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A New Analytical Method for Erythromycin in Fish by Liquid Chromatography/Tandem Mass Spectrometry

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Abstract Erythromycin has been used to treat Streptococosis, Edwardsiellosis, Vibriosis, Bacterial enteritis in the cultured fish. In this study, a rapid and effective erythromycin analysis method with new sample treatment protocol and liquid chromatography/tandem mass spectrometry (LC/MS/MS) system for fish products was developed. For the erythromycin extraction from fish muscle, the solvent mixture composed of 0.2% meta-phosphoric acid and methanol (6:4) showed good recovery rate, and the optimum extraction solvent volume was 20 mL. Erythromycin detection using LC/MS/MS were carried out under electrospray ionization (ESI) positive condition and erythromycin mass value 576.2 and 157.9. And the detection limit of the established method was 0.005 mg/kg in fish products. The recovery rate of the developed method applied to the fish species were as following, olive flounder, 87.6±5.0%; black rockfish, 87.2±6.4%; eel, 85.2±4.8%; and rainbow trout, 86.0±6.2%. In the established method in this study, the correlation of coefficient values (R^2) of erythromycin calibration curve (n=11) was 0.9998.

Keywords: cultured fish, erythromycin, analysis method, liquid chromatography/tandem mass spectrometry (LC/MS/MS), detection limit

Introduction

According to the report 'State of World Aquaculture' issued in 2006 by Food and Agriculture Organization (FAO), the cultured fish occupied 43% of whole fish consumption in the world (1). In Korea, about 91,002 tons of cultured fish were produced in 2006 (2) and about 24 kinds of antibiotics have been used to prevent and treat the bacterial fish disease in the aquaculture industry (3). Many studies have been carried out to identify and evaluate the antibiotics in the cultured fish as food hazard because of the diversification and increment of the antibiotics use in the fish culture industry (4-6). The study area concerned about antibiotics was concentrated on the establishment of analysis method and withdrawal time in Korea after government study of antibiotics level control for the cultured fish was started at 2000. From the study results, among 24 kinds of antibiotics being distributed in the market place, 7 group 19 kinds of antibiotics analysis method were established (6-8).

Erythromycin is being used to prevent and treat for vibriosis in the olive flounder and black rock fish that occupied more than 80% of total cultured fish production in Korea. The total used amount of erythromycin was reported as 10,545 kg in 2004, and ranked the third place followed to oxytetracycline and amoxicillin (3). Erythromycin is also regarded as a food safety hazard that needs to be controlled to secure the food safety of the cultured fish.

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Many analytical methods for detection of erythromycin from the food products have been reported (7,9-16). But the reported methods of erythromycin detection by high performance liquid chromatography (HPLC) that have been reported by other researchers for fish products have a few week points like time consuming and high cost due to extract clean up using cartridge. And the suggested methods are not supposed to be applied for food products analysis because of low sensitivity. So the existing HPLC methods have to be improved in the effectiveness aspect such as cost, time, and sensitivity. In Korea, two different methods, bacterial method using antibiotics sensitive bacteria and HPLC method, have adopted for erythromycin analysis from food products for standard method (7). But the bacterial method has shown low sensitivity and is not proper for quantitative measure. The HPLC method has some problems such as time consuming for sample treatment, low recovery, and low detection limit (10 mg/mL).

In this study, a simple and low cost liquid chromatography/tandem mass spectrometry (LC/MS/MS) method of erythromycin detection for in the fish products was developed based on the existing methods to secure food safety of the cultured fish products.

Material and Methods

Reagent and sample Erythromycin standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, hexane, and methanol used for the erythromycin extraction and making the mobile phase were HPLC grade and purchased from Merck Co. (Darmstadt, Germany). The meta-phosphoric acid and formic acid used for antibiotics

extraction were purchased from Sigma-Aldrich. The distilled water (DW) used for reagent and mobile phase was prepared using the Puris esse-up water system (Up Multi-TOC; MiraeST Co., Ltd., Anyang, Korea). Four kinds of fish species, olive flounder (*Paralichthys olivaceus*), black rockfish (*Sebastes schlegeli*), eel (*Anguilla japonica*), and rainbow tout (*Oncorhynchus mykiss*) were supplied for establishment of erythromycin analyzing method and recovery test.

Stock solution of erythromycin Ten mg of erythromycin was dissolved into about 10 mL of methanol and made up to 100 mL with same solvent with final concentration of 100 μg/mL. Stock solution was kept under 4°C during whole experiment.

Working solution Erythromycin working solutions ranged 10-0.005 μg/mL were prepared using stock solution and methanol with gradient dilution.

Preparation of calibration curve Calibration curve was made based on the chromatogram that were produced from 3 times injection of same concentration ranged 10-0.005 μg/mL of erythromycin.

Extraction of erythromycin from fish muscle Extraction procedure of erythromycin from fish muscle was established based on the method reported by Horie *et al.* (16). The detail extraction procedure is shown in Fig. 1. For antibiotics extraction, the prepared fish were peeled and filleted, and then ground whole fish meat together. Twenty mL mixture of 0.2% meta-phosphoric acid and methanol (6:4) was added to 20 g of ground fish meat and mixed with homogenizer (DIAX 600; Heidolph, Kelheim, Germany) for 2 min at 8,000 rpm. The homogenate was centrifuged at 270×g for 15 min. The supernatant was added 10 mL of hexane and centrifuged again at 270×g for 15 min to remove lipids in the extracts. After removing the lipids, the aqueous layer were filtered with 0.2 μm syringe filter and analyzed with LC/MS/MS.

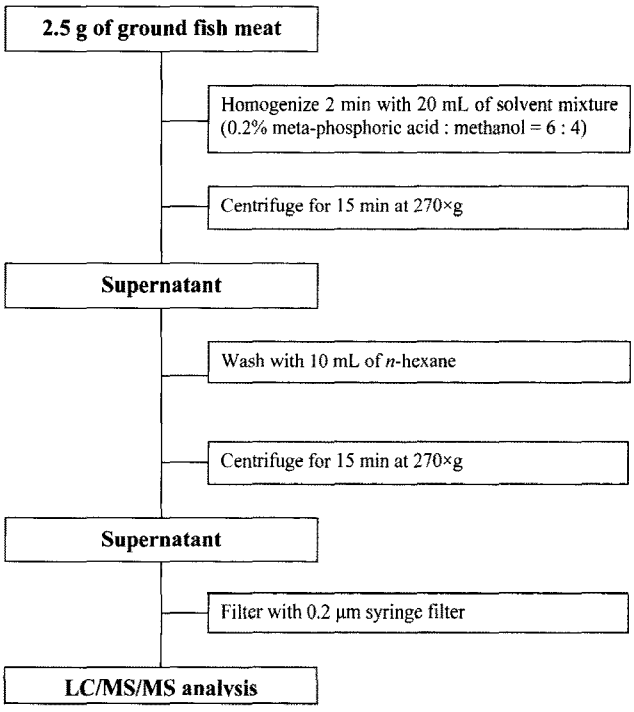

Fig. 1. Extraction and clean up procedures for the analysis of erythromycin in the fish.

Recovery rate of the established protocol Four fish species, olive flounder, back rockfish, eel, and rainbow trout were used for the recovery test. To identify the recovery rate of the antibiotics extraction protocol established in this study, erythromycin standard solution was added to fish meat with the final concentration of 0.1, 0.2, and 0.5 mg/kg in each sample. Recovery rate was calculated as follows;

$$\text{Recovery rate (\%)} = \frac{\text{detected erythromycin concen. in the spiked sample}}{\text{added erythromycin concentration}} \times 100$$

Equipment and operating condition of LC/MS/MS To detect the erythromycin standard and the erythromycin in the sample extract, the MS/MS system (Quattro Premier XE; Waters, Milford, MA, USA) equipped with HPLC system (Acquity; Waters) and HPLC exclusive column (Acquity UPLC™ BEH C_{18}, 1.7 μm, 100×2.1 mm i.d., Waters) were used. The mobile phase for erythromycin detection was composed with DW and acetonitrile including 0.1% formic acid. A gradient flow method was adopted and the flow rate was 0.3 mL/min for the effective analysis (Table 1). The LC/MS/MS operation condition for erythromycin detection was shown in Table 1. The multiple reactions monitoring (MRM) method was adopted for the erythromycin peak detection using MS/MS. Nitrogen gas and argon gas were used for erythromycin ionizing. The ionized erythromycin was detected using electrospray ionization (ESI) under positive ion mode. The target mass of

Table 1. Operation conditions of LC/MS/MS for detection of erythromycin

MS/MS conditions			
Ionization	ESI, positive		
MRM transition (*m/z*)	734→576.2 734→157.9		
Cone voltage (V)	36		
Collision energy (eV)	28, 20		
Desolvation gas flow (L/hr)	800		
UPLC conditions			
Column	Acquity UPLC BEH C18 1.7 μm (100×2.1 mm i.d.)		
Mobile phase A: 0.1% formic acid in DW B: 0.1% formic acid in Acetonitrile	Time (min)	A (%)	B (%)
	Initial	90	10
	1.0	90	10
	5.0	10	90
	5.1	90	10
	5.1	90	10
	6.0	90	10
Flow rate	0.3 mL/min		
Oven temperature	50°C		
Injection volume	5 μL		

Table 2. Recovery rate[1] under the different solvent condition

Solvent	Average (%)
0.2% Meta-phosphoric acid : methanol (6:4)	89.3±4.6
Methanol : DW (7:3)	40.1±0.9
5% Ammonium acetate : methanol (5:5)	74.6±10.0

[1]Recovery rate was obtained from 3 replications.

Table 3. The recovery rate[1] under different extraction volume

0.2% Meta-phosphoric acid : methanol (6:4)	Average (%)
10 mL	70.0±6.0
15 mL	83.8±1.7
20 mL	92.0±2.0
25 mL	86.6±4.6

[1]Recovery rate was obtained from triplicate.

Table 4. Recovery rate[1] of the established protocol for erythromycin analysis in the fish species

Fishes	Fortification level (mg/kg)			
	0.05	0.1	0.2	Average (%)
P. olicaceus	83.0±6.0	85.6±4.5	91.1±4.3	87.6±5.0
CV[2]	6.97	5.26	4.72	5.80
S. schlegeli	85.3±6.1	83.3±6.5	93.0±2.7	87.2±6.4
CV	7.16	7.80	2.99	7.37
A. japonica	82.6±6.4	83.6±2.5	89.3±2.4	85.2±4.8
CV	7.77	3.00	2.76	5.64
O. mykiss	82.0±3.4	83.3±5.8	92.6±2.4	86.0±6.2
CV	4.22	7.03	2.66	7.20

[1]Recovery rate was obtained from triplicate.
[2]Coefficient of variation.

the erythromycin was selected as parent ion Mw 734.3 and daughter ion Mw 576.3, 158.1.

Results and Discussion

Optimum extraction solvent To select optimum extraction solvent, 3 kinds of solvent compositions that are good for ionization in the MS/MS system were prepared and compared. The composition was as following; 0.2% meta-phosphoric acid: methanol (6:4, v/v), methanol : DW (7:3, v/v), 5% ammonium acetate : methanol (5:5, v/v). The recovery rate was checked in the flounder sample using each solvent composition (20 mL) and the spiked sample 0.5 mg/kg. From the 3 times repeated try, the solvent composition of 0.2% meta-phosphoric acid : methanol (6:4) showed the highest recovery rate as 89.33% (Table 2).

Optimum extraction volume The optimum extraction volume of the solvent was also checked using the spiked sample 0.5 mg/kg and the results were presented in Table 3. Among the extraction volume rage from 10 to 25 mL, the recovery rate was highest as 92% under 20 mL (Table 3).

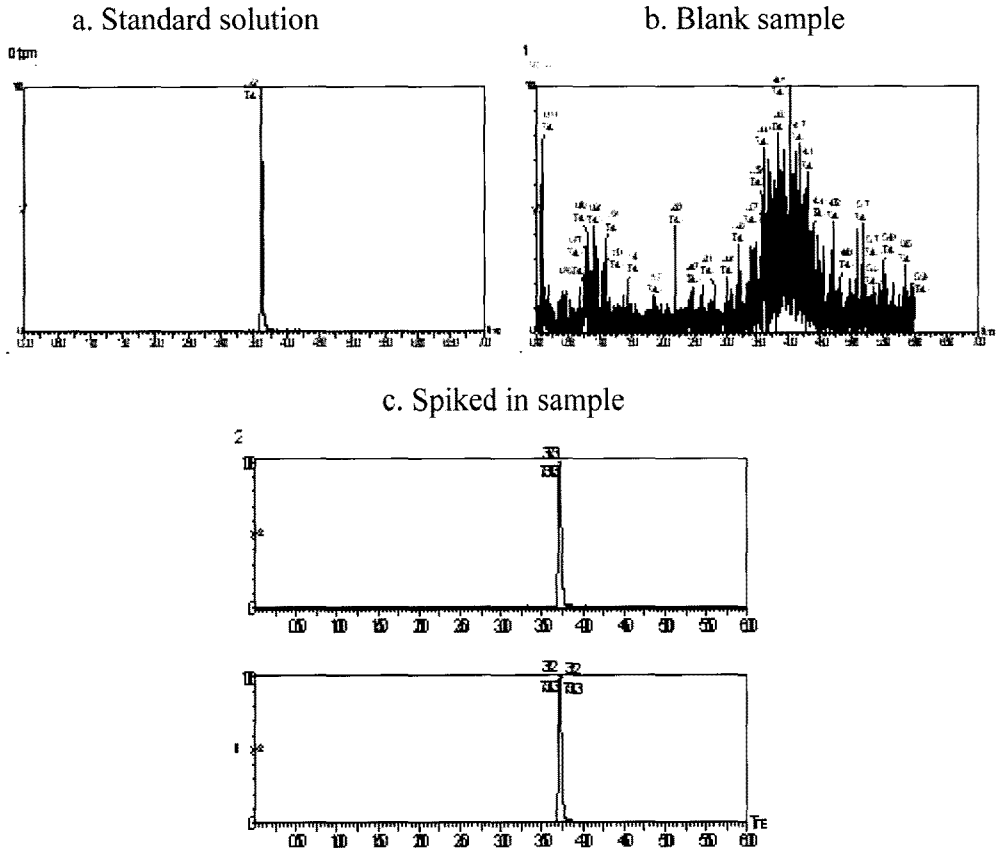

Fig. 2. Chromatogram of erythromycin under 0.1 mg/kg in the olive flounder.

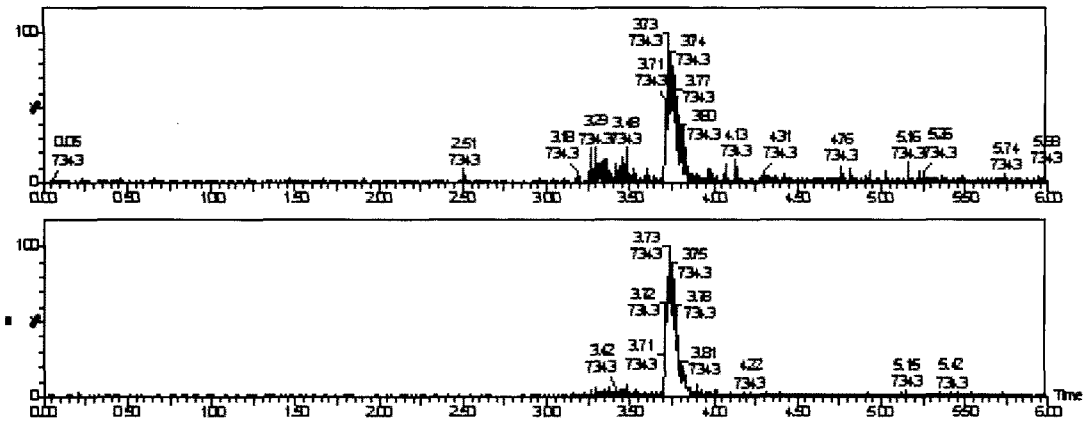

Fig. 3. Chromatogram of erythromycin under 0.005 mg/kg in the olive flounder.

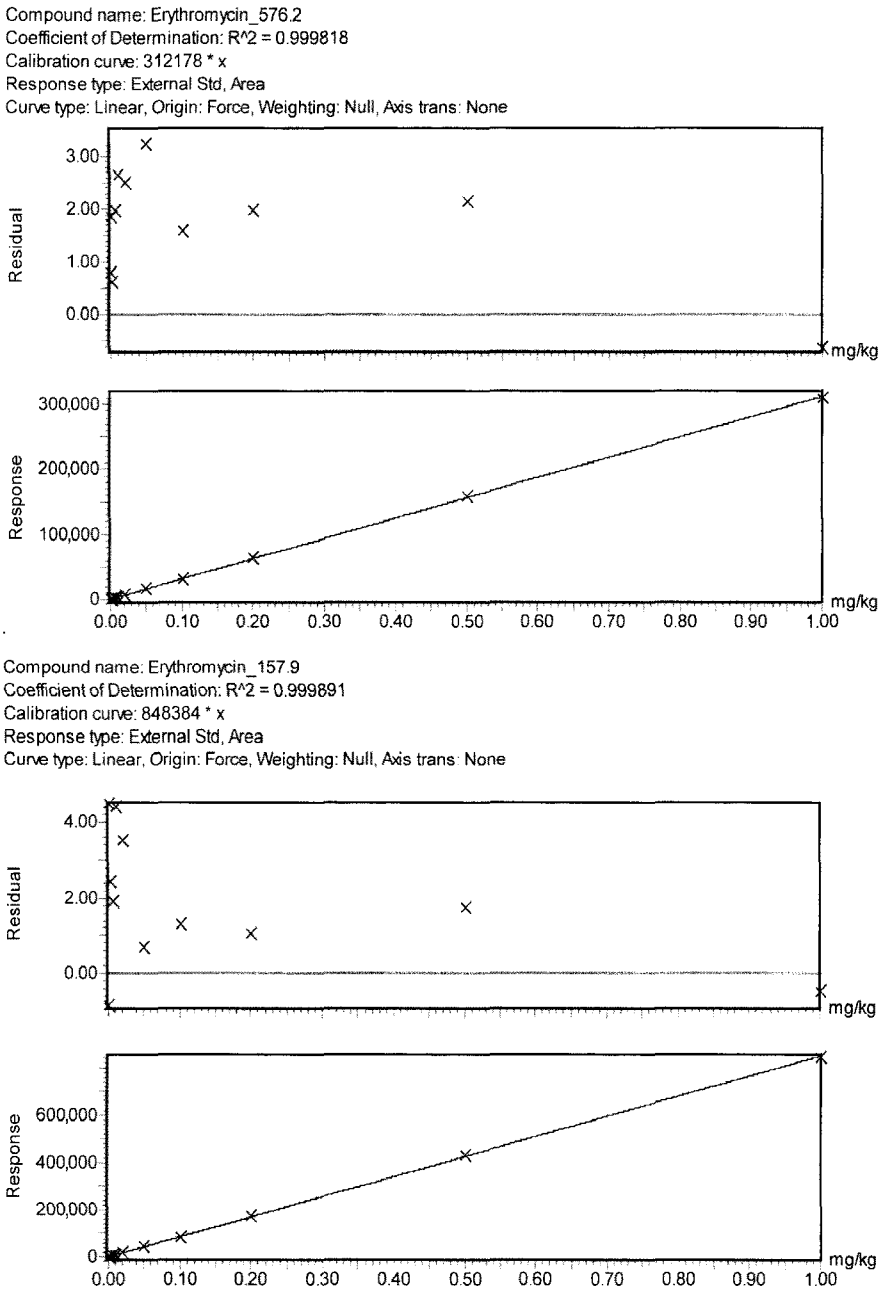

Fig. 4. Standard calibration curve for determination of erythromycin.

Recovery rate in different fish species Under established solvent condition and volume, the recovery rate in different fish species were checked. Four kinds of fish, olive flounder, black rockfish, eel, and rainbow trout were prepared and the standard stock solution were added into each fish muscle and the final concentration were adjusted as 0.1, 0.2, and 0.5 mg/kg, respectively. The recovery tests were repeated 3 times and the checked results were presented in Table 4.

The recovery rates in the olive flounder, black rockfish, eel, and rainbow trout were 87.6±5.0, 87.2±6.4, 85.2±4.8, and 86.0±6.2%, respectively. The coefficient of variation (CV) value ranges of them were 4.72-6.97, 2.99-7.80, 2.76-7.77, and 2.66-7.03%, respectively. Horie *et al.* (16) reported they adopted 0.2% meta-phosphoric acid extraction and Hyflo Super-Cel cartridge concentration and clean-up step, the recovery rate in the yellow tail and red sea bream was 82.1±4.3 and 83.0±4.5%, respectively.

From the verified test results described above, the established method in this study was confirmed more effective and time saving compared to the existing method. Money saving is also benefit in the suggested method here because clean up step in the antibiotics extraction procedure can be omitted (Fig. 2).

Limit of detection and quantitative measure To identify the detection limit in the suggested method, the signal vs. noise rate comparison protocol was adopted. The signal from the low concentration positive sample was compared to the signal from blank sample. Generally, the concentration that the signal (S) to noise (N) rate (S/N ratio) is 3/1 has been considered as a limit of detection.

The response rate of noise level of the negative sample to positive sample signal was established as 3:1 in this study. Under the fixed condition, the detection limit in the fish species, flounder, black rockfish, eel, and rainbow trout was identified as 0.005 mg/kg. The peak at 0.005 mg/kg was clear and distinguishable (Fig 3).

But Horie *et al.* (16) reported that the detection limit of erythromycin using LC/MS/MS system for the fish products, yellowtail, and red sea bream was 0.01 μg/g. And in the erythromycin analysis report for beef products written by Ryu *et al.* (15), the detection limit was 0.02 μg/g when they adopted gas mass spectrometry system and sample extract clean up step.

The U.S. Food and Drug Administration (FDA) recommend that the concentration in sample should be 3 times higher than detection limit in a specific method of quantitative measure (17). Erythromycin, therefore, can be detectable regardless fish species if its concentration in the sample were more than 0.015 mg/kg by method in this study. Many countries have tolerance limit of erythromycin for food products. Especially, Europe Union (EU) has adopted the maximum residue limit for fish products as 200 μg/kg. However, most of the countries in EU regard erythromycin as a kind of off-label antibiotic that is not allowed to fish culture (18) and the withdrawal time of erythromycin needed to no detectable level in fish products was suggested as 500°C-day (19,20). Li *et al.* (21) reported the detection limit and recovery rate of erythromycin in human plasma using LC/MS/MS system were 0.5 ng/mL and 88-105%, respectively. Horie *et al.* (16) reported that the detection limit in fish products by LC/MS/MS with concentration and clean up was 0.01 μg/g. Although many researches adopted a concentration and clean up step, they reported that the method was time consuming and expensive. The new method established in this study is very reasonable and effective for time and cost. Furthermore the method will be able to adopt by regulatory authority and industry for inspection and monitoring.

Calibration curve To verify analyzing condition of the established method, the calibration curve was made with standard working solution ranged from 0.0005 to 1.0 mg/kg. From the test results as following, R^2=0.9998 under erythromycin mass value 576.2 and R^2=0.9999 under erythromycin mass value 157.9 (Fig. 4). These results mean that the established method is very stable for continuing analysis.

References

1. FAO Fisheries Department. State of World Aquaculture 2006. Food and Agriculture Organization, Rome, Italy. pp. 5-16 (2006)
2. MOMAF. Survey on the Status of Fish Culture. Ministry of Maritime Affairs & Fisheries, Seoul, Korea. pp. 15-29 (2006)
3. KFDA. Approval of Registration of the Animal Drug and Usage Status. Korea Food & Drug Administration, Seoul, Korea. pp. 15-183 (2006)
4. MOMAF. Study on the Development on HACCP System for Fishery Products During Production and before Market. Ministry of Maritime Affairs & Fisheries. Busan, Korea. pp. 90-113 (2001)
5. KFDA. Establishment of Antimicrobials Management System for Aquaculture. Korea Food & Drug Administration, Seoul, Korea. pp. 24-54 (2003)
6. NFRDI. Antibiotics Analysis Methods for Fish Products. National Fisheries Research and Development Institute, Busan, Korea. pp. 1-25 (2000)
7. KFDA. Food Code. Korea Food & Drug Administration, Seoul, Korea. pp. 1002-1151 (2006)
8. KFDA. Permission Level of the Veterinary Drugs in Human Food. Korea Food & Drug Administration, Seoul, Korea. pp. 3-192 (2004)
9. Bernabu JA, Camacho MA, Gil-Alegre ME, Ruz V, Torres-Suárez AI. Microbiological bioassay of erythromycin thiocyanate: Optimisation and validation. J. Pharmaceut. Biomed. 21: 347-353 (1999)
10. Lingerfelt B, Scoot CW. Macrolide and ketolide antibiotic separation by reversed phase high performance liquid chromatography. J. Pharmaceut. Biomed. 20: 459-469 (1999)
11. Horie M, Saito K, Ishii R, Yoshida T, Haramaki Y, Nakazawa H. Simultaneous determination of five macrolide antibiotics in meat by high performance liquid chromatography. J. Chromatogr. A 812: 295-302 (1998)
12. Leal C, Codony R, Compañó R, Granados M, Dolors Prat M. Determination of macrolide antibiotics by liquid chromatography. J. Chromatogr. A 910: 285-290 (2001)
13. Dehouck P, Vander Heyden Y, Smeyers-Verbeke J, Massart DL, Marini RD, Chiap P, Hubert Ph, Crommen J, Van de Wauw W, De Beer J, Cox R, Mathieu G, Reepmeyer JC, Voigt B, Estevenon O, Nicolas A, Van Schepdael A, Adams E, Hoogmartens J. Interlaboratory study of a liquid chromatography method for erythromycin: Determination of uncertainty. J. Chromatogr. A 1010: 63-74 (2003)
14. Hanada E, Ohtani H, Kotaki H, Sawada Y, Iga T. Determination of erythromycin concentration in rat plasma and liver by high performance liquid chromatography with amperometric detection. J. Chromatogr. B 592: 478-482 (1997)
15. Ryu JC, Song YS, Yang JS, Seo JW, Kim MS, Park JS. A study on the determination of residual antibiotics and synthetic antibacterial agents in meat (III): Simultaneous gas chromatography/mass spectrometry analysis of erythromycin and tylosin. Korean J. Food

Hyg. 8: 17-23 (1993)
16. Horie M, Takegami H, Toya K, Nakazawa H. Determination of macrolide antibiotics in meat and fish by liquid chromatography-electrospray mass spectrometry. Anal. Chim. Acta 492: 187-197 (2003)
17. FDA. Validation of Analytical Procedures: Methodology. No. 64. Recommended for implementation at step 7 of the VICH process by the VICH Steering Committee, Department of Health & Human Services, Washington DC, USA. pp. 1-13 (1998)
18. EEC. Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Council Regulation (EEC) No. 2377/1990. European Economic Commission, Brussels, Belgium. Off. J. European Commun. 224: 1-8 (1990)
19. EEC. Directive of the European Parliament and of the Council of 6 November 2001 on the community code relating to veterinary medicinal products. Council Directive (EEC) No. 82/2001. European Economic Commission, Brussels, Belgium. Off. J. European Commun. 311: 0001-0066 (2001)
20. Esposito A, Fabrizi L, Lucchetti D, Marvasi L, Coni E, Guandalini E. Orally administered erythromycin in rainbow trout (*Oncorhynchus mykiss*): Residues in edible tissues and withdrawal time. Antimicrob. Agents Ch. 51: 1043-1047 (2007)
21. Li YX, Neufeld K, Chastain J, Curtis A, Velagaleti P. Sensitive determination of erythromycin in human plasma by LC/MS/MS. J. Pharmaceut. Biomed. 16: 961-970 (1998)