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Simultaneous Determination of Quinolones in Livestock and Marine Products by High Performance Liquid Chromatography

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ABSTRACT

A high performance liquid chromatographic (HPLC) method was developed for the simultaneous determination of nalidixic acid, flumequine, oxolinic acid, piromidic acid, danofloxacin, enrofloxacin and sarafloxacin in chicken, pork and fish. These seven quinolone antibacterials were extracted with 0.3% metaphosphoric acid: acetonitrile (1:10, v/v), followed by a Bond Elut C18 cartridge clean up procedure. The HPLC separation was achieved on a Cosmosil 5C18-AR-II column (5 μm, 4.6 mm i.d. × 250 mm) with acetonitrile: 0.05M NaH_2PO_4 (pH 2.5)(35:65, v/v) containing 3.5 mM sodium dodecyl sulfate as a mobile phase, and detection was performed with photodiode array and fluorescence detector (by using wavelength programming). Good linearity was observed from the calibration plot at concentrations from 0.05 to 10.0 mg/mL (0.005~2.0 μg/mL for danofloxacin). Recovery studies of the analytes were performed at 0.01, 0.1, 0.4 and 2.0 ppm (0.001, 0.01, 0.04 and 0.20 ppm for danofloxacin) spiked levels. Average recoveries of low concentration ranged from 74.3 to 85.5% and those of the rest ranged from 80.1 to 99.9% with coefficients of variation less than 5.8%. The detection limits of quinolones were 0.01~0.04 ppm with photodiode array detection and 0.0006~0.05 ppm with fluorescence detection. The coefficients of variation of intra-day and inter-day assays were lower than 3.29% and 5.23%, respectively. These results indicated that the developed method had an acceptable precision. Using this method to detect quinolones in sixty three samples purchased from various markets in Taipei, we found that nine wu ku chicken muscles contained enrofloxacin residues ranged from 0.08 to 4.04 ppm, four wu ku chicken liver muscles contained enrofloxacin residues ranged from 0.01 to 0.27 ppm, and oxolinic acid residues in three sweet fish samples ranged from 0.13 to 0.35 ppm. The results indicated that 25.4% of samples violated the regulation set by the Department of Health. The enrofloxacin residue in wu ku chicken muscle is very stable even after a long period of refrigeration at 4°C.

Key words: Veterinary drugs; Quinolone antibacterials; Chicken; Pork; Fish; High performance liquid chromatography.

INTRODUCTION

Recently the scale of chicken, swine, and fish farming has been expanded. The density is so high that infection often occurs. Large amount of quinolone antibacterials applied to prevent infectious diseases. When the lung, urinary, or digestive system of an animal is infected, it can be treated by quinolone antibacterials by inhibition of DNA gyrase[1,2] which is a type II topoisomerase, an essential enzyme for forming DNA supercoils[3].

Quinolones are a series of synthetic antibacterials derived from nalidixic acid. Their common structure is composed by 1-substituted-1,4-dihydro-4-oxopyridine-3-carboxylic moiety and aromatic groups (single or multiple rings). It has been reported that such molecules have antibacterial activity[4,5]. Although some scholars have classified quinolones as the first, second, and third generations based on their antibacterial spectrum, potency, and pharmacology, there is no widely-accepted classification at present[3, 6]. Based on chemical structures, quinolone antibacterials are divided into two categories. The first category (the first generation) includes antibaterials containing pyridonecarboxylic acid, such as nalidixic acid, flumequine, oxolinic acid and piromidic acid, which have good antibacterial activity against gram-negative bacteria[5]. They have been used on fishes such as eel and yellow fish[7]. Their antibacterial effect is no longer good as drug-resistant bacteria have evolved[8]. The second category (the second and third generation) includes fluoroquinolones containing fluorine at C-6 position and piperazinyl at C-7 position, such as oxfloxacin, danofloxacin, enrofloxacin, marbofloxacin, and sarafloxacin, which have broad antibacterial spectrum against gram-negative bacteria, gram-positive bacteria, and mycoplasma, so their antibacterial activity is better[1, 8, 9]. Quinolones are safe antibacterials. Side effects usually relate to central nerve system including gastrointestinal discomfort, dizziness, insomnia, and headache[10].

The usage of quinolone antibacterials in chicken, swine, and fish has increased dramatically in the past 10 years. Health authorities should monitor the consequences caused by residues of such drugs. Our laboratory has analyzed the amount of residual oxolinic acid in chicken,

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pork, and fish purchased from local markets. The drug was found in 7 out of 90 samples (7.7%) and the amount was between 0.065 and 0.072 ppm[11]. Horie *et al*. have screened chicken, pork, and fish in Japan for residual oxolinic acid. It was found in 7 of 10 eel samples with a concentration ranged from 0.01 to 0.1 ppm (average 0.05 ppm)[7]. Also Horie *et al*. reported in 1995 that oxolinic acid was detected in eel and sweet fish[8]. After further analysis, fish liver contained the highest amount of residual oxolinic acid (3.86 to 9.05 ppm), followed by kidney (2.60 to 5.05 ppm), then muscle (0.74 to 1.90 ppm). Stability tests were performed as well. After heating process, the residual oxolinic acid in sweet fish remained almost the same showing its high stability[8]. Sheu *et al*. have randomly screened 112 samples of chicken muscle purchased from markets at 7 areas in Taiwan. Enrofloxacin was found in 19.6 % samples ranged from 0.095 to 0.005 ppm, and ofloxacin was found in 2.7% samples ranged from 0.099 to 0.017 ppm)[12].

For Taiwan's accession to WTO, the Department of Health announced the revised "Tolerances for Residues of Veterinary Drugs" in January 2001[13]. According to this regulation, limits for residues of danofloxacin, flumequine, and sarafloxacin were set, while other quinolones are not allowed at all. At present, no analytical method has been announced by the Department of Health. Therefore, it is an important issue to establish a standard analytical method for monitoring the residual quinolones in livestock and marine products.

Methods for analyzing quinolones include thin layer chromatography (TLC)-fluorescence[10], high performance liquid chromatography (HPLC) with ultraviolet (UV) or fluorescence detection [1,2,4,7,8, 14 to 18], capillary electrophoresis[6,9,19,20], HPLC-mass spectrometry (HPLC-MS)[21,22], and gas chromatography-mass spectrometry (GC-MS)[23]. Among these methods, HPLC is the most popular one. Quinolones in samples were extracted with basic acetonitrile solution[1], 1 M hydrochloric acid[2], metaphosphoric acid: acetonitrile solution[8,16], n-hexane: ethyl acetate solution[14], or water : acetonitrile solution[18], then cleaned up with C18[1,2,8,16] or amino cartridge[14], followed by HPLC-UV or fluorescence analysis[1,8,16].

The purpose of this research is to establish an HPLC analytical method to simultaneously determine 7 quinolones including nalidixic acid, flumequine, oxolinic acid, piromidic acid, danofloxacin, enrofloxacin and sarafloxacin. Quinolones were extracted with solvent, followed by a solid-phase extraction clean-up procedure. Confirmation and quantification were performed by photodiode array (PDA) detector and a fluorescence detector. The specificity, sensitivity, and accuracy of this method were evaluated. Subsequently this method was applied to analyze livestock and marine products purchased from markets to understand how prevalent residual quinolone antibacterials are. Results can be provided to health authorities as a reference for administration and regulation.

MATERIALS AND METHODS

I. *Sample Collection*

There were 63 samples randomly purchased from supermarkets and traditional markets in Taipei City and County, including 18 chicken muscles (8 from ordinary chicken and 10 from wu ku chicken), 15 chicken livers (10 from ordinary chicken and 5 from wu ku chicken), 10 swine muscles, 10 swine livers, 5 eels, and 5 sweet fish. All samples were stored at -20˚C until analyzed.

II. *Chemicals*

Nalidixic acid (NA, 100.0% purity), oxolinic acid (OXA, 99.2%), and piromidic acid (PMA, 99.9%) were all purchased from Wako Chem. (Osaka, Japan). Flumequine (FMQ, 100.7%) and sarafloxacin hydrochloride (SRFX, 99.5%) were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.) and Abbott laboratories (North Chicago, IL, U.S.A.), respectively. Danofloxacin (DNFX, 77.74%) and enrofloxacin (ERFX, 100.1%) were provided by the Institute of Animal Drug Inspection (Chunan Branch, Taiwan). The chemical structures of all 7 quinolones are shown in Figure 1. Sodium hydroxide (NaOH), sodium dihydrogen phosphate (NaH_2PO_4), metaphosphoric acid (MPA), phosphoric acid, n-propanol, and sodium dodecyl sulfate (SDS) were of ultra high grade purchased from Nacalai Tesque Inc. (Kyoto, Japan). Methanol, acetonitrile, and n-hexane were of HPLC grade purchased from Labscan Co., Ltd. (Bankok, Thailand).

III. *Instrument and Apparatus*

(I) *HPLC*

A Shimadzu (Shimadzu Corporation, Kyoto, Japan) instrument includes Shimadzu LC-10AT pump system, SPD-M6A Photodiode Array UV-Vis Detector (PDA), and RF- 551 Fluorescence HPLC Monitor. The data processing system is Class-LC10 control and integration software from Shimadzu.

(II) *Fluorescence spectrophotometer*

F-4500, Hitachi Ltd., Tokyo, Japan

(III) *Homogenizer*

ACE, Nihonseiki Kaisha Ltd., Tokyo, Japan

IV. *Preparation of Standard Solutions*

To prepare a stock of standard solutions, 10 mg of NA, OXA, FMQ, PMA, and SRFX were weighed and dissolved in 0.01N NaOH: methanol (2:8, v/v) then brought up to 100 mL, while 10 mg of ERFX and 12.9 mg of DNFX (77.74% purity) were dissolved in methanol, then brought to 100

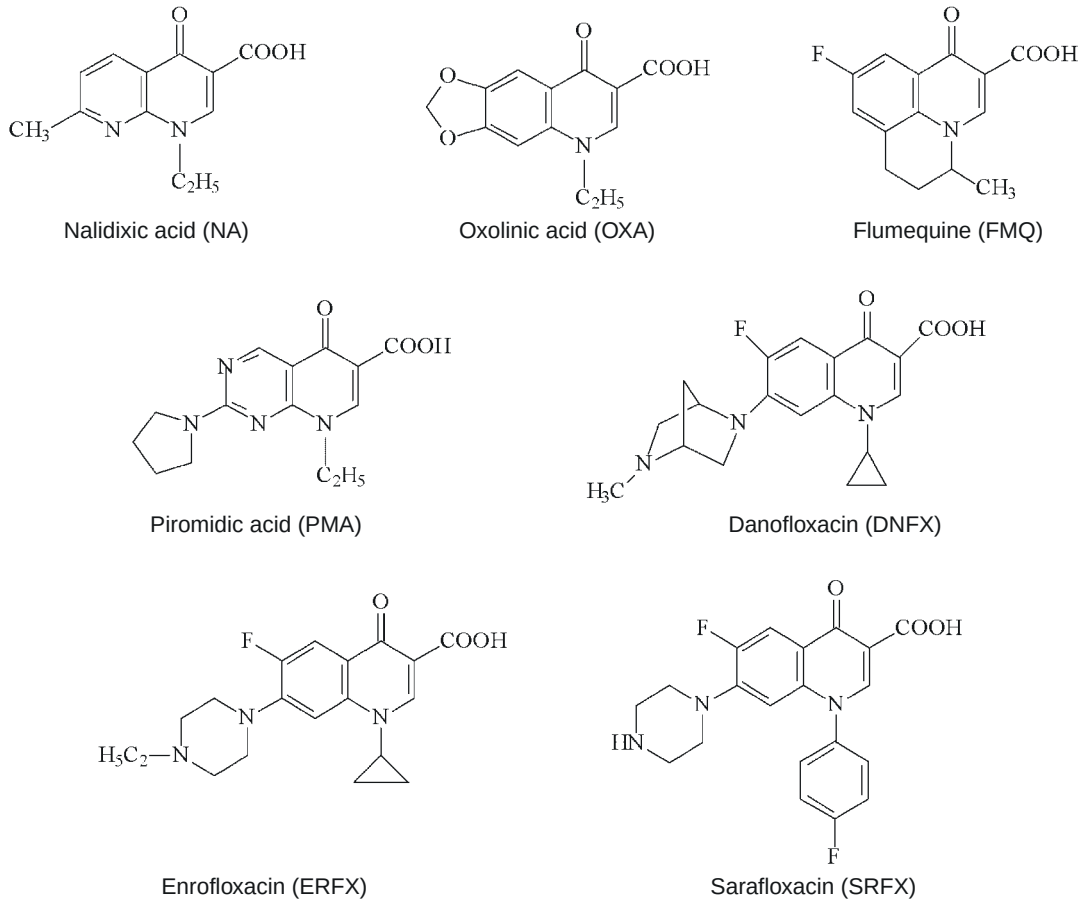

Figure 1. Chemical structures of 7 quinolone antibacterials.

mL, respectively. Working standard solutions were diluted from the stock of standard solutions to a serial mixtures of various concentrations with mobile phase (acetonitrile: 0.05M NaH_2PO_4 (pH 2.5) (35:65, v/v) containing 3.5 mM SDS) upon use.

V. *Preparation of Samples*

(I) *Extraction*

After homogenized, 5 g of sample was weighed and then transferred to the homogenizer with 30 mL of 0.3% metaphosphoric acid: acetonitrile (1:10, v/v), followed by homogenizing for 3 min. The mixture was filtered under suction by Buchner funnel, then shaken for 5 min with 50 mL of n-hexane saturated by acetonitrile in a separatory funnel. The acetonitrile layer was added with 5 mL of n-propanol to inhibit sudden boiling in a concentration bottle, followed by depressurized concentration to dryness in a 40˚C water bath.

(II) *Clean up*

The concentrated material was dissolved in 10 mL of water, then loaded to Bond Elut C18 cartridge (200 mg, Varian, Harbor City, CA, U.S.A.), which was previously activated with 5 mL of methanol and rinsed with 10 mL of water. The original concentration bottle was washed twice with 5 mL of 10% methanol. The washing solution was loaded to a cartridge, and the flow-through was discarded. Finally, the bottle was washed twice with 5 mL of methanol: 0.05 M NaH_2PO_4 (pH 2.5) (7:3, v/v). This washing solution was loaded to cartridge for elution. The eluent was collected and dried by depressurized concentration at 40˚C. The residue was ready for HPLC analysis after dissolved in 1 mL of mobile phase, then filtered by 0.45 μm membrane (nylon, Micron Separations Inc., West Borough, MA, U.S.A.).

VI. *HPLC Analysis*

(I) *Analytical condition*

The column for separating quinolones was Cosmosil 5C18-AR-II (5 μm, 4.6 mm i.d. × 250 mm; Nacalai Tesque Inc., Kyoto, Japan). The detectors were PDA and a fluorescence detector. The scan range of PDA was 220 to 400 nm, and the detection wavelengths were 260 and 286 nm. The excitation (Ex) and emission (Em) wavelengths of fluorescence detector were set to time program. The sensitivity was set to low and the gain was set to ×4. Mobile phase was acetonitrile: 0.05M NaH_2PO_4 (pH 2.5) (35:65, v/v)

containing 3.5 mM SDS. Flow rate was 1.0 mL/min. The injected volume of samples was 20 μL.

(II) *Standard Curves*

The stock of standard quinolones was diluted with mobile phase to 6 different concentrations of mixed standard solutions, 0.05, 0.1, 0.5, 2.0, 5.0, and 10.0 μg/mL (0.005, 0.01, 0.05, 0.2, 1, and 2 μg/mL for DNFX). These solutions were analyzed 3 times by HPLC-PDA and fluorescence detector. The standard curves were plotted with peak areas of quinolones versus concentrations.

(III) *Identification and quantitative analysis*

Quinolones were identified by: (1) comparing the retention time of unknown substance with standard substance, (2) comparing each peak against the spectra from PDA of standards.

Samples and mixed standard solutions were analyzed by HPLC-PDA and a fluorescence detector. The identification was determined by comparing the retention time, area, and spectra of peaks. Quantitative analysis of each quinolone was calculated from standard curves as below:

$$\text{Amount of each quinolone in samples (ppm)} = \frac{C \times V}{W}$$

C: concentration of each quinolone calculated from each standard curve (μg/mL)
V: volume of samples (mL)
W: weight of samples (g)

VII. *Recovery Test*

To each 5 g of homogenized chicken muscle, chicken liver, swine muscle, swine liver, eel, and sweet fish, standard solutions of 7 quinolones (without SDS) were spiked to make final amounts equivalent to 0.01, 0.1, 0.4, and 2.0 ppm (0.001, 0.01, 0.04, and 0.20 ppm for DNFX). Recovery tests were conducted for 3 times as well as the blank test to collect the rate of additive recovery.

VIII. *Limits of Detection*

Samples, spiked with different concentrations of standard solutions respectively, were analyzed by the method established in this research. Detection wavelengths of PDA were 260 and 268 nm. The detection wavelength of fluorescence detector was set to program. Limits of detection were estimated using a peak area ratio of signal and noise (S/N ratio) greater than 3 as the criteria for determination.

RESULTS AND DISCUSSION

I. *Study on the HPLC Conditions*

(I) *Column selection*

In the reverse phase HPLC analysis, quinolones are absorptive to ODS column causing peak tailing. It may be due to metal impurity or silanol residues of stationary phase[14,24,25]. Horie *et al.*[7] presumed that it was caused by the chelate formation from bonding of metal ion with the carboxyl group of C-3 and the oxygen atom of C-4, while Ikai *et al.*[14] considered that it was caused by silanol residues of stationary phase. To eliminate this problem, we used the column that uses high purity of silane base or is end capped was used for this kind of experiment[4,7,8,14]. Preliminarily 3 kinds of columns, Cosmosil 5C18-AR-II (Nacalai Tesque), LiChrospher RP-18 (Merck), and Luna 5 μ C18[2] (phenomenex), were compared. It was found that Cosmosil 5C18-AR-II had a better separating results and peak shapes whereas LiChrospher RP-18 resulted in severe peak tailing because it is not end capped.

(II) *Mobile phase selection*

Addition of ionic or acidic modifiers such as acetic acid, hyperchloric acid, or quaternary ammonium salt was able to reduce peak tailing effects[4,14,26]. Horie *et al.* added ion-pairing reagents to mobile phase for analyzing quinolones[8]. According to the method described by Horie *et al.*[8], various concentrations of SDS (2 to 5 mM) were added to acetonitrile: 0.05M NaH_2PO_4 (pH 2.5) (35:65, v/v) to study its effect of separating quinolones. The first generation quinolones are more hydrophobic because they are neutral compound (pka $\approx$ 6) in acidic condition, so they are not affected by ion-pairing reagents. On the other hand, the second generation quinolones contain two ionizable groups, carboxylic acid and piperazine, so they are in the form of cation in acidic condition, and their polarity is stronger. The capacity factor (K') will increase along with SDS concentration. The result showed that the addition of 3.5 mM SDS has better separating effect (Figure 2).

(III) *Selection of detection wavelengths*

The most common detection methods for analyzing quinolones by HPLC are with a PDA and a fluorescence detector[1,2,8]. Except PMA, other quinolones all have fluorescence characteristic[8]. After spectrum scanning on quinolones by PDA and fluorescence spectrophotometer, the maximum absorptive wavelength of UV as well as the excitation and emission wavelength of fluorescence are shown in Table 1. In the case of the maximum absorptive wavelength of UV, besides it is 286 nm for PMA, it is between 241 to 267 nm for the first generation and 284 to 287 nm for the second generation quinolones. To consider the entire situation, detection wavelengths were set to 260 and 286 nm. The excitation/emission wavelength of the first generation quinolones, OXA, NA, and FMQ, are all Ex 327/Em 367 nm. Besides it is Ex 278/Em 442 nm for SRFX, it is both Ex 295/Em 446 nm for the second generation quinolones, DNFX and ERFX. The fluorescence

Table 1. Optimal detection wavelength for the 7 quinolone antibacterials using the UV and the fluorescence

Quinolone	Peak number	UV (nm)	Fluorescence (nm)	
			Excitation	Emission
OXA	1	267	327	367
NA	2	258	327	367
FMQ	3	241	327	367
DNFX	4	287	295	446
PMA	5	286	*	*
ERFX	6	284	295	446
SRFX	7	287	278	442

* PMA's fluorescence quantum yield too low to be detected.

wavelength in this experiment was set to time program in 3 Ex/Em detection wavelengths, Ex327/Em 367 nm for OXA, NA, and FMQ, Ex 295/Em 446 nm for DNFX and ERFX, and Ex 278/Em 442 nm for SRFX. PMA has a very weak fluorescence characteristic and cannot be detected, but the other 6 quinolones can be detected simultaneously at maximum sensitivity. Figure 3 shows the chromatograms of 7 quinolones standard solutions by UV at 260 and 268 nm and fluorescence detection. The peaks are symmetrical and the separating results are good.

II. *Sample Preparation*

(I) *Solvent extraction*

According to literature, acetonitrile has the best capability to extract quinolones while ethyl acetate, dichloromethane, and methanol do not[1,16,27]. Preliminarily 2 ppm (0.2 ppm of DNFX) of quilonones was spiked into 5 g of chicken muscle, chicken liver, and eel, then extracted with 30 mL of acetonitrile with mix and homogenization. After partitioned to remove fat by acetonitrile-saturated n-hexane, the lower layer was dried by depressurized concentration then dissolved in mobile phase followed by HPLC analysis. The results (Figure 4) show that average recoveries of the first generation quinolones (95.9%) are better than the second generation (84.6%). It is caused by piperazini(y)l moiety of the second generation quinolones. Since piperazini(y)l moiety has two pka ($pKa_1 \approx 6$ and $pka_2 \approx 9$), it is in zwitterionic form at neutral condition so it is difficult to be extracted with polar solvent (such as acetonitrile)[1]. However, acetonitrile containing water is an effective solvent for extraction in acidic condition (cationic form) and basic condition (anionic form)[1,8,16]. Recoveries of the extraction method, which used basic solvent (acetonitrile and buffer of pH 9.1) by Yorke and Froc were between 59 and 77%[1]. It cannot match the criteria for establishing an analytical method. After comparing the extraction capability of two acidic solvents, 0.2% metaphosphoric acid: acetonitrile (7:3, v/v)[8] and 0.3% metaphosphoric acid: acetonitrile (1:10, v/v)[16], the pH values of those two solvents are 2.7 and 4.1, respectively, the result shows that 0.3% metaphosphoric acid: acetonitrile (1:10, v/v) was better (Figure 4). Recoveries of 7 quinolones spiked into chicken muscle, chicken liver and eel were between 91.0 to 99.8 %.

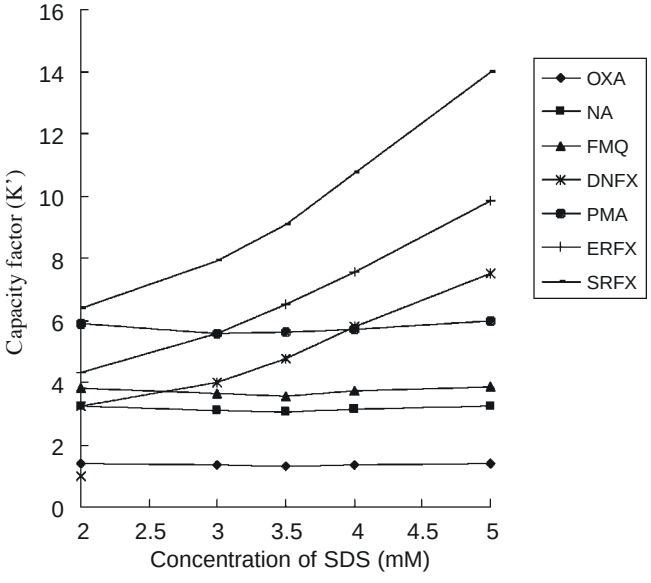

Figure 2. Effect of SDS concentration on the retention time of 7 quinolone antibacterials.
HPLC conditions: Cosmosil-5C18-AR-II; mobile phase, acetonitrile: 0.05 M NaH_2PO_4 (pH 2.5) (35:65) containing 2-5 mM SDS; flow rate, 1.0 mL/min.

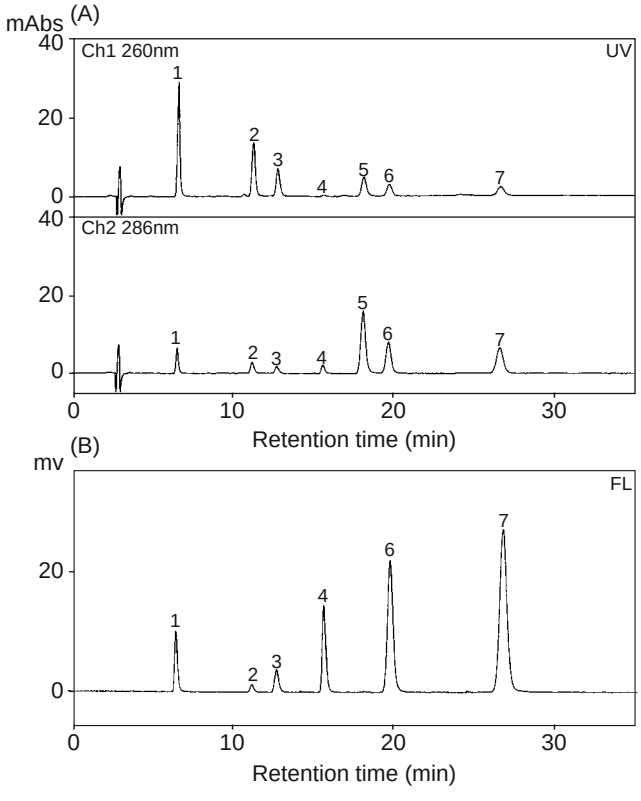

Figure 3. HPLC chromatograms of 7 quinolone antibacterials by (A) photodiode array detector and (B) fluorescence detector.
HPLC conditions: Cosmosil 5C18-AR-II; mobile phase, acetonitrile : 0.05M NaH_2PO_4 (pH 2.5)(35:65) containing 3.5 mM SDS; flow rate, 1.0 mL/min. Peak identification: 1, OXA (40 ng); 2, NA (40 ng); 3, FMQ (40 ng); 4, DNFX (4 ng); 5, PMA (40 ng); 6, ERFX (40 ng); 7, SRFX (40 ng).

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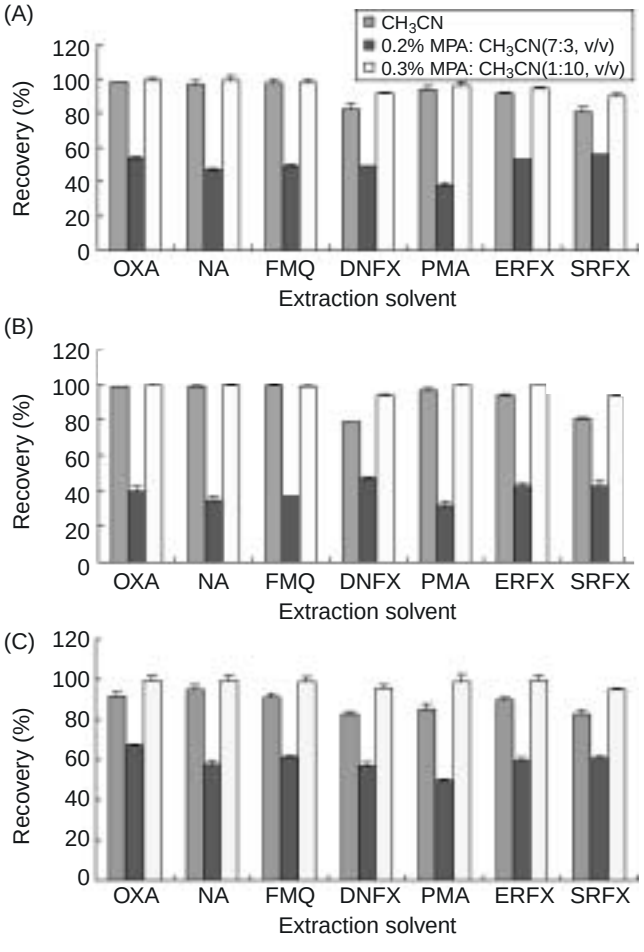

Figure 4. Recoveries of 7 quinolone antibacterials, each at 2 ppm (0.2 ppm for DNFX) spiked amount into (A) chicken muscle, (B) chicken liver and (C) eel with various solvents.

The pH values of samples in this solvent are all approximately 6, which means the addition of metaphosphoric acid with acetonitrile has the capability of extracting and removing protein.

To study the result of various extraction methods, 2 ppm (0.2 ppm of DNFX) of quilonones was spiked into 5 g of chicken muscle, chicken liver, and eel then 30 mL of 0.3% metaphosphoric acid: acetonitrile (1:10, v/v) was added followed by (1) mixing for 3 min at 10,000 rpm in a homogenizer (Nihonseiki Kaisha Ltd., Tokyo, Japan), (2) shaking on a shaker (Hsiangtai Machinery Ind. Co., Ltd., Taipei County) for 10 min, and (3) vortex mixing for 10 min on a vortex mixer (Thermolyne, Iowa, USA), respectively. It was found that the extraction method of mixing in a homogenizer presents the best recovery result (90.0 to 99.2%). Although the shaker and vortex mixer were easy to operate, the recoveries were relatively low (76.7 to 93.5% and 64.4 to 90.9%, respectively). Therefore, the method of mixing in a homogenizer was used for extraction in this research.

(II) *Clean up procedure*

Most clean-up procedures included n-hexane to remove fat and fat-soluble impurity then a cartridge to clean up. Previously cartridges applied were C18[2,8,20] or Baker 10 amino[14], and the elution solutions were methanol[8], 70% methanol[20], or methanol: 1 mM KH_2PO_4 (pH 2.5) (1:1, v/v)[2].

Samples of chicken liver were used for studying the method of clean-up and elution because livers contain the most impurity among animal tissues. Samples of 5 g chicken liver were extracted based on procedures described above. To avoid errors caused by extraction procedures, 2 ppm (0.2 ppm of DNFX) of quilonones was spiked into samples after extraction. Preliminarily the clean up method by Horie *et al.*[8] was adopted. The extracted solution was concentrated to approximately 10 mL then loaded to Bond Elut C18 cartridge. After the sample was washed with water and eluted with methanol, the eluent was analyzed by HPLC. It was found that quinolones were not completely absorbed by Bond Elut C18 cartridge. It may be caused by residual acetonitrile in concentrated solution. Therefore, the extracted solution was dried then dissolved in water. After washed with 10% methanol, quinolones were absorbed by cartridge completely, also the interference of impurity decreased. Subsequently methanol[8] and 70% methanol[20] were tested for elution, respectively. Figure 5 shows that both solvents were good for eluting the first generation quinolones (recoveries were greater than 87.1%), but were not good for eluting the second generation quinolones (recoveries were between 54.3 to 75.9%). Presumably it is caused by strong absorption to cartridge from the zwitterionic form of piperazini (y) l moiety at neutral condition. Thus 70% methanol was slightly modified by substituting water with phosphate buffer in mobile phase, that is, the cartridge was eluted with methanol: 0.05 M NaH_2PO_4 (pH 2.5) (70:30, v/v). The results showed that recoveries of all 7 quinolones increased (88.8 to 99.3%) (Figure 5). It may be caused by weak absorption to cartridge from the cationic form of the second generation quinolones at acidic condition, so they are easily

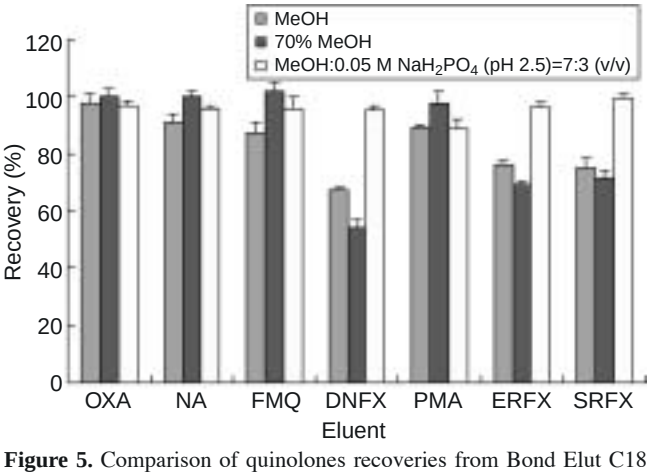

Figure 5. Comparison of quinolones recoveries from Bond Elut C18 cartridge eluted with various solvents.

eluted from Bond Elut C18.

In this research, the retention capability to quinolones of 4 kinds of C18 cartridges was compared, including Bond Elut (200 mg and 500 mg), Sep-Pak (360 mg), and J. T. Baker (500 mg). It was found that quinolones were strongly absorbed by all 4 kinds of cartridges. Among the same brand of cartridges such as Bond Elut, small bed volume (200 mg) and large bed volume (500 mg) have the similar retention capability. However, Sep-Pak C18 cartridge (360 mg) is not as good. It appeared that the retention capability to quinolones is not determined only by the amount of bed volume. The particle shape, pore size, carbon amount, and end capping of packing material have to be considered as well. Based on the discussion above, Bond Elut C18 was used for this research. In addition, elution with methanol: 0.05 M NaH_2PO_4 (pH 2.5) (70:30, v/v) was also tested. The eluent was collected in sequential fractions followed by HPLC analysis. Quinolones were almost all eluted within 8 mL. Thus 10 mL of methanol: 0.05 M NaH_2PO_4 (pH 2.5) (70:30, v/v) was used for elution in this research. In conclusion, the overall scheme of this method was shown in Figure 6.

(III) *Standard curves*

The mixed standard solution of quinolones was prepared to concentrations of 0.05, 0.1, 0.5, 2, 5, and 10 μg/mL (0.005, 0.01, 0.05, 0.2, 1, and 2 μg/mL for DNFX). Standard curves were plotted based on the HPLC analysis with PDA and fluorescence detector according to procedures described in the section of Materials and Methods. Because the PDA is less sensitive, 0.005 mg/mL of mixed standard solution was not included. Linear regression equations of standard curves for 7 quinolone from the HPLC analysis with these two detectors were shown in Table 2. Correlation coefficients (R^2) were all above 0.999, which means the linear correlation is good.

(IV) *Recovery and limit of detection*

To chicken muscle, chicken liver, swine muscle, swine liver, eel, and sweet fish, 0.01, 0.1, 0.4, and 2.0 ppm (0.001, 0.01, 0.04, and 0.20 ppm for DNFX) of 7 quinolones were spiked respectively, then the recovery tests were performed based on the method established in this research. Recoveries were calculated base on the best detection condition, that is, UV 260 and 286 nm for NA and PMA, respectively, and fluorescence detection for the other 5 quinolones. To match the limits for residue of SRFX in chicken muscle (below 0.01 ppm)[13], that concentration level was the lowest additive amount. NA, FMQ, and PMA cannot be detected because their concentrations were lower than the limit of detection. Results (Table 3) show that average recoveries at low concentration were between 74.3 and 85.5%, while the average recoveries of the other 3 concentrations were between 80.1 and 99.9%. All coefficients of variation were less than 5.8%. Figure 7 illustrates the HPLC chromatograms of 7 quinolones spiked into chicken

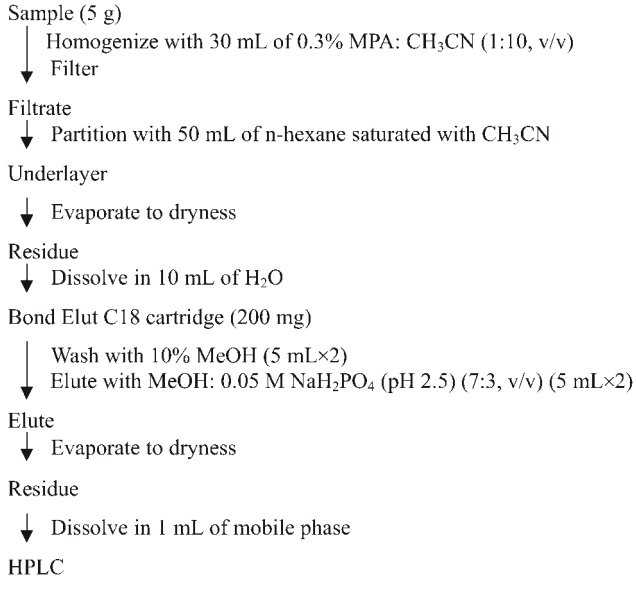

Figure 6. Analytical procedure for quinolone antibacterial residues.

Table 2. Linear regression equations and correlation coefficients of calibration curves for quinolone antibacterials

Quinolone[a]	Detection	Linear equation	Correlation coefficient
	Fluorescence		
OXA	Ex 327/Em 367 nm	$Y = 6.70\times10^4 X - 54$	0.9994
NA	Ex 327/Em 367 nm	$Y = 1.08\times10^4 X - 1014$	1.0000
FMQ	Ex 327/Em 367 nm	$Y = 4.10\times10^4 X - 4766$	0.9996
DNFX	Ex 295/Em 446 nm	$Y = 1.19\times10^6 X - 12233$	1.0000
ERFX	Ex 295/Em 446 nm	$Y = 2.66\times10^5 X - 13016$	0.9999
SRFX	Ex 278/Em 442 nm	$Y = 3.97\times10^5 X - 18657$	0.9999
	Ultraviolet		
OXA	260 nm	$Y = 1.90\times10^5 X + 4687$	0.9995
NA	260 nm	$Y = 1.33\times10^5 X - 6318$	0.9999
FMQ	260 nm	$Y = 8.64\times10^4 X - 1665$	0.9999
DNFX	286 nm	$Y = 1.78\times10^5 X - 1378$	1.0000
PMA	286 nm	$Y = 2.26\times10^5 X - 30396$	0.9996
ERFX	286 nm	$Y = 1.14\times10^5 X - 7362$	1.0000
SRFX	286 nm	$Y = 1.09\times10^5 X - 8211$	1.0000

a: The concentration ranges from 0.005 to 2.0 μg/mL for DNFX and from 0.05 to 10.0 μg/mL for the rest.

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Table 3. Recoveries of 7 quinolones spiked into chicken muscle, chicken liver, swine muscle, swine liver, eel and sweet fish

Tissue source	Quinolone	Recovery [a] (%)			
		0.01 ppm	0.1 ppm	0.4 ppm	2.0 ppm
Chicken muscle	OXA	81.4 (3.8)[b]	85.2 (5.4)	82.5 (0.3)	93.1 (2.1)
	NA	N.D.[c]	86.6 (3.3)	90.8 (4.1)	94.0 (1.9)
	FMQ	N.D.	95.5 (1.5)	92.2 (0.5)	96.9 (1.7)
	DNFX [d]	83.2 (2.1)	89.2 (0.9)	84.3 (0.3)	86.8 (1.2)
	PMA	N.D.	90.1 (1.5)	90.8 (1.8)	93.7 (1.4)
	ERFX	84.5 (3.2)	89.8 (1.4)	87.2 (0.6)	90.2 (1.2)
	SRFX	81.3 (3.5)	87.8 (1.4)	85.4 (1.0)	88.2 (1.5)
Chicken liver	OXA	82.9 (5.4)	83.0 (1.5)	89.7 (2.5)	93.4 (1.3)
	NA	N.D.	82.7 (3.2)	97.3 (3.2)	96.6 (1.7)
	FMQ	N.D.	85.4 (1.5)	96.1 (3.9)	93.8 (2.3)
	DNFX [d]	75.2 (5.8)	86.8 (3.2)	84.2 (3.1)	84.9 (1.0)
	PMA	N.D.	92.0 (0.4)	88.2 (2.9)	91.6 (1.5)
	ERFX	80.2 (4.8)	90.4 (2.8)	88.5 (3.8)	91.0 (0.9)
	SRFX	78.7 (5.0)	82.2 (2.3)	82.7 (1.4)	84.6 (1.2)
Swine muscle	OXA	81.2 (3.8)	85.4 (3.4)	98.3 (1.3)	94.5 (0.3)
	NA	N.D.	91.4 (1.7)	98.5 (2.1)	94.5 (0.5)
	FMQ	N.D.	94.6 (2.6)	92.4 (2.2)	94.9 (0.7)
	DNFX [d]	79.9 (5.1).	85.5 (1.1)	88.2 (0.3)	85.7 (0.2)
	PMA	N.D.	90.5 (1.1)	89.3 (2.1)	91.9 (1.2)
	ERFX	85.5 (4.7)	90.7 (3.2)	93.6 (2.8)	90.9 (0.3)
	SRFX	80.2 (3.2)	86.2 (1.5)	84.5 (2.7)	87.1 (0.6)
Swine liver	OXA	76.9 (3.4)	89.6 (3.2)	89.2 (2.8)	91.9 (3.5)
	NA	N.D.	88.4 (4.3)	97.2 (3.9)	92.0 (2.3)
	FMQ	N.D.	96.1 (5.0)	93.6 (3.0)	92.5 (4.4)
	DNFX [d]	81.1 (5.4)	81.2 (3.9)	80.5 (1.6)	82.1 (1.1)
	PMA	N.D.	96.4 (4.6)	88.2 (2.2)	89.8 (2.2)
	ERFX	82.2 (3.9)	87.6 (1.8)	89.1 (2.5)	89.7 (1.3)
	SRFX	79.9 (4.2)	80.3 (0.8)	80.1 (1.3)	82.2 (1.7)
Eel	OXA	75.4 (2.1)	82.0 (4.1)	85.9 (4.1)	94.9 (0.3)
	NA	N.D.	94.1 (2.3)	83.1 (4.1)	93.3 (0.4)
	FMQ	N.D.	93.3 (3.3)	92.2 (0.1)	94.8 (0.3)
	DNFX [d]	82.3 (3.2)	90.7 (2.8)	87.2 (1.2)	87.4 (0.5)
	PMA	N.D.	91.0 (0.7)	92.3 (2.0)	93.1 (0.7)
	ERFX	79.5 (2.7)	89.9 (0.8)	91.2 (0.2)	92.6 (0.5)
	SRFX	80.3 (4.2)	83.3 (1.5)	85.5 (0.2)	88.4 (0.5)
Sweet fish	OXA	74.3 (4.6)	84.0 (4.8)	99.3 (3.1)	96.3 (2.1)
	NA	N.D.	95.1 (3.0)	98.2 (2.9)	93.8 (3.0)
	FMQ	N.D.	98.6 (3.3)	99.9 (0.4)	94.3 (0.4)
	DNFX [d]	80.6 (4.2)	86.4 (4.7)	92.9 (2.1)	86.5 (1.8)
	PMA	N.D.	92.7 (1.6)	90.6 (3.8)	93.1 (1.4)
	ERFX	80.2 (3.8)	89.6 (2.0)	91.3 (1.2)	92.2 (0.5)
	SRFX	83.3 (2.9)	82.5 (3.0)	88.6 (0.6)	87.3 (0.3)

a: Average of triplicate. b: Value in parenthesis is the coefficient of variation (CV, %).
c: Not detected. d: The spiked levels were 0.001, 0.01, 0.04 and 0.20 ppm.

muscles and chicken livers. Separation results are good without interference from impurity.

Limits of detection shown in Table 4 were determined by setting a S/N ratio greater than 3 as the criteria. The limits with UV detection were between 0.01 ppm (OXA) and 0.04 ppm (FMQ), while the limits with fluorescence detection were between 0.0006 ppm (DNFX) and 0.05 mg/g (NA). All are lower than the limits for residue of quinolones set by the Department of Health[13]. Except NA and PMA, the sensitivity of fluorescence detector is better than UV detector for all other quinolones (Table 4). Especially for the second generation quinolones, the UV/FL ratios range from 10 times for ERFX and SRFX to 33 times

Table 4. Detection limits of 7 quinolones with UV and fluorescence methods

Quinolone	UV		Programmable fluorescence		UV/FL ratio
	Wavelength (nm)	Detection limit (ppm)	Ex/Em (nm)	Detection limit (ppm)	
OXA	260	0.01	327/367	0.01	1
NA	260	0.02	327/367	0.05	0.4
FMQ	260	0.04	327/367	0.02	2
DNFX	286	0.02	295/446	0.0006	33
PMA	286	0.02	295/446	N.D.[a]	–
ERFX	286	0.02	295/446	0.002	10
SRFX	286	0.02	278/442	0.002	10

a: Not detected.

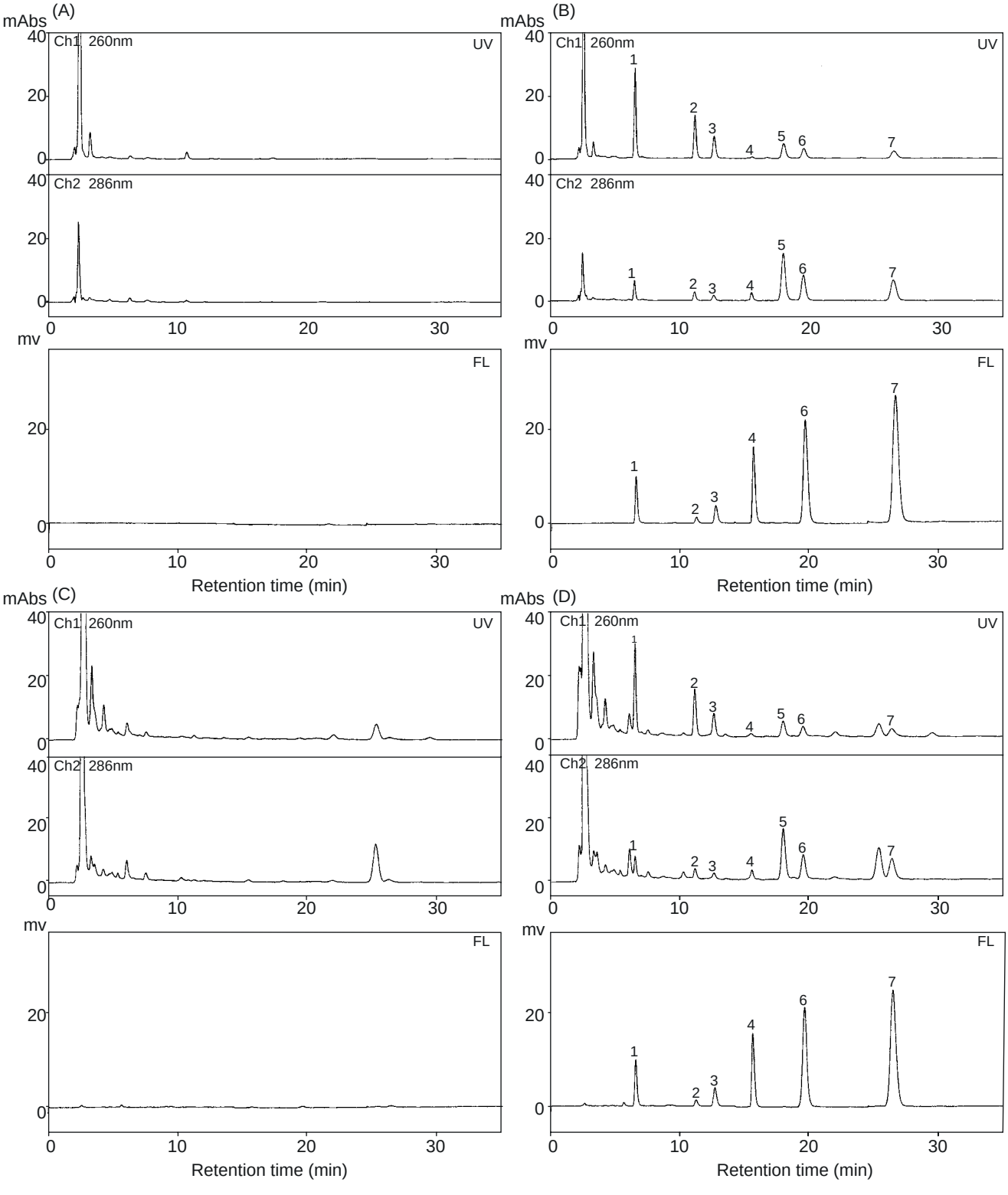

Figure 7. HPLC chromatograms of 7 quinolones spiked into samples at the 0.4 ppm level (0.04 ppm for DNFX). (A) Blank chicken muscle, (B) spiked chicken muscle, (C) blank chicken liver, (D) spiked chicken liver. The performance conditions and peak identification are the same as Figure 3.

for DNFX. However, for the first generation quinolones, the sensitivity differences are smaller, ranging from 0.4 times for NA to 2 times for FMQ. Figure 8 shows the HPLC chromatograms for the detection limits of all 7 quinolones in chicken muscles and chicken livers. This was in contrast with literature where the detection limits of the method published by Hori *et al.*[8] using UV and fluorescence detectors to analyze 8 quinolones were all 0.01

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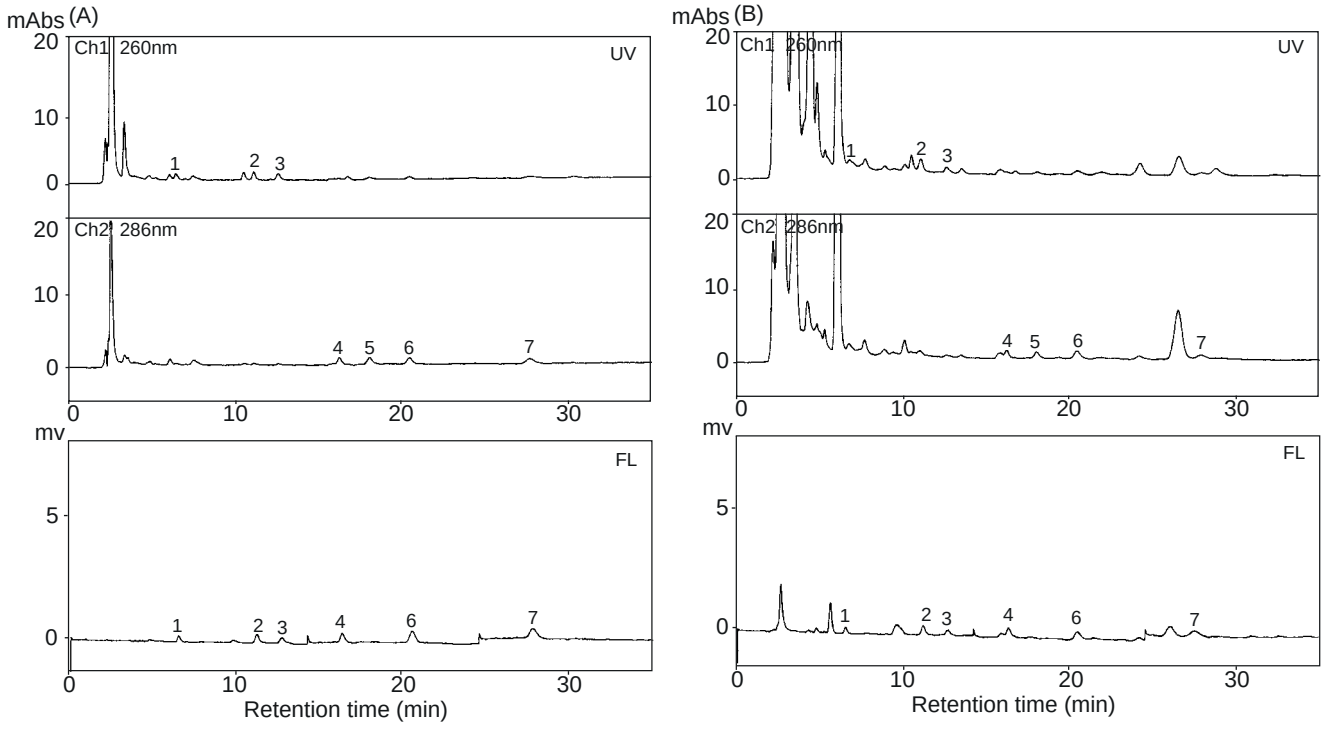

Figure 8. HPLC chromatograms for the detection limits of 7 quinolone antibacterials. (A) Chicken muscle, (B) chicken liver. The performance conditions and peak identification are the same as Figure 3.

ppm. The sensitivities of the method in this present research by PDA (most of them are 0.02 ppm) are lower than the method by Hori *et al*.. It may be due to the lower sensitivity of PDA than UV detector, when fluorescence detection is better than the result of Hori *et al*.. Compared with other methods also using PDA, the detection limit of ERFX (0.02 ppm) is similar to the method published by Gigosos *et al*.[2]. The sensitivities of fluorescence detection are similar to the method by Yorke and Forc (0.0005 to 0.035 ppm) as well[1].

(V) *Intra-day and inter-day repeatability*

To find out more about the stability of this analysis system, 3 concentrations of mixed standard solutions of quinolones were used for analyzing the intra-day and inter-day repeatability. Each concentration was analyzed 3 times for intra-day repeatability. For inter-day repeatability, each concentration was analyzed 3 times for 3 days using daily prepared standard solutions and mobile phase, that is, each concentration was analyzed for 9 times. Results (Table 5) show that intra-day coefficients of variation were less than 3.29% and inter-day coefficients of variation were less than 5.23%, which means the precision of the method in this research is acceptable.

(VI) *Analysis of the contents of quinolone residues in livestock and marine products purchased from markets*

Ten samples each of swine muscle and swine liver, 18 samples of chicken muscle (8 from ordinary chicken and 10 from wu ku chicken), 15 samples of chicken liver (10 from ordinary chicken and 5 from wu ku chicken), 5 eels, and 5 sweet fish were analyzed by the method described in this research. Results (Table 6) show that ERFX ranging from 0.08 to 4.04 ppm (average 0.12 ± 0.13 ppm) was found in 9 wu ku chicken muscle samples. The ratio of positive

Table 5. Intra-day and inter-day repeatability of 7 quinolones

Quinolone	Concentration (μg/mL)	Intra-day (CV, %)(n=3)	Inter-day (CV, %)(n=9)
OXA	0.5	2.04	3.25
	2.0	0.43	1.85
	10.0	0.42	0.50
NA	0.5	2.43	1.83
	2.0	0.66	1.37
	10.0	1.48	0.19
FMQ	0.5	3.29	5.23
	2.0	1.77	2.43
	10.0	1.81	4.54
DNFX	0.05	2.07	2.43
	0.2	0.35	0.66
	2.0	0.23	1.48
PMA	0.5	1.02	1.13
	2.0	0.40	0.95
	10.0	0.10	0.28
ERFX	0.5	0.93	1.10
	2.0	0.12	0.28
	10.0	0.36	1.62
SRFX	0.5	1.06	3.20
	2.0	0.25	0.62
	10.0	0.17	0.33

Table 6. Summary of quinolone residues in chicken muscle, chicken liver, swine muscle, swine liver, eel and sweet fish purchased from various markets

Category	Type of quinolone detected	No. of violated samples	Ratio of violated samples (%)	Quinolone[a] (ppm)
Chicken muscle	ERFX	9 (18)[b]	50	1.08 ± 1.23[c] (0.08~4.04)[d]
Chicken liver	ERFX	4 (15)	26.7	0.12 ± 0.13 (0.01~0.27)
Swine muscle	N.D.[e]	0 (10)	0	N.D.
Swine liver	N.D.	0 (10)	0	N.D.
Eel	N.D.	0 (5)	0	N.D.
Sweet fish	OXA	3 (5)	60	0.24 ± 0.11 (0.13~0.35)
Total		16 (63)	25.4	

a: Average of triplicate.
b: Total number of samples.
c: Mean ± S.D. of detected samples.
d: Data represent the range of values from detected samples.
e: Not detected.

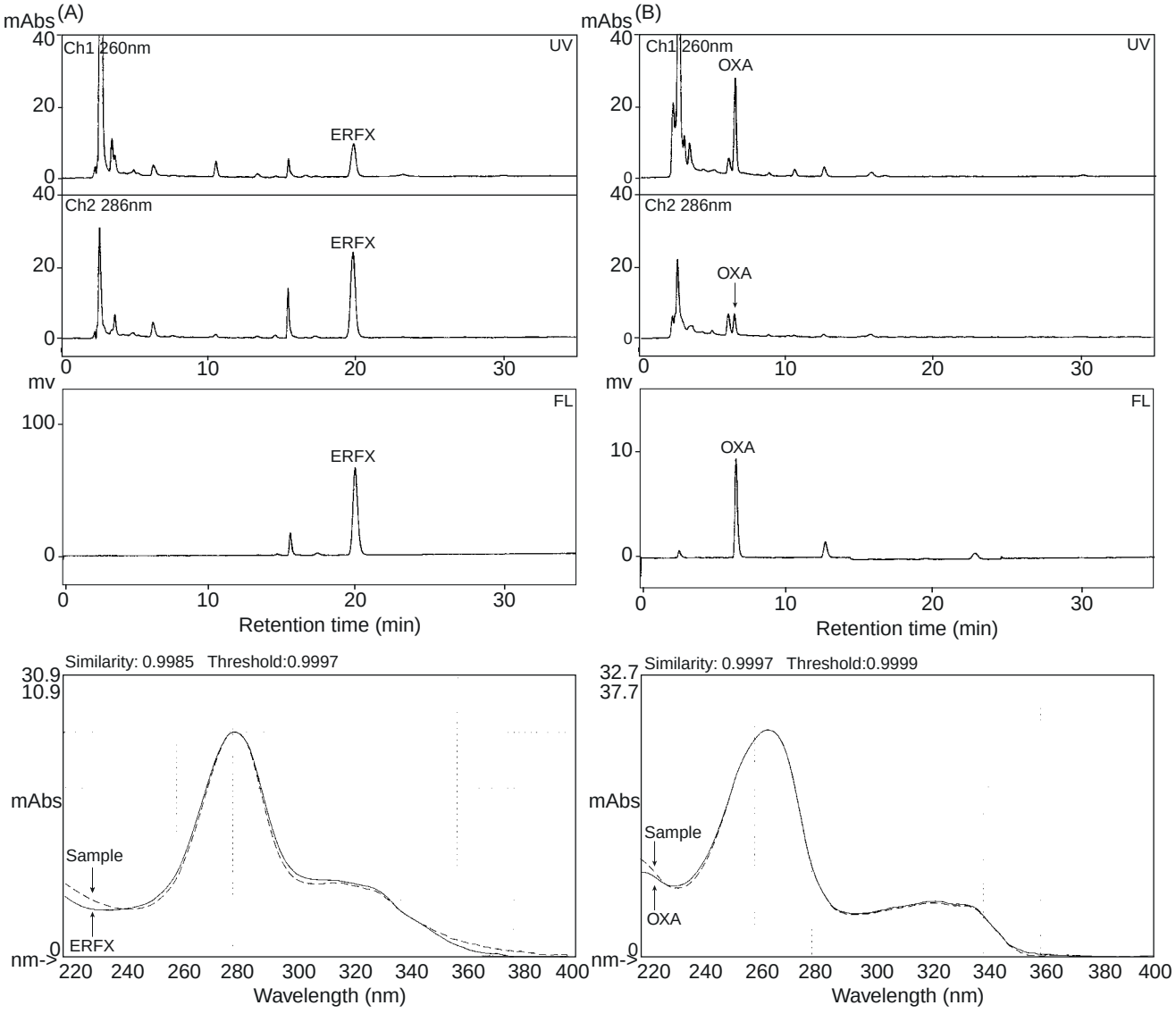

Figure 9. HPLC chromatograms of wu ku chicken muscle sample with ERFX residue of 1.08 ppm (A), and sweet fish sample with OXA residue of 0.35 ppm (B). UV-absorption spectrum of the peak obtained from chicken muscle sample (dashed line) compared with standard ERFX (solid line)(C), and spectrum of the peak obtained from sweet fish sample (dashed line) compared with standard OXA (solid line)(D).

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ERFX identification is 50% of all chicken muscle samples. Also ERFX ranging from 0.01 to 0.27 ppm (average 0.12 ± 0.13 ppm) were found in all 4 wu ku chicken livers. That is, samples tested positive for ERFX are all from we ku chicken. In addition, 0.13, 0.25, and 0.35 ppm (average 0.24 ± 0.11 ppm) of OXA were found in 3 samples of sweet fish, respectively. Quinolone residues were not found in all other samples. HPLC chromatograms and UV-absorption spectra of chicken muscle and sweet fish samples positive for ERFX or OXA are shown in Figure 9. PDA was applied in this method. Compared the UV-absorption spectra with the standard solutions, the similarity reaches 0.9985 for ERFX and 0.9997 for OXA. These are further criteria for identification.

According to regulations in the "Tolerances for residues of veterinary drugs"[13] promulgated by the Department of Health, residues of ERFX and OXA are not allowed in chicken, pork, and fish. Thus 25.4% of samples violated the regulations, that is, some farmers did not follow regulations for veterinary drugs regarding application, dosage, and off-drug[28]. At present, the Codex Alimentarius Commission has not set limits for residues of OXA and ERFX[29]. However, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at its 48th meeting in 1997 has decided that the acceptable daily intake (ADI) of ERFX is 0~2 μg/kg bw[30].

(VII) *Tissue levels and stability of residual ERFX after refrigeration in wu ku chicken muscles*

Among various tissues of farmed sweet fish, liver contains the highest amount of OXA residues, followed by kidney, then muscle[8]. The authors have not found any report related to residual ERFX in chicken, pork, or fish. Thus the following discussion is focused on ERFX positive samples of wu ku chicken muscle.

Among 4 samples of wu ku chicken, tissue levels of ERFX in skin, muscle, and liver are shown in Table 7. Chicken skin contains the highest amount of ERFX (0.21 to 2.87 ppm), followed by muscle (0.08 to 1.54 ppm), then liver (0.01 to 0.27 ppm). These results are different from drug levels found in animal tissues, among which liver usually contains a higher amount. Subsequently 4 samples of wu ku chicken muscle above were stored at 4°C for 1, 4, and 7 days to test the stability of ERFX. The level of residual ERFX in samples did not decrease after 7 days (Figure 10). That is, the residual ERFX in samples of wu ku chicken muscle did not easily degrade during refrigerated transportation and storage (4°C) process.

Table 7. ERFX levels in wu ku chicken tissue

No. of sample	Concentration[a] (ppm)		
	Muscle	Skin	Liver
1	1.16 (4.8)[b]	2.37 (1.3)	0.27 (0.8)
2	1.54 (3.9)	2.87 (4.3)	0.18 (3.7)
3	0.10 (4.9)	0.25 (2.5)	0.01 (2.1)
4	0.08 (3.9)	0.21 (4.3)	0.01 (3.9)

a: Average of triplicate.

b: Value in parenthesis is the coefficient of variation (CV, %).

CONCLUSION

A high performance liquid chromatographic (HPLC) method for simultaneous determination of OXA, NA, FMQ, PMA, DNFX, ERFX, and SRFX in livestock and marine products was developed. The peaks are symmetrical. The separation results and the repeatability are good. Average recoveries of low concentration of all 7 quinolones ranged from 74.3 to 85.5% and those of the rest ranged from 80.1 to 99.9% with coefficients of variation less than 5.8%. The detection limits of quinolones were 0.01~0.04 ppm with UV detection and 0.0006~0.05 ppm with fluorescence detection. Using this method to detect quinolones in 63 samples of chicken, pork, and fish purchased from markets, ERFX residue was found in 9 wu ku chicken muscle samples and 4 wu ku chicken liver samples. Three sweet fish samples contained OXA residue. The results indicated that 25.4% of the samples violated the regulations. The residual ERFX in wu ku chicken muscle is very stable even after long refrigeration at 4°C. The method in this research is accurate and reliable, so it can be applied to routine analytical work. It is suggested that this method be promulgated as the standard analytical method for laboratories which are responsible for such work. In addition, the screening study showed that residual quinolone antibaterials were found in livestock and marine products purchased from markets.

These findings can be provided to health authorities as a reference for improvements on administration and regulation.

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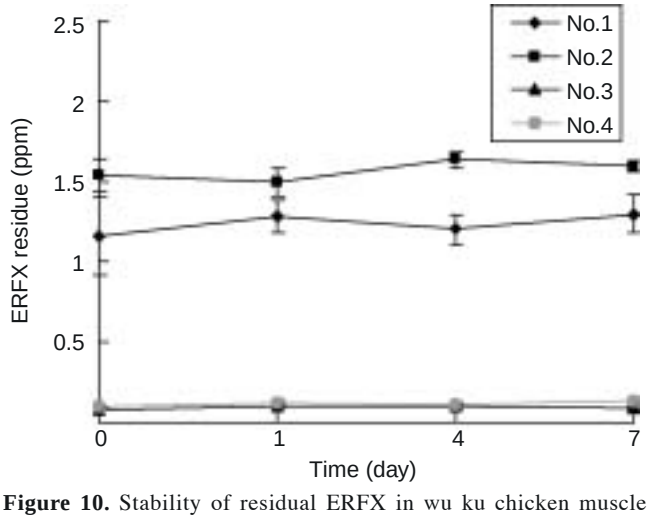

Figure 10. Stability of residual ERFX in wu ku chicken muscle samples during refrigeration at 4°C.

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