As. J. Food Ag-Ind. **2012**, 5(01), 11-28

OPEN ACCESS

Asian Journal of Food and Agro-Industry

ISSN 1906-3040

Available online at www.ajofai.info

Research Paper

Fast scanning stripping square wave voltammetry analysis of erythromycin A in tilapia with a dropping mercury electrode

Nguyen Phuoc Minh

Department of Food Technology, Ho Chi Minh City University of Technology, 268 Ly Thuong Kiet Street, District 10, Ho Chi Minh City, Vietnam.

Email: stapimex@gmail.com

Abstract

A new sensitive analytical approach for determination of erythromycin A in tilapia (*Oreochromis niloticus)* by a fast scanning stripping square wave voltammetry using the dropping mercury electrode (PSA-F) was developed and validated. Optimum parameters for erythromycin A quantification were: Ammonium acetate buffer 0.1 M, pH 8.0 as supporting electrolyte; acetonitrile as the solvents for disolving erythromycin standard; forward scanning; V_{start}: -400 mV; V_{stop}: -1700 mv; V_{step}: 6 mV; V_{pulse}: 40 mV; T_{dop}: 5000 ms; $V_{electrolide}$: -1100 mV; $T_{electrolide}$: 5s. Peak of erythromycin A appeared at $E_{1/2}$ = -1430 mV, separated and distinguished with peaks of other antibiotics. This assay showed high recovery (> 85.07 %), high sensitivity (detection limit 0.52 µg/kg), high precision (RSD, 0.8 ÷ 2.1 %), high accuracy (relative error - RE, 85.07÷ 88.56 %) as well as excellent linearity ($r^2_{adjusted}$ = 1.0). Simpler, reagent-saving and time-saving were other advantages of this assay method. An equivalence of analyzing results between PSA-F and LC-MS/MS could be obviously seen.

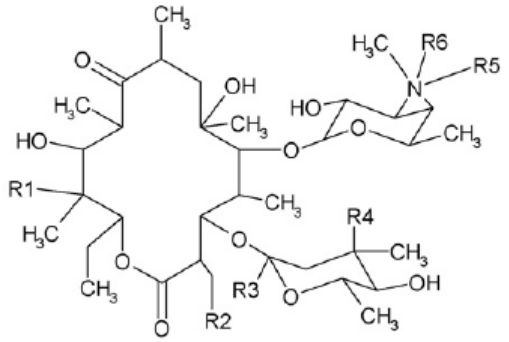
Keywords: Erythromycin A; tilapia; stripping square wave voltammetry; dropping mercury electrode, *Oreochromis niloticus*, Vietnam.

Introduction

Tilapias are known to have been an important component of fisheries in the Mekong River Delta. The most significant diseases in Nile tilapia (*Oreochromis niloticus)* culture are caused by *Streptococcus iniae, Aeromonas hydrophila*, *Trichodina* sp., *Flexibacter Columnaris, Edwardsiella* spp. *Streptococcocus* spp, gram positive bacteria, have become a major problem for tilapia farmers and there is still no effective commercial vaccine available that can be used to

prevent *Streptococcocus* spp. in tilapia. They can cause mass death in tilapia farms, and unlike many other tilapia diseases it will affect even large and otherwise healthy fish. The macrolide antibiotic erythromycin has long been the chemotherapeutant of choice to prevent and control *Streptococcocus* spp.

Erythromycins are broad spectrum antibiotics that exhibit high activity against nearly all Gram-positive and Gram-negative bacteria. Erythromycin A consists of a polyhydroxylactone and two sugars. The aglycone part of all erythromycin molecules, the erythronolide, is a 14-membered lactone ring (Figure 1). Depending on the type of erythromycin this lactone ring is substituted via 4-position with a cladinose in case of erythromycin A, erythromycin B, erythromycin E, erythromycin F and with a mycarose in case of erythromycin C and erythromycin D. All erythromycin molecules contain the aminosugar d-desosamine, which is β-glycosidic linked to the 6 position of the lactone ring.



Erythromycin	Formula	Molecular mass	R_1	R_2	R_3	R_4	R_5
A	$C_{37}H_{57}NO_{13}$	**734**	**OH**	**H**	**H**	OCH_3	CH_3
B	$C_{37}H_{57}NO_{12}$	**718**	**H**	**H**	**H**	OCH_3	CH_3
C	$C_{38}H_{55}NO_{13}$	**720**	**OH**	**H**	**H**	**OH**	CH_3
D	$C_{36}H_{65}NO_{12}$	**704**	**H**	**H**	**H**	**OH**	CH_3
E	$C_{37}H_{67}NO_{13}$	**748**	**OH**	**-O-**		OCH_3	CH_3
F	$C_{37}H_{67}NO_{14}$	**750**	**OH**	**OH**	**H**	CH_3	CH_3

Figure 1. Structural formula of erythromycin A and related substances.

The minimum inhibited concentration of erythromycins A, B, C, and D and some of their derivatives were determined against 21 gram-positive and 15 gram-negative microorganisms. Antibacterial activity was confined to gram-positive and very few gram-negative bacteria. Erythromycin B was somewhat less active than erythromycin A, and erythromycin C and D showed about half that activity or even less [11].

Owing to their extensive use in infectious disease therapy, several procedures have been reported for its determination (Table 1).

Table 1. Some typical papers published recently.

Year	Author	Sample	Method	LoD (µg/kg)
1994	Zierfels G [22]	Egg, muscle, milk, liver, kidney of swan	HPLC	<10
1998	Yong-Xi Li [21]	Human plasma	LC-MS/MS	LoQ 0.5
1999	Kondo T [13]	Human plasma	LC-MS/MS	LoQ 0.05
2000	Dreassi E [4]	Plasma: beef, pork, poultry	HPLC-UV	LoQ 250
		Milk	HPLC-UV	LoQ 25
		Kidney, liver, muscle, gan, fat of beef, pork, poultry.	HPLC-UV	LoQ 125
2000	Huaisheng Wang [9]	Drug, urine.	ASV-PGCE	5
2001	Carmen Leal [1]	Chicken	LC-FL	400
2001	R. Draisci [5]	Beef	ELISA	0.4
		Muscle and liver of beef	LC-MS/MS	LoQ 50
		Kidney of beef	LC-MS/MS	LoQ 80
2003	Stanley M. Billedeau [18]	Salmon	LC - ESI/MS	LoD: 5, LoQ: 16
2003	Horie Masakazu [7]	Meat and seafood	LC- ESI-MS	10
2003	Michael P. Sche [15]	Manure	HPLC-MS/MS	0.4-11
2005	W. Xiao [20]	Drugs (propionate, base)	HPLC–ESI-MS	1
2006	A. Deube [2]	Muscle	LC-MS/MS	0.25
2006	Hui Yun – Hua [10]	Tilapia	HPLC	400
2006	Jian Wang [12]	Fresh milk	LC-ESI/MS/MS	0.07
2006	Tang HP [19]	Meat	LC-MS/MS	0.1
2007	Deng B [3]	Rat plasma	ECL	0.35
2008	Berrada Houda [8]	Meat and seafood	LC-ESI/MS	25
2009	Granja R [6]	Honey	LC-MS/MS	LoD 1.27, LoQ 5.0
2009	P. Norouzi [17]	Human plasma, urine.	CV	LoD 2.4, LoQ 7.0

The aim of this work was to determine the feasibility of square wave voltammetry for the direct detection and quantification of erythromycin A in tilapia tissue.

Materials and Methods

Reagents and materials

All chemicals and reagents were HPLC grade or p.a. Double-distilled water (DDW) was used throughout the study. The high purity antibiotic standards (>99%) of erythromycin A, erythromycin A, chloramphenicol, furazolidone, florfenicol, ciprofloxacin, colistin, malachite green were purchased from Vietnam Central Institute of Pharmacy.

Apparatus
A fast scanning stripping square wave voltammetry at the slowly dropping mercury electrode was performed in the ANALYZER SQF-505. The mercury dropping electrode was used as a working electrode, silver/silver chloride (saturated KCl) as a reference electrode and a platinum wire as an auxiliary one.

Sample extraction and clean-up procedure
❖ *Primary extraction:*
A 5 g aliquot of a blank or spiked minced tissue sample was mixed with a small volume of erythromycin standard. After a 15-min equilibration period, the tissues were mixed vigorously for 15 min with 25 ml Tris buffer (0.1M; pH 10.5). After a 10-min centrifugation at 3000 g and 4°C, the supernatant was transferred to a polypropylene tube and the solid residue extracted a second time with 25 ml Tris buffer.

Acetic acid (600 µl) and 5 ml sodium tungstate buffer (0.15M) were added to precipitate the proteins. After equilibration for one hour at 4°C, the samples were centrifuged at 3000 g for 10 min. The supernatants were further filtered through a plug of glass wool.

❖ *Solid phase extraction:*
The 6-cm3 HLB OASIS extraction cartridges (200 mg) were prepared and conditioned with 10 ml methanol and 10 ml water. The biological samples were placed at the top of the column. Two wash solution volumes were applied before erythromycin elution: 20 ml methanol-water (5:95, v/v) and 5 ml hexane.

After the last washing step, the OASIS columns were vacuum-dried for 10 min. Erythromycin was finally eluted with 5 ml methanol-ammonia 30 % (95:5, v/v) and evaporated dry under a nitrogen flow. The extracts were dissolved in 500 µl NH_4AC-ACN (80/20 v/v), transferred to Eppendorf tubes and centrifuged at 3000 g for 10 min. Aliquots of the supernatant were transferred into the voltammetric cell with 2,500 mL of ammonium acetate 0.1 M, pH 8.0 before being quantified by Analyzer SQF-505 machine in mode stripping square wave voltammetry.

Results and Discussion

Voltammetric behavior of erythromycin at the slowly dropping electrode
✓ **Effect of Supporting Electrolytes and pH values.**
The adsorptive peak current of erythromycin has been strongly affected by the type of supporting electrolyte. To study the adsorptive behavior of erythromycin, different supporting electrolytes including sodium acetate, ammonium acetate, citrate-phosphate, borax, Tris buffers were examined. Ammonium acetate buffer was recommended to complete these studies where erythromycin showed the highest peak current and the best peak shape (Table 2 and Figure 2).

The effect of pH of ammonium acetate buffer on the peak current was examined from 7.0 to 10.0. Erythromycin showed highest peak current at pH 8.0 ($E_{1/2}$ = -1438 mV, I = 351.7 ± 5.7 nA). Hence ammonium acetate buffer (pH 8.0) was selected for further investigations.

Table 2. Peak current of erythromycin A was affected by supporting electrolytes, pH values.

pH	5.0		6.0		7.0		8.0		9.0		10.0	
Supporting electrolytes	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Natri acetate	56.67^{b} ± 1.1	1.9	35.5^{a} ± 2.3	6.6	255.5^{d} ± 7.3	2.8	333.4^{e} ± 4.7	1.4	236.2^{c} ± 7.7	3.3	254^{d} ± 4.7	1.9
Ammonium acetate					210.3^{a} ± 5.4	2.6	**351.7^{d} ± 5.7**	1.6	216.5^{b} ± 2.7	1.2	263.1^{c} ± 1.6	0.6
Citrat-phosphate			207.7^{d} ± 6.0	2.9	168.2^{a} ± 2.3	1.4	194.4^{b} ± 2.6	1.4	229.4^{e} ± 2.1	0.9	200.9^{c} ± 2.0	1.0
Tris					180.0^{d} ±13.1	7.3	27.5^{a} ± 0.3	1.1	53.4^{b} ± 3.6	6.7	126.4^{c} ± 9.7	7.7
Borax							168.1^{a} ± 1.6	0.9	255.5^{c} ± 6.5	2.6	173.9^{b} ± 1.8	1.0

** Each value was the mean of 5 samples (n = 5)*

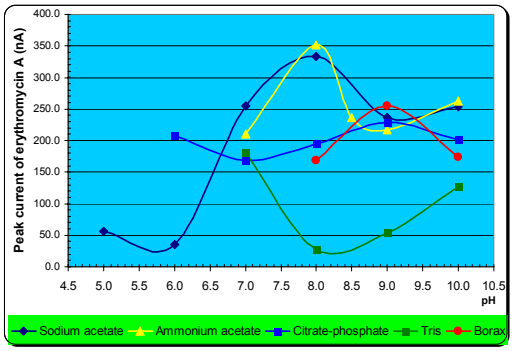


Figure 2. **Effect of supporting electrolytes, pH values on peak current of erythromycin A.**

✓ **The effect of the ionic strength of supporting electrolyte** was examined at pH 8.0 over the range from 0.05÷0.25 M. Erythromycin showed highest peak current at ammonium acetate 0.1M ($E_{1/2}$ = -1438 mV, I = 254.8 ± 10.2 nA). So this value was selected for further studies (Table 3 and Figure 3).

Table 3. Erythromycin A peak current was affected by ionic strength of ammonium acetate.

Ionic strength (M)	0.05		0.1		0.15		0.2		0.25	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
	210^{c} ± 7.1	3.4	**254.8^{d} ± 10.2**	4.0	213.7^{c} ± 10.1	4.7	192.1^{b} ± 2.2	1.1	173.2^{a} ± 1.8	1.0

** Each value was the mean of 5 samples (n = 5)*

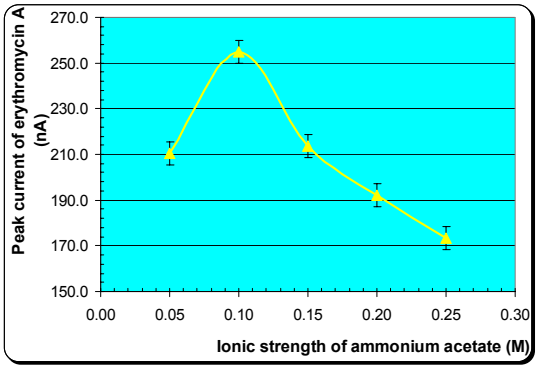


Figure 3. Effect of ionic strength of ammonium acetate on peak current of erythromycin A.

✓ **Effect of the solvents** for disolving erythromycin A standard on the peak current were examined. Among methanol, acetonitril, ethyl acetate, the peak current increased with a maximum at acetonitril ($E_{1/2}$ = -1438 mV, I = 189.2 ± 3.5 nA). So acetonitril was selected for subsequent work (Table 4 and Figure 4).

Table 4. Peak current of erythromycin A was affected by solvents.

Ethyl acetate		Acetonitril		Methanol	
Mean± SD	RSD (%)	Mean± SD	RSD (%)	Mean ± SD	RSD (%)
183.6^{b} ± 1.5	0.8	**189.2^{c} ± 3.5**	1.9	166.3^{a} ± 5.5	3.3

** Each value was the mean of 5 samples (n = 5)*

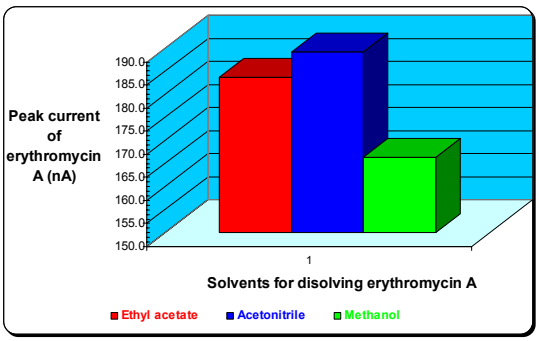


Figure 4. Effect of solvents for dissolving erythromycin A on peak current.

Optimization of measurement conditions

✓ **Effect of forward scanning (0 to -1800 mV) and reverse scanning (-1800mV to 0)** on the peak current signal was examined. The forward scanning **(0 to -1800 mV)** showed high peak. Meanwhile, the peak current of the reverse scanning (-1800mV to 0) was too low. So the forward scanning **(0 to -1800 mV)** was chosen for further investigations.

✓ **Effect of V_{start}**. Mode PSA-F, forward scanning, V_{stop}: -1800 mV, V_{step}: 4 mV, V_{pulse}: 30mV, T_{drop}: 3000ms, $V_{electrolise}$: -700mV, $T_{electrolise}$: 6s, $T_{stabilize}$: 1s. Examining V_{start} from -400 mV to -1100 mV. V_{start} was optimum at **-400mV** ($E_{1/2}$ = -1430 mV, I = **106.5 ± 4.7** nA) (Table 5 and Figure 5).

Table 5. Peak current of erythromycin A was affected by V_{start}

V_{start} (mV)	-400	-500	-600	-700	-800	-900	-1000	-1100
Mean ± SD	**106.5^{g} ± 4.7**	97^{f} ± 3.4	79.5^{e} ± 0.8	71.5^{d} ± 4.3	93.3^{f} ± 4.0	45.0^{c} ± 2.6	23.4^{b} ± 1.0	14.8^{a} ± 0.9
RSD (%)	4.4	3.5	1.0	6.0	4.3	5.8	4.3	6.2

** Each value was the mean of 5 samples (n = 5)*

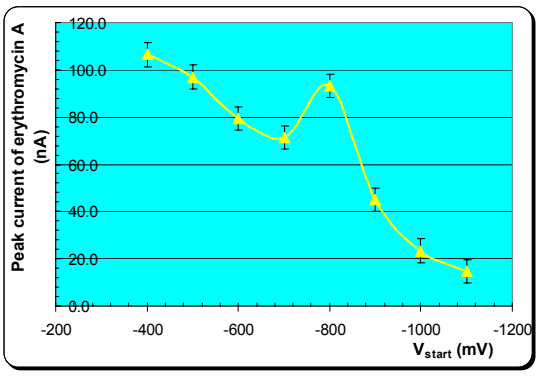


Figure 5. Effect of V_{start} on erythromycin A peak current.

✓ **Effect of V_{stop}.** Mode PSA-F, forward scanning, V_{start}: -400 mV, V_{step}: 4 mV, V_{pulse}: 30 mV, T_{drop}: 3000 ms, $V_{electrolise}$: -700 mV, $T_{electrolise}$: 6 s, $T_{stabilize}$: 1 s. Examining V_{stop} from -1700 mV to -2000 mV. V_{start} was optimum at **-1700 mV** ($E_{1/2}$ = -1430 mV, I = **136.7 ± 3.9** nA) (Table 6 and Figure 6).

Table 6. Peak current of erythromycin A was affected by V_{stop}

V_{stop} (mV)	-1500.0	-1600.0	-1700.0	-1800.0
Mean ± SD	104^{a} ± 1.0	118.2^{b} ± 1.4	**136.7^{c} ± 3.9**	120.1^{b} ± 1.1
RSD (%)	0.9	1.2	2.9	0.9

** Each value was the mean of 5 samples (n = 5)*

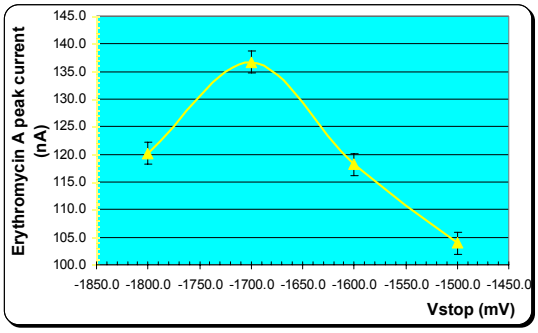


Figure 6. Effect of V_{stop} on erythromycin A peak current.

✓ **Effect of V_{step}.** Mode PSA-F, forward scanning, $V_{start:}$ -400 mV, V_{stop}: -1700 mV, V_{pulse}: 30 mV, $T_{drop:}$ 3000 ms, $V_{electrolise}$: -700 mV, $T_{electrolise}$: 6 s, $T_{stabilize}$: 1 s. Examining V_{step} from 4 mV to 10 mV. **V_{step}** was optimum at **6.0 mV** ($E_{1/2}$ = -1430 mV, I = 214.6 ± 13.1 nA) (Table 7 and Figure 7).

Table 7. Peak current of erythromycin A was affected by V_{step}

V_{step} (mV)	4.0	6.0	8.0	10.0
Mean ± SD	162.8^{a} ± 5.8	**214.6^{c} ± 13.1**	176.9^{b} ± 8.3	165.0^{ab} ± 10.9
RSD (%)	3.5	6.1	4.7	6.6

** Each value was the mean of 5 samples (n = 5)*

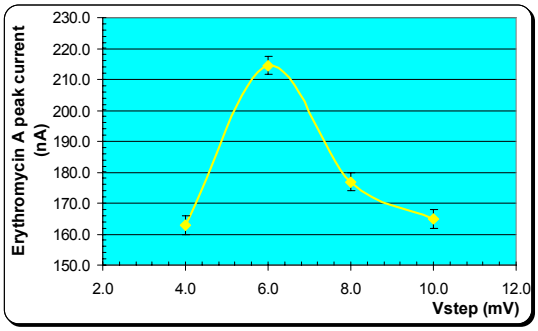


Figure 7. Effect of V_{step} on erythromycin A peak current.

✓ **Effect of V_{pulse}.** Mode PSA-F, forward scanning, V_{start}: -400 mV, V_{stop}: -1700 mV, V_{step}: 6.0 mV, T_{drop}: 3000 ms, $V_{electrolise}$: -700 mV, $T_{electrolise}$: 6 s, $T_{stabilize}$: 1 s. Examining V_{pulse} from 10 mV to 40mV. **V_{pulse}** was optimum at **40 mV** ($E_{1/2}$ = -1430 mV, I = 692.6 ± 14.9 nA) (Table 8 and Figure 8).

Table 8. Peak current of erythromycin A was affected by V_{pulse}

V_{pulse} (mV)	10	20	30	40
Mean ± SD	230.6^{a} ± 2.7	388.4^{b} ± 12.7	528.0^{c} ± 8.0	**692.6^{d} ± 14.9**
RSD (%)	1.2	3.3	1.5	2.2

** Each value was the mean of 5 samples (n = 5)*

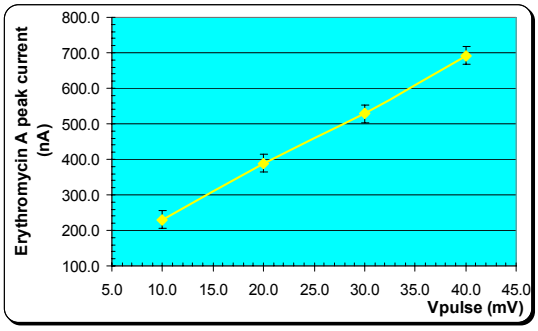


Figure 8. Effect of V_{pulse} on erythromycin A peak current.

✓ **Effect of T_{drop}** Mode PSA-F, forward scanning, V_{start}: -400 mV, V_{stop}: -1700 mV, V_{step}: 6 mV, V_{pulse}: 40 mV, $V_{electrolise}$: -700 mV, $T_{electrolise}$: 6 s, $T_{stabilize}$: 1 s. Examining T_{drop} from 1000 ms to 5,000 ms. **T_{drop}** was optimum at **5,000 ms** ($E_{1/2}$ = -1430 mV, I = 381.3 ± 2.9 nA) (Table 9 and Figure 9).

Table 9. Peak current of erythromycin A was affected by T_{drop}

T_{drop} (ms)	1,000	2,000	3,000	4,000	5,000
Mean ± SD	128.3[a] ± 1.2	197.9[b] ±1.9	269.1[c] ± 12.9	323.2[d] ±3..3	**381.3[e] ± 2.9**
RSD (%)	0.9	1.0	4.8	1.0	0.8

** Each value was the mean of 5 samples (n = 5)*

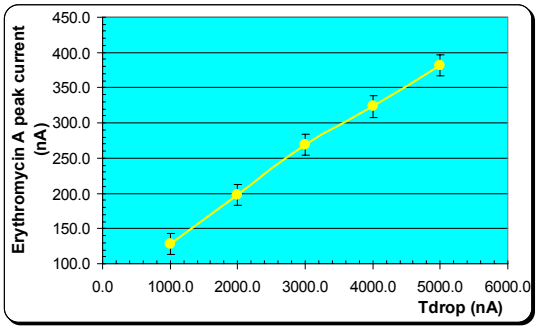


Figure 9. Effect of T_{drop} on erythromycin A peak current.

✓ **Effect of $T_{electrolise}$** Mode PSA-F, forward scanning, V_{start}: -400 mV, V_{stop}: -1700 mV, V_{step}: 6 mV, V_{pulse}: 40 mV, T_{drop}: 5,000 ms, $V_{electrolise}$: -700 mV, $T_{stabilize}$: 1 s. Examining $T_{electrolise}$ from 3s to 6s. **$T_{electrolise}$** was optimum at **5s** ($E_{1/2}$ = -1430 mV, I = 1717.0 ±13.7 nA) (Table 10 and Figure 10).

Table 10. Peak current of erythromycin A was affected by $T_{electrolise}$

$T_{electrolise}$ (s)	3	4	5	6
Mean ± SD	1353.6[a]± 10.8	1555.4[b] ±13.0	**1717.0[d] ±13.7**	1655.4[c] ±5.0
RSD (%)	0.8	0.8	0.8	0.3

** Each value was the mean of 5 samples (n = 5)*

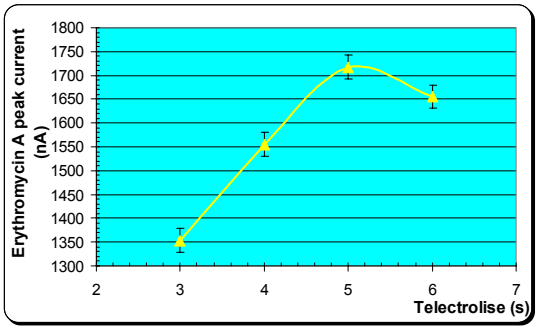


Figure 10. **Effect of $T_{electrolise}$ on erythromycin A peak current.**

✓ **Effect of $V_{electrolise}$** Mode PSA-F, forward scanning, V_{start}: -400 mV, V_{stop}: -1700 mV, V_{step}: 6 mV, V_{pulse}: 40 mV, T_{drop}: 5,000 ms, $T_{electrolise}$: 5 s, $T_{stabilize}$: 1 s. Examining $V_{electrolise}$ from -400 mV to -1400 mV. $V_{electrolise}$ was optimum at **-1100 mV** ($E_{1/2}$ = -1438 mV, I = 1863.2 ± 24.1 nA) (Table 11 and Figure 11).

Table 11. Peak current of erythromycin A was affected by $V_{electrolise}$

$V_{electrolise}$ (Mv)	-400.0	-900.0	-1100.0	-1400.0
Mean ± SD	1709.0^{b}± 17.3	1815.0^{c} ±3.7	1863.2^{c} ± 24.1	1593.4^{a} ±13.5
RSD (%)	1.0	0.2	1.3	0.8

** Each value was the mean of 5 samples (n = 5)*

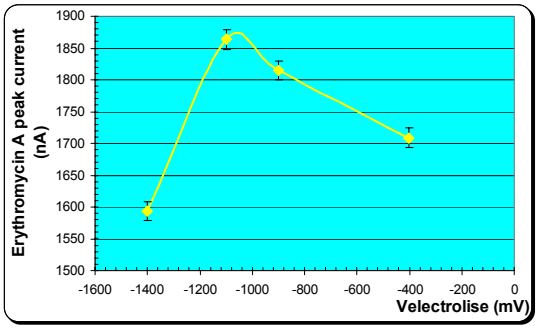


Figure 11. **Effect of $V_{electrolise}$ on erythromycin A peak current.**

Calibration

✓ **Calibration curves and detection Limit:** A 25 mL supporting electrolyte ammonium acetate 0.1M, pH 8.0 was transferred to the cell and spiked with 5 µL, 10 µL, 20 µL, 30 µL, 40 µL, 50 µL, 60 µL, 70 µL, 80 µL, 90 µL of stock 250 ppm solution of erythromycin in pure acetonitril. The concentrations of erythromycin in the cell were 50 µg/kg, 100 µg/kg, 200 µg/kg, 300 µg/kg, 400 µg/kg, 500 µg/kg, 600 µg/kg, 700 µg/kg, 800 µg/kg, 900 µg/kg respectively. Mode PSA-F, V_{start}: -400mV, V_{stop}: -1700 mV, V_{step}: 6 mV, V_{pulse}: 40 mV, T_{drop}: 5000 ms, $T_{electrolise}$: 5s, $V_{electrolise}$: - 1100 mV, $T_{stabilize}$: 1s.

A detection limit of 0.57 µg/kg was obtained for erythromycin. A linear behaviour was also observed with a correlation coefficient r^2_{adjust} = 1.0 (Figure 12).

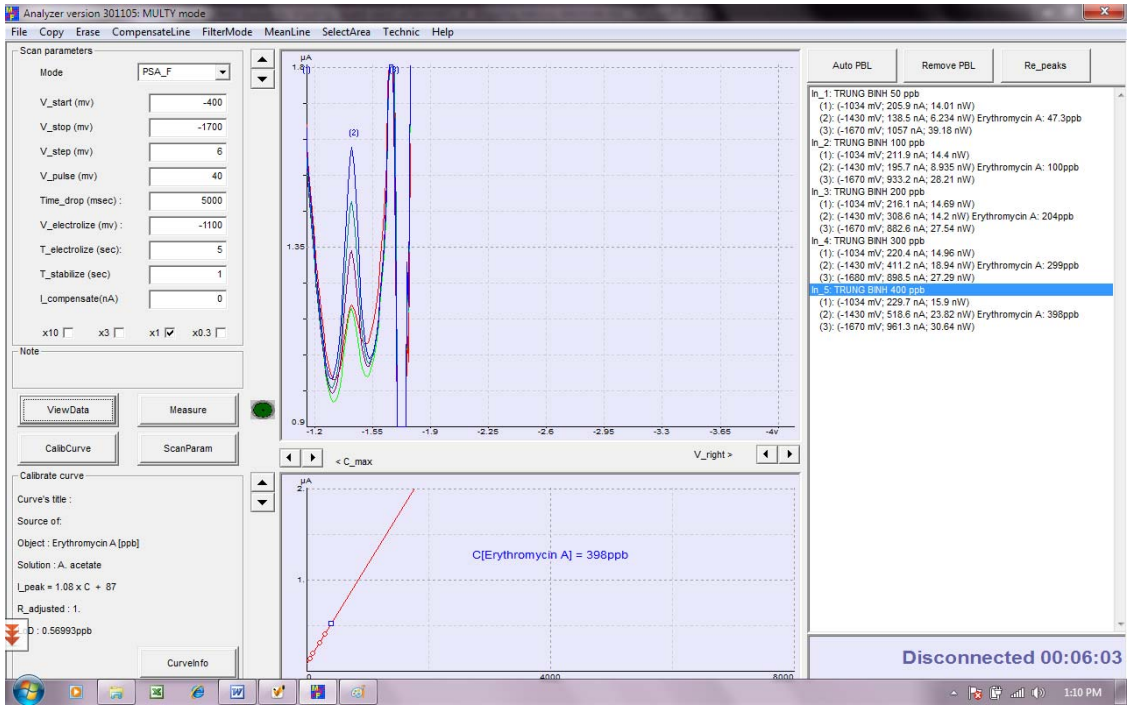


Figure 12. Calibration curve of erythromycin A.

Interference

✓ Effect of Interferences. The effect of co-existing ions was examined by introducing different concentrations of some ions to the voltametric cell and recording the corresponding voltammogram using the conditions selected above. It was observed that the additions of 0÷5ppm K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Cl^-, SO_4^{2-}, HPO_4^{2-} ions have no effect (< ±5%) on the peak response.

Sample analysis

✓ In tilapia samples:

Recovery rate: 85.07 ÷ 88.56%, MDL: 0.52 µg.kg^{-1}, R_adjust: 1.0, RSD: 0.80 ÷ 2.10 %. (Fig. 13).

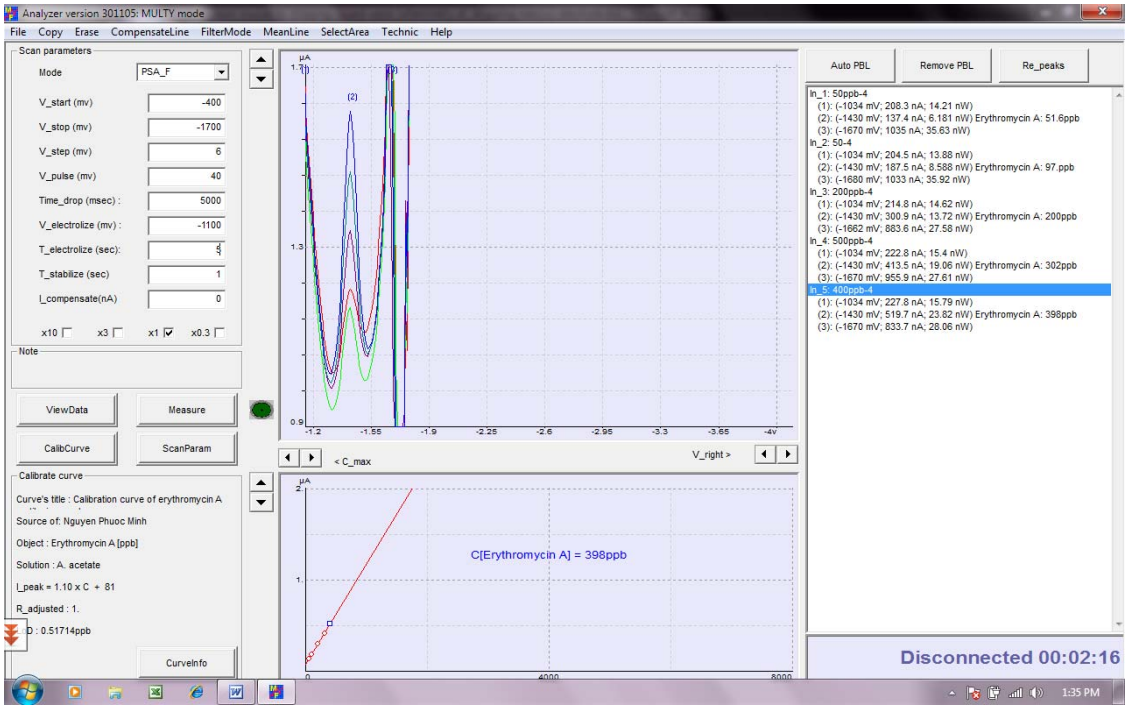


Figure 13. Calibration curve of erythromycin A in tilapia sample.

✓ Comparison results between SWV and LC-MS/MS method

Incurred tilapia samples were obtained through medication at 100 mg erythromycin·kg^{-1} fish body weight^{-1}·d^{-1} for 7 days; sampled at 7, 8 and 9 days post-dosing. These incurred samples (high, medium, and low) were divided in two groups: samples in group A were analyzed by Square Wave Voltammetry, samples in group B were controlled by LC-MS/MS via Intertek Vietnam Ltd. A closely homogeneity could be obviously seen in comparison between two analyzing methods. SWV seems to be superior and more accurate to LC-MS/MS (Table 12)

Table 12. Comparison of homogeneity of two analytical methods.

Sample I.D	SWV (LOD = 0.52 µg*kg^{-1})			LC-MS/MS (LOD = 10 µg*kg^{-1})		
	Result (mg*kg^{-1})	Mean ± SD (mg*kg^{-1})	R.S.D (%)	Result (mg*kg^{-1})	Mean ± SD (mg*kg^{-1})	R.S.D (%)
TL – Blank	N.D	N.A	N.A	N.D	N.A	N.A
TL – Blank	N.D			N.D		
TL – Blank	N.D			N.D		
TL – I	1.31	1.29^{a} ± 0.02	1.61	1.23	1.26^{a} ± 0.03	2.10
TL – I	1.27			1.28		
TL – I	1.30			1.27		
TL – II	1.95	2.02^{b} ± 0.07	3.47	3.14	2.72^{b} ± 0.43	15.64
TL – II	2.09			2.72		
TL – II	2.02			2.29		
TL – III	2.80	2.78^{c} ± 0.03	1.25	2.80	2.80^{b} ± 0.01	0.21
TL – III	2.80			2.80		
TL – III	2.74			2.81		

**LOD: Limit of detection **N.D: Not detected ***N.A: No application*

Validation of quantification method

Linearity

The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method. The calibration curves constructed for erythromycin were linear over the concentration range of 50 ÷ 400 µg/kg. Peak areas of erythromycin were plotted versus its concentration and linear regression analysis performed on the resultant curve. A correlation coefficient of R^2_{adjust} = 1.0 with %R.S.D. values ranging from 0.4 ÷ 3.7 % across the concentration range studied were obtained following linear regression analysis (Table 13). Typically, the regression equation for the calibration curve was found to be Y=1.08*X + 87.

Table 13. Linear range in regression analysis of erythromycin.

Erythromycin A concentration (ppb)	**50.0**	**100.0**	**200.0**	**300.0**	**400.0**
Peak current (Mean ± SD)	**138.5[a] ± 5.1**	194.0[b] ± 3.9	305.2[c] ± 5.2	411.2[d] ± 1.8	524.8[e] ± 14.3
RSD (%)	3.7	2.0	1.6	0.4	2.7

** Each value was the mean of 5 samples*

LOD

The LOD was the lowest amount of measured analyte that may be detected to produce a response which is significantly different from that of a blank. Limit of detection was approved by calculations based on the standard deviation of the response (δ) (here the current) which is obtained from blank with 5 replicas and (S) is the slope of the calibration curve according to equation LOD=3.3(δ/S). The LOD for erythromycin was 0.57 µg/kg (Figure 13).

Precision, Accuracy and Recovery

Precision was investigated by the intra- and inter-day (n = 6) assays at three different concentrations with respect to both repeatability and reproducibility. Repeatability was investigated by injecting six replicate samples of each of the 100, 200, 300 µg/kg standards. Inter-day precision was assessed by injecting the same three concentrations over 3 consecutive days. Accuracy (relative error, RE, %) was calculated by assessing the agreement between measured and nominal concentrations of the fortified samples. Recovery was assessed at erythromycin A, concentrations of 100, 200, 300 µg/kg and the mean value was calculated (Table 14 and Figure 14).

Table 14. Precision (RSD%), accuracy (RE%) and recovery of erythromycin A in tilapia muscles.

Day	Spike level (µg*kg^{-1})	Measured concentration (mean ± SD, µg*kg^{-1})	RSD (%)	RE (%)
1	100	85.07^{a} ± 1.26	1.48	85.07
2	100	85.34^{a} ± 1.79	2.10	85.34
3	100	85.31^{a} ± 1.24	1.45	85.31
1	200	173.25^{b} ± 2.34	1.35	86.63
2	200	173.03^{b} ± 2.09	1.21	86.51
3	200	172.82^{b} ± 1.39	0.80	86.41
1	300	261.62^{c} ± 4.53	1.73	87.21
2	300	262.57^{c} ± 3.42	1.30	87.52
3	300	265.68^{c} ± 5.38	2.02	88.56

** Each value was the mean of 6 samples*

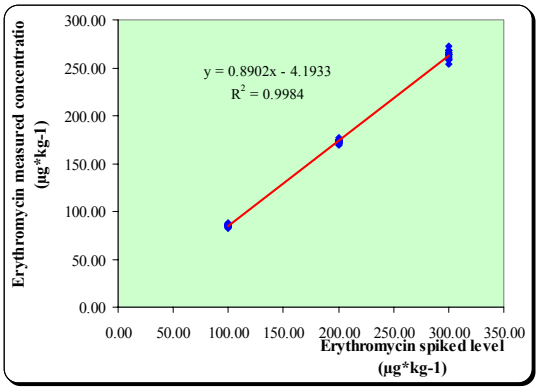


Figure 14. Erythromycin concentration was detected on fish samples at different times and levels.

Differentiation

The differentiation of the method was checked by monitoring standard solutions of erythromycin A in the presence of other antibiotic components. The peak response ($E_{1/2}$) of erythromycin A ($E_{1/2}$ = -1430 mV) was separated, independent and distinguished from ones obtained in chloramphenicol ($E_{1/2}$ = -196 mV), furazolidone ($E_{1/2}$ = -1152 mV), florfenicol ($E_{1/2}$ = -78 mV), enrofloxacin, ciprofloxacin ($E_{1/2}$ = -1336 mV), colistin ($E_{1/2}$ = -1120 mV) malachite green ($E_{1/2}$ = -1228 mV). Hence, the determination of erythromycin by SWV was considered having not only "screening" but also "confirming" abilities.

Application

Tilapia samples from ten provinces in the Mekong River Delta were taken and analyzed to survey the erythromycin residue. Residual results could be obviously seen in Table 15 and Figure 15.

Table 15. Erythromycin residue in tilapia samples from ten provinces, three districts in each province in the Mekong River Delta.

No	Province	District	Tilapia						
			M1	M2	M3	M4	M5	Mean ($\mu g*kg^{-1}$)	RSD (%)
1	Dong Thap	Cao Lanh	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Tam Nong	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Thap Muoi	N.D	N.D	N.D	N.D	N.D	N.D	N.D
2	Kien Giang	Go Quao	289.4	287.5	290.3	279.9	280.8	285.6^{d}	1.71
		Giong Rieng	175.2	177.9	176.3	175.9	177.8	176.6^{c}	0.67
		Vinh Thuan	N.D	N.D	N.D	N.D	N.D	N.D	N.D
3	An Giang	Thoai Son	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Long Xuyen	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Phu Tan	N.D	N.D	N.D	N.D	N.D	N.D	N.D
4	Vinh Long	Tam Binh	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Binh Minh	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Long Ho	N.D	N.D	N.D	N.D	N.D	N.D	N.D
5	Ben Tre	Mo Cay	713.0	719.2	720.0	715.8	716.9	716.9^{f}	0.39
		Chau Thanh	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Giong Trom	72.5	73.1	72.8	74.0	72.7	73.0^{b}	0.81
6	Tra Vinh	Duyen Hai	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Tra Cu	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Cang Long	N.D	N.D	N.D	N.D	N.D	N.D	N.D
7	Hau Giang	Phung Hiep	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Long My	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Vi Thuy	N.D	N.D	N.D	N.D	N.D	N.D	N.D
8	Soc Trang	My Tu	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		My Xuyen	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Ke Sach	N.D	N.D	N.D	N.D	N.D	N.D	N.D
9	Can Tho	Vinh Thanh	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		O Mon	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Co Do	45.6	47.1	44.9	45.2	45.0	45.6^{a}	1.98
10	Bac Lieu	Phuoc Long	327.4	328.4	319.7	326.6	326.9	325.8^{e}	1.07
		Hong Dan	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		Gia Rai	N.D	N.D	N.D	N.D	N.D	N.D	N.D

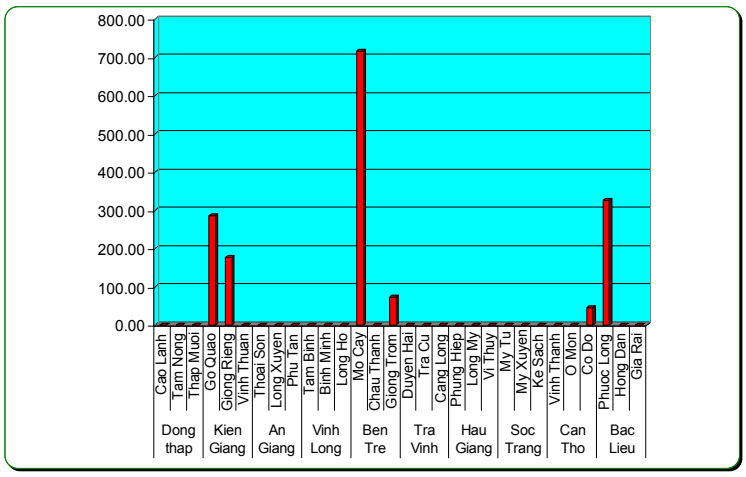


Figure 15. Erythromycin surveillance in tilapia aquaculture at ten provinces, three districts in each province of Mekong River Delta.

Conclusion

A new analytical procedure based on Square Wave Voltammetry has been developed for determination of erythromycin in tilapia. The proposed method was simple, quick, economical and sensitive. It should be extensively used for veterinary drug residue screening in food surveillance programs.

Acknowledgements

This work was supported by Dr Tran Bich Lam – HCM University of Technology, Dr Nguyen Trong Giao – Viet Nam_Russia Tropical Center, South Branch, Viet Nam.

References

1. Carmen Leal, Rosa Codony, Ramon Compañó, Mercè Granados and M. Dolors Prat (2001). Determination of macrolide antibiotics by liquid chromatography. ***Journal of Chromatography A*,** 910, 285-290.
2. Deubel A, Fandiño AS, Sörgel F and Holzgrabe U. (2006). Determination of Erythromycin and related substances in commercial samples using liquid chromatography/ion trap mass spectrometry. ***Journal of Chromatography A*,** 1136, 39-47.
3. Deng B, Kang Y, Li X and Xu Q. (2007). Determination of Erythromycin in rat plasma with capillary electrophoresis-electrochemiluminescence detection of tris (2, 2'-bipyridyl) ruthenium (II). ***Journal of Chromatography B*,** 857, 136-141.
4. Dreassi E, Corti P, Bezzini F and Furlanetto S. (2000). High-performance liquid chromatographic assay of erythromycin from biological matrix using electrochemical or ultraviolet detection. ***Analyst,*** 125, 1077-1081.
5. Draisci R, Delli Quadri F, Achene L, Volpe G, Palleschi L and Palleschi G. (2001). A new electrochemical enzyme-linked immunosorbent assay for the screening of macrolide antibiotic residues in bovine meat. ***Analyst*,** 126, 1942–1946.

6. Granja R, Niño AM, Zucchetti R, Niño RM, Patel R and Salerno AG (2009). Determination of erythromycin and tylosin residues in honey by LC-MS/MS. ***Journal of AOAC International*****,** 92, 975-980.
7. Horie Masakazu, Ishii Rie, Yoshida Terumitsu, Hoshino Yoji and Nakazawa Hiroyuki (1999). Determination of erythromycin and oleandomycin in meat and fish by LC/MS. ***Journal of the Food Hygienic Society of Japan*****,** 40, 309-313.
8. Houda Berrada, Francesc Borrull, Guillermina Font and Rosa Maria Marcé (2008). Determination of macrolide antibiotics in meat and fish using pressurized liquid extraction and liquid chromatography–mass spectrometry. ***Journal of Chromatography A*****,** 1208, 83-89.
9. Huaisheng Wang, Aimei Zhang, Hui Cui, Daojie Liu and Renmin Liu (2000). Adsorptive stripping voltammetric determination of erythromycin at a pretreated glassy carbon electrode. ***Microchemical Journal*****,** 64, 67-71.
10. Hui Yun-Hua, Yu Hui-juan, Cai You—Xiong and Zhou Pei—Ge (2006). Residual determination of erythromycin in tilapia by high performance liquid chromatography. ***Marine Fisheries*****,** 28, 321-325.
11. Isaac Ongubo Kibwage,Jos Hoogmartens, Eugene Roets, Hubert Vanderhaeghe, Ludo Verbist, Maurice Dubost,V Claude Pascal, Pierre Petitjean, and Georgette Levol, 1985. Antibacterial activities of erythromycins A, B, C, and D and some of their derivatives. ***Antimicrobial Agents and Chemotherapy*****,** 28, 630-633.
12. Jian Wang, Daniel Leung and Steven P. Lenz (2006). Determination of five macrolide antibiotic residues in raw milk using liquid chromatography−electrospray ionization tandem mass spectrometry. ***Journal of Agricultural and Food Chemistry*****,** 54, 2873–2880.
13. Kondo T, Dote N, Hagimoto T, Yoshimura Y. (1999). Application of liquid chromatography-turbo ion spray tandem mass spectrometry for quantitative analysis of a potent motilin receptor agonist, EM574, and its metabolites in human plasma. ***Journal of Chromatography B,*** 29, 734, 101-112.
14. Masakazu Horie, Harumi Takegami, Kazuo Toya and Hiroyuki Nakazawa (2003). Determination of macrolide antibiotics in meat and fish by liquid chromatography–electrospray mass spectrometry. ***Analytica Chimica Acta*****,** 492, 187–197.
15. Michael P. Schlüsener, Kai Bester and Michael Spiteller (2003). Determination of antibiotics such as macrolides, ionophores and tiamulin in liquid manure by HPLC-MS/MS. ***Analytical and Bioanalytical Chemistry*****,** 375, 942–947.
16. Nagla. F. Galal, Safinaz G.M. Ismail, R.H.Khalil and M.K. Soliman (2005). Studies on *Edwardsiella* infection in *Oreochromis niloticus*. ***Egyptian Journal of Aquatic Research*****,** 31, 460 – 471.
17. P. Norouzi, P. Daneshgar and M.R. Ganjali (2009). Electrochemical evaluation of non-electroactive drug Erythromycin in trace amount at biological samples by continuous cyclic voltammetry. ***Materials Science and Engineering: C*****,** 29, 1281-1287.

18. Stanley M. Billedeau, Thomas M. Heinze and Paul H. Siitonen (2003). Liquid chromatography analysis of erythromycin A in salmon tissue by electrochemical detection with confirmation by electrospray ionization mass spectrometry. ***Journal of Agricultural and Food Chemistry*****,** 51, 1534–1538.
19. Tang HP, Ho C and Lai SS. (2006). High-throughput screening for multi-class veterinary drug residues in animal muscle using liquid chromatography/tandem mass spectrometry with on-line solid-phase extraction. ***Rapid Communications in Mass Spectrometry*****,** 20, 2565 – 2572.
20. Xiao W, Chen B, Yao S and Cheng Z. (2005). Simultaneous determination of Erythromycin propionate and base in human plasma by high-performance liquid chromatography–electrospray mass spectrometry. ***Journal of Chromatography B*****,** 817, 153–158.
21. Yong-Xi Li, Kathy Neufeld, Jim Chastain, Alletta Curtis and Poonam Velagaleti (1998). Sensitive determination of erythromycin in human plasma by LC-MS/MS. ***Journal of Pharmaceutical and Biomedical Analysis*****,** 16, 961-970.
22. Zierfels G and Petz M. (1994). Fluorimetric determination of Erythromycin residues in foods of animal origin after derivatization with FMOC and HPLC separation. ***Z Lebensm Unters Forsch.*****,** 198, 307-312.