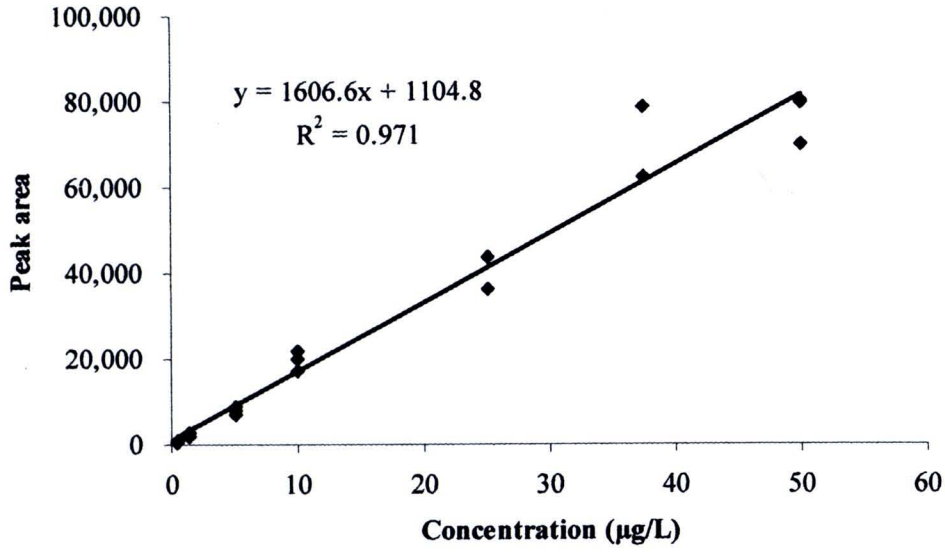

Figure 4.14 Standard calibration curve of tylosin (TYL) in HF-LPME analysis.

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

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

4.2.2 Linearity

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

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

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

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

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

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

4.2.4 Enrichment factor

The enrichment ability of this method under optimized HF-LPME conditions was determined in eight replicates and each analyte was spiked in water at 25 and 50 μg/L. After HF-LPME analysis, the enrichment factor from two spiking level was determined as seen in Table 4.13. The results indicated the highest enrichment factors of the four

macrolides with HF-LPME from two spiking levels are 13.10 (ERY), 38.54 (SPI), 30.24 (TIL), and 31.51 (TYL).

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

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

4.2.5 Accuracy

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

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

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

4.2.6 Precision

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

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

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

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

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

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

4.3.1 Water sample

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

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

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

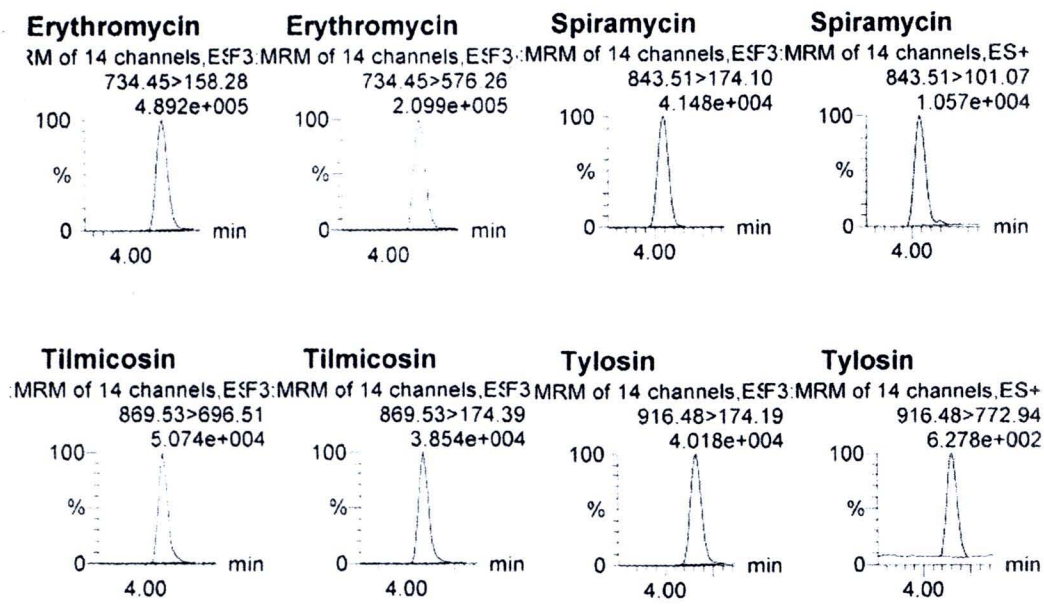

Figure 4.15 Example LC-ESI-MS/MS chromatogram of ERY, SPI, TIL and TYL spiking 20 μg/L in water sample after HF-LPME method at MS quantification and conformation transition.

4.3.2 Poultry sample

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

4.3.2.1 Extraction Method I

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

From the results, the four analytes spiked at 1 mg/L could not be enriched or extracted with directly using donor solution as extracting solution with three pathways. Due to

high protein and fat content in chicken sample, analytes were blocked to be enriched or extracted. However, the enrichment tendency could suggest that centrifugation is necessary to precipitate interferences or sample matrices and is better than the other pathways. A centrifugation step was used for further optimization.

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

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

4.3.2.2 Extraction Method II

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

factor between donor solution modification and not, the results showed that the modification with donor in meta-phosphoric acid-methanol give a better results because the existence of donor solution can comfortably transfer or extract analytes in the similar way with HF-LPME process.

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

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

4.3.2.3 Extraction Method III

The extracting solution of Method III was based on McIlvaine buffer. McIlvaine buffer consists of citric acid monohydrate, disodium hydrogenphosphate (Na_2HPO_4), and ethylenediaminetetraacetic acid disodium salt (Na_2EDTA). It is one of the most popular protein precipitating agents in the extraction from biomatrices. The presence of EDTA and citric acid prevents the adsorbing sites of analytes from chelating with cations that can affect to extraction efficiency. Hence, McIlvaine buffer is an alternative way to precipitate protein in matrix. The extraction with McIlvaine buffer was investigated

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

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

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

4.3.2.4 Extraction Method IV

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

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

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

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

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

4.3.2.5 Extraction Method V

The extracting solution of Method V was based on KH_2PO_4-ACN. This solution with organic solvent and the concept were adapted from *(47)*. The modification of organic solvent in extracting solution may induce transport of analytes through organic phase in hollow fiber membrane pores. The organic solvent used in modification must be more miscible with water than the organic phase to protect leakage of organic phase from hollow fiber during the preconcentration process. Acetonitrile was added to KH_2PO_4 to perform as extracting solution. This type of extracting solution was studied with two compositions. Each composition was tested in two replicates and the results were determined as seen in Table 4.21. The enrichment factors obtained indicated that the addition of organic solvent in extracting solution did not significantly improved the

extraction or preconcentration; even if in donor modification the extraction efficiency could be increased.

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

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

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

4.3.3 Method performance in water and poultry sample application

4.3.3.1 Water sample application

In real sample analysis, the recovery represents the method accuracy and the limit of detections refers to the method lowest concentration detection. Both parameters were defined in eight replicates at spiking level of four macrolides 20 μg/L in water sample followed by preconcentrated with optimized HF-LPME method and detection with LC-ESI-MS/MS to evaluate the method effectiveness. The result of recovery and limits of detections was shown in Table 4.22 All values of recovery and LODs of four macrolides gained are in the same tendency and range as the method validation results.

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

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

4.3.3.2 Poultry sample application

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

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

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