

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Cyclic Voltammetric Study

The cyclic voltammetric (I-E) responses for a solution containing 1 mM tetracycline in phosphate buffer – acetonitrile (80:20, v/v) solution together with the corresponding background voltammogram at the Au electrode and at the anodized BDD electrode were shown in Figure 4.1 (A) and (B). The results obtained from the other antibiotics, i. e., oxytetracycline, chlortetracycline, and doxycycline were analogous to the tetracycline response and were shown in APPENDIX A.

In the presence of tetracyclines at Au electrode, the anodic signal for the oxidation of tetracyclines was observed on the positive scan in the region of ca. +1.0 to +1.3 V versus Ag/AgCl. The cathodic peak was observed on the negative scan in the region of ca. +0.9 to +0.5 V versus Ag/AgCl, due to the reduction of activity of the oxide covered gold surface.

At the anodized BDD, the oxidation of tetracyclines occurred in the region of ca. +1.2 to +1.6 V versus Ag/AgCl and cathodic peak was not observed on the negative scan in the region of ca. +0.8 to 0.0 V versus Ag/AgCl.

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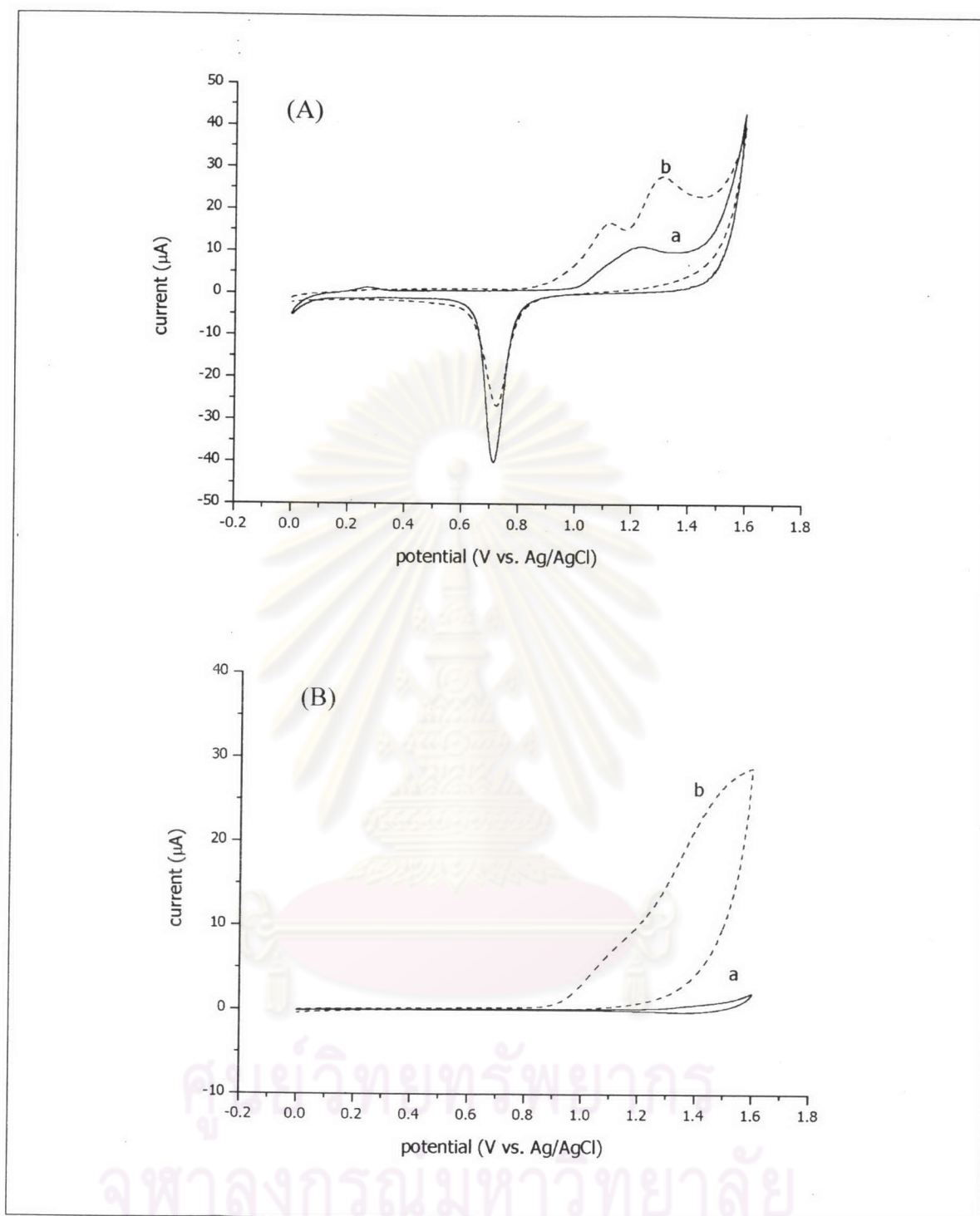


Figure 4.1 Cyclic voltammograms for 1 mM tetracycline (line b) in 0.01 M phosphate buffer (pH 2.5) – acetonitrile (80:20, v/v) at (A) Au electrode and (B) anodized BDD electrode. The sweep rate was 50 mV/s. Background voltammograms are also shown in the figure (line a).

4.2 PAD Optimization

In this part, the PAD waveform parameters were optimized by injection of 5 ppm mixed standard solution using HPLC conditions. The order of optimized PAD waveform parameters were detection potential (E_{det}), delay time (t_{del}), integration time (t_{int}), oxidation potential (E_{oxd}), oxidation time (t_{oxd}), reduction potential (E_{red}), and reduction time (t_{red}), respectively. The potential range used for E_{det} optimization was chosen from the potential region in the cyclic voltammogram (Figure 4.1), which the oxidation of tetracyclines occurred. The E_{det} was varied from +1.0 to +1.4 V versus Ag/AgCl at the Au electrode and +1.25 to +1.55 V versus Ag/AgCl at the anodized BDD in intervals of 500 mV. Then the t_{del} was varied from 100 to 900 msec in intervals of 100 msec and the t_{int} was varied from 30 to 180 msec in 30 msec increments. The results of PAD waveform in the detection step at the Au electrode and anodized BDD were shown in Figure 4.2 and Figure 4.5, respectively.

To receive reproducible signals, the electrode should be pulsed adequately more positive potential to remove adsorbed species. The E_{oxd} optimization was varied from +1.7 to +2.0 V versus Ag/AgCl intervals of 100 mV at both Au and anodized BDD electrode. The variation of t_{oxd} was optimized from 100 to 900 msec. The results of PAD waveform in the oxidation step at the Au electrode and the anodized BDD were shown in Figure 4.3 and Figure 4.6, respectively.

Because of the formation of oxide on the electrode surface, it was essential that the E_{red} and t_{red} were optimized to achieve complete reductive dissolution of the surface oxide. In the reactivation step, the E_{red} was varied in the +0.1 to +0.4 V versus Ag/AgCl in intervals of 100 mV for several values of t_{red} in the range 100 to 600 msec. The results of PAD waveform in the reduction step at the Au electrode and anodized BDD were shown in Figure 4.4 and Figure 4.7, respectively. The optimal PAD waveform parameters for determination tetracyclines were summarized in the Table 4.1.

Table 4.1 Optimal PAD waveform parameters for the developed method at the Au electrode and the anodized BDD electrode

Parameter	Au	Anodized BDD
1. Detection step		
E_{det} (V vs. Ag/AgCl)	1.3	1.5
t_{det} (sec)	0.8	0.2
t_{int} (sec)	0.12	0.09
2. Oxidation step		
E_{oxd} (V vs. Ag/AgCl)	2.0	2.0
t_{oxd} (sec)	0.9	0.2
3. Reduction step		
E_{red} (V vs. Ag/AgCl)	0.1	0.4
t_{red} (sec)	0.3	0.2

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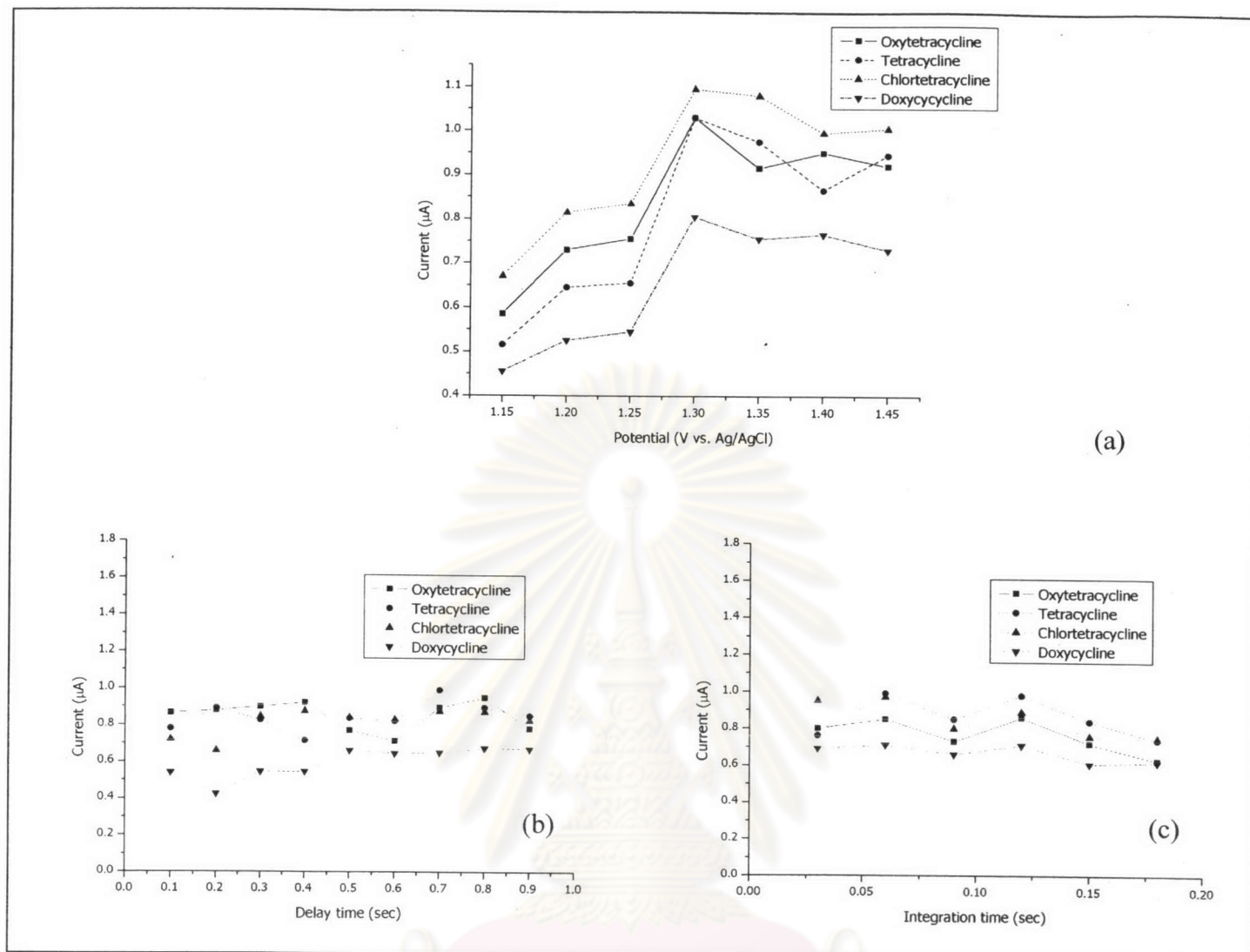


Figure 4.2 HPLC-PAD response as a function of (a) E_{det} , (b) t_{del} and (c) t_{int} for 5 ppm of oxytetracycline, tetracycline, chlortetracycline and doxycycline at the Au electrode.

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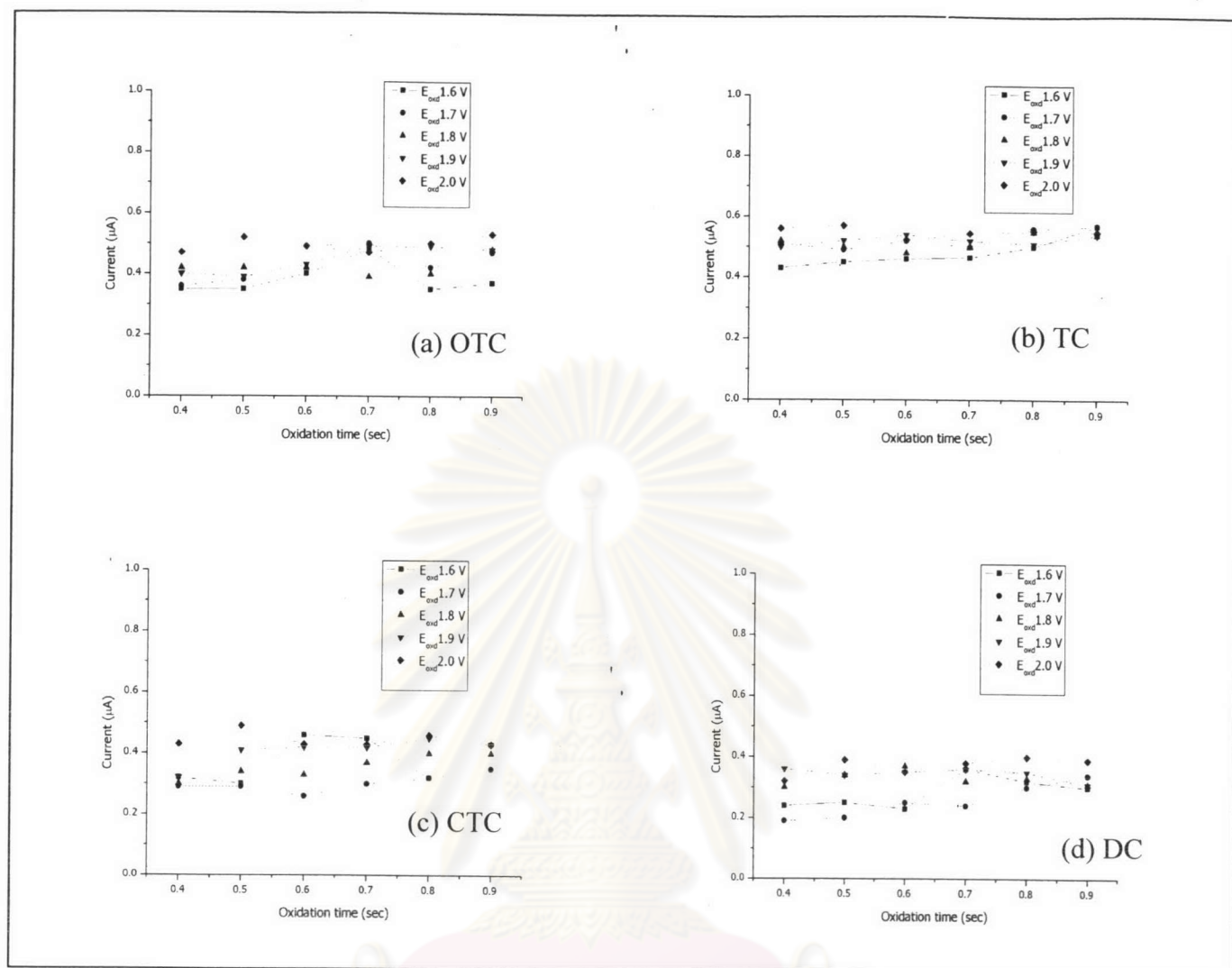


Figure 4.3 HPLC-PAD response as a function of E_{oxd} , and t_{oxd} for 5 mg/kg of (a) oxytetracycline (OTC), (b) tetracycline (TC), (c) chlortetracycline (CTC) and (d) doxycycline (DC) at the Au electrode.

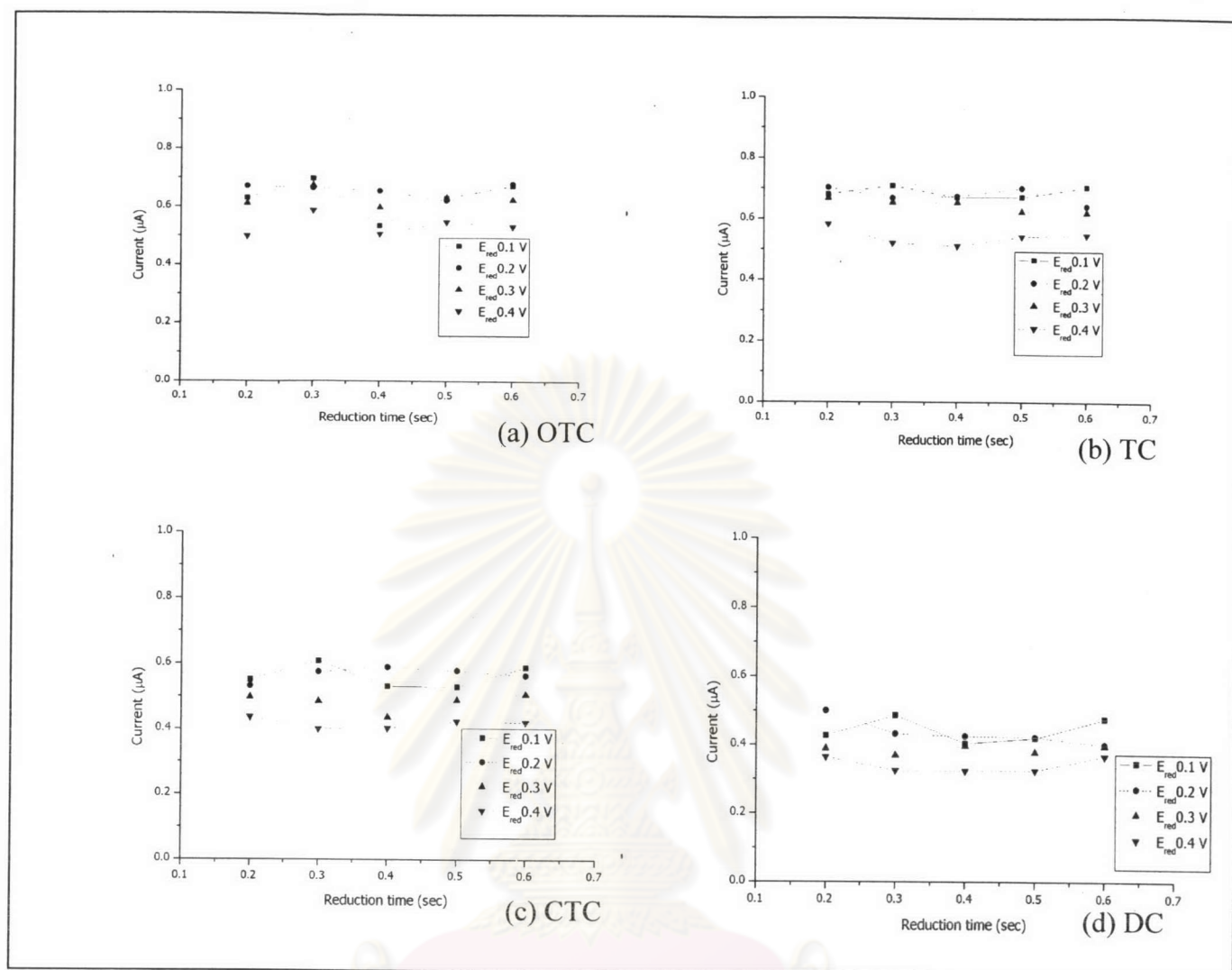


Figure 4.4 HPLC-PAD response as a function of E_{red} and t_{red} for 5 ppm of (a) oxytetracycline (OTC), (b) tetracycline (TC), (c) chlortetracycline (CTC) and (d) doxycycline (DC) at the Au electrode.

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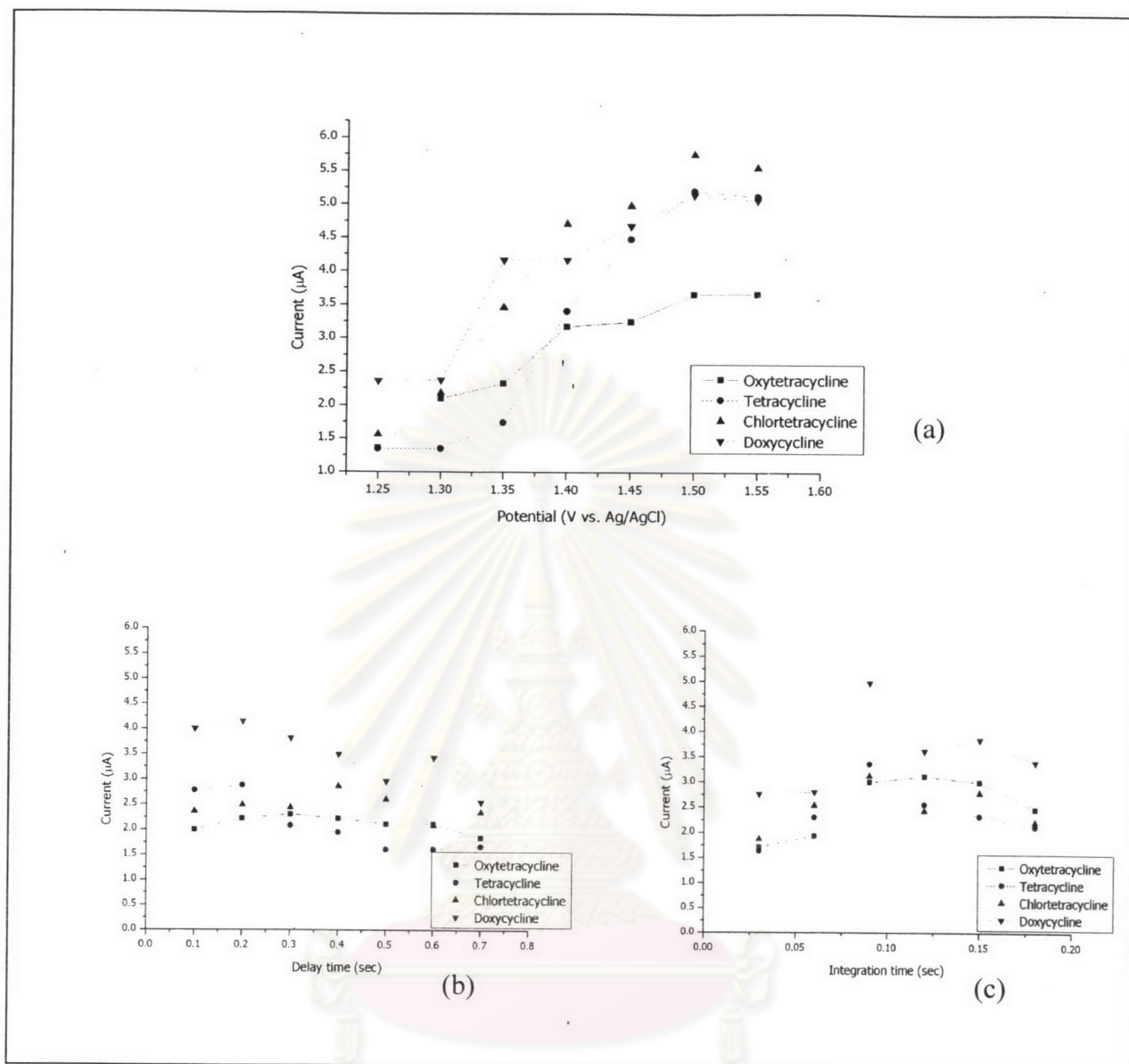


Figure 4.5 HPLC-PAD response as a function of (a) E_{det} , (b) t_{del} and (c) t_{int} for 5 ppm of oxytetracycline, tetracycline, chlortetracycline and doxycycline at the anodized BDD electrode.

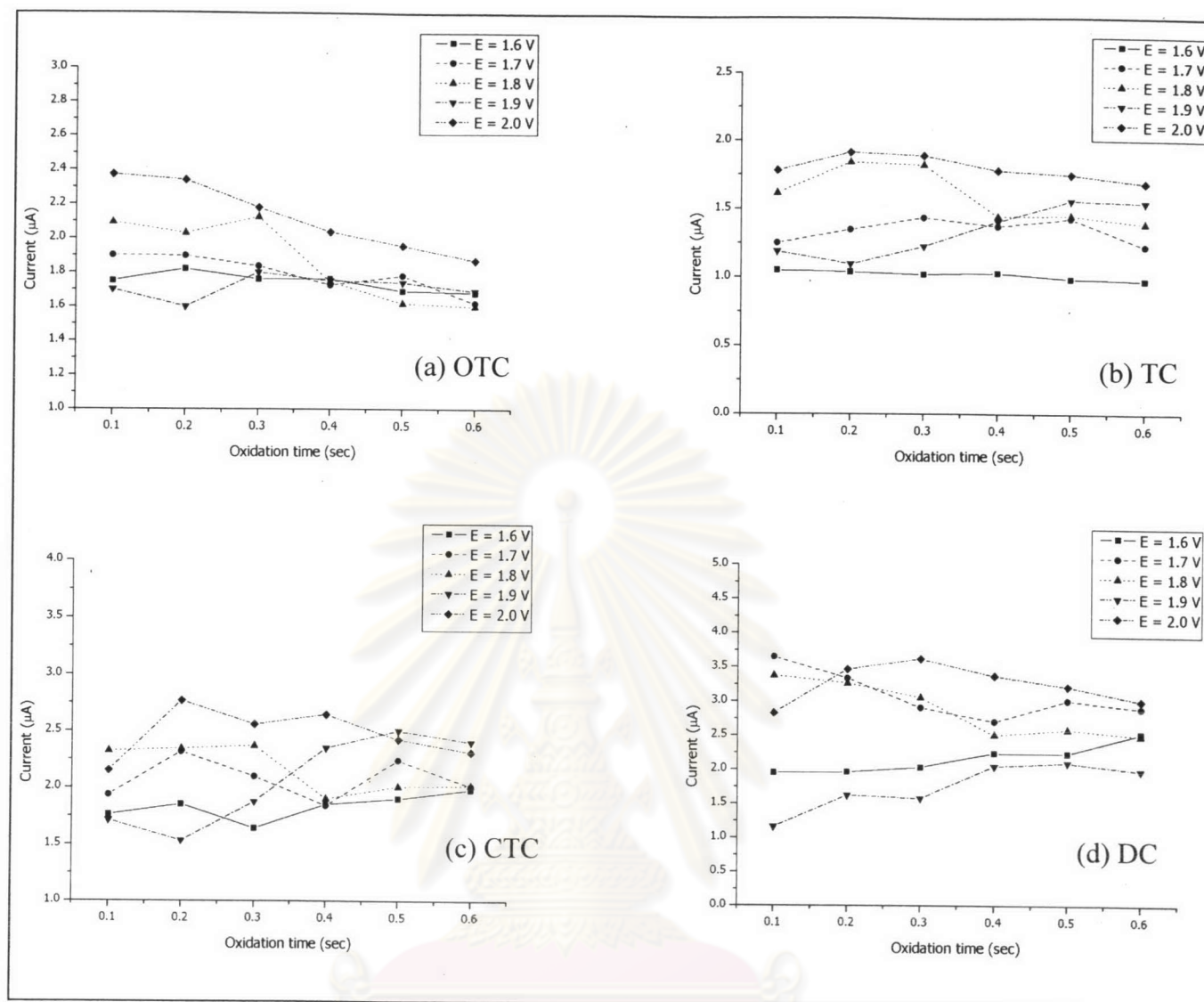


Figure 4.6 HPLC-PAD response as a function of E_{oxd} , and t_{oxd} for 5 ppm of (a) oxytetracycline (OTC), (b) tetracycline (TC), (c) chlortetracycline (CTC) and (d) doxycycline (DC) at the anodized BDD electrode.

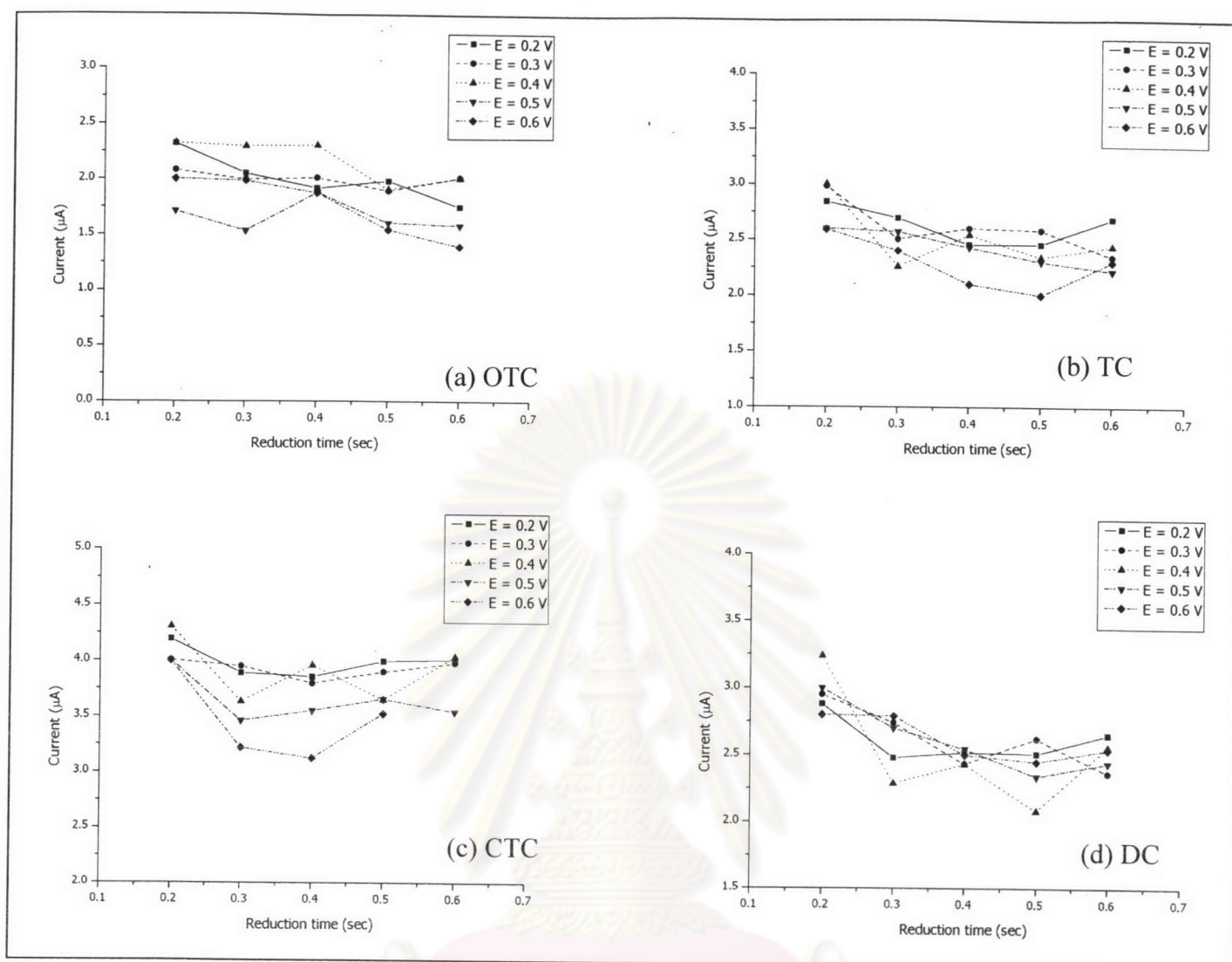


Figure 4.7 HPLC-PAD response as a function of E_{red} and t_{red} for 5 ppm of (a) oxytetracycline (OTC), (b) tetracycline (TC), (c) chlortetracycline (CTC) and (d) doxycycline (DC) at the anodized BDD electrode.

4.3 Optimal HPLC Conditions

The optimal HPLC conditions that could separate 4 tetracyclines were shown in Table 4.2. The chromatograms of mixed standard solutions and spiked sample were shown in Figure 4.8. The selectivity of HPLC conditions can be determined by retention time and resolution value of critical pairs (R_s) of each peak. From Table 4.3, all of interesting peaks could be separated and there is no interference of the analysis of tetracyclines in a shrimp sample.

For the detection of tetracyclines, the optimal PAD parameters and HPLC conditions were summarized in Table 4.1 and Table 4.2, respectively.

Table 4.2 The HPLC conditions for the detection of 4 tetracyclines

HPLC parameters	HPLC conditions
Stationary phase	GL Science Inertsil-3 C ₁₈ , 5 μ m, 4.6 mm x 25 cm
Mobile phase	Phosphate buffer (0.01M, pH 2.5) – acetonitrile (80:20, v/v)
Flow rate	1 mL/min
Injection volume	20 μ L
Temperature	Room temperature
Detector	Pulsed Amperometric Detection (PAD)

Table 4.3 Retention time and resolution of 4 tetracyclines under HPLC conditions in Table 4.2.

Compounds	Retention time (min)	Resolution
Oxytetracycline	5.6	-
Tetracycline	7.5	4.58
Chlortetracycline	17.5	10.19
Doxycycline	26.0	8.27

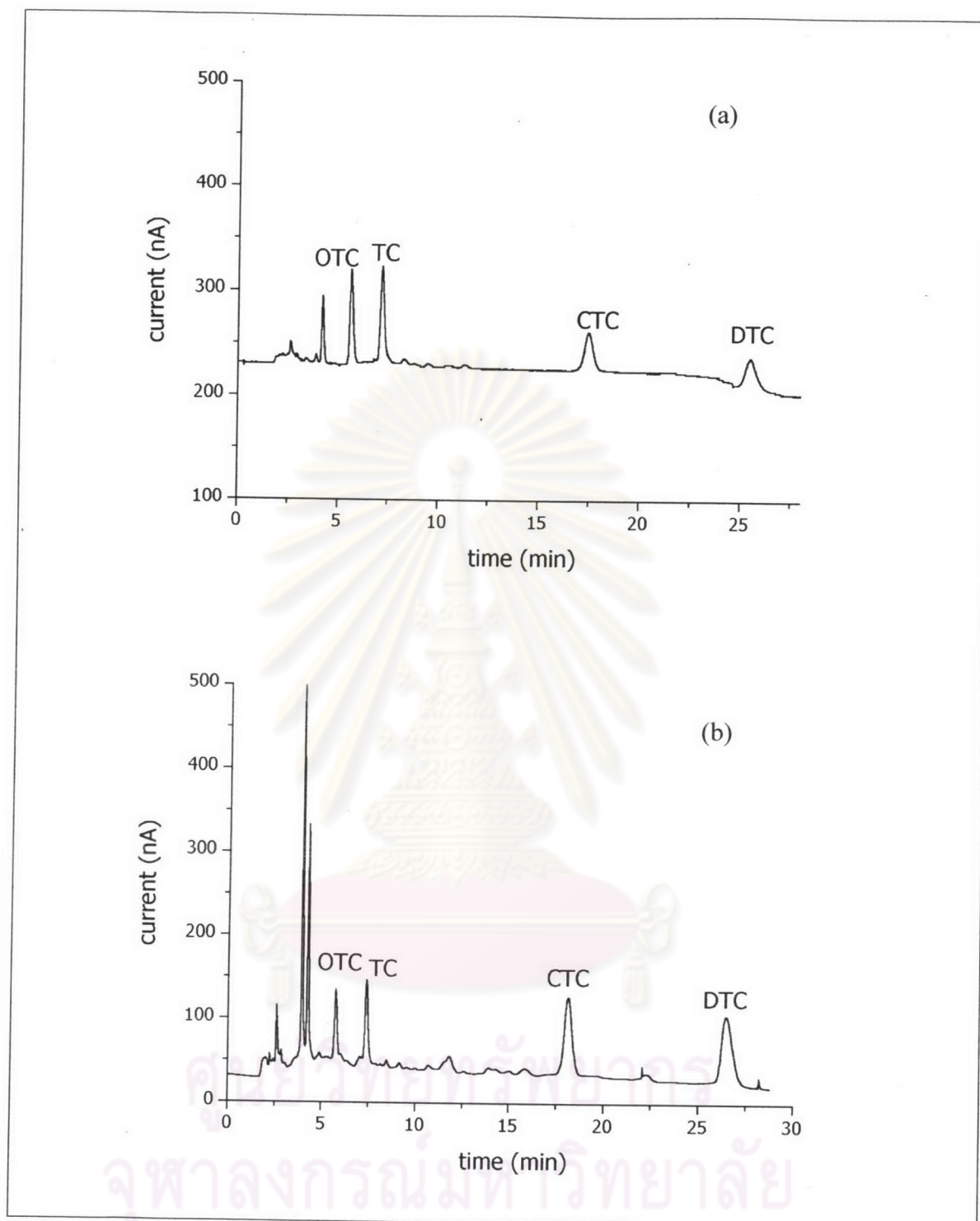


Figure 4.8 HPLC-PAD chromatograms of a standard mixture containing 10 $\mu\text{g/mL}$ concentrations of OTC, TC, CTC and DTC (a) and a shrimp sample spiked with 10 $\mu\text{g/mL}$ of OTC, TC, CTC and DTC (b).

4.4 Calibration and Linearity

The current responses of OTC, TC, CTC, and DC varied linearly with 9 standard concentrations, covering the range of 0.1 – 100 ppm. The 9 points of calibration curves when using the Au and anodized BDD electrode in HPLC-PAD system, shown in Figure 4.9 and 4.10, respectively, were plotted by peak areas versus concentrations. The calibration characteristics of OTC, TC, CTC, and DC at the Au electrode and anodized BDD electrode were shown in Table 4.4 and Table 4.5, respectively.

From the results, it was found that the current responses were observed using the anodized BDD electrode at the low concentration of TCs at 0.05 ppm, but the Au electrode failed to detect TCs at the concentration of 1.0 ppm. Using the anodized BDD in HPLC-PAD system, the linear concentration of TCs was obtained at 0.1 to 100 ppm, which was provided wider linear range than the one using the Au electrode.

Table 4.4 Calibration characteristics of oxytetracycline, tetracycline, chlortetracycline and doxycycline by the HPLC conditions and the optimal PAD waveform parameters in Table 4.1 at the Au electrode

Compounds	Slope (peak area units/ ppm)	Intercept	R ²
Oxytetracycline	17.52	30.18	0.9942
Tetracycline	11.14	30.58	0.9899
Chlortetracycline	6.90	31.45	0.9993
Doxycycline	9.76	39.89	0.9937

Table 4.5 Calibration characteristics of oxytetracycline, tetracycline, chlortetracycline and doxycycline by the HPLC conditions and the optimal PAD waveform parameters in Table 4.1 at the anodized BDD electrode

Compounds	Slope (peak area units/ ppm)	Intercept	R ²
Oxytetracycline	64.91	189.72	0.9940
Tetracycline	109.31	326.20	0.9960
Chlortetracycline	134.22	315.97	0.9931
Doxycycline	100.81	107.69	0.9972



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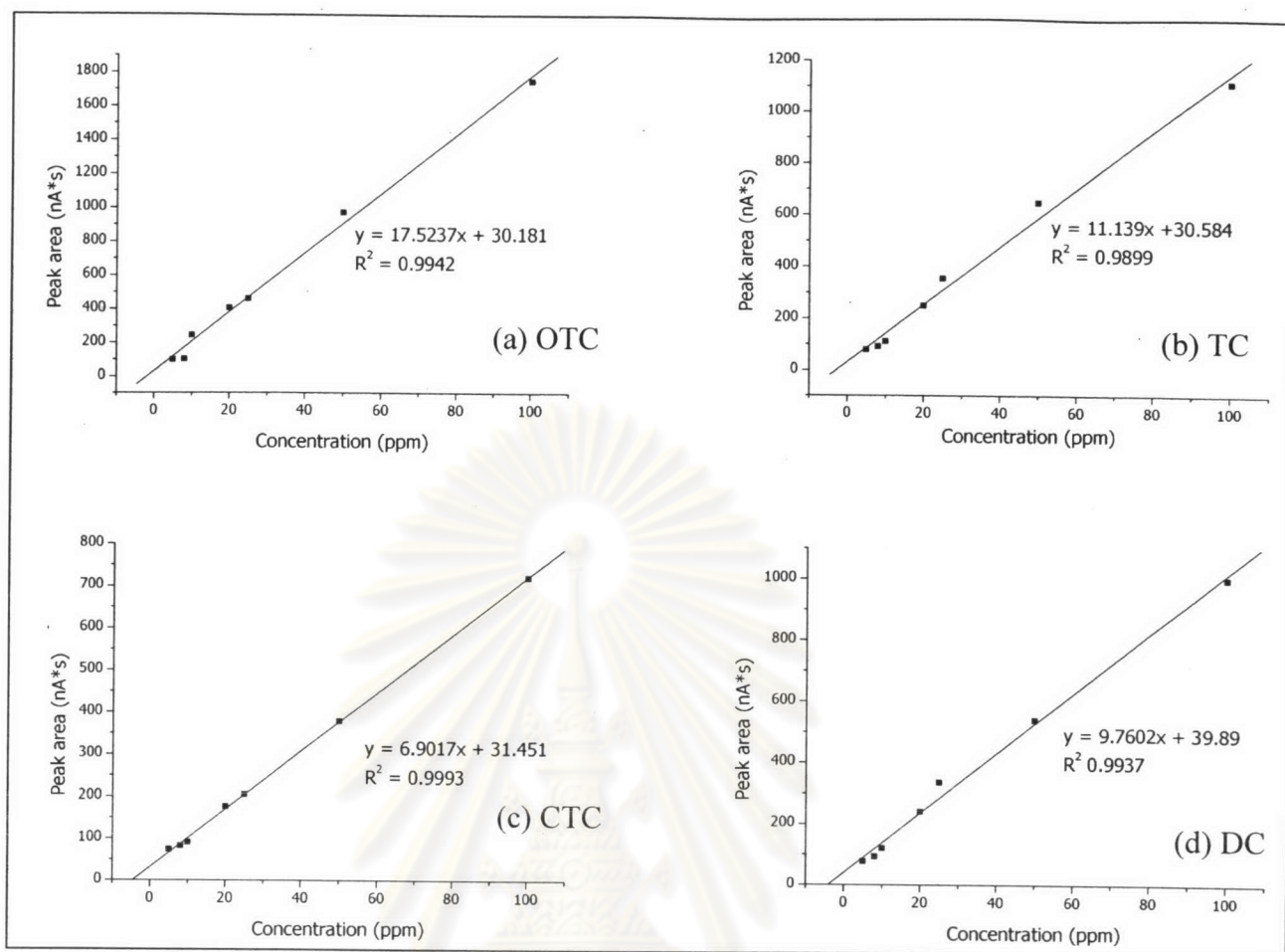


Figure 4.9 The calibration curve of mixed standard solutions by HPLC-PAD using the Au electrode (a) OTC, (b) TC, (c) CTC and (d) DC.

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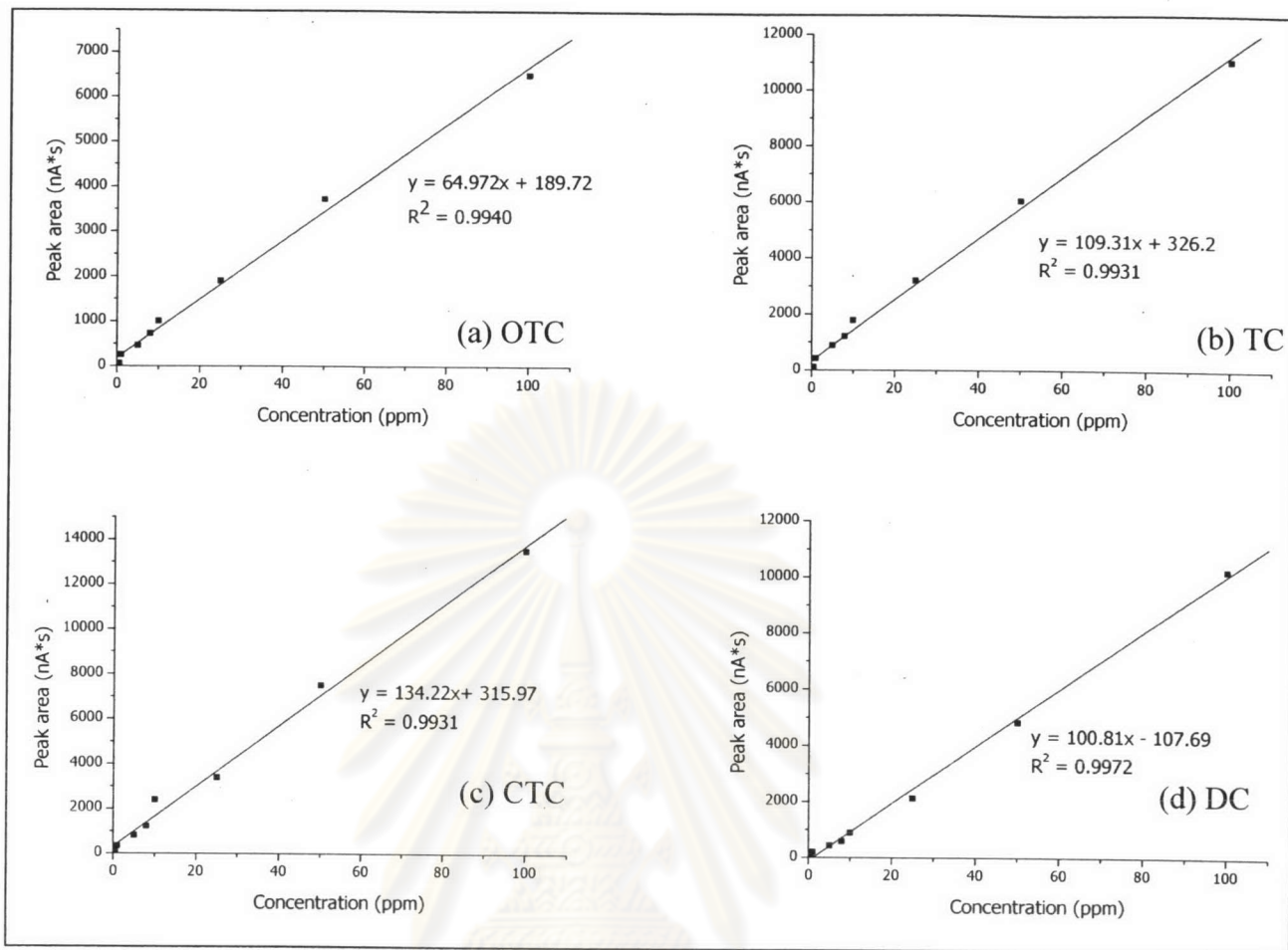


Figure 4.10 The calibration curve of mixed standard solutions by HPLC-PAD using the anodized BDD electrode (a) OTC, (b) TC, (c) CTC and (d) DC

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4.5 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) were investigated by examine various concentrations of standard TCs in matrix sample from 0.01-2.00 ppm. LOD was determined under the definition of 3 times of signal to noise ratio ($LOD = 3S/N$).

Table 4.6 summarized the limit of detection and limit of quantitation of OTC, TC, CTC, and DC at both Au and anodized BDD electrode. From the results, the anodized BDD electrode provided lower detection limit than the Au electrode. Therefore, the anodized BDD electrode was chosen to use in HPLC-PAD systems in the developed method.

Table 4.6 The limit of detection and limit of quantitation of OTC, TC, CTC and DC.

Compound	Au electrode		Anodized BDD electrode	
	LOD (ppm)	LOQ (ppm)	LOD (ppm)	LOQ (ppm)
Oxytetracycline	0.80	1.00	0.01	0.05
Tetracycline	0.80	1.00	0.01	0.05
Chlortetracycline	1.25	2.00	0.05	0.10
Doxycycline	1.25	2.00	0.05	0.10

4.6 Accuracy and Precision

The known concentrations of TCs spiked in shrimp sample at 0.5, 1.0, 5.0 and 10 mg/kg were used to study accuracy and precision of the developed method. For the intra-day precision, each of spiked samples was investigated in the one day. The results were shown in Table 4.7-4.9. For the inter-day precision, each of spiked samples was investigated for 3 days. The results were shown in Table 4.10. Table 4.11 summarized the values of recovery at spiking of 0.5, 1.0, 5.0 and 10 mg/kg.

The values of recovery were found in the range of 75.0 to 98.4% with RSD < 10%, indicating high accuracy and precision of the HPLC-PAD method, respectively.

Table 4.7 % Recovery of OTC, TC, CTC and DC at the spiking level 0.5, 1.0, 5.0 and 10 mg/kg in shrimp sample in the first day (n=3)

Compounds	% Recovery			Mean	SD	%RSD
	1	2	3			
Spiking 0.5 mg/kg						
OTC	72.5	74.3	75.5	74.1	1.5	2.0
TC	80.5	81.4	79.9	80.4	1.0	1.2
CTC	72.5	75.3	76.4	74.7	2.0	2.7
DC	80.5	74.9	81.5	79.0	3.6	4.5
Spiking 1 mg/kg						
OTC	79.4	80.3	81.4	80.4	1.0	1.2
TC	75.9	82.1	85.1	81.0	4.7	5.8
CTC	80.4	79.9	82.5	80.9	1.4	1.7
DC	78.9	89.3	85.4	84.5	5.3	6.2
Spiking 5 mg/kg						
OTC	85.9	90.7	92.4	89.7	3.4	3.8
TC	90.4	91.5	92.4	91.4	1.0	1.1
CTC	90.9	93.5	98.9	94.4	4.1	4.3
DC	92.1	93.1	95.5	93.6	1.7	1.9
Spiking 10 mg/kg						
OTC	92.5	100.7	97.5	96.5	4.1	4.3
TC	100.2	97.5	95.5	97.8	2.4	2.5
CTC	94.5	93.5	97.3	95.1	2.0	2.1
DC	99.7	96.5	95.5	97.2	2.2	2.3

Table 4.8 % Recovery of OTC, TC, CTC and DC at the spiking level of 0.5, 1.0, 5.0 and 10 mg/kg in shrimp sample in the second day (n=3)

Compounds	% Recovery			Mean	SD	%RSD
	1	2	3			
Spiking 0.5 mg/kg						
OTC	77.5	73.5	75.2	75.4	2.0	2.7
TC	75.1	82.7	84.3	80.7	4.9	6.1
CTC	72.1	73.4	75.5	73.7	1.7	2.3
DC	73.5	72.5	77.1	74.4	2.4	3.3
Spiking 1 mg/kg						
OTC	80.4	79.5	85.3	81.7	3.1	3.81
TC	89.9	90.5	82.5	87.6	4.5	5.1
CTC	84.4	79.1	80.2	81.2	2.8	3.4
DC	80.5	82.4	85.4	82.8	2.5	3.0
Spiking 5 mg/kg						
OTC	89.3	89.9	92.1	90.4	1.5	1.6
TC	91.5	92.5	93.5	92.5	1.0	1.1
CTC	94.3	92.5	89.5	92.1	2.4	2.6
DC	85.9	92.5	93.5	90.6	4.1	4.6
Spiking 10 mg/kg						
OTC	92.5	100.7	97.5	96.9	4.1	4.3
TC	100.2	97.5	95.5	97.8	2.4	2.5
CTC	94.5	93.5	97.3	95.1	2.0	2.1
DC	99.7	96.5	95.5	97.2	2.2	2.3

Table 4.9 % Recovery of OTC, TC, CTC and DC at the spiking level of 0.5, 1.0, 5.0 and 10 mg/kg in shrimp sample in the third day (n=3)

Compounds	% Recovery			Mean	SD	%RSD
	Day 1	Day 2	Day 3			
Spiking 0.5 mg/kg						
OTC	74.5	75.2	76.7	75.5	1.1	1.5
TC	80.5	76.4	77.4	78.1	2.1	2.7
CTC	82.5	84.1	72.3	79.6	6.4	8.0
DC	79.5	80.2	72.4	77.4	4.3	5.6
Spiking 1 mg/kg						
OTC	89.2	85.1	84.3	86.2	2.6	3.0
TC	85.3	82.1	89.5	85.6	3.7	4.3
CTC	90.1	85.4	87.3	87.6	2.4	2.7
DC	89.1	87.2	84.1	86.8	2.5	2.9
Spiking 5 mg/kg						
OTC	92.1	95.7	94.5	94.1	1.8	1.9
TC	90.9	93.2	94.1	92.7	1.7	1.8
CTC	92.4	93.5	99.5	95.1	3.8	4.0
DC	92.	93.1	90.5	92.0	1.4	1.5
Spiking 10 mg/kg						
OTC	104.5	99.5	97.3	100.4	3.7	3.7
TC	100.4	98.3	97.2	98.6	1.6	1.6
CTC	95.2	101.7	94.7	97.2	3.9	4.0
DC	95.9	97.1	80.5	94.2	4.1	4.3

Table 4.10 % Recovery of OTC, TC, CTC and DC at the spiking level of 0.5, 1.0, 5.0 and 10 mg/kg in shrimp sample for 3 days (inter-day precision, n=3)

Compounds	% Recovery			Mean	SD	%RSD
	Day 1	Day 2	Day 3			
Spiking 0.5 mg/kg						
OTC	74.1	75.4	75.5	75.0	2.9	1.2
TC	80.4	80.7	78.1	79.7	3.0	1.3
CTC	74.7	73.7	79.6	76.0	4.1	3.6
DC	79.0	74.4	77.4	76.9	3.7	2.9
Spiking 1 mg/kg						
OTC	80.4	81.7	86.2	82.8	3.4	4.1
TC	81.0	87.6	85.6	84.8	4.7	5.6
CTC	80.9	81.2	95.1	84.7	3.8	4.6
DC	84.5	82.8	92.0	84.7	1.4	4.3
Spiking 5 mg/kg						
OTC	89.7	90.4	94.1	91.4	2.9	3.2
TC	91.4	92.5	92.7	92.2	1.2	1.3
CTC	94.4	92.1	95.1	93.9	3.3	3.6
DC	93.6	90.6	92.0	90.1	2.7	2.9
Spiking 10 mg/kg						
OTC	97.9	96.9	100.4	98.4	3.6	3.6
TC	95.4	97.8	98.6	97.3	2.8	2.9
CTC	95.4	95.1	97.2	95.9	2.8	2.9
DC	96.8	97.2	94.2	96.1	3.1	3.3

Table 4.11 Accuracy of the developed method at the spiking level of 0.5, 1.0, 5.0 and 10 mg/kg

Compound	Mean of % Recovery			
	Spiking 0.5 mg/kg	Spiking 1.0 mg/kg	Spiking 5.0 mg/kg	Spiking 10 mg/kg
Oxytetracycline	75.0±1.5	82.8±3.4	91.4±2.9	98.4±3.6
Tetracycline	79.7±3.0	84.8±4.7	92.2±1.2	97.3±2.6
Chlortetracycline	76.0±4.4	83.3±3.8	93.9±3.3	95.9±2.8
Doxycycline	76.9±3.7	84.7±3.6	92.1±2.7	96.1±3.1

4.7 Applications

4.7.1 Comparison of Using the Au and Anodized BDD Electrode

Table 4.12 showed the results of the determination of tetracyclines in shrimps when using the Au and anodized BDD electrode in HPLC-PAD system. From the results, tetracycline and oxytetracycline could be determined by HPLC-PAD using the anodized BDD electrode, but tetracyclines, could not be detected by the Au electrode.

Table 4.12 The results of determination of TCs by the developed method using the Au and the anodized BDD electrodes

Compound	Concentration of TCs in shrimp (mg/kg)			
	Sample 1*		Sample 2*	
	Au	Anodized BDD	Au	Anodized BDD
Oxytetracycline	ND**	0.04	ND**	0.06
Tetracycline	ND**	0.06	ND**	ND**
Chlortetracycline	ND**	ND**	ND**	ND**
Doxycycline	ND**	ND**	ND**	ND**

* Farming-shrimp

** Not detectable

4.7.2 Comparison of Methods between the HPLC-PAD and the AOAC Official Method

The HPLC-PAD method for the detection of tetracyclines in shrimp was compared to the standard method, AOAC Official method. The AOAC Official method detects only three tetracyclines; tetracycline, oxytetracycline and chlortetracycline. For the AOAC method, HPLC conditions and sample preparation were carried out using the procedure as described in Section 3.12. Figure 4.11 and 4.12 showed HPLC chromatograms when using HPLC-PAD and AOAC method, respectively. From the chromatograms, HPLC-PAD method gave higher the current signal than the AOAC method.

From Table 4.13 and 4.14, the values of recovery at spiking of 0.5, 1.0, 5.0 and 10 mg/kg from AOAC method were less than HPLC-PAD method, due to the loss of TCs during the extraction of AOAC method prior to HPLC injection. The sample preparation procedures as described in Section 3.3.5 used in the developed method were more suitable to detect TCs in shrimp because it gave higher the recovery than the preparation of AOAC method. Moreover, using PAD at the anodized BDD in HPLC system provided greater sensitivity than the UV detector at 350 nm. Therefore, the developed method can be used for the determination of 4 tetracyclines in shrimps.

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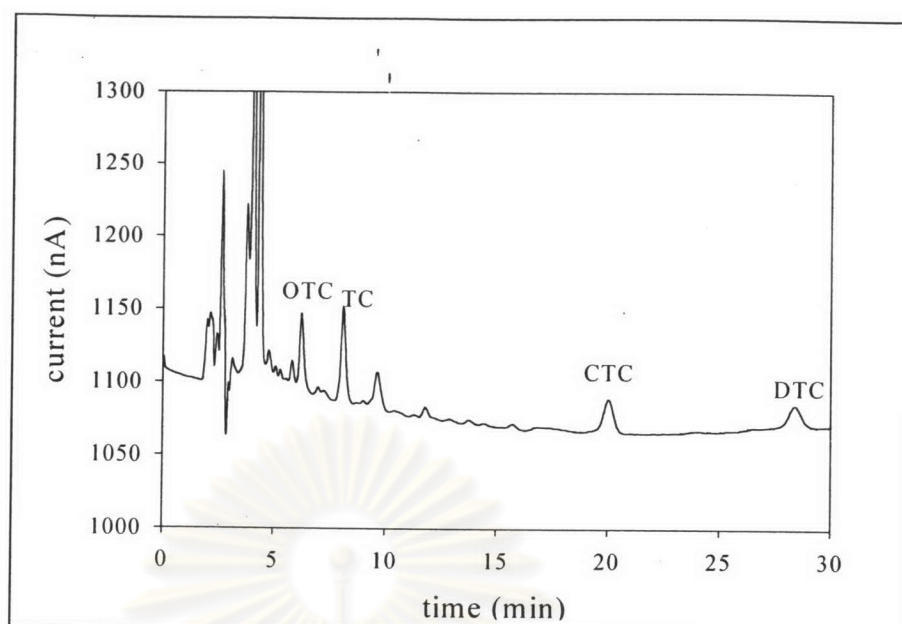


Figure 4.11 HPLC chromatogram of shrimp sample spiked with mixed standard 0.5 mg/kg using HPLC-PAD method.

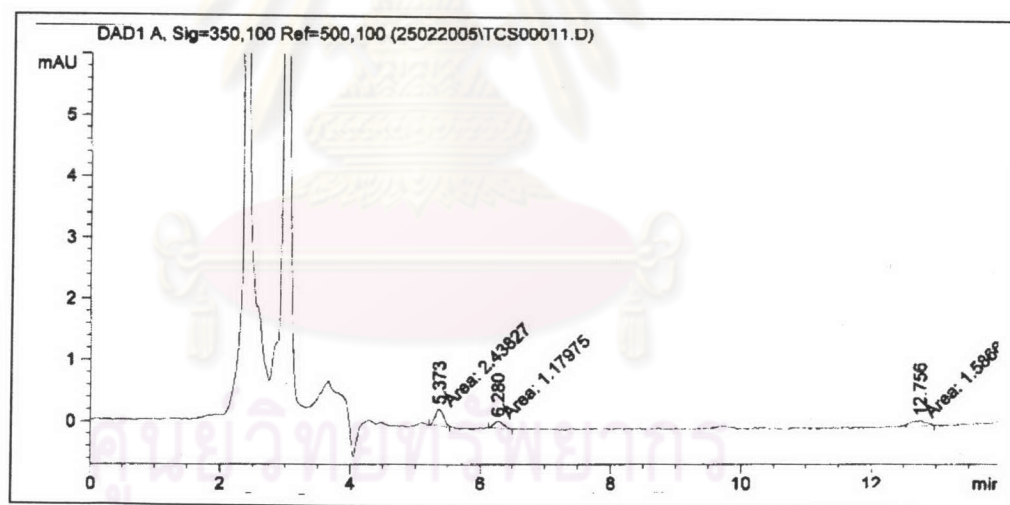


Figure 4.12 HPLC chromatogram of shrimp sample spiked with mixed standard 0.5 mg/kg using AOAC official method.

Table 4.13 Comparison of %recovery of spiked sample between the HPLC-PAD method and the AOAC Official method for shrimp^a

	% Recovery	
	Developed method	AOAC Official method*
Spiking at level 0.5 mg/kg		
Oxytetracycline	74.8±1.7	71.3±2.0
Tetracycline	80.6±3.2	73.1±4.3
Chlortetracycline	74.2±1.8	73.7±1.9
Doxycycline	76.7±3.7	-
Spiking at level 1.0 mg/kg		
Oxytetracycline	81.1±2.2	80.5±3.3
Tetracycline	84.3±5.5	78.5±4.0
Chlortetracycline	93.3±2.0	77.0±6.2
Doxycycline	92.1±3.8	-
Spiking at level 5.0 mg/kg		
Oxytetracycline	90.1±2.4	84.6±4.6
Tetracycline	92.0±1.1	83.9±3.7
Chlortetracycline	93.3±3.3	81.2±4.1
Doxycycline	92.1±3.3	-
Spiking at level 10 mg/kg		
Oxytetracycline	97.4±3.3	89.5±3.5
Tetracycline	96.6±2.8	91.2±3.6
Chlortetracycline	95.2±2.2	85.8±3.6
Doxycycline	97.0±2.4	-

^a sea-shrimp

* AOAC official method 995.09 (Chlortetracycline, Oxytetracycline and Tetracycline in edible animal tissues)

Table 4.14 Comparison of %recovery of spiked sample between the HPLC-PAD and the AOAC Official method for shrimp^a

	% Recovery	
	Developed method	AOAC Official method*
Spiking at level 0.5 mg/kg		
Oxytetracycline	76.3±2.4	76.2±4.0
Tetracycline	79.0±3.4	73.1±3.6
Chlortetracycline	78.3±4.8	71.8±2.4
Doxycycline	79.9±7.8	-
Spiking at level 1.0 mg/kg		
Oxytetracycline	89.3±3.8	81.0±3.8
Tetracycline	88.8±4.3	79.3±2.4
Chlortetracycline	88.4±2.4	75.4±2.5
Doxycycline	87.3±2.9	-
Spiking at level 5.0 mg/kg		
Oxytetracycline	95.3±2.8	83.7±4.3
Tetracycline	95.0±3.9	83.6±3.9
Chlortetracycline	91.8±5.1	81.1±2.1
Doxycycline	94.1±3.1	-
Spiking at level 10 mg/kg		
Oxytetracycline	100.2±2.6	90.7±5.5
Tetracycline	97.9±3.5	89.1±3.2
Chlortetracycline	96.2±4.5	90.1±3.2
Doxycycline	94.3±6.9	-

^a sea-shrimp

* AOAC official method 995.09 (Chlortetracycline, Oxytetracycline and Tetracycline in edible animal tissues)

4.7.3 Determination of TCs in commercial shrimps

Two kinds of shrimp samples; farming shrimp sample and sea shrimp sample, were used to detect TCs by the developed method, the AOAC Official method. Moreover, the determination of TCs in these samples was also detected by Laboratory Center for Food and Agricultural Products Company Limited (LCFA). The results are shown in Table 4.15 and 4.16. The results showed that OTC, TC and DC were not found in both samples, except CTC was found to be 0.07 mg/kg in sea shrimp by LCFA. TCs in both samples were not found when using HPLC-PAD and AOAC Official method.

Table 4.15 The comparison of TCs in the farming shrimp

Compound	Concentration of TCs in shrimp (mg/kg)		
	Developed method	AOAC**	LCFA***
Oxytetracycline	ND*	ND*	ND*
Tetracycline	ND*	ND*	ND*
Chlortetracycline	ND*	ND*	ND*
Doxycycline	ND*	-	-

Table 4.16 The comparison of TCs in the sea shrimp

Compound	Concentration of TCs in shrimp (mg/kg)		
	Developed method	AOAC**	LCFA***
Oxytetracycline	ND*	ND*	ND*
Tetracycline	ND*	ND*	ND*
Chlortetracycline	ND*	ND*	0.07
Doxycycline	ND*	-	-

* Not detectable

** AOAC official method 995.09 (Chlortetracycline, Oxytetracycline and Tetracycline in edible animal tissues)

*** Result of Laboratory Center for Food and Agricultural Product Co.,Ltd (LCFA)