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**A Dissertation of the Degree of Doctor of Philosophy**

**Development of multi-residue analytical methods for  
quinolones, cephalosporins, and trimethoprim and  
application to the residue monitoring in livestock  
and marine products in Korea**

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항생제에 대한 동시분석법 개발 및 잔류실태조사

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## **ABSTRACT**

### **Development of multi-residue analytical method for quinolones, cephalosporin, and trimethoprim and application to the residue monitoring in livestock and marine products in Korea**

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Three reliable analytical methods were developed based on a simple and rapid sample preparation followed by ultra performance liquid chromatography with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) for the determination of 13 quinolones, 9 cephalosporins, and trimethoprim in livestock and marine products (beef, pork, chicken, milk, egg, flatfish, jacobever, common eel, and shrimp). The proposed methods were validated according to the CODEX guidelines and applied in the survey of in a total of 1012 samples of livestock and marine products commercialized in Korea.

In the first study, a simple and specific analytical method was developed for the simultaneous determination of 13 quinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, ofloxacin,

orbifloxacin, oxolinic acid, pefloxacin, and sarafloxacin) using UPLC-ESI-MS/MS and all results fully complied with CODEX recommendation. Good linearities were achieved and the correlation coefficients were ranged between 0.9992 and 0.9999. The recoveries of 13 quinolones were higher than 80%, the limit of detection (LOD) and limit of quantitation (LOQ) were lower than 0.1 and 0.4 µg/kg, respectively. Besides, the matrix-matched calibration curve and internal standard (IS) played a significant role in compensating for the matrix effects. A survey for 13 quinolones residues was conducted on 310 livestock and marine products. Oxolinic acid, enrofloxacin, and flumequine were the most commonly detected antibiotics. The residues of quinolones were detected on 104 samples (33.5% incidence) but residue levels were below the MRLs. Detected concentrations were 0.1-100.0 µg/kg and the risk value (EDI/ADI, %) were safe levels between 0.0005% and 0.612%. However, pefloxacin was detected in one common eel sample above the legal residue limit and the detected concentration was 62.4 µg/kg.

In the second study, a sensitive and reliable method was developed and validated for the simultaneous determination of 9 cephalosporins (cefacetrile, cefazolin, cephapirin, desacetyl cephapirin, cefalexin, cefalonium, cefoperazone, cefuroxime, and cefquinome)

and all obtained results were satisfied with CODEX recommendation. The use of UPLC-ESI-MS/MS with polarity switching ionization mode improved the sensitivity and reduced analysis time, allowing the identification and quantification of 9 cephalosporins in 5 min. Because of the matrix effects, matrix matched calibration curves with IS were used for quantification to determine cephalosporin residues in samples. Good linearities were acquired and the LOD and LOQ were lower than 8 and 25  $\mu\text{g}/\text{kg}$ , respectively. The survey for the 9 cephalosporins residues was conducted on 333 livestock and marine products. The residues were detected in only 12 livestock products (3.6% incidence) but residue levels were below the MRLs and the detected cephalosporins were cefalonium in beef and cefquinome in milk. Detected concentrations were from 1.10 to 9.77  $\mu\text{g}/\text{kg}$  in beef and 7.50  $\mu\text{g}/\text{kg}$  in milk. The risk value was very safe level of 0.02% in cephalonium and 0.01% in cefquinome.

In the third study, a specific and simple analytical method was developed using UPLC-ESI-MS/MS for the determination of trimethoprim. The sample preparation through the ultrasonic-assisted extraction and SPE clean-up procedure was improved the recovery and reduced the matrix effect. The recoveries were higher than 70% and the LOD and LOQ

were lower than 0.3 and 1.0 µg/kg, respectively. The survey for the trimethoprim residue was conducted on 369 livestock and marine products. The residues of trimethoprim were detected in 7 marine products (1.9% incidence) but residue levels were below the MRL. Detected concentrations were 1.17 to 16.43 µg/kg in jacoever, 40.0 µg/kg in flatfish, and 13.3 µg/kg in common eel. The risk value was safe level of 0.13%.

In conclusion, the methods developed in this study were more reliable and accurate for screening, quantification, and identification of 13 quinolones, 9 cephalosporins, and trimethoprim residues in livestock and marine products and could be successfully applicable in field samples. The resulting residue level of 13 quinolones, 9 cephalosporins, and trimethoprim appeared to be relatively safe. However, the quinolones and 3<sup>rd</sup> - & 4<sup>th</sup> - generation cephalosporins were classified as critically important antimicrobials for human medicine (CIA) and trimethoprim and 1<sup>st</sup> - & 2<sup>nd</sup> -generation cephalosporins were classified as highly important (HIA) from World Health Organization (WHO). In addition, quinolones, cephalosporins, and trimethoprim were classified as veterinary critically important antimicrobials (VCIA) from the Office International des Epizooties (OIE). Thus, a strict guideline for the use of antibiotics and continuous survey on antibiotic

residues is needed to ensure the safety of animal origin foods. The methods developed in this study will help to implement such guidelines of CODEX on the use of antimicrobials.

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Keywords: UPLC-MS/MS, mass spectrometry, monitoring, antibiotic residue

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## ABBREVIATION

ADI	Acceptable daily intake
CIA	Critically important antimicrobials for human medicine
EDI	Estimated daily intake
ESI	Electrospray ionization
HLB	Hydrophile-lipophile balance
IS	Internal standard
LOD	Limit of detection
LOQ	Limit of quantitation
MFDS	Ministry of food and drug safety
MRL	Maximum residue limit
MRM	Multiple reaction monitoring
MSPD	Matrix solid-phase dispersion
OIE	Office international des epizooties
QuEChERS	Quick easy cheap effective rugged and safe
RSD	Relative standard deviation
SPE	Solid-phase extraction
UPLC-ESI-MS/MS	Ultra performance liquid chromatography with electrospray ionization tandem mass spectrometry
VCIA	Veterinary critically important antimicrobials

## General Introduction

As the food culture in Korea changes from traditional Korean style to western one, the consumption of foods originated from livestock, poultry, and fish increased dramatically for last decades, and the transition forced livestock and aquaculture industry turned into large and dense breeding system. Antibiotics have been administered to animals for prevention and treatment of infectious diseases and widely used at sub-therapeutic levels for growth promotion of livestock and aquaculture farms. The global consumption of antibiotics for animals has been estimated to be between 100,000 and 200,000 tons/year (Zucatto et al., 2010). Although the annual sales of antibiotic in Korea was greatly decreased from 1,450 ton in 2006 to 630 ton in 2014 (KAHPA, 2015), the sales of phenicols and cephalosporins increased by 1.9 and 2.3 times from 2006 to 2014, respectively (QIA, 2015). Antimicrobial treatments have several benefits, but when large quantities of specific types of antibiotics are supplied, adverse effects, such as appearance of drug resistant microorganisms, can be manifested (Blasco et al., 2009). In Korea, resistance against tetracyclines have remained high ( $\geq 75\%$ ) and resistances against

ciprofloxacin, chloramphenicol, ceftiofur, and trimethoprim/sulfamethoxazole have increased steadily since 2008 (QIA, 2015).

Moreover, misuse and overuse of antibiotics could be caused by livestock owners. Since antibiotics have been used with long-term administration in low concentrations, the presence of antibiotic residues in animal originated foods may increase antibiotic resistance of pathogens and can threaten public health. To minimize the exposure of antibiotics to humans, many countries established maximum residue limits (MRLs) for antibiotics residue in food producing animals. The European Union (EU, Commission regulation No. 37/2010, 2010) and the Joint FAO/WHO Expert Committee on Food additives have set up the MRLs in animals producing milk, muscle, fat, liver, and kidney. The Positive List System of Japan contains MRLs in animal and marine products. In Korea, the MRLs have been established from 1990s by the Ministry of Food and Drug Safety and currently, have been set for 185 kinds of antibiotics. Due to the expansion of international regulations on MRLs in recent years, more sensitive and specific analytical methods are required for the determination of antibiotic residues in animal originated foods.

In these studies, multi-residue analytical methods were developed for the determination of 13 quinolones, 9 cephalosporins, and trimethoprim in livestock and marine products using UPLC-ESI-MS/MS. The high selectivity, sensitivity, and versatility of UPLC-ESI-MS/MS have allowed the successful quantification of antibiotic residues in livestock and marine products. Moreover, these studies were applied to monitoring of antibiotic residues in field samples and risk assessments for multi-antibiotic residues were also performed.

# **CHAPTER I**

## **Development of multi-residue analytical method for 13 quinolones and application to the residue monitoring in livestock and marine products in Korea**

## **ABSTRACT**

The aim of this study was to develop and validate a specific ultra performance liquid chromatography with electrospray ionization tandem mass spectrometric method (UPLC-ESI-MS/MS) for simultaneous determination of 13 quinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, ofloxacin, orbifloxacin, oxolinic acid, pefloxacin, and sarafloxacin) in livestock and marine products. The analytical method involved the sample preparation based on solvent extraction without further clean up procedure. All samples were deproteinized with acidified acetonitrile, followed by defatting with acetonitrile-saturated hexane. Because of matrix effects, matrix matched calibrations with internal standard were used for quantification during the determination of the quinolones residues in samples.

The proposed method was validated according to the CODEX guidelines and all results fully complied with CODEX recommendation. Good linearities were achieved and the correlation coefficients ranged between 0.9992 and 0.9999 depending upon antibiotics.

Both the limit of detection and limit of quantitation for this assay were lower than 0.1 and 0.4 µg/kg, respectively, and these values were below the maximum residue limits (MRLs) established by the Korean Food Code. Recoveries (%) were in the ranged of 87.5 to 104.7% for livestock products and 83.0 to 100.9% for marine products, respectively. The relative standard deviations (RSD, %) of repeatability ranged from 0.33 to 3.72 and reproducibility ranged from 0.71 to 6.75 depending upon sample species. This quantitative method has many advantages including simple preparation step, rapid determination, and high sensitivity, which could be applied to the determination and quantification of quinolones residues in livestock and marine products. The monitoring results revealed that quinolones were detected in 104 out of 310 livestock and marine products (33.5% incidence) but residue levels were below the MRLs in the Korean Food Code and the risk value was very safe level. Of samples, 1 common eel containing pefloxacin above the legal residue limit was found, and the detected concentration was 62.4 µg/kg.

## INTRODUCTION

The quinolones and fluoroquinolones are a family of synthetic broad spectrum antibiotics and the most frequently employed. These drugs are administered at therapeutic doses to treat bacterial infections in food-producing animals, such as cattle, swine, turkey, and chicken (Junza et al., 2011) and also used at sub-therapeutic doses as prophylactics. They are active against Gram-negative bacteria as well as Gram-positive bacteria by inhibiting DNA topoisomerase (Scortichini et al., 2009). Nalidixic acid is the first generation of the quinolones approved on 1963, by Food and Drug Administration for the treatment of urinary tract infections (Eleni et al., 2007) and structural modifications have resulted in the second, third, and fourth generations of the (fluoro)quinolones which have improved coverage of Gram-positive organisms (Roberta et al., 2009). Nowadays, they are widely used in the treatment of respiratory diseases and enteric bacterial infections in humans and food-producing animals and aqua-cultured fish (Hermo et al., 2006). Since 2000 there has been a significant progressive increase in the use of the quinolones in animal production (Strolker and Brinkman, 2005; Di Corcia and Nazzari, 2002). It has produced more chance of their residue in the animal origin foods and led to the

emergence of fluoroquinolone-resistant bacterial strains in animals (Rodriguez et al., 2011). It could cause many health problems in humans, such as the transmission of fluoroquinolone-resistant bacteria (e.g. fluoroquinolone-resistant *Campylobacter* and *Salmonella*) to humans since the early 1990s (Fabrega et al., 2011; Okeke et al., 2005).

To minimize the exposure of antibiotics to humans, many countries have been establishing MRLs for quinolones residue in foodstuffs (Table 1). The European Union (EU) has established the MRLs of veterinary drugs in animal origin foods, and some of them are quinolones (Council Regulation No. 2377/90). In addition, the EU and the Joint FAO/WHO Expert Committee on Food additives have set up the MRLs in several animal tissues, milk, and eggs (Rubies et al., 2007). The Italian National Residue Control Plan is very precise: samples taken at slaughterhouse are screened for the presence of residues/metabolites on the basis of MRLs (Luca et al., 2015). According to the Australia and New Zealand Food Standards Code, residues of quinolones cannot be detected in any foodstuff for domestic consumption and the only exception is set for oxolinic acid in pacific salmon at 0.01 mg/kg (Lesley et al., 2002). In Korea, the 13 quinolones are

permitted for prevention and treatment of animal disease. The MRLs and the withdrawal period were established by the Ministry of Food and Drug Safety (MFDS) (Table 2).

As the MRLs of veterinary drugs are very low level, they have been demanded to improve the specific and sensitive analytical methods for the multi-residue analysis. Many published papers have been reported for analysis of the (fluoro)quinolones residues in livestock and marine products. Liquid chromatography (LC) is the most frequently used for separation (Belal et al., 1999; Hernandez-Arteseros et al., 2002), mainly coupled with ultraviolet (UV) (Eleni et al., 2007; Bailac et al., 2004; Pecorelli et al., 2003), or fluorescence detector (Herranz et al., 2007; Zeng et al., 2005). However, some methods either covered only several compounds of (fluoro)quinolone or were time-consuming for sample preparation. Immunoassay (Ashwin et al., 2009; Anne-Catherine et al., 2006) have been sometimes used for analysis of (fluoro) quinolones and provided a rapid screening test. But it is difficult to apply a multi-residual detection. Thin-layer chromatography (TLC) (Juhel-Gaugain and Abjean, 1998), gas chromatography (GC) (Asami et al., 2000), and capillary electrophoresis (CE) (Ana et al., 2006) have been rarely applied.

Recently, several reports are focused on accurate and sensitive confirmatory quantification methods, such as liquid chromatography combined to tandem mass spectrometry (LC-MS/MS). These procedures included only one sample of interest, for instance, milk (Junza et al., 2014; Lina et al., 2011; Hong et al., 2009; Sara Bogialli, 2008), gilthead sea bream (Romero-Gonzalez et al., 2007), egg (Anna et al., 2012; Antonia et al., 2010; Sara et al., 2009), shrimp (Christine et al., 2007), bovine muscle (Rubies et al., 2007), turkey (Lorena et al., 2015), and swine plasma (Garces et al., 2006). Besides, compared to LC, UPLC increased resolution and sensitivity, as well as considerably decreased sample analysis time and mobile phase solvent consumption (Junza et al., 2011; Stolker et al., 2005).

Therefore, the purpose of this study was to develop a rapid and reliable analytical method for the simultaneous determination of 13 quinolones in 9 species of livestock and marine products (beef, pork, chicken, egg, milk, flatfish, jacobever, common eel, and shrimp) using UPLC-ESI-MS/MS. Furthermore, this paper was investigated the residues of 13 quinolones in livestock and marine products commercialized in Korea using UPLC-ESI-MS/MS.

## MATERIALS & METHODS

### *Chemical and materials*

Ciprofloxacin (CIP), danofloxacin (DAN), difloxacin hydrochloride (DIF), enrofloxacin (ENR), flumequine (FLU), marbofloxacin (MAR), nalidixic acid (NAL), norfloxacin (NOR), ofloxacin (OFL), orbifloxacin (ORB), oxolinic acid (OA), pefloxacin mesylate dehydrate (PEF), and sarafloxacin (SAR) were supplied by Sigma-Aldrich (St Louis, MO, USA). Piromidic acid (PIR) from Sigma-Aldrich was used as an internal standard (IS). The molecular structures were described in Fig. 1.

HPLC grade acetonitrile and methanol were obtained from Burdick & Jackson (Ulsan, Korea). Hexane and formic acid (98%) were purchased from Merck (Darmstadt, Germany). Acetic acid (99.9%, HPLC grade) was supplied by J.T Baker (NJ, USA) and trichloroacetic acid (99.0%) by Sigma-Aldrich (St Louis, MO, USA). Ultrapure water was purified with a MILLI-Q system (Milipore, Bedford, MA, USA). All solutions prepared for UPLC-MS/MS were passed through a 0.45 µm nylon filter before use.

### ***Preparation of standard solutions***

Each individual standard, such as CIP, DAN, DIF, ENR, FLU, MAR, NAL, NOR, OFL, ORB, OA, PEF, and SAR, was made by dissolving in methanol. Stock standard solutions (50 µg/mL) were obtained and stored at 4 °C in the dark for no longer than 2 months. Working standard solutions (1 µg/mL) were prepared daily by mixing individual stock solutions and diluted in 0.1% formic acid in 10% acetonitrile (v/v). Piromidic acid (IS) was prepared by dissolving solution at the concentration of 100 ng/mL.

### ***Instrumentation***

An AT 261 analytical balance (Mettler Toledo, Greifensee, Switzerland) was used in the preparation of standard solutions. A REAX TOP vortex mixer (Heidolph, Schwabach, Germany), POWER SONIC 520 sonicator (Hwashin Tech., Seoul, Korea), and Allegra X-22R centrifuge (Beckman Coulter, Alle, USA) were used in sample extraction.

UPLC equipment was an Acquity ultra performance LC (Waters, MA, USA) and ESI-MS/MS measurements were performed using a Quattro premier XE (Waters). Data was collected using MassLynx 4.1 software (Waters) on a personal computer.

### ***Sample collection***

Sampling was set in accordance with the 「Sampling with probability proportionate to size」 and species and number of samples were determined on the basis of the consumption of food according to the 「National Health and Nutrition Examination Survey, 2011」. Each sample was purchased at least two and sampling points within the sampling area were not biased.

As a result, livestock and marine products (n=310) including beef (n=59), pork (n=49), milk (n=29), egg (n=27), chicken (n=34), flatfish (n=26), jacoever (n=29), common eel (n=27), and shrimp (n=30) were purchased from different markets in Seoul, Busan, Incheon, Daegu, Daejeon, Gwangju, and Ulsan. Details were given in Fig. 2. All solid samples were finely ground with blender and stored in a freezer at -20°C until use.

### ***Preparation of spiked samples and standard***

One sample of each matrix was repeatedly measured to confirm that no 13 quinolones were present and was used for the preparation of matrix-matched calibration standards and fortified samples for the validation of the proposed method.

Blank samples of beef, pork, chicken, egg, milk, flatfish, jacoever, common eel, and shrimp were homogenized and stored at -20°C until use. Fortified samples were prepared by spiking 100 µL of diluted working solutions at the concentration of 0.2, 0.4, and 10 µg/kg for DIF, ENR, FLU, NAL, OFL, and SAR, and 0.4, 0.8, and 10 µg/kg for CIP, DAN, MAR, NOR, ORB, OA, and PEF by using 0.1% formic acid in 10% acetonitrile (v/v).

### ***Matrix-matched calibration curves***

Quantification was carried out using matrix-matched calibration curves with IS. The blank samples (beef, pork, chicken, milk, egg, flatfish, jacoever, common eel, and shrimp) were used as matrix and fortified with working standard solutions.

The seven point matrix-matched calibration curves were prepared with the blank samples spiked with 0, 0.2, 0.4, 1.0, 2.0, 4.0, and 10.0 µg/kg for DIF, ENR, FLU, NAL, OFL, and SAR, and 0, 0.4, 0.8, 2.0, 4.0, 8.0, 10.0, and 20.0 µg/kg for CIP, DAN, MAR, NOR, ORB, OA, and PEF. Piromidic acid (IS) was spiked in all of these samples at the concentration of 4 µg/kg. Each sample was submitted to the full extraction procedures.

The calibration curves for 13 quinolones were constructed by plotting the response factor (the ratio of peak area of analyte vs. peak area of internal standard) as a function of the analyte concentration. Separate calibration curves were used for each sample.

### ***Extraction procedure***

Homogenized sample (1 g) of beef, pork, egg, chicken, flatfish, jacobever, common eel, and shrimp and 1 mL of milk were accurately weighed and taken into a 50 mL disposable polypropylene centrifuge tube. Selected quinolones standard solutions (only for the matrix-matched calibration curves) and IS were directly spiked. The spiked samples were stood in the dark at room temperature, for at least 10 min, to allow the interaction between the quinolones and the matrix. The samples were added with 2.5% trichloroacetic acid (1 mL) and shaken on a vortex mixer (Heidolph Reax top, Heidolph, Schwabach, Germany) for 1 min. After addition of 1% acetic acid in acetonitrile (15 mL), the samples were mixed again for 1 min and centrifuged (Allegra X-22R, Beckman Coulter, Brea, CA, USA) at 1000×g for 15 min to induce the precipitation of proteins. The supernatant was carefully filtered through a syringe filter (Whatman<sup>®</sup>, PVDF memb-

rane, pore size 0.45  $\mu\text{m}$ ) and poured into a separating funnel. Acetonitrile-saturated hexane (15 mL) was added and intensionally agitated for 5 min. The lower layer was transferred into a 20 mL glass tube and evaporated to dry at 45  $^{\circ}\text{C}$  under a stream of nitrogen (EYELA GM-2200, Tokyo, Japan). The dried residue was dissolved in 1 mL of 0.1% formic acid in acetonitrile and filtered through a syringe filter (Whatman<sup>®</sup>, PVDF membrane, pore size 0.2  $\mu\text{m}$ ) prior to UPLC injection. A schematic diagram of the sample preparation was presented in Fig. 3. All samples were analyzed in triplicate and results were expressed as a mean value of the total residue concentration  $\pm$  standard deviation (SD).

### ***UPLC-ESI-MS/MS conditions***

The quinolones were separated on an Acquity UPLC BEH C<sub>18</sub> column (2.1 mm  $\times$  100 mm; 1.7  $\mu\text{m}$  particle size, Waters). Mobile phase A was purified water containing 0.1% formic acid (v/v), and mobile phase B was acetonitrile. The gradient conditions were initiated with 90% mobile phase A followed by a linear decrease to 30% in 5 min, 0% in 0.5 min, and maintained for 1 min at 0%. At 6.6 min, the gradient was programmed to re-

equilibrate the column for 1.5 min under initial conditions. The run time for each injection was 8 min. The flow rate was 0.3 mL/min and the injection volume was 20  $\mu$ L in full-loop mode. Details were given in Table 3.

MS determination was performed with MRM experiments in ESI positive ion mode combined with monitoring of the most abundant MS/MS (precursor $\rightarrow$ product) ion transitions using a dwell time of 0.03s. Detailed parameters for MRM acquisition were presented in Table 4. The MS parameters were as follow: capillary voltage, 3.0 kV; source temperature, 150 $^{\circ}$ C; desolvation temperature, 350 $^{\circ}$ C; cone gas (N<sub>2</sub>) flow, 50 L/h; and desolvation gas (N<sub>2</sub>) flow, 800 L/h.

### ***Method validation***

The method was validated according to the CODEX guideline (FAO, 2009). The validation parameters to evaluate the method were linearity, LOD, LOQ, precision (repeatability and reproducibility), and accuracy (Table 5).

### ***Linearity***

Linearity was evaluated using the squared correlation coefficients ( $r^2$ ) of 7-points

matrix-matched standards curves, which were based on extracts of blank samples (beef, pork, milk, egg, chicken, flatfish, jacobever, common eel, and shrimp). The calibration ranges were from 0 to 10 µg/kg for DIF, ENR, FLU, NAL, OFL, and SAR, and from 0 to 20.0 µg/kg for CIP, DAN, MAR, NOR, ORB, OA, and PEF, respectively.

### ***Limit of detection and quantitation***

The LOD is the lowest concentration of analyte that an analytical process can reliably differentiate from background levels, while the LOQ is the lowest concentration of analyte that can be quantified. LOD and LOQ values were calculated based on signal-to-noise ratio (S/N) values of 3.3 and 10, respectively.

### ***Precision***

The precision was expressed as a percentage of RSD (%), which was performed in a single laboratory. Intra-day and inter-day precision (repeatability and reproducibility) were performed at 3 different concentrations (1.0, 4.0, and 10.0 µg/kg). The intra-day analysis was achieved through six replicates, and the inter-day analysis was obtained through repeating three consecutive days at each concentration level.

### ***Accuracy***

The accuracy was expressed as a percentage of recovery, which was performed in a single laboratory. Accuracy was evaluated through 3 different fortification levels; LOQ (0.2 or 0.4 µg/kg), 2×LOQ (0.4 or 0.8 µg/kg), and 10 µg/kg. Six replicates per each sample were performed and the results were expressed as a mean value of the total concentration ± SD.

## RESULTS & DISCUSSION

### *Method validation*

One sample of each matrix was repeatedly measured to confirm that no 13 quinolones were present and was used for the preparation of matrix-matched calibration standards and fortified samples for the validation of the method. The proposed method was validated with UPLC-ESI-MS/MS according to the CODEX guideline (FAO, 2009) for quantitative methods. All obtained results were satisfied with CODEX recommendation and indicated the stability of the proposed method.

As shown in Table 6, linearities, LOD, and LOQ for each species of matrices were summarized. The linearity was checked with the matrix-matched calibration curves using 7 calibration points. The linearities were calibrated from 0 to 10 ng/mL for DIF, ENR, FLU, NAL, OFL, and SAR and 0 to 20 ng/mL for CIP, DAN, MAR, NOR, ORB, OA, and PEF, respectively. Good linearities were obtained and the correlation coefficients ranged between 0.9992 and 0.9999 depending upon antibiotics. Furthermore, piromidic acid as IS, which significantly increased the linearity of the matrix-matched calibration

curves of 13 quinolones. Based on these results, a good linearity was proven and allowed coverage of 13 quinolones within the working range.

The LOD ranged from 0.06 to 0.1  $\mu\text{g}/\text{kg}$  and LOQ ranged from 0.2 to 0.4  $\mu\text{g}/\text{kg}$  depending upon species of samples. The results of LOD and LOQ showed that the method were enough to determine the MRLs values in animal origin samples.

The repeatability and reproducibility (the intra-day and inter-day precision) were evaluated in beef and flatfish. The repeatability was evaluated by spiking 2 blank samples (n=6 per each matrix) at the validation level (1, 4, and 10  $\mu\text{g}/\text{kg}$ ) and the reproducibility was carried out on 3 consecutive days. Repeatability and reproducibility results are presented in Table 7. The RSD (%) of repeatability ranged from 0.33 (Danofloxacin) to 3.72 (Orbifloxacin) in beef and from 0.43 (Marbofloxacin) to 3.33 (Danofloxacin) in flatfish, respectively. The RSD (%) of reproducibility ranged from 0.68 (Marbofloxacin) to 4.86 (Oxolinic acid) in beef and from 0.71 (Oxolinic acid) to 6.75 (Flumequine) in flatfish, respectively. These results indicate the good precision and reliability of the developed method. In addition, the repeatability values expressed as RSD (%) were lower

than 30%, which is within the acceptance criteria of the CODEX and satisfied the validating analytical methods (Table 5).

To evaluate method accuracy, recoveries were calculated at 3 different spiking levels. Besides, matrix-matched calibration curves were used with IS. Average recoveries of each analyte were obtained performing the analysis in 6 replicates for each matrix. Accuracy data were recorded in Table 8 and 9.

Satisfactory results were acquired, with the percentage of recoveries (%) was between  $87.5 \pm 5.0$  and  $104.7 \pm 3.7$  in livestock products and between  $83.0 \pm 2.8$  and  $100.9 \pm 1.1$  in marine products, respectively. The results were within the acceptance criteria (60-120%) of the CODEX validating analytical method (Table 5). The RSD (%) ranged from 0.4 to 6.0 and from 0.9 to 5.7, respectively. The maximum recoveries were obtained under acidic conditions and in the presence of a high percentage of acetonitrile (Rodriguez et al., 2011; Marazuela and Moreno-Bondi, 2004). In this work, acetonitrile and trichloroacetic acid played an important role in precipitating proteins, which leading to obtained higher recovery.

In this study, excellent validation parameters were acquired, which indicated that the method was suitable for the analysis of quinolones. Furthermore, the use of matrix-matched calibration curves with IS were able to minimize the difference in the signal output of analyte between sample extract solvent and a pure solvent.

### ***Optimization of sample preparation***

The simple and specific analytical method was developed. Sample preparation was focused on the establishment of the most suitable conditions used for the simultaneous determination of 13 quinolones from livestock and marine products.

Several extraction solvent mixtures were evaluated. Beef and flatfish blank samples fortified with 4 µg/kg of 13 quinolones were extracted with solution of acetonitrile with or without 1% acetic acid, and additionally added the solution of trichloroacetic acid in different ratio from 1% to 5% (v/v). As shown in Table 10, the combination of 1% acetic acid in acetonitrile (v/v) and 2.5% trichloroacetic acid was shown to be the most effective recoveries and the lowest RSD (%) of the 13 quinolones.

The precipitation of proteins is achieved in many cases by adding a strong acid, such as

trichloroacetic acid in combination with organic solvents (Andreia et al., 2013). Furthermore, acetonitrile and methanol are more efficient as extraction solvents as they can simultaneously precipitate the proteins, denature enzymes, and extract the target analyte. Many authors prefer acetonitrile over methanol as extraction solvent. Besides, methanol extracts too many matrix compounds, complicating the following clean-up steps (Marilena and Nikolaos, 2015). In this work, acetic acid and trichloroacetic acid was added to samples and acetonitrile was used as the extracting solvent, which promoted the precipitation of proteins. Thus, it could be assumed that the matrix components responsible for possible interference (such as, signal suppression or enhancement) were removed. The obtained results demonstrated acceptable recoveries of all of the quinolones, ranging from 91.93% to 102.20% in beef and from 94.06% to 110.80% in flatfish.

In order to accomplish the deproteinization and defatting of samples, the extraction with acetonitrile-saturated hexane was performed after centrifugation at 1000×g. Moreover, instead of clean-up step, e.g. using SPE cartridge, the solvent extraction including the steps of syringe filtration, centrifugation, and agitation step was applied to

the samples, thereby simplifying sample handling and reducing the time of sample preparation. Antonia et al (2010) reported that solvent extraction provided the good recovery for quinolones among the result of comparing extraction procedures, for instance solvent extraction, QuEChERS procedure, MSPD, and SPE.

Furthermore, the residue concentration of veterinary drugs could be different in the egg white (albumen) and egg yolk (ovum) (Cornelis and Michael, 2000; Antonia et al., 2010). For accurate results, all samples were sufficiently homogenized (at least 3 min). Besides, because target analytes have different polarities, and consequently a different distribution between albumin and yolk, the method was performed on the whole egg.

### ***UPLC-ESI-MS/MS optimization***

Chromatographic conditions of UPLC were evaluated in order to obtain the good separation and retention for the analytes. Several experiments were conducted testing mobile phases consisting of distilled water with different concentrations of formic acid (0.01, 0.05, and 0.1%, v/v) and acetonitrile (80, 90, 100%, v/v).

As a result, the addition of 0.1% formic acid in distilled water and 100% acetonitrile (v/v)

allowed the best resolution, as well as it enhanced the ionization efficiency. Formic acid and acetonitrile were important roles for the formation of well distributed peak and sharp peak shape (Lina et al., 2011; Junza et al., 2014).

Other parameter such as flow rate, injection volume, and gradient profile were studied in order to obtain a fast and reliable separation. The best results were acquired when 0.30 mL was used as flow rate and 20  $\mu$ L were injected. Several gradient profiles were tested, and good response was obtained with the gradient described in Table 3. Besides, in the first 2 min of the run time, major interferences are presented due to polar matrix constituents that are eluted and co-elution with the analytes would lead to large signal suppression (Marilena et al., 2015). Thus, it was necessary that the 13 quinolones began to elute after the 2 min of the run time.

In this work, 8 min of run time was achieved with the optimum conditions for the separation of 13 quinolones. The optimal chromatographic separation of 13 quinolones was performed with a 2.1 mm  $\times$  100 mm column. The UPLC columns consisting of a smaller particle size are the possibility of having high efficiency in peak separation, sharp peaks, and also a reduction in run time when compared with common HPLC columns

(Andreia et al., 2013). The acquired UPLC chromatograms showed a good resolution for all the quinolones (Fig. 4) and indicated the excellent specificity of the developed method. In addition, the use of an acidic mobile phase (acetonitrile) adjusted with 0.1% formic acid promoted positive ionization and maximized sensitivity and resolution, which improved the detection of 13 quinolones.

In MS/MS system, the protonated molecular ion  $[M+H]^+$  was selected as precursor ion for 13 quinolones, and the product ions were obtained according to the collision energy (eV). The MRM mode was used to increase the sensitivity and selectivity of the determination. To achieve maximum sensitivity for 13 quinolones, MS/MS conditions (for example, capillary voltage, source temperature, and desolvation temperature) were optimized by direct infusion into the detector of working standard solutions. The results of UPLC-ESI-MS/MS ion reconstituted chromatograms for 13 quinolones spiked to bovine muscle were presented in Fig. 4. As showed in Table 4, MRM interferences between FLU and OXO were observed because the precursor ion and one of two product ions were common to FLU and OXO. Therefore, the  $[M + H]^+ \rightarrow [M + H-H_2O]^+$  was applied to transition for quantification of FLU and OXO. Also, the transition  $[M + H]^+ \rightarrow$

$[M + H-OC_3H_8]^+$  ( $m/z$  262  $\rightarrow$ 202) was used for FLU and  $[M + H]^+ \rightarrow [M + H-OCH_2O]^+$  ( $m/z$  262  $\rightarrow$ 216) was used for OXO, respectively (Hermo et al (2006); Rubies et al (2007); Christine et al (2007)). The results of ions transition, obtained from the bovine muscle fortified with 10  $\mu$ g/kg of FLU and 20  $\mu$ g/kg of OXO, were described in Fig. 5. Additionally, for separating FLU and OXO, the gradient condition was increased the percentage of mobile B from 10 to 70%. Therefore, there was no interference to be observed with the monitored MS reactions.

To obtain an adequate number of data acquisition points related to peak shape and reproducible chromatographic signals, different dwell times (between 0.01 and 0.1 s) were evaluated. Good peak shape and reproducible data without a decrease of the sensitivity were acquired using a dwell time 0.03 s in 10 quinolones, whereas FLU, NAL, OA, and PIR (IS) were obtained using a dwell time of 0.1 s.

### ***Application of the proposed method to animal products***

Quinolones were analyzed in 310 livestock and marine products including 59 beef, 49 pork, 34 chicken, 29 milk, 29 egg, 26 flatfish, 29 jacobever, 27 common eel, and 30 shrimp (Table 11). The results are shown in Table 12. The residues of quinolones were

detected in 39 out of 198 livestock product samples (19.7% incidence) and 65 out of 112 marine product samples (58.0% incidence); 7 out of 59 beef samples (11.9% incidence), 7 out of 49 pork samples (14.3%), 22 out of 34 chicken samples (64.7%), 2 out of 29 milk samples (6.9%), 1 out of 27 egg samples (3.7%), 12 out of 26 flatfish samples (46.2%), 16 out of 27 common eel samples (59.3%), 27 out of 29 jacopever samples (93.1%), and 10 out of 30 shrimp samples (33.3%) (Fig. 2). The incidence of 13 quinolones residues followed the order; jacopever > chicken > common eel > flatfish > shrimp > pork > beef > milk > egg.

Oxolinic acid, enrofloxacin, and flumequine were the most common antibiotics found in the detected samples. The kinds of the detected quinolones in livestock and marine products are summarized in Table 13. As seen in Table 13, the detected milk samples contained only one antibiotic, while residues of more than one antibiotic were found in the detected beef, pork, chicken, egg, flatfish, jacopever, common eel, and shrimp. It indicated that quinolones were employed as multiple uses in livestock and marine products.

Detected concentrations were 0.2 to 20.0 µg/kg in beef, 0.6 to 4.1 µg/kg in pork, 0.1 to 47.0 µg/kg in chicken, 0.7 to 0.9 µg/kg in milk, 0.3 to 0.5 µg/kg in egg, 0.3 to 21.1 µg/kg in flatfish, 0.1 to 100.0 µg/kg in common eel, 0.3 to 22.8 µg/kg in jacopever, and 0.2 to 5.1 µg/kg in shrimp, respectively. The risk value based on the quinolones exposure by food intake was calculated using the EDI (estimated daily intake) and ADI (acceptable daily intake). Compared with ADI, the risk value was very safe level of 0.205% in sarafloxacin, 0.007% in marbofloxacin, 0.612% in oxolinic acid, 0.027% in flumequine, 0.090% in enrofloxacin, and 0.004% in difloxacin, respectively. Details were given in Table 14 and the food contribution for EDI was described in Fig. 6.

The detected concentration of pefloxacin in common eel was 62.4 µg/kg. The level of residue concentration was above the MRL (not detected in livestock product, fish, and shellfish) for pefloxacin in Korean Food Code. Fig. 7 showed the UPLC-ESI-MS/MS chromatograms of pefloxacin in detected sample. We assumed that non-observance of the required withdrawal period caused the illegal residue of pefloxacin, or the antibiotics were used during the transporting after shipment. Besides, the regular and massive use of veterinary medicines was seen as a risk factor for direct contamination of products

(Mitema et al., 2001; Won et al., 2011). Thus, treatments were carried out in accordance with proper usage of veterinary drugs and withdrawal periods were observed.

In Korea, the annual per capita consumption of livestock and marine products is growing and reached 53.5 kg in marine products, 11.5 kg in chicken, 20.9 kg in pork, 10.3 kg in beef, and 71.6 kg in milk (MAFRA, 2015). Besides, in Korea, many people commonly consume sashimi of jacobever and flatfish and enjoy roasted eel. Intensive farming, which can lead to a high potential for spread of infectious disease, has been maintained to satisfy an increasing demand for marine products. Therefore, the survey of quinolone residues in livestock and marine products is significant in Korea. In particular, quinolones classified as VCIA (veterinary critically important antimicrobials) from the OIE (Office International des Epizooties), and as CIA (critically important antimicrobials for human medicine) from WHO (World Health Organization). Considering the possibility of antibiotic cross-resistance between human and animal, it requires a proper use of antibiotics and continuous monitoring.

## CONCLUSION

A sensitive and specific method was developed and validated for the simultaneous determination of 13 quinolones in livestock and marine products using UPLC-ESI-MS/MS. The recoveries (%) of 13 quinolones were higher than 80%, the LOD and LOQ values were lower than the established MRLs in Korea, and other validation parameters were also in accordance with CODEX guideline. The proposed method using UPLC-ESI-MS/MS involved significant advantages with respect to simple preparation step, high sensitivity, speed, and resolution, making it an attractive choice for the analysis of quinolones in livestock and marine products. However, the fast gradient used in UPLC promoted matrix effects by reducing chromatographic separation between analytes and endogenous compound. As a result, the matrix-matched calibration and IS were used, which play very important roles in compensating for the matrix effects.

The livestock and marine products (n=310) commercialized in Korea were analyzed and quantified by proposed method. The residues of quinolones were detected in 104 samples (33.5% incidence) but residue levels were below the MRLs in Korea Food Code.

The risk value was safe level of 0.205% in sarafloxacin, 0.007% in marbofloxacin, 0.612% in oxolinic acid, 0.027% in flumequine, 0.090% in enrofloxacin, and 0.004% in difloxacin, respectively. However, pefloxacin was detected in one common eel above the legal residue limit and the detected concentration was 62.4 µg/kg.

Although this survey results appear to be relatively safe levels, antibiotic residues are exposed to consumers. Therefore, we proposed that the monitoring of quinolones in livestock and marine products was continued.

Table 1. Global organization for registration and establishment of MRLs for veterinary drugs

Country	Registration	Establishment of MRLs
Korea	Animal and Plant Quarantine Agency (QIA)	Ministry of Food and Drug Safety (MFDS)
Japan	The Ministry of Agriculture, Forestry and Fisheries (MAFF)	Ministry of Health, Labor and Welfare (MHLW)
USA	Center for Veterinary Medicine (CVM)	United States Food and drug Administration (FDA)
EU	Committee for Medicinal Products for Veterinary use (CVMP)	European Medicines Agency (EMA)
Australia	Australian Pesticides and Veterinary Medicines Authority (APVMA)	Food Standards Australia New Zealand (FSANZ)
New Zealand	New Zealand Food Safety Authority (NZFSA)	Food Standards Australia New Zealand (FSANZ)

Table 2. Maximum residues limits of 13 quinolones in Korea

Compound	Food	mg/kg
Enrofloxacin+Ciprofloxacin	Cattle, Pig, Sheep, Goat, Poultry	0.1~0.3
	Egg	Not detected
	Fish, Crustacean	0.1
	Milk	0.05
Danofloxacin	Cattle, Poultry	0.1~0.4
	Pig	0.05~0.2
	Milk	0.03
Difloxacin	Cattle, Pig, Sheep, Goat	0.4~1.4
	Poultry	0.3~1.9
	Fish, Crustacean	0.3
Flumequine	Cattle, Pig, Sheep	0.2~1.5
	Poultry	0.4~1.0
	Fish, Crustacean	0.5
Marbofloxacin	Cattle, Pig	0.05~0.15
	Milk	0.075
Nalidixic acid	Cattle, Fish	0.03
Orbifloxacin	Cattle, Pig, Milk	0.02
Oxolinic acid	Cattle, Pig	0.05
	Chicken	0.15~0.1
	Fish, Crustacean	0.1
Ofloxacin, Pefloxacin, Norfloxacin	Livestock products, Fish, Crustacean	Not detected
Sarafloxacin	Poultry	0.01~0.08

Table 3. Analytical conditions of UPLC-ESI-MS/MS for analysis of 13 quinolones

Items		Conditions		
LC	Instrument	Acquity ultra performance LC		
	Column	Acuity UPLC BEH C <sub>18</sub> (2.1×10 mm, 1.7 μm)		
	Mobile phase	A: 0.1% formic acid in DW		
		B: Acetonitrile		
		Time (min)	A (%)	B (%)
		Initial	90	10
		5.0	30	70
	Gradient	5.5	0	100
		6.5	0	100
		6.6	90	10
8.0		90	10	
	Flow rate	0.3 mL/min		
	Injection volume	20 μL		
MS	Instrument	Quattro Premier XE		
	Ionization mode	ES+		
	Capillary voltage	3.0 kV		
	Source temperature	150 °C		
	Desolvation temperature	350 °C		

Table 4. MRM conditions of the MS/MS for the detection of 13 quinolones

Compound	MRM transition (m/z) (Precursor ion > product ion)	Cone voltage (V)	Collision energy (eV)
Ciprofloxacin	332 > 245	35	26
	332 > 288		18
Danofloxacin	358 > 283	30	22
	358 > 314		18
Difloxacin	400 > 299	35	32
	400 > 356		20
Enrofloxacin	360 > 316	28	20
	360 > 342		22
Flumequine	262 > 202	25	34
	262 > 244		16
Marbofloxacin	363 > 72	30	20
	363 > 320		15
Nalidixic acid	233 > 187	25	26
	233 > 215		14
Norfloxacin	320 > 233	35	24
	320 > 276		18
Ofloxacin	362 > 261	35	28
	362 > 318		20
Orbifloxacin	396 > 295	35	24
	396 > 352		20
Oxolinic acid	262 > 216	25	28
	262 > 244		20
Pefloxacin	334 > 233	35	26
	334 > 290		18
Piromidic acid (IS)	289 > 243	25	30
	289 > 271		20
Sarafloxacin	386 > 299	40	26
	386 > 342		20

Table 5. Accuracy and precision ranges as requirements in CODEX guidelines

Concentration ( $\mu\text{g}/\text{kg}$ )	Coefficient of variability (CV) (Repeatability, %)	Acceptable recovery (%)
$\leq 1$	35	50 - 120
1 to 10	30	60 - 120
10 to 100	20	70 - 120
100 to 1000	15	70 - 110
$\geq 1000$	10	70 - 110

Table 6. Validation parameters for detection of 13 quinolones using UPLC-MS/MS

Compound	Calibration equation	Determination coefficient ( $r^2$ )	LOD ( $\mu\text{g/kg}$ )	LOQ ( $\mu\text{g/kg}$ )
Ciprofloxacin	$y = 0.137x + 0.004$	0.9998	0.1	0.4
Danofloxacin	$y = 0.031x + 0.001$	0.9998	0.1	0.4
Difloxacin	$y = 0.105x + 0.007$	0.9996	0.07	0.2
Enrofloxacin	$y = 0.106x + 0.002$	0.9999	0.06	0.2
Flumequine	$y = 0.322x + 0.077$	0.9992	0.06	0.2
Marbofloxacin	$y = 0.122x + 0.035$	0.9998	0.1	0.4
Nalidixic acid	$y = 0.284x + 0.003$	0.9999	0.06	0.2
Norfloxacin	$y = 0.042x + 0.009$	0.9999	0.1	0.4
Ofloxacin	$y = 0.081x + 0.004$	0.9993	0.07	0.2
Orbifloxacin	$y = 0.218x + 0.050$	0.9995	0.1	0.4
Oxolinic acid	$y = 0.061x + 0.009$	0.9998	0.1	0.4
Pefloxacin	$y = 0.080x + 0.123$	0.9992	0.1	0.4
Sarafloxacin	$y = 0.042x + 0.029$	0.9996	0.07	0.2

Table 7. Intra- and inter-day precision of 13 quinolones at 3 different concentration levels.

Compound	Spiked conc. (µg/kg)	Intra-day precision (n = 6)		Inter-day precision (n = 3)	
		RSD(%) <sup>1)</sup>		RSD (%) <sup>1)</sup>	
		beef	flatfish	beef	flatfish
Ciprofloxacin	1.0	1.06	0.96	3.35	3.75
	4.0	3.36	0.57	2.90	2.91
	10.0	1.19	2.20	3.68	1.74
Danofloxacin	1.0	1.63	1.08	2.07	2.02
	4.0	1.08	3.33	3.18	2.22
	10.0	0.62	1.41	1.45	2.14
Difloxacin	1.0	0.33	0.69	2.12	2.76
	4.0	0.84	0.90	5.25	2.20
	10.0	1.30	1.63	1.12	3.95
Enrofloxacin	1.0	0.52	0.57	1.44	1.51
	4.0	2.00	0.75	2.41	0.95
	10.0	1.69	1.16	1.75	1.29
Flumequine	1.0	1.41	0.49	1.77	2.94
	4.0	0.45	2.23	3.85	2.00
	10.0	1.07	1.59	1.91	2.53
Marbofloxacin	1.0	1.68	2.47	3.97	6.75
	4.0	1.89	1.33	2.20	3.13
	10.0	3.48	0.90	0.68	1.14
Nalidixic acid	1.0	0.79	0.43	2.62	1.01
	4.0	1.64	3.30	4.71	0.80
	10.0	2.35	0.62	1.78	1.94
Norfloxacin	1.0	0.85	0.93	1.38	0.98
	4.0	1.60	0.91	4.13	4.12
	10.0	1.69	1.97	2.28	2.18
Ofloxacin	1.0	1.00	1.44	0.92	1.92
	4.0	3.49	1.22	3.75	1.67
	10.0	1.26	1.16	1.55	1.67
Orbifloxacin	1.0	1.46	0.65	2.80	3.05
	4.0	1.87	2.13	1.84	1.99
	10.0	3.72	1.54	0.96	1.02
Oxolinic acid	1.0	1.78	2.12	2.22	1.10
	4.0	0.49	0.76	4.86	4.54
	10.0	1.12	0.93	2.01	0.98
Pefloxacin	1.0	0.65	0.47	4.60	0.71
	4.0	1.46	0.69	4.27	3.31
	10.0	1.98	1.16	1.00	1.28
Sarafloxacin	1.0	0.52	0.58	2.15	1.06
	4.0	1.18	1.20	3.54	3.80
	10.0	2.48	0.95	3.02	1.43

<sup>1)</sup> Relatively standard deviation

Table 8. Average recoveries and RSD (%) measured in livestock products

Com- pound	Spiked conc. (µg/kg)	Beef		Pork		Chicken		Milk		Egg	
		M <sup>1)</sup>	RSD (%)								
CIP	0.4	94.0±3.7	3.9	93.8±3.2	3.5	97.0±2.4	2.5	96.9±2.6	2.7	100.4±2.2	2.2
	0.8	94.2±3.7	3.8	96.7±3.8	3.9	97.2±4.1	4.2	96.9±2.5	2.6	101.5±2.9	2.9
	10.0	99.1±2.8	2.9	97.0±2.8	2.9	100.2±1.6	1.6	98.2±2.7	2.7	100.6±1.8	1.8
DAN	0.4	98.8±5.6	5.7	95.8±4.7	4.9	94.6±2.2	2.4	97.0±2.4	2.5	98.3±3.4	3.5
	0.8	96.0±2.5	2.6	96.8±3.7	3.8	93.8±4.7	5.0	97.0±3.3	3.4	96.9±3.1	3.2
	10.0	97.5±4.3	4.4	91.3±3.1	3.3	94.2±2.3	2.5	98.2±2.1	2.1	100.4±1.2	1.2
DIF	0.2	95.6±2.2	2.3	96.1±3.1	3.2	99.4±2.0	2.0	96.3±3.5	3.6	97.8±2.1	2.1
	0.4	93.3±2.2	2.4	96.3±4.8	5.0	97.6±4.1	4.2	98.2±2.1	2.2	95.5±4.1	4.3
	10.0	96.0±4.6	4.8	97.1±2.3	2.4	99.5±1.6	1.6	100.0±0.5	0.5	102.6±1.6	1.5
ENR	0.2	91.0±4.5	5.0	89.0±3.7	4.2	88.4±2.9	3.2	101.0±3.1	3.1	89.2±5.2	5.9
	0.4	90.0±3.4	3.8	94.1±5.6	6.0	95.3±2.5	2.6	98.6±3.4	3.4	93.1±5.0	5.4
	10.0	99.1±3.1	3.1	97.8±3.8	3.9	98.0±2.2	2.3	97.6±1.0	1.1	100.9±2.7	2.6
FLU	0.2	100.2±2.8	2.8	94.4±3.4	3.6	96.2±3.1	3.2	96.3±3.6	3.7	93.0±2.0	2.2
	0.4	95.6±3.1	3.3	94.6±2.3	2.4	98.1±2.3	2.3	98.1±2.4	2.5	96.6±1.8	1.9
	10.0	99.1±2.3	2.3	100.0±2.7	2.8	99.0±1.6	1.6	100.0±0.8	0.8	99.1±2.7	2.8
MAR	0.4	95.4±2.2	2.3	104.3±5.6	5.4	94.7±3.5	3.7	98.8±1.9	1.9	99.3±2.2	2.2
	0.8	96.6±3.1	3.2	100.1±3.3	3.3	90.9±4.3	4.8	98.7±1.6	1.6	93.7±1.6	1.7
	10.0	93.8±2.3	2.2	100.0±3.7	3.7	93.5±2.4	2.5	96.9±1.1	1.2	99.3±2.4	2.5

<sup>1)</sup> Mean recoveries (% , n = 6) ± Standard deviation

Table 8. (Continued)

Com- pound	Spiked conc. ( $\mu\text{g}/\text{kg}$ )	Beef		Pork		Chicken		Milk		Egg	
		M <sup>1)</sup>	RSD (%)								
NAL	0.2	95.3 $\pm$ 2.0	2.0	100.0 $\pm$ 3.5	3.5	97.3 $\pm$ 1.8	1.9	96.4 $\pm$ 3.4	3.6	96.7 $\pm$ 1.2	1.2
	0.4	96.5 $\pm$ 1.5	1.6	97.4 $\pm$ 3.0	3.1	95.3 $\pm$ 4.6	4.8	98.2 $\pm$ 1.0	1.1	96.3 $\pm$ 3.3	3.5
	10.0	98.0 $\pm$ 1.8	1.9	97.5 $\pm$ 2.7	2.8	97.6 $\pm$ 3.0	3.1	99.2 $\pm$ 3.9	4.0	98.9 $\pm$ 0.4	0.4
NOR	0.4	94.4 $\pm$ 4.6	4.9	93.6 $\pm$ 3.9	4.1	97.1 $\pm$ 4.4	4.9	100.9 $\pm$ 1.9	1.9	95.1 $\pm$ 4.7	5.0
	0.8	96.1 $\pm$ 2.8	2.9	97.3 $\pm$ 4.3	4.4	94.9 $\pm$ 4.0	4.2	96.5 $\pm$ 4.7	4.9	95.2 $\pm$ 2.5	2.7
	10.0	96.2 $\pm$ 4.1	4.3	101.7 $\pm$ 5.1	5.0	98.2 $\pm$ 1.8	1.9	96.9 $\pm$ 3.8	3.9	101.5 $\pm$ 1.7	1.6
OFL	0.2	94.0 $\pm$ 4.4	4.7	102.8 $\pm$ 4.1	4.0	87.5 $\pm$ 5.0	5.8	95.3 $\pm$ 5.7	6.0	92.8 $\pm$ 3.1	3.4
	0.4	90.0 $\pm$ 3.5	3.9	94.6 $\pm$ 4.6	4.9	87.8 $\pm$ 4.7	5.3	91.4 $\pm$ 0.9	1.0	98.8 $\pm$ 4.7	4.8
	10.0	96.1 $\pm$ 3.2	3.3	103.9 $\pm$ 3.1	3.0	96.5 $\pm$ 1.4	1.5	97.7 $\pm$ 2.4	2.4	98.7 $\pm$ 2.6	2.6
ORB	0.4	94.2 $\pm$ 3.0	3.2	93.2 $\pm$ 2.6	2.8	93.1 $\pm$ 2.1	2.2	92.4 $\pm$ 1.5	1.6	92.0 $\pm$ 2.4	2.6
	0.8	93.4 $\pm$ 2.0	2.1	92.9 $\pm$ 2.4	2.6	95.0 $\pm$ 0.7	0.7	92.9 $\pm$ 2.9	3.2	92.5 $\pm$ 2.2	2.4
	10.0	96.8 $\pm$ 2.1	2.2	94.1 $\pm$ 2.9	3.1	93.6 $\pm$ 2.0	2.2	93.8 $\pm$ 1.5	1.6	94.8 $\pm$ 2.7	2.9
OXO	0.4	96.5 $\pm$ 5.4	5.6	92.5 $\pm$ 4.3	4.6	90.8 $\pm$ 3.6	4.0	98.8 $\pm$ 4.1	4.2	97.5 $\pm$ 4.9	5.0
	0.8	94.9 $\pm$ 2.3	2.4	94.7 $\pm$ 3.7	3.9	88.9 $\pm$ 3.2	3.6	97.8 $\pm$ 0.6	0.6	98.9 $\pm$ 4.2	4.3
	10.0	99.2 $\pm$ 1.5	1.6	97.5 $\pm$ 2.4	2.5	97.0 $\pm$ 3.0	3.1	99.4 $\pm$ 3.0	3.0	103.2 $\pm$ 0.8	0.7
PEF	0.4	97.3 $\pm$ 1.7	1.7	96.9 $\pm$ 4.6	4.7	88.7 $\pm$ 4.3	4.9	90.8 $\pm$ 3.9	4.3	94.7 $\pm$ 3.0	3.2
	0.8	92.8 $\pm$ 4.0	4.3	95.4 $\pm$ 3.2	3.4	96.0 $\pm$ 1.9	2.0	93.4 $\pm$ 5.3	5.7	97.4 $\pm$ 3.5	3.6
	10.0	91.0 $\pm$ 2.3	2.6	99.4 $\pm$ 2.5	2.5	97.2 $\pm$ 5.6	2.7	93.5 $\pm$ 5.5	5.9	103.3 $\pm$ 1.3	1.3
SAR	0.2	95.9 $\pm$ 5.7	5.9	104.7 $\pm$ 3.7	3.6	102.2 $\pm$ 3.4	3.3	98.2 $\pm$ 4.8	4.9	91.4 $\pm$ 4.9	5.2
	0.4	94.5 $\pm$ 5.5	5.8	96.1 $\pm$ 4.1	4.3	99.5 $\pm$ 3.6	3.7	99.3 $\pm$ 4.4	4.5	96.2 $\pm$ 5.3	5.5
	10.0	95.8 $\pm$ 5.3	5.6	93.1 $\pm$ 4.2	4.5	96.3 $\pm$ 2.7	2.8	96.9 $\pm$ 2.1	2.2	101.6 $\pm$ 3.4	3.3

<sup>1)</sup> Mean recoveries (%; n = 6)  $\pm$  Standard deviation

Table 9. Average recoveries and RSD (%) measured in marine products

Compound	Spiked conc. (µg/kg)	Flatfish		Common eel		Jacopever		Shrimp	
		M <sup>1)</sup>	R <sup>2)</sup>						
CIP	0.4	89.9±3.7	4.0	98.9±0.8	0.8	89.6±3.8	4.3	92.4±1.6	1.8
	0.8	93.4±4.5	4.8	97.8±1.3	1.3	92.1±2.3	2.5	95.7±1.0	1.1
	10.0	95.0±2.6	2.7	95.9±3.4	3.5	98.2±2.1	2.1	97.2±2.2	2.3
DAN	0.4	88.9±1.7	1.9	93.7±3.2	3.4	95.0±1.7	1.8	93.0±1.9	2.0
	0.8	92.8±1.2	1.3	95.0±1.6	1.7	92.9±2.8	3.1	92.7±3.0	3.2
	10.0	97.7±3.8	3.9	97.4±2.7	2.8	96.6±2.0	2.0	96.0±3.3	3.5
DIF	0.2	90.4±4.5	4.9	97.3±3.9	4.0	90.9±3.8	4.2	95.0±2.5	2.6
	0.4	92.7±4.4	4.8	97.5±3.3	3.3	89.2±3.5	3.9	96.5±3.0	3.1
	10.0	92.7±1.3	1.4	95.2±2.4	2.7	88.3±1.1	1.3	96.9±3.6	3.7
ENR	0.2	88.5±4.3	4.8	94.7±3.2	3.3	90.6±4.1	4.5	96.9±3.4	3.5
	0.4	90.3±4.9	5.5	95.1±3.0	3.2	94.8±4.4	4.6	97.3±2.6	2.7
	10.0	91.1±1.2	1.3	96.2±2.5	2.6	94.9±1.4	1.5	97.7±2.9	3.0
FLU	0.2	86.0±2.7	3.1	94.8±2.7	2.9	97.3±2.9	3.0	95.8±2.3	2.4
	0.4	93.5±3.8	4.1	95.2±3.2	3.4	93.4±3.4	3.6	96.5±2.5	2.6
	10.0	96.9±1.8	1.9	96.7±2.3	2.4	98.7±1.8	1.8	98.8±2.5	2.5
MAR	0.4	98.4±3.2	3.3	97.1±1.7	1.7	87.7±3.0	3.4	96.0±2.6	2.7
	0.8	93.1±3.7	3.9	97.1±1.5	1.6	93.7±2.1	2.2	96.1±3.5	3.7
	10.0	96.8±2.2	2.3	97.1±3.0	3.1	91.7±2.7	3.0	96.1±2.6	2.7
NAL	0.2	87.6±2.2	2.6	93.5±2.2	2.3	88.3±3.3	3.7	93.3±1.2	1.3
	0.4	90.9±4.3	4.7	94.1±1.4	1.5	88.8±2.4	2.7	95.8±2.4	2.5
	10.0	94.6±1.6	1.7	95.1±2.9	3.1	92.6±3.0	3.2	96.8±2.7	2.8
NOR	0.4	89.9±2.9	3.2	95.8±1.8	1.9	89.2±2.7	3.0	93.7±3.3	3.5
	0.8	83.0±2.8	3.0	96.4±2.5	2.6	94.6±2.7	2.9	97.4±3.5	3.6
	10.0	95.3±2.4	2.5	95.6±0.9	0.9	96.5±1.1	1.1	97.4±2.7	2.7
OFL	0.2	87.3±3.9	4.5	94.2±1.5	1.6	88.7±5.0	5.6	97.8±2.4	2.4
	0.4	90.9±5.2	5.7	95.1±1.4	1.5	88.7±3.2	3.6	96.9±2.5	2.6
	10.0	94.9±3.6	3.8	97.0±2.8	2.9	92.2±1.8	2.0	97.2±3.0	3.1
ORB	0.4	87.7±2.4	2.8	93.5±3.1	3.3	89.6±4.9	5.4	93.2±1.8	1.9
	0.8	88.1±3.2	3.7	94.2±3.0	3.2	93.2±2.3	2.5	93.2±1.7	1.8
	10.0	94.3±2.0	2.1	94.2±1.3	1.4	94.0±2.1	2.2	95.1±2.1	2.2
OXO	0.4	90.1±4.2	4.7	96.2±2.0	2.1	94.1±1.7	1.8	97.0±2.8	2.9
	0.8	93.6±3.4	3.6	93.6±1.7	1.8	93.7±2.8	3.0	96.3±2.5	2.6
	10.0	97.1±2.2	2.3	94.2±1.8	1.9	100.9±1.1	1.0	96.8±2.2	2.2
PEF	0.4	91.3±3.8	4.1	98.3±2.3	2.3	92.9±3.8	4.1	93.4±0.8	0.9
	0.8	93.3±4.4	4.7	97.7±0.9	0.9	93.4±1.8	2.0	97.3±2.4	2.4
	10.0	95.7±1.8	1.9	95.8±1.9	1.9	92.1±1.4	1.5	97.9±2.6	2.7
SAR	0.2	87.9±4.3	4.8	95.9±2.9	3.1	88.6±4.2	4.7	94.5±2.1	2.2
	0.4	92.6±4.9	5.3	96.1±3.5	3.7	89.2±3.2	3.6	96.2±3.8	4.0
	10.0	93.9±2.2	2.3	95.4±2.1	2.2	88.2±3.9	4.4	98.3±1.5	1.5

<sup>1)</sup> Mean recoveries (% , n = 6) ± Standard deviation

<sup>2)</sup> Relative standard deviation (%)

Table 10. Comparison of the solvent extraction effects on the recoveries (%) from beef and flatfish fortified with 4 µg/kg of 13 quinolones.

Sample	TCA <sup>1)</sup>	Acetonitrile					1% acetic acid in acetonitrile				
		Min. (%)	Max. (%)	Aver. <sup>2)</sup> (%)	SD <sup>3)</sup>	RSD <sup>4)</sup>	Min. (%)	Max. (%)	Aver. <sup>2)</sup> (%)	SD <sup>3)</sup>	RSD <sup>4)</sup>
Beef	1%	77.00	105.30	90.21	7.05	7.82	96.94	109.90	104.60	3.59	3.43
	2%	85.8	111.80	98.58	8.66	8.78	98.85	117.20	104.20	4.63	4.44
	2.5%	89.21	115.10	101.02	6.92	6.85	91.93	102.20	96.10	3.20	3.33
	3%	69.73	112.30	88.62	12.58	14.20	103.70	116.80	110.25	3.72	3.38
	4%	66.72	113.00	94.27	14.35	15.22	96.35	112.70	103.35	4.12	3.99
	5%	87.14	124.80	107.35	8.68	8.09	92.75	121.20	102.76	7.00	6.81
Flatfish	1%	86.08	117.10	100.98	8.26	8.18	91.02	159.6	106.62	17.33	16.25
	2%	92.18	113.70	102.38	7.08	6.92	93.56	124.90	102.43	8.42	8.23
	2.5%	92.28	113.80	103.66	5.95	5.74	94.06	110.80	100.51	4.80	4.78
	3%	113.10	139.40	127.03	8.33	6.56	89.04	114.00	100.96	6.98	6.91
	4%	95.54	131.40	108.34	8.62	7.96	91.03	116.30	99.45	6.93	6.97
	5%	90.23	117.40	105.61	7.57	7.17	98.98	112.40	106.37	5.30	4.98

<sup>1)</sup> Trichloroacetic acid

<sup>2)</sup> Average of recovery rate (%)

<sup>3)</sup> Standard deviation

<sup>4)</sup> Relative standard deviation (%)

Table 11. Number of samples according to the sampling area

	Seoul	Busan	Incheon	Daegu	Gwangju	Daejeon	Ulsan	Total
Beef	21	8	5	7	6	6	6	59
Pork	17	6	7	5	4	5	5	49
Chicken	13	5	5	3	3	2	3	34
Milk	7	6	4	3	3	2	4	29
Egg	5	5	4	2	3	4	4	27
Flatfish	5	5	4	2	3	3	4	26
Common eel	6	5	3	3	4	3	3	27
Jacopever	6	5	4	3	4	4	3	29
Shrimp	7	5	4	4	3	4	3	30
Total	87	50	40	32	33	33	35	(n=310)

Table 12. Incidence and range of 13 quinolones levels in 310 livestock and marine products

Sample category	Number of analyzed samples	Number of detected samples	Below LOQ <sup>1)</sup>	Range of quinolone level (µg/kg)	Incidence of quinolone (%)
Beef	59	7	8	0.2~20.0	11.9
Pork	49	7	5	0.6~4.1	14.3
Chicken	34	22	3	0.1~47.0	64.7
Milk	29	2	8	0.7~0.9	6.9
Egg	27	1	4	0.3~0.5	3.7
Flatfish	26	12	1	0.3~21.1	46.2
Common eel	27	16	5	0.1~100.0	59.3
Jacopever	29	27	1	0.3~22.8	93.1
Shrimp	30	10	11	0.2~5.1	33.3

<sup>1)</sup> LOQ: Limit of quantification

Table 13. The kinds of the detected quinolones in livestock and marine products

Number of detected samples	Beef	Pork	Chicken	Milk	Egg	Flatfish	Common eel	Jacopever	Shrimp
Difloxacin	-	-	-	-	1	-	-	-	5
Enrofloxacin	-	-	8	-	-	2	-	7	1
Ciprofloxacin	-	-	-	-	-	1	-	11	-
Enrofloxacin+ciprofloxacin	-	-	11	-	-	-	-	7	-
Flumequine	-	2	6	-	1	4	3	8	-
Marbofloxacin	-	-	-	1	-	2	-	-	-
Nalidixic acid	-	-	-	-	-	-	-	1	-
Orbifloxacin	4	-	-	1	-	1	-	1	-
Oxolinic acid	1	4	1	-	-	7	14	11	7
Pefloxacin	-	-	-	-	-	-	1 <sup>1)</sup>	-	-
Sarafloxacin	2	2	1	-	-	1	2	1	1

<sup>1)</sup> Above the legal residue limit

Table 14. Assessment of dietary intake and risk value of detected antibiotics

Compound	Food	Frequency <sup>2)</sup>	Detected Conc. (mg/kg)	Food intake <sup>3)</sup> (kg/person/day)	EDI <sup>4)</sup> (mg/person/day)	EDI/ADI <sup>5)</sup> (%)
Sarafloxacin ※ ADI <sup>1)</sup> =0.0004	Beef	2/59	0.0002-0.0007	30.36	0.00000002	0.004
	Pork	2/49	0.007-0.0041	56.19	0.0000002	0.050
	Chicken	1/34	0.0014	78.44	0.00000006	0.014
	Flatfish	1/26	0.0003	58.93	0.00000001	0.003
	Common eel	2/27	0.0007-0.0036	54.30	0.00000013	0.003
	Jacopever	1/29	0.0228	36.23	0.00000052	0.129
	Shrimp	1/30	0.0006	18.45	0.00000001	0.002
					∑EDI=0.00000077	∑EDI/ADI=0.205
Marbofloxacin ※ ADI=0.0045	Milk	1/29	0.0007	217.61	0.0000001	0.002
	Flatfish	2/26	0.0011-0.0015	58.93	0.0000002	0.005
	Shrimp	2/30	0.0003-0.0004	18.45	0.00000002	0.0003
					∑EDI=0.00000033	∑EDI/ADI=0.0073

<sup>1)</sup> ADI is expressed in terms of mg/kg weight/day by a person of 55 kg

<sup>2)</sup> Number of detected sample/total sample

<sup>3)</sup> From Korean National Health and Nutrition Examination Survey

<sup>4)</sup> EDI (mg/person/day) = Food intake (kg/person/day) × detected concentration in food (mg/kg)

<sup>5)</sup> Risk value (%)

Table 14. (Continued)

Compound	Food	Frequency <sup>2)</sup>	Detected Conc. (mg/kg)	Food intake <sup>3)</sup> (kg/person/day)	EDI <sup>4)</sup> (mg/person/day)	EDI/ADI <sup>5)</sup> (%)
Oxolinic acid ※ ADI <sup>1)</sup> =0.0004	Beef	1/59	0.002	30.36	0.00000002	0.00008
	Pork	3/49	0.001-0.003	56.19	0.00000004	0.0002
	Chicken	1/34	0.0009	78.44	0.00000004	0.0002
	Flatfish	7/26	0.0008-0.021	58.93	0.0000092	0.036
	Common eel	14/27	0.0001-0.1	54.30	0.000138	0.553
	Jacopever	10/29	0.0003-0.005	36.23	0.0000045	0.018
	Shrimp	7/30	0.001-0.005	18.45	0.00000102	0.004
					ΣEDI=0.00015	ΣEDI/ADI=0.612
Flumequine ※ ADI=0.00825	Pork	2/49	0.0004-0.0006	56.19	0.000000042	0.0005
	Egg	1/27	0.0005	28.90	0.000000010	0.0001
	Chicken	6/34	0.00003-0.0005	78.44	0.000000242	0.003
	Flatfish	3/26	0.0006-0.0035	58.93	0.000000618	0.007
	Jacopever	7/29	0.0002-0.0037	36.23	0.000000986	0.012
	Common eel	3/27	0.0004-0.001	54.30	0.000000362	0.004
					ΣEDI=0.0413	ΣEDI=0.027

Table 14. (Continued)

Compound	Food	Frequency <sup>2)</sup>	Detected Conc. (mg/kg)	Food intake <sup>3)</sup> (kg/person/day)	EDI <sup>4)</sup> (mg/person/day)	EDI/ADI <sup>5)</sup> (%)
Enrofloxacin ※ ADI <sup>1)</sup> =0.0062	Chicken	8/34	0.0004-0.0037	78.44	0.000004	0.064
	Flatfish	2/26	0.0012	58.93	0.0000002	0.003
	Jacopever	7/29	0.0006-0.0037	36.23	0.0000014	0.022
	Shrimp	1/30	0.0004	18.45	0.000000004	0.0001
					∑EDI=0.000006	∑EDI/ADI=0.0895
Difloxacin ※ ADI=0.001	Egg	1/27	0.0003	28.90	0.000000006	0.0006
	Shrimp	3/30	0.0002-0.0006	18.45	0.000000037	0.0037
					∑EDI=0.000000043	∑EDI=0.0043

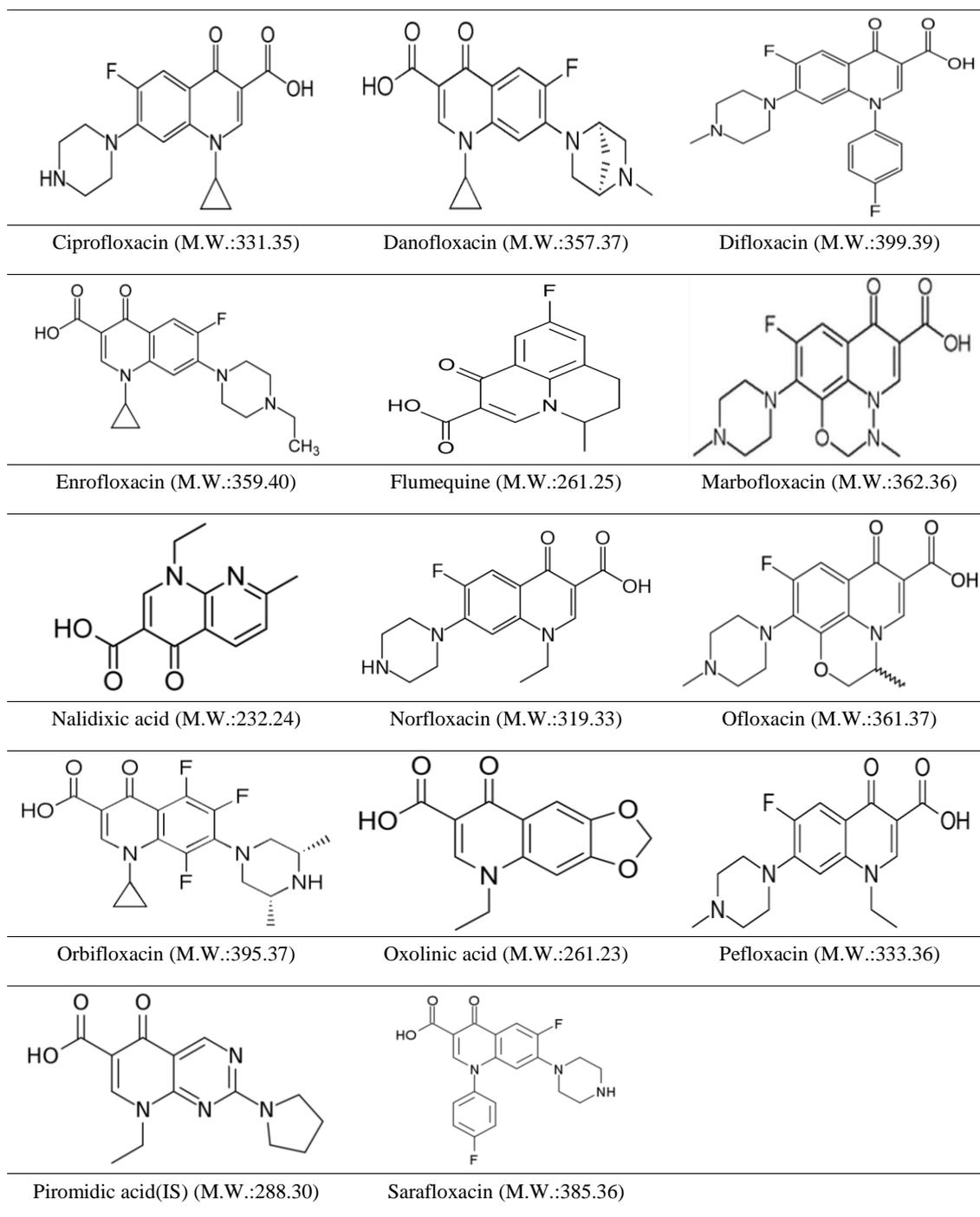
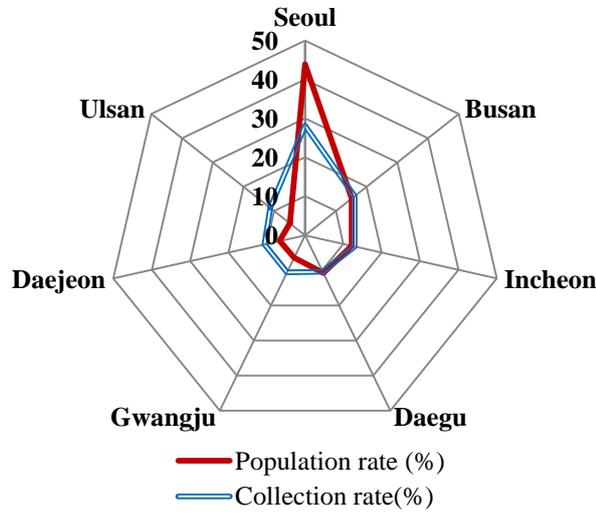


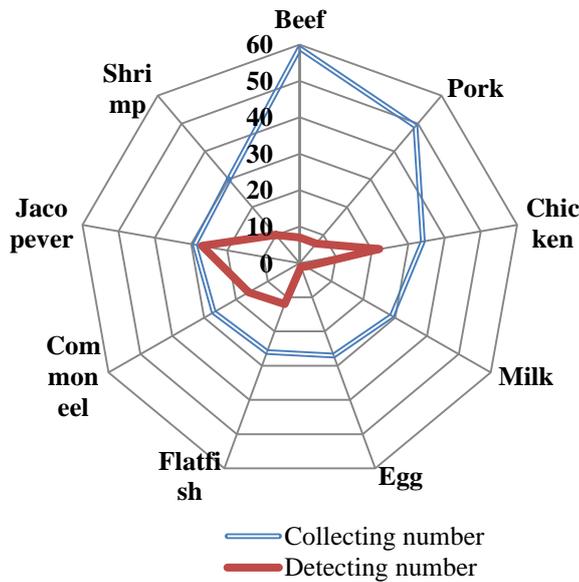
Fig. 1. Molecular structure of 13 quinolones and piromidic acid (IS).

(A)



City	Population (unit:1000)	Collecting rate (%)
Seoul	10,206	43.9
Busan	3,359	15.2
Incheon	2,840	12.2
Daegu	2,508	10.8
Gwangju	1,471	6.4
Daejeon	1,525	6.6
Ulsan	1,147	4.9
Total	23,236	100

(B)



Species	Collecting number	Detecting number
Beef	59	7
Pork	49	7
Milk	34	22
Chicken	29	2
Egg	27	1
Flatfish	26	12
Common eel	27	16
Jacopever	29	27
Shrimp	30	10
Total	310	104

Fig. 2. The sampling number by area according to the 「Sampling with probability proportionate to size」 (A) and distribution of samples containing quinolones residues by region (B).

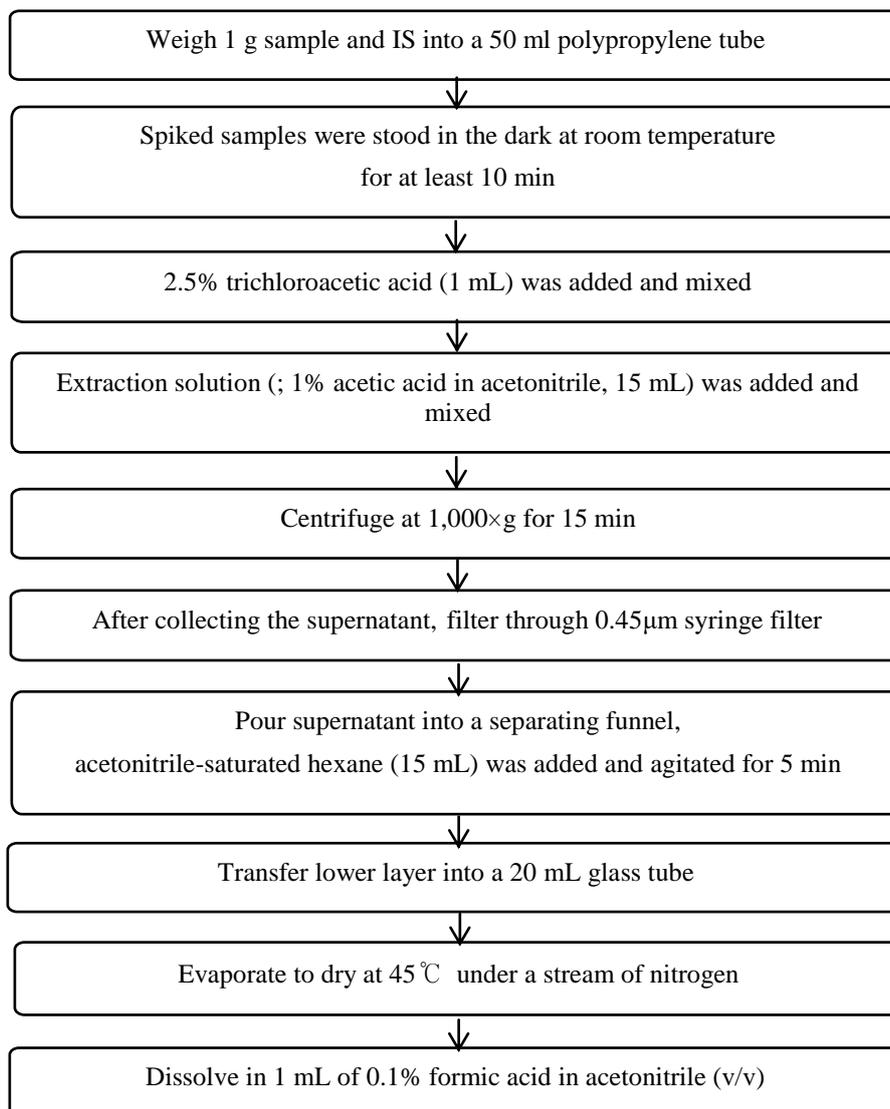


Fig. 3. Schematic diagram of the extraction procedure for the determination of quinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, ofloxacin, orbifloxacin, oxolinic acid, pefloxacin, and sarafloxacin) in livestock and marine products.

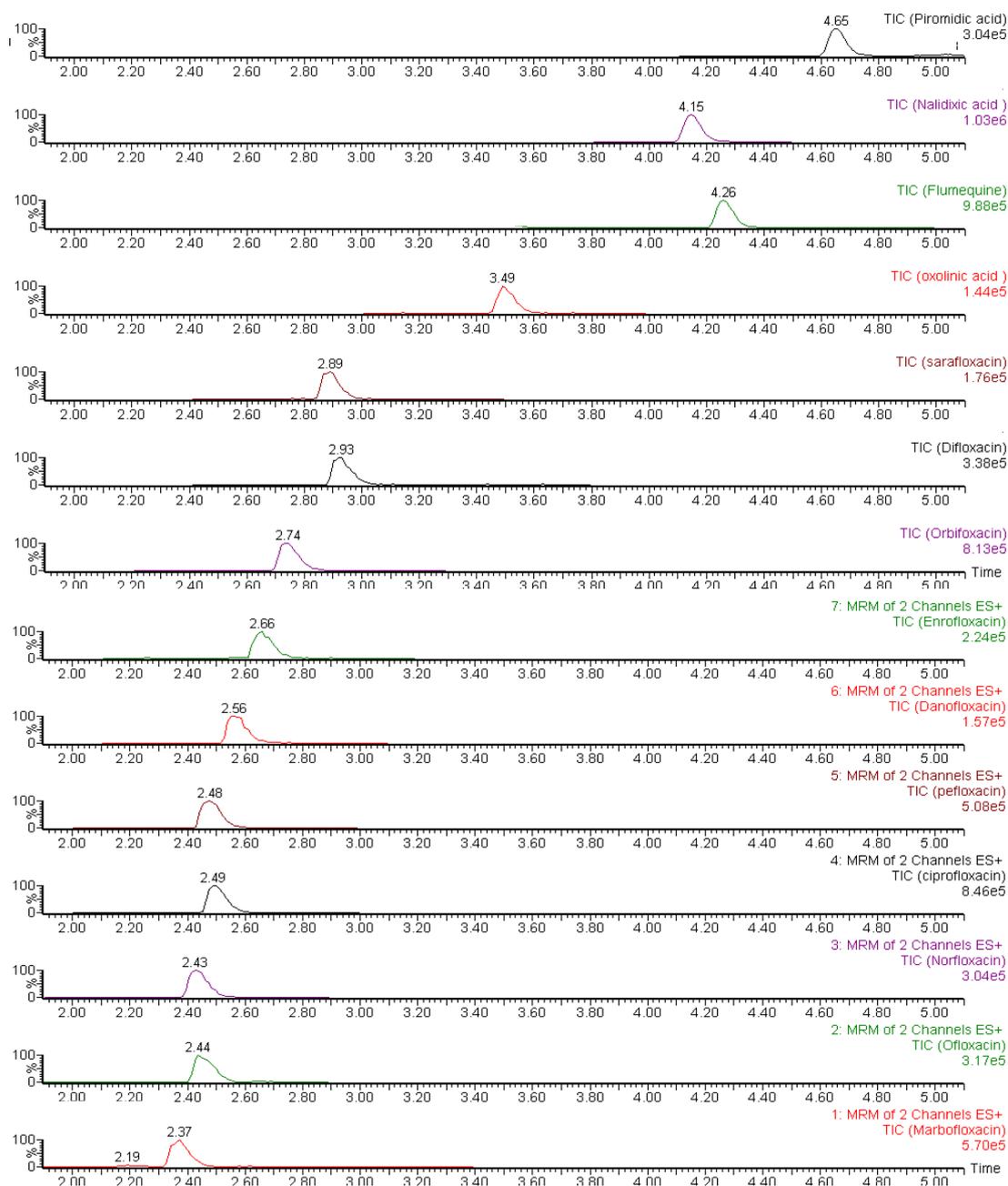


Fig. 4. UPLC-ESI-MS/MS chromatograms of a blank bovine muscle sample spiked at 10 µg/kg for DIF, ENR, FLU, NAL, OFL, and SAR, 20 µg/kg for CIP, DAN, MAR, NOR, ORB, OA, and PEF, and 4 µg/kg for PIR (IS).

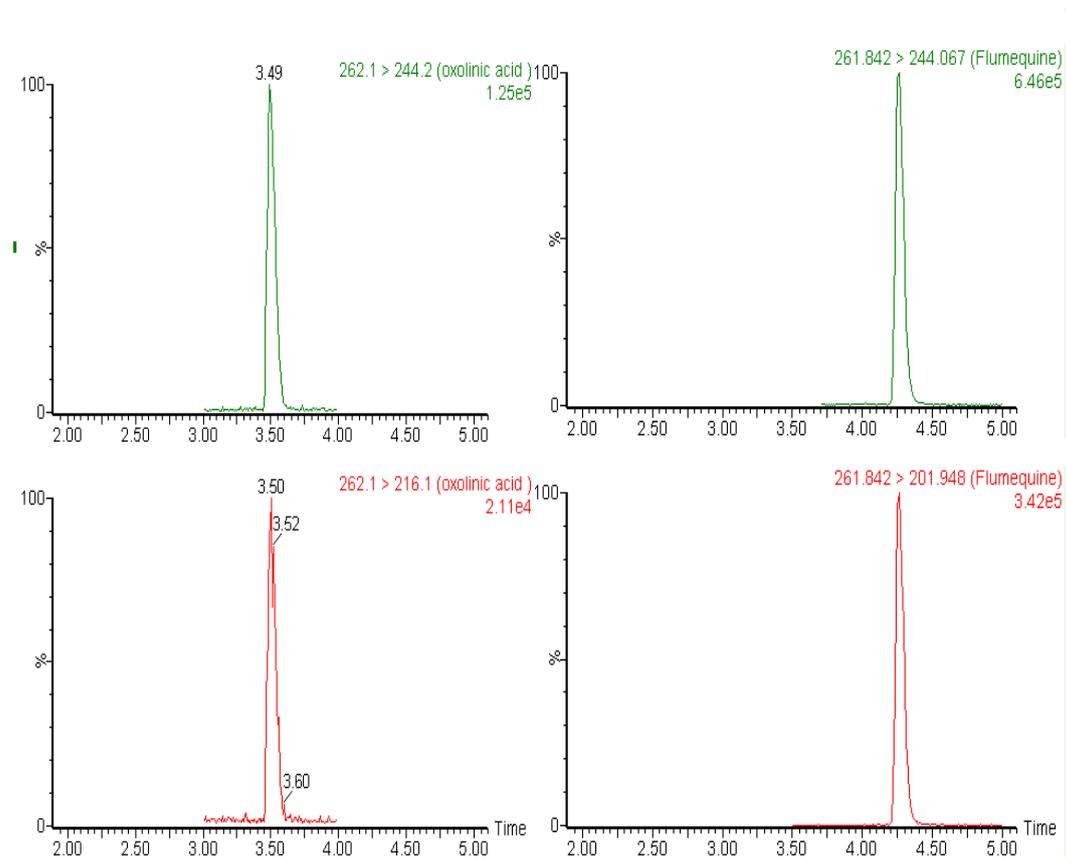
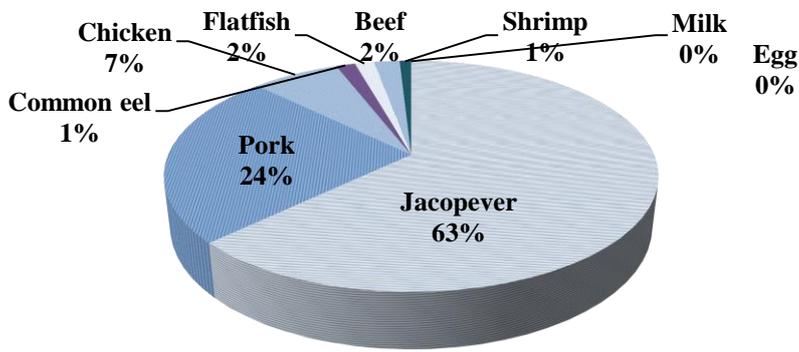
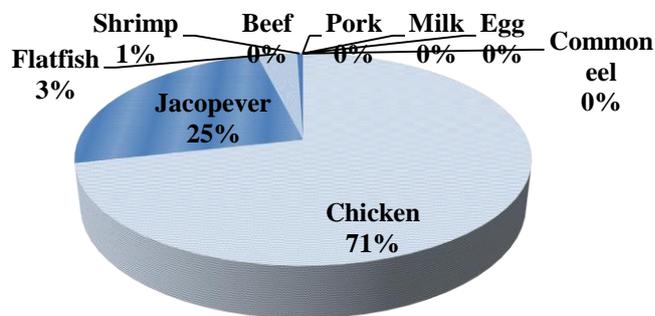


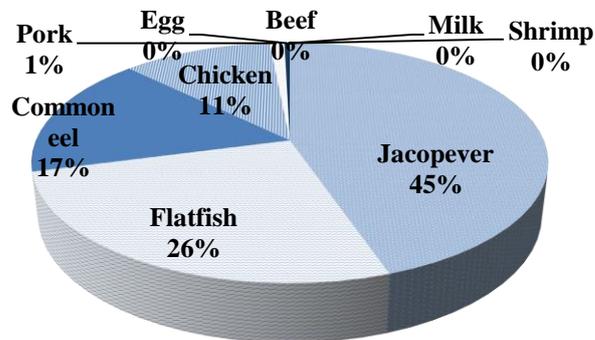
Fig. 5. Comparison of ion transition between flumequine and oxolinic acid. Chromatograms, obtained from the bovine muscle fortified with 10  $\mu\text{g}/\text{kg}$  of flumequine and 20  $\mu\text{g}/\text{kg}$  of oxolinic acid, were described. The transition  $[\text{M} + \text{H}]^+ \rightarrow [\text{M} + \text{H-OC}_3\text{H}_8]^+$  ( $m/z$  262  $\rightarrow$  202) was used for flumequine and  $[\text{M} + \text{H}]^+ \rightarrow [\text{M} + \text{H-OCH}_2\text{O}]^+$  ( $m/z$  262  $\rightarrow$  216) was used for oxolinic acid, respectively.



(A) Sarafloxacin

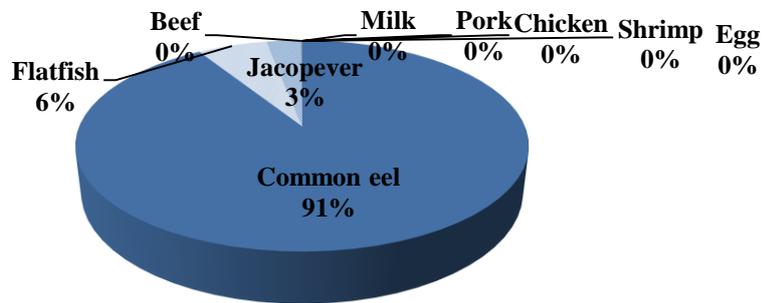


(B) Enrofloxacin

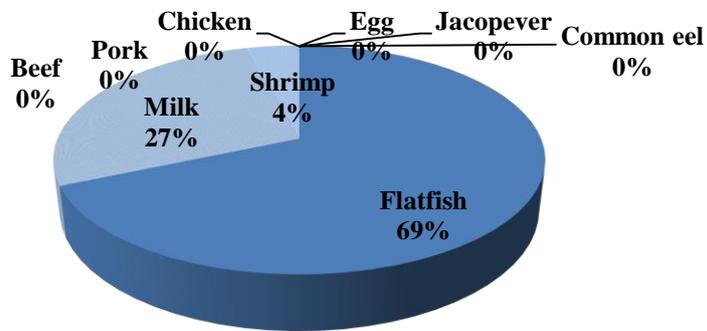


(C) Flumequine

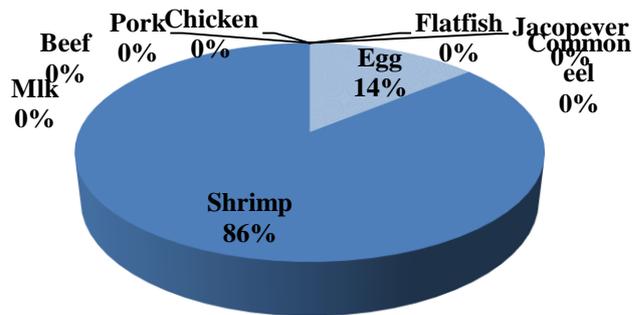
Fig. 6. The food contribution for estimated daily intake from quinolones residues. (A) Sarafloxacin, (B) Enrofloxacin, (C) Flumequine, (D) Oxolinic acid, (E) Marbofloxacin, (F) Difloxacin



(D) Oxolinic acid



(E) Marbofloxacin



(F) Difloxacin

Fig. 6. (Continued)

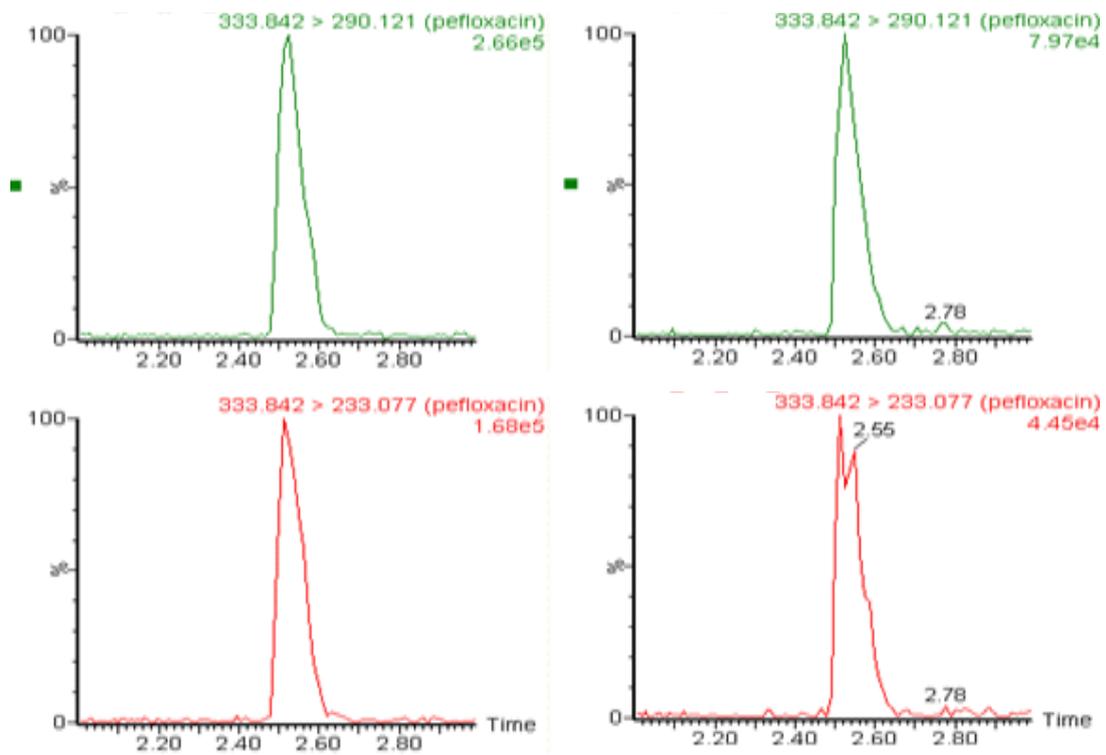


Fig. 7. UPLC-ESI-MS/MS chromatogram of pefloxacin standard solution (left) and detected common eel (right). The detected concentration of pefloxacin was 62.4  $\mu\text{g}/\text{kg}$ .

## **CHAPTER II**

### **Development of multi-residue analytical method for 9 cephalosporins and application to the residue monitoring in livestock and marine products in Korea**

## ABSTRACT

A sensitive ultra performance liquid chromatography with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) method was developed for the simultaneous quantitative analysis of 9 cephalosporins in 8 species of livestock and marine products (beef, pork, chicken, milk, flatfish, jacopeer, common eel, and shrimp). Multiple reaction monitoring scanning was performed for quantification with switching electrospray ion source polarity between positive and negative modes in a single run time of 5 min. Highest sensitivity for the analytes was obtained when cefazolin, cephapirin, desacetyl-cephapirin, cephalixin, cefalonium, cefoperazone, and cefuroxime were measured in the positive electrospray ionization mode (ESI(+)) and cefquinome and brucine (as internal standard) were measured in the negative electrospray ionization mode (ESI(-)). The analytical method involved the sample preparation based in a combination of solvent extraction and ultrasonic-assisted extraction. Besides, matrix matched calibration curve with internal standard was used for quantification during the determination of the cephalosporin residues in samples.

The proposed method was validated according to the CODEX guideline and all results fully complied with CODEX recommendation. Good linearities were obtained and the

correlation coefficients ranged from 0.9990 to 0.9997 depending upon antibiotics. The limit of detection and limit of quantitation were lower than 8 and 25  $\mu\text{g}/\text{kg}$ , respectively.

A survey for 9 cephalosporins was conducted using 333 livestock and marine products distributed in Korea. Cefalonium was detected in 11 beef samples and cefquinome was detected in 1 milk sample. Detected concentrations were from 1.10 to 14.7  $\mu\text{g}/\text{kg}$  in beef and 7.50  $\mu\text{g}/\text{kg}$  in milk. All the detected levels were below the legal residue limits of 10-600  $\mu\text{g}/\text{kg}$  in the Korean Food Code and the risk values were very safe levels.

## INTRODUCTION

Cephalosporins are  $\beta$ -lactamic antibiotics that consist of the 7-amino cephalosporonic acid nucleus and a six membered dihydrothiazine ring fused to the  $\beta$ -lactam portion and they act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls (Hou et al., 2013). They are most widely used in veterinary medicine for livestock farming and bovine milk production and used for the prevention and treatment of bacterial infection, such as respiratory, urinary, and skin infections (Hermo et al., 2013). However, it is well known that  $\beta$ -lactam antibiotics possess an allergic potential in pre-sensitized people and therefore it cannot completely be excluded that high residue levels in food of animal origin will evoke allergic reactions in highly sensitive individuals. For these reasons, no feeding before shipping, the proper dosage, the observance of withdrawal period, and the accurate pharmaceutical feeding are major factors to prevent possible resistances.

The EU has established maximum residue limits (MRLs) for cephalosporins in foodstuffs of animal origin (EU, 2009). The MRLs ranges are between 20 and 1,000  $\mu\text{g}/\text{kg}$  for mammalian food producing species, such as milk, muscle, fat, and so on. In Korea, cefacetrile, cefazolin, cephapirin, cefalexin, cefalonium, cefoperazone, cefuroxime, cefquinome, and ceftiofur were licensed for the treatment of bacterial infections of animals used in livestock farming. The MRLs of cephalosporins were established by the

Ministry of Food and Drug Safety (MFDS) (Table 1) and ranged between 0.1 and 1 mg/kg for mammalian food producing species, such as cattle, pig, sheep, etc (MFDS, 2014). The residue levels below the MRLs are considered as safe. In this sense, the simultaneous determination of cephalosporins residues at MRL levels requires reliable and efficient analytical methods to comply with current legislations.

Several analytical methods have been described for the determination of cephalosporins included capillary electrophoresis (Solangi et al., 2007), luminescence (Yunjuan and Jiuru, 2006), spectrophotometry (Saleh et al., 2001; Saleh et al., 2003), and liquid chromatography (Virginia et al., 2000; De Diego et al., 2005; Zhu and Row, 2009; Karaqeorqou and Samanidou, 2010). However, the problems of the methods related to specificity and selectivity. Analytical detection limits of HPLC-UV method are generally limited by significant signal interference associated with UV spectral overlaps with other food constituents (Hui et al., 2013). Nowadays, LC-MS/MS has become the major analytical method for identifying cephalosporins due to its higher selectivity and sensitivity than other methods. Several methods have been described applying LC-MS/MS in bovine milk (Bruno et al., 2001; Hou et al., 2013), muscle (Matthias et al., 2004; Perez-Burgos et al., 2012), and kidney (Fagerquist and Lightfield, 2003; Clifton et al., 2005; Katerina and Alan, 2008). Although other mass spectrometers can be applied,

triple quadrupole mass spectrometers are excellent for quantification because they isolate and target biomolecules of interest, exclude signal from the background matrix, and are sensitive, fast-scanning and reproducible with high dynamic range. For this reason, triple quadrupoles are routinely used for developing mass spectrometry-based quantitative assays via MRM (Min et al., 2012). However, there is still a need for multi-residue methods capable of confirming and quantifying most of cephalosporins for which MRL values were set in different matrices. Moreover, compared to LC, UPLC improved resolution and sensitivity, as well as significantly reduced sample analysis time and mobile phase solvent consumption (Junza et al., 2011; Stolker et al., 2005).

Therefore, the purpose of this work was to develop a simultaneous method based in a combination of solvent extraction and ultrasonic-assisted extraction followed by UPLC-ESI-MS/MS for the detection and quantification of 9 cephalosporins (cefacetrile, cefazolin, cephalpirin, desacetylcephalpirin, cefalexin, cefalonium, cefoperazone, and cefuroxime) in livestock and marine products. Moreover, the proposed method was applied to the monitoring on residues of 9 cephalosporins in livestock and marine products commercialized in Korea.

## MATERIALS & METHODS

### *Chemicals and materials*

Cefacetrile and desacetyl-cephapirin were obtained from Toronto Research Chemical (Ontario, Canada). Cephalixin, cefalexin, cefalonium, cefoperazone, cefuroxime, and cefquinome were purchased from Fluka and, cefazolin was supplied by Sigma-aldrich (MO, USA) (Fig. 1). Brucine from Sigma-aldrich was used as internal standard (IS). Acetonitrile, hexane, and methanol were HPLC grade and obtained from Merck (Darmstadt, Germany).

### *Sample collection*

Sampling area was set in accordance with the 「Sampling with probability proportionate to size」 and species and number of samples were determined on the basis of the consumption of food according to the 「National Health and Nutrition Examination Survey, 2011」. Each sample was purchased at least two and sampling points within the sampling area were not biased.

As a result, a total of 333 livestock products including beef (n=75), pork (n=63), milk (n=77), chicken (n=21), flatfish (n=23), jacoever (n=27), common eel (n=22), and

shrimp (n=25) were purchased from different markets in Seoul, Busan, Incheon, Daegu, Daejeon, Gwangju, Ulsan, and Jeju. Details were given in Fig. 2. All solid samples were finely ground with blender and stored in a freezer (-20°C) until use.

### ***Preparation of standard solution***

Each individual standard, for example, cefacetriple, desacetyl-cephapirin, cephapirin, cefalexin, cefalonium, cefoperazone, cefuroxime, and cefquinome was dissolved in methanol. Stock standard solution (100 µg/mL) were obtained and stored at 4°C in the dark for no longer than 2 months. Working standard solutions were prepared daily by mixing individual stock solutions and diluted in distilled water. Brucine (IS) was diluted in distilled water at the concentration of 1 µg/ mL.

### ***Preparation of spiked samples and standard***

One sample of each matrix was repeatedly measured to confirm that no 8 cephalosporins were present and was used for the preparation of matrix-matched calibration standards and fortified samples for the validation of the proposed method.

Each blank sample of beef, pork, chicken, milk, flatfish, jacobever, common eel, and shrimp was homogenized and stored at -20°C until use. Fortified samples were prepared

by spiking 100  $\mu$ L of diluted working solutions at the concentration of 1/2 MRL, MRL, and 2 MRL for cephalosporins.

### ***Matrix-matched calibration curves***

For quantification, matrix-matched calibration curves with IS were performed. The blank samples (beef, pork, chicken, milk, flatfish, jacopecover, common eel, and shrimp) were used as matrix and fortified with working standard solutions. Each sample was submitted to the full extraction procedure. The six-point matrix-matched calibration curves in livestock and marine products (except milk) were prepared with the blank samples spiked with 0, 0.25, 0.05, 0.1, 0.2, and 0.3 mg/kg for cefacetile, desacetyl-cephapirin, cephapirin, cefoperazone, and cefquinome, and 0, 0.05, 0.1, 0.2, 0.4, and 0.6 mg/kg for cefalexin and cefazolin, and 0, 0.005, 0.01, 0.02, 0.04, and 0.06 mg/kg for cefalonium, and 0, 0.01, 0.02, 0.04, 0.08, and 0.12 mg/kg for cefuroxime, respectively.

In milk, the curves were prepared with the blank samples spiked with 0, 0.025, 0.05, 0.1, 0.2, and 0.3 mg/kg for cefacetile and cefazolin, and 0, 0.015, 0.03, 0.06, 0.12, and 0.18 mg/kg for cephapirin, desacetyl-cephapirin, and cefoperazone, and 0, 0.05, 0.1, 0.2, 0.4, and 0.6 mg/kg for cefalexin, and 0, 0.005, 0.01, 0.02, 0.04, and 0.06 mg/kg for cefalonium, and 0, 0.01, 0.02, 0.04, 0.08, 0.12 mg/kg for cefuroxime and cefquinome,

respectively. Brucine (IS) was spiked in all of these samples at the concentration of 20  $\mu\text{g}/\text{kg}$ . The calibration curves for 9 cephalosporins were constructed by calculating the ratio of each peak area relative to the corresponding IS. Separate calibration curves were used for each sample.

### ***Extraction procedure***

Homogenized sample (2 g) of beef, pork, chicken, flatfish, jacoever, common eel, and shrimp and 2 mL of milk was taken into a 50 mL disposable polypropylene centrifuge tube and IS solution (100  $\mu\text{L}$ ) was directly spiked. 5 mL of acetonitrile was added to the sample and followed by shaking during 2 min on a vortex mixer (Heidolph Reax top, Heidolph, Schwabach, Germany). The samples were homogenized again for 10 min with ultra-sonicator (Power sonic 520, Hwashin, Seoul, Korea) and centrifuged (Allegra X-22R, Beckman Coulter, Brea, CA, USA) at  $1,000\times g$  for 10 min to induce the precipitation of proteins. The supernatant was poured into a separating funnel and repeat the same step once more. The supernatant obtained two times was moved in a 50 mL centrifuge tube, 10 mL of hexane was added to the supernatant extract in order to remove the lipid. After vortex-mixing for 1 min and centrifugation at  $1,000\times g$  for 10 min, the lower layer was filtered through a syringe filter (Whatman<sup>®</sup>, PVDF membrane, pore size 0.45 $\mu\text{m}$ ) and

transferred into the 15-mL glass test tube. The extract was evaporated to a proper volume (about 1 mL) under a stream of nitrogen (EYELA GM-2200, Tokyo, Japan) and subsequently methanol (1 mL) was added and evenly mixed. After that, the extract was evaporated until dryness under a gentle nitrogen stream at 45°C. The residue was reconstituted with 400 µL of 0.1% formic acid in 10% acetonitrile (v/v) and filtered through a syringe filter (Whatman®, PVDF membrane, pore size 0.2 µm) prior to UPLC-ESI-MS/MS injection (Fig. 3). Every sample was analyzed three times and the result was expressed as the mean of the total residue concentration.

### ***UPLC-ESI-MS/MS conditions***

UPLC equipment was an Acquity ultra performance LC-Waters system equipped with an autosampler (Waters, MA, USA) and ESI-MS/MS measurements were performed using a Quattro premier XE (Waters, MA, USA). Cephalosporins were separated on an Acquity UPLC BEH C<sub>18</sub> column (2.1 mm × 100 mm, 1.7 µm particle size, Waters). Data was controlled by MassLynx 4.1 software (Waters) in a personal computer.

The binary mobile phase of UPLC-MS/MS composed of deionized water and acetonitrile with 0.1% formic acid in both solvents was used. The gradient conditions were initiated with 95% mobile phase A followed by a linear decrease to 10% in 3 min,

and maintained for 0.5 min at 10%. Then mobile phase A was increased to 95% in 0.1 min and holding at 95% for 1.4 min. The run time for each injection was 5 min, the flow rate was 0.35 mL/min, and the injection volume was 20  $\mu$ L in the full-loop mode. Details were given in Table 2.

The mass spectrometer was operated with ESI source in the polarity switching mode. MS determination was performed with MRM experiments combined with monitoring of the most abundant MS/MS (precursor $\rightarrow$ product) ion transitions using a dwell time of 0.03s. Detailed parameters for MRM acquisition are presented in Table 3. The MS parameter were as follow: capillary voltage, 3.5 kV; source temperature, 120 $^{\circ}$ C; desolvation temperature, 350 $^{\circ}$ C; cone gas flow, 50 L/h; and desolvation gas flow, 800 L/h.

### ***Method validation***

The method was validated according to the CODEX guideline (FAO, 2009) for a quantitative confirmation method. The validation parameters to evaluate the method were linearity, limit of detection (LOD), limit of quantitation (LOQ), precision (repeatability and reproducibility), and accuracy.

## RESULTS & DISSCUSION

### *Method validation*

Each matrix was repeatedly measured to confirm that no 9 cephalosporins were detected and was used for the preparation of matrix-matched calibration standards and fortified samples for the validation of the method. The developed method was validated with UPLC-ESI-MS/MS according to the CODEX guidelines (FAO, 2009) for quantitative methods. All obtained results were satisfied with CODEX recommendation and indicated the stability of the proposed method.

The linearities, LOD, and LOQ for each species of matrices were shown in Table 4. The linearity was measured with the matrix-matched calibration curves using 6 calibration points. Good linearities were obtained and the correlation coefficients ranged between 0.9990 and 0.9997 depending upon antibiotics. Besides, brucine as IS, which considerably increased the linearity of the matrix-matched calibration curves of 9 cephalosporins.

The LOD and LOQ values were measured based on signal-to-noise ratio (S/N) values of 3.3 and 10, respectively. The LOD ranged from 0.3 to 6 µg/kg and LOQ ranged from 1 to 20 µg/kg in livestock (except milk) and marine products, respectively. In milk, the LOD ranged from 0.8 to 8 µg/kg and LOQ ranged from 8 to 25 µg/kg, respectively. The results

of LOD and LOQ demonstrated that the method were enough to determine the MRLs values in animal origin products.

The repeatability and reproducibility (the intra-day and inter-day precision) were evaluated in 8 livestock and marine products. These validation parameters are expressed as relative standard deviation (RSD, %). The repeatability was evaluated by spiking 8 blank samples (n=6, for each matrix) at the MRL level and the reproducibility was carried out on 3 consecutive days. Repeatability and reproducibility results were presented in Table 5 and 6. The RSD (%) of repeatability ranged between 3.07 (Cefuroxime) in pork and 11.68 (Desacetyl-cephapirin) in jacopever. The RSD (%) of reproducibility ranged between 0.88 (Cefalexin) in beef and 10.92 (Desacetyl-cephapirin) in shrimp. It could be observed that RSD (%) were always lower than 12%. The repeatability values expressed as RSD (%) were lower than 15%, which is within the acceptance criteria of CODEX for validating analytical methods.

The accuracy of the method was estimated through recovery studies. The percentage of recoveries were calculated at 3 different spiking levels ( $1/2 \times \text{MRL}$ , MRL, and  $2 \times \text{MRL}$ ). The matrix-matched calibration curves were used with IS. Average recoveries (%) of each analyte were obtained performing the analysis in 6 replicated for each matrix. The recovery data were presented in Table 7 and 8. The acquired results were satisfied, with

the percentage of recoveries (%) ranged from  $63.82 \pm 8.00$  (cefacetrile) to  $92.89 \pm 4.09$  (cefalonium) in livestock products and from  $73.16 \pm 2.63$  to  $95.13 \pm 7.24$  (cefalexin) in marine products. The RSD (%) ranged from 3.27 to 12.54 in livestock products and from 3.06 to 7.61 in marine products. The results were within the acceptance criteria of the CODEX validating analytical method (Fig. 4).

In this study, good validation parameters were obtained indicating the suitability of the proposed method for the multi-residue analysis of 9 cephalosporins. Besides, the use of matrix-matched calibration curves with IS enabled to reduce the difference in the signal output of analyte between a sample extract solvent and a pure solvent.

### ***Optimization of sample preparation***

In this work, the reliable and specific method for livestock and marine products was developed. Extraction procedure was focused on the establishment of the most suitable conditions used for the simultaneous determination of 9 cephalosporins from animal origin products.

Several extraction solvents were evaluated in order to maximize the recovery and minimize preparation time. Beef, flatfish, and milk blank samples fortified with 9 cephalosporins were extraction with solution of 100% acetonitrile (v/v), 100% methanol

(v/v), and acetonitrile: distilled water (80:20, v/v) and consecutively extracted with hexane or not. As a result, 100% acetonitrile (v/v) and additional hexane extraction were shown to the most effective recoveries and lowest RSD (%) in 3 species of matrices (Fig. 5). Acetonitrile was preferred than methanol as an extraction solvent. Because acetonitrile could simultaneously precipitate the proteins, denature enzymes, and extract the target analytes. The extraction of hexane and syringe filtration was useful to achieve the deproteinization and defatting of samples. Moreover, after the solvent extraction of acetonitrile, a continuative ultrasonic-assisted extraction was conducted in order to acquire higher recovery. Ultrasound could be regarded as a useful alternative for solid sample pretreatment because the energy accelerated the steps of sample preparation, such as dissolution, fusion, and leaching (De Castro and da Silva 1997; Marilena and Nikolaos, 2015).

In addition, a pre-concentration step was evaluated. To carry out this step, methanol (1 mL) was added or not during evaporation under a stream of nitrogen. Consequently, when the methanol (1 mL) was added to tube in pre-concentration step, the recoveries (%) were improved by 14% in beef (cefuroxime), 45.7% in flatfish (cefoperazone), and 37% in milk (cefalexin), respectively. Details were given in Fig. 6.

When ESI is used as ionization technique, one of the main problems is the signal suppression or enhancement of the analytes due to the other components present in the matrix (Rocui et al., 2014). Because the existence of matrix effects, matrix matched calibrations were used for quantification during the determination of the 9 cephalosporins in livestock and marine products. Besides, quantification by matrix-matched calibration curves using IS allowed to monitor the efficiency of the extraction procedure and also to correct possible matrix effects (Andreia et al., 2014). Thus, this proposed method could be applied to the diverse matrices.

### ***Optimization of UPLC-ESI-MS/MS***

Chromatographic conditions of UPLC were studied in order to achieve the best separation and retention for the analytes, minimizing analysis time. Several experiments were conducted testing different mobile phases consisting of acetonitrile as organic phase and water with different concentrations of formic acid (0.05 and 0.1%) or ammonium formate (5 mM). As a result, the addition of 0.1% formic acid allowed better results than ammonium formate, and it enhanced the ionization efficiency. Especially, formic acid and acetonitrile were significant roles for the formation of well distributed peak and sharp peak shape (Lina et al., 2011; Junza et al., 2014). Moreover, 5 min of run time was

acquired with the optimum conditions for the separation of 9 cephalosporins. Good response was obtained with the gradient described in Table 2. Furthermore, in the first 1 min of the analysis time, main interference are presented due to polar matrix constituents that are eluted and co-elution with the analytes would lead to large signal suppression (Marilena et al., 2015). As a result, it was important that 9 cephalosporins began to elute after the 1 min of the analysis time. Other parameter such as flow rate, and injection volume were studied in order to obtain a fast and reliable separation. The best results were acquired when 0.35 mL was used as flow rate and 20  $\mu$ L were injected, respectively. The chromatographic separation of 9 cephalosporins was performed with a 2.1 mm $\times$  100 mm column, which was consisting of a smaller particle size than common HPLC column (Andreia et al., 2013). Thus, it could be acquired the high efficiency in peak separation and good peak resolution.

In MS/MS system, at first, the selection and tuning of the precursor and product ions were performed. For polarity switching ionization mode, direct infusion of each standard solution (1 $\mu$ g/mL) was carried out, depending on if the determination of the analyte is achieved in positive or negative ionization mode, respectively. As a result, the protonated ( $[M+H]^+$ ) or deprotonated ( $[M-H]^-$ ) molecular ions were selected as the precursor ion. Most of cephalosporins antibiotics could be detected as protonated molecular ions

$[M+H]^+$  using the positive electrospray ionization mode (ESI(+)). However, the negative electrospray ionization mode (ESI(-)) was found to be the most sensitive electrospray condition. Consequently, in this study, 8 cephalosporins (cefacetrile, cefazolin, cephalirin, desacetyl-cephalirin, cefalexin, cefalonium, cefoperazone, and cefuroxime) had better sensitivity and reproducibility in the positive ion mode, and negative ion mode was suitable for the analysis of cefquinome and brucine. Especially, the ESI(-) mode resulting in a much better signal to noise ratio of the molecular ions (Matthias et al., 2004).

Then, collision energies were evaluated in order to trace the most abundant product ions, selecting the most sensitive transition for quantification purposed and a second one for confirmation. Table 3 presented MS/MS transitions as well as cone voltage and collision energy values optimized for the 9 cephalosporins.

The system was operated in the MRM mode acquiring two diagnostic product ion transitions for each cephalosporin. Two product ion transitions (quantifier ion, qualifier ion) are monitored and the ratio between the monitored fragment ions is calculated and compared with the ratio obtained from the analytical standard. To achieve maximum sensitivity for all analytes, sufficiently long dwell times are required for each daughter ion transition measured. As a result, the chromatography gradient conditions were

adjusted to obtain a nearly complete separation of 9 cephalosporins. Chromatograms of 9 cephalosporin antibiotics were presented in Fig. 7.

### ***Application of the proposed method to animal products***

Cephalosporins were analyzed in 333 livestock and marine products including 75 beef, 63 pork, 77 milk, 21 chicken, 23 flatfish, 27 jacoever, 22 common eel, and 25 shrimp (Table 9). The results were described in Table 10. The residues of cephalosporins were detected in only 12 livestock product samples (3.6% incidence), such as, beef and milk. 11 out of 75 beef samples (14.7% incidence) and 1 out of 77 milk samples (1.3% incidence) showed cephalosporins residues (Fig. 2). The detected cephalosporins in livestock products were cefalonium in beef and cefquinome in milk, respectively. Detected concentrations were from 1.10 to 9.77  $\mu\text{g}/\text{kg}$  in beef and 7.50  $\mu\text{g}/\text{kg}$  in milk.

Cephapirin is rapidly and quantitatively metabolized to desacetylcephapirin and acetic acid in spiked muscle and kidney homogenate. Thus, only desacetyl cephapirin could be determined and validated in tissue homogenate (Matthias et al., 2004). This metabolism was not observed for spiked raw milk. However, it has to be considered that after intramammary infusion of cephapirin, cephapirin can be detected in milk. The council regulation (EEC) 2377/90 and the Korea Food Code set MRLs for the sum of cephapirin

and desacetylcephapirin. Thus, the proposed method has been developed so that cephapirin and desacetylcephapirin are simultaneously determined in all samples.

Furthermore, there are no MRLs for the fisheries and marine products in Korea, except for cefalexin. In veterinary field, cephalosporin antibiotics are mostly applied to the livestock, and especially cefalonium was widely used as a therapeutic agent for bovine mastitis (Yim, 2014). In this study, among cephalosporin antibiotics, only cefalonium and cefquinome were detected in livestock products below the MRL. Thus, the risk value based on the cephalosporins exposure by food intake was calculated using the EDI (estimated daily intake) and ADI (acceptable daily intake). Compared with ADI (0.0153 mg/kg b.w./ day in cephalonium and 0.0038 mg/kg b.w./ day in cefquinome), the risk value was safe level of 0.02% in cephalonium and 0.01% in cefquinome, respectively. Details were given in Table 11.

However, cephalosporin were classified as veterinary critically important antimicrobials (VCIA) from the Office International des Epizooties (OIE) and also 1- and 2-generation cephalosporins were classified as highly important antimicrobials (HIA), and 3- and 4-generation cephalosporin was classified as critically important antimicrobials (CIA) for human medicine) from the World Health Organization (WHO). Although cephalosporin residues appeared to be at very save levels in livestock and marine products, the

possibility of antibiotic misuse, such as violation of withdrawal period, pharmaceutical feed by mistake, and excess dosage, could occur. Therefore, since cephalosporin antibiotic resistance is very important in terms of public health, continuous management and monitoring are required.

## CONCLUSION

A specific and reliable method was developed and validated for the simultaneous determination of 9 cephalosporins in livestock and marine products using UPLC-ESI-MS/MS. The use of UPLC-ESI-MS/MS improved the sensitivity and reduced analysis time, allowing the identification and quantification of 9 cephalosporins in 5 min. Acquired validation parameters fully complied with CODEX recommendation. Besides, the matrix-matched calibration curves and internal standard play significant roles in compensating for the matrix effects.

In this work, a large number of animal origin products (n=333) commercialized in Korea were analyzed and quantified. The residues of 9 cephalosporins were detected in 12 samples (3.6% incidence) but residue levels were below the MRLs. The detected cephalosporins were cefalonium in beef and cefquinome in milk, respectively. Furthermore, the risk value was safe level of 0.02% in cephalonium and 0.01% in cefquinome. Even though the monitoring results appear to be considerably safe levels, antibiotic residues are still exposed to consumers. Therefore, the survey of cephalosporins residues in livestock and marine products was continued.

Table 1. Maximum residues limits of cephalosporins in Korea

Compound	Food	mg/kg
Cefacetrile	Milk	0.05
Cefazolin	Cattle, Pig, Sheep, Goat, Milk	0.05
Cephapirin	Cattle	0.05~0.1
+desacetylcephapirin	Milk	0.03
Cefalexin	Cattle, Pig, Sheep, Goat, Equine, Chicken	0.2~1.0
	Fish	0.2
	Milk	0.1
Cefalonium	Cattle	0.01
Cefoperazone	Milk	0.03
Cefuroxime	Cattle, Milk	0.02
Cefquinome	Cattle, Pig, Equine	0.05~0.2
	Milk	0.02
Ceftiofur	Cattle, Pig	1.0~6.0
	Milk	0.1

Table 2. Analytical conditions of LC-ESI-MS/MS for analysis of cephalosporins

	Items	Conditions	
LC	Instrument	Acquity ultra performance LC	
	Column	Acuity UPLC BEH C <sub>18</sub> (2.1×10 mm, 1.7 um)	
	Mobile phase	A: 0.1% formic acid in DW B: 0.1% formic acid in acetonitrile	
	Gradient	Time(min)	A (%)      B (%)
		Initial	95          5
		3.0	10          90
		3.5	10          90
3.6		95          5	
5.0	95          5		
MS	Flow rate	0.35 mL/min	
	Injection volume	20 µL	
	Instrument	Quattro Premier XE	
	Ionization mode	Polarity switching mode	
	Capillary voltage	3.5 kV	
	Cone voltage	17 V	
	Source temperature	120 °C	
	Desolvation temperature	800 °C	

Table 3. Multiple reaction monitoring conditions of the MS/MS for the detection of 9 cephalosporins

Analyte	Ionization mode	Transition		Cone volt. (V)	Collision E. (eV)
		Precursor ion (m/z)	Product ion (m/z)		
Cefacetile	ESI(+)	362	178	20	13
			258		12
Cefazolin	ESI(+)	455	156	15	11
			323		17
Cephapirin	ESI(+)	424	292	20	15
			152		23
Desacetyl- cephapirin	ESI(+)	382	152	25	25
			292		14
Cefalexin	ESI(+)	348	174	14	12
			158		8
Cefalonium	ESI(+)	459	152	15	11
			337		19
Cefoperazone	ESI(+)	644	188	18	20
			528		10
Cefuroxime	ESI(+)	423	207	15	8
			318		15
Cefquinome	ESI(-)	529	134	20	15
			125		65
Brucine (IS)	ESI(-)	395	244	49	35
			170		65

Table 4. Validation parameters on the correlation coefficient ( $r^2$ ), LOD, and LOQ of 9 cephalosporins

Compound	Determination coefficient ( $r^2$ )	Livestock & marine products		Milk	
		LOD <sup>1)</sup> (µg/kg)	LOQ <sup>2)</sup> (µg/kg)	LOD <sup>1)</sup> (µg/kg)	LOQ <sup>2)</sup> (µg/kg)
Cefacetrole	0.9990	-	-	8	25
Cefazolin	0.9994	3	10	1.5	5
Cephapirin	0.9997	6	20	1.5	5
Desacetyl- cephapirin	0.9992	1.5	5	1.5	5
Cefalexin	0.9991	6	20	1.5	5
Cefalonium	0.9995	0.3	1	0.8	2.5
Cefoperazone	0.9996	-	-	3	10
Cefuroxime	0.9994	3	10	4	12.5
Cefquinome	0.9991	1.5	5	1.5	5

<sup>1)</sup> Limit of detection

<sup>2)</sup> Limit of quantitation

Table 5. Intra-day precision of 9 cephalosporins at MRL levels

Compound	Intra-day precision (n = 6), RSD (%) <sup>1)</sup>							
	Beef	Pork	Milk	Chicken	Flatfish	Jacopever	Common eel	Shrimp
Cefacetrile			7.91					
Cefazolin	7.40	8.29	6.83	6.62	9.37	6.61	9.47	8.86
Cephapirin	8.26	5.70	8.74	9.93	11.39	9.93	7.76	6.34
Desacetylcephapirin	8.21	5.54	6.27	9.13	5.66	11.68	7.63	6.50
Cefalexin	6.28	10.50	3.74	5.10	3.93	6.92	3.21	6.65
Cefalonium	4.73	5.82	9.51	3.20	10.76	8.96	8.43	8.26
Cefoperazone	6.23	6.95	6.97	8.79	8.39	9.67	10.97	8.48
Cefuroxime	7.57	3.07	8.74	10.15	9.99	8.85	4.92	9.93
Cefquinome	5.80	4.95	6.76	2.40	9.85	9.01	7.87	10.63

<sup>1)</sup> Relatively standard deviation (%)

Table 6. Inter-day precision of 9 cephalosporins at MRL levels

Compound	Inter-day precision (n = 3), RSD (%) <sup>1)</sup>							
	Beef	Pork	Milk	Chicken	Flatfish	Jacopever	Common eel	Shrimp
Cefacetrile	-	-	8.16	-	-	-	-	-
Cefazolin	4.83	4.01	8.42	5.28	4.63	3.59	10.38	9.50
Cephapirin	3.21	4.55	4.70	4.90	5.83	5.27	4.82	5.75
Desacetylcephapirin	3.83	4.65	5.58	1.10	3.58	7.47	6.00	10.92
Cefalexin	0.88	4.35	3.62	4.73	3.88	2.10	0.92	4.61
Cefalonium	4.83	2.95	9.15	8.92	4.22	5.70	2.94	5.81
Cefoperazone	6.52	4.78	4.84	8.37	5.12	7.62	10.52	5.99
Cefuroxime	6.49	8.30	4.70	9.61	9.29	2.80	5.25	6.14
Cefquinome	4.71	4.97	8.02	5.82	6.21	5.17	4.83	6.12

<sup>1)</sup> Relatively standard deviation (%)

Table 7. Average recoveries and RSD (%) measured in livestock products

Com-pound	Spiked conc. (µg/kg)	Beef		Pork		Chicken		Milk	
		Mean±SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>
Cefacetrile	1/2 MRL	-	-	-	-	-	-	63.82±8.00	12.54
	MRL	-	-	-	-	-	-	79.17±6.26	7.91
	2 MRL	-	-	-	-	-	-	76.28±6.28	8.23
Cefazolin	1/2 MRL	86.35±4.68	5.42	78.84±3.04	3.86	-	-	90.60±7.59	8.37
	MRL	87.91±6.50	7.40	83.16±6.90	8.29	-	-	90.55±6.19	6.83
	2 MRL	78.87±5.09	6.45	78.87±4.01	5.09	-	-	72.99±6.42	8.80
Cephapirin	1/2 MRL	79.28±4.55	5.74	-	-	-	-	76.13±4.00	5.25
	MRL	85.48±7.06	8.26	-	-	-	-	85.20±7.45	8.74
	2 MRL	83.85±4.30	5.13	-	-	-	-	82.83±4.06	4.90
Desacetyl-cephapirin	1/2 MRL	78.51±7.47	9.52	-	-	-	-	77.58±5.42	6.98
	MRL	86.16±7.07	8.21	-	-	-	-	88.72±5.56	6.27
	2 MRL	82.71±4.75	5.74	-	-	-	-	82.52±2.62	3.17
Cefalexin	1/2 MRL	80.26±4.56	6.25	70.04±2.99	4.27	87.42±7.15	8.18	78.24±7.03	8.99
	MRL	84.99±4.02	7.57	70.42±8.10	10.50	79.91±4.08	5.10	71.87±2.69	3.74
	2 MRL	81.49±2.66	3.27	72.31±8.19	10.33	83.02±3.62	4.36	72.07±5.71	7.92

<sup>1)</sup> Standard deviation

<sup>2)</sup> Relatively standard deviation (%)

Table 7. (Continued)

Compound	Spiked conc. (µg/kg)	Bovine		Swine		Chicken		Milk	
		Mean±SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>
Cefalonium	1/2 MRL	80.44±4.56	5.67	-	-	-	-	71.62±6.23	8.69
	MRL	84.99±4.02	4.73	-	-	-	-	79.72±7.58	9.51
	2 MRL	91.22±6.59	7.23	-	-	-	-	92.89±4.09	4.41
Cefoperazone	1/2 MRL	-	-	-	-	-	-	77.50±4.27	5.52
	MRL	-	-	-	-	-	-	87.17±6.07	8.74
	2 MRL	-	-	-	-	-	-	82.60±3.57	4.90
Cefuroxime	1/2 MRL	76.05±4.76	6.25	-	-	-	-	76.13±4.00	5.25
	MRL	88.98±6.74	7.57	-	-	-	-	85.20±7.45	8.74
	2 MRL	81.49±2.66	3.27	-	-	-	-	82.83±4.06	4.90
Cefquinome	1/2 MRL	90.59±5.11	5.64	76.20±2.87	3.77	-	-	88.39±7.62	8.62
	MRL	81.24±4.71	5.80	78.19±5.18	6.63	-	-	71.88±4.86	6.76
	2 MRL	84.87±5.24	6.17	79.93±3.95	4.95	-	-	89.12±8.66	9.72

<sup>1)</sup> Standard deviation

<sup>2)</sup> Relatively standard deviation

Table 8. Average recoveries and RSD (%) measured in marine products

Com-pound	Spiked conc. (µg/kg)	Flatfish		Common eel		Jacopever		Shrimp	
		Mean±SD <sup>1)</sup>	RSD(%) <sup>2)</sup>						
	1/2 MRL	80.03±2.44	3.05	75.70±4.33	5.71	78.57±4.17	5.31	73.16±2.63	3.60
Cefalexin	MRL	87.87±3.45	3.93	80.27±2.58	3.21	89.36±6.18	6.92	77.12±5.13	6.65
	2 MRL	94.77±5.49	5.79	84.65±5.76	6.80	95.13±7.24	7.61	81.53±5.74	7.06

<sup>1)</sup> Standard deviation

<sup>2)</sup> Relatively standard deviation

Table 9. Number of samples according to the sampling area

	Seoul	Busan	Incheon	Daegu	Gwangju	Daejeon	Ulsan	Jeju	Total
Beef	33	14	8	8	3	3	4	2	75
Pork	28	11	7	6	3	3	2	3	63
Milk	34	14	9	7	4	4	2	3	77
Chicken	6	3	2	2	2	2	2	2	21
Flatfish	6	3	2	2	2	2	2	4	23
Common eel	5	4	2	2	3	2	2	2	22
Jacopever	8	4	2	2	2	2	3	4	27
Shrimp	8	4	2	2	2	2	2	3	25
Total	128	57	34	31	21	20	19	23	333

Table 10. The incidence and detection levels of 9 cephalosporins in 333 livestock and marine products

Sample category	Analyzed samples	Detected sample	Below LOQ <sup>1)</sup>	Range of TMP level. (µg/kg)	Incidence of cephalosporins (%)	Kinds of cephalosporins
Beef	n=75	11	5	1.10-9.77	14.7	cefalonium
Pork	n=63	-	-			
Chicken	n=21	-	-			
Milk	n=77	1	-	7.50	1.30	cefquinome
Flatfish	n=23	-	-			
Common eel	n=22	-	-			
Jacopever	n=27	-	-			
Shrimp	n=25	-	-			

Table 11. Assessment of dietary intake and risk value of detected antibiotics

Compound	ADI <sup>1)</sup>	Food	Frequency <sup>2)</sup>	Detected Conc. (mg/kg)	Food intake <sup>3)</sup> (kg/person/day)	EDI <sup>4)</sup> (mg/person/day)	EDI/ADI <sup>5)</sup> (%)
Cephalonium	0.00153	Beef	11/75	0.0011-0.0098	30.36	0.0000030	0.02
Cefquinome	0.0038	Milk	1/77	0.0075	217.61	0.0000004	0.01

<sup>1)</sup> ADI is expressed in terms of mg/kg weight/day by a person of 55 kg.

<sup>2)</sup> Number of detected sample/total sample

<sup>3)</sup> From Korean National Health and Nutrition Examination Survey

<sup>4)</sup> EDI (mg/person/day) = Food intake (kg/person/day) × detected concentration in food (mg/kg)

<sup>5)</sup> Risk value (%)

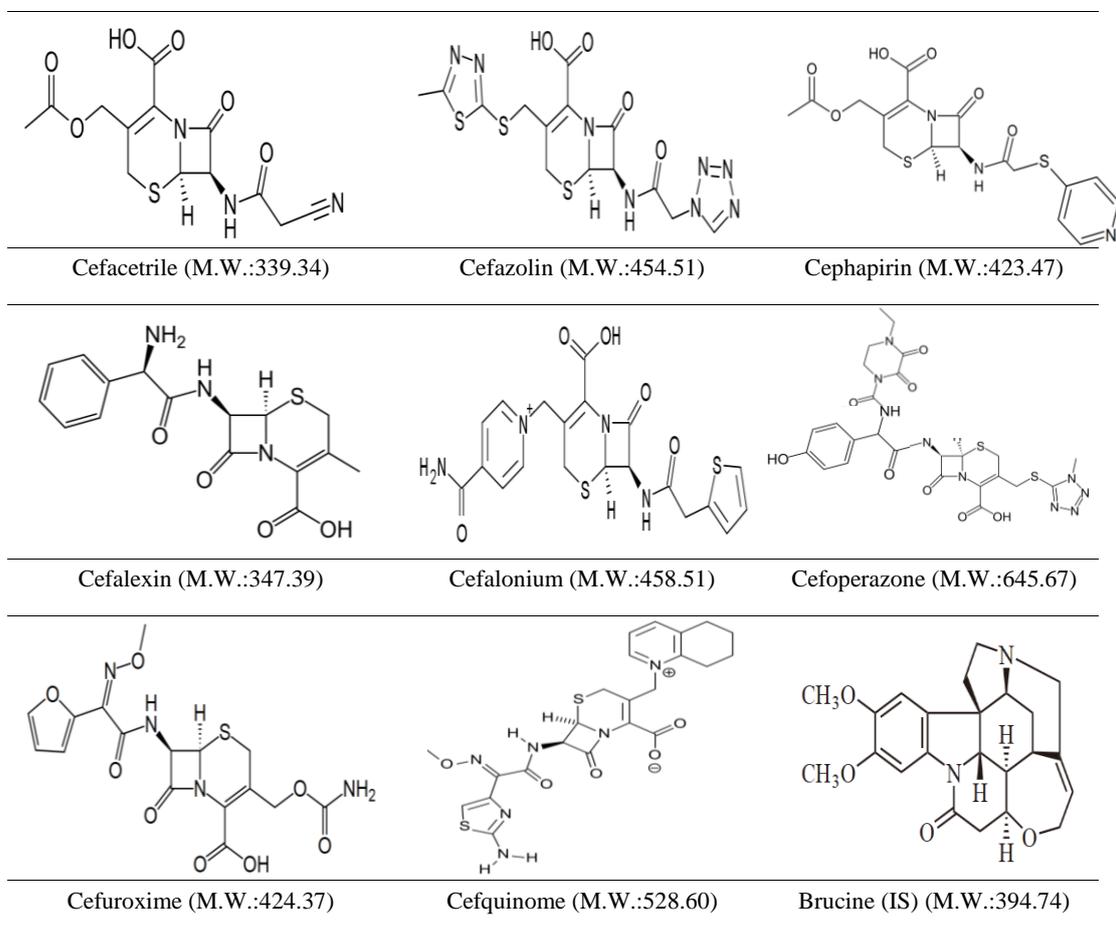
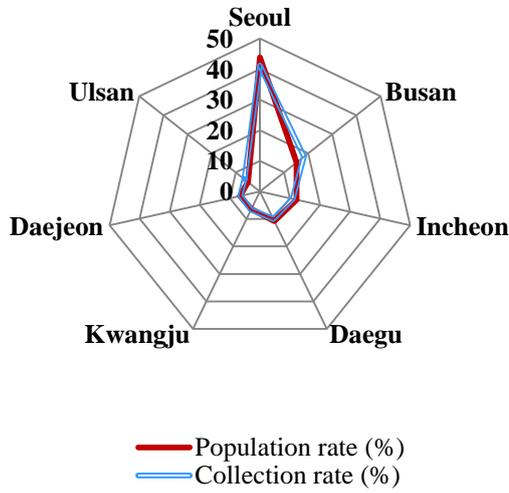


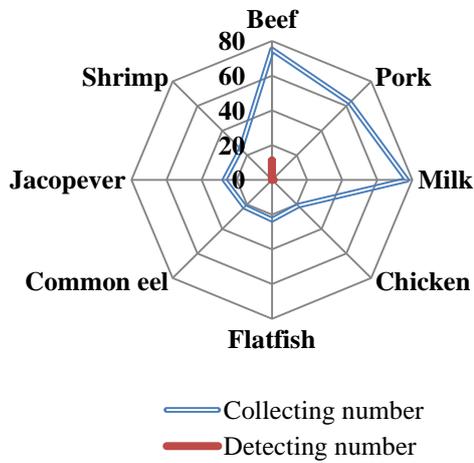
Fig. 1. Molecular structure of cephalosporins and brucine (IS).

(A)



City	Population (unit:1000)	Collecting rate (%)
Seoul	10,206	43.9
Busan	3,359	15.2
Incheon	2,840	12.2
Daegu	2,508	10.8
Gwangju	1,471	6.4
Daejeon	1,525	6.6
Ulsan	1,147	4.9
Total	23,236	100

(B)



Species	Collecting number	Detecting number
Beef	75	11
Pork	63	0
Milk	77	1
Chicken	21	0
Flatfish	23	0
Common eel	22	0
Jacopever	27	0
Shrimp	25	0
Total	333	12

Fig. 2. The number of collecting number according to the 「Sampling with probability proportionate to size」 (A) and distribution of samples containing cephalosporins residues by region (B).

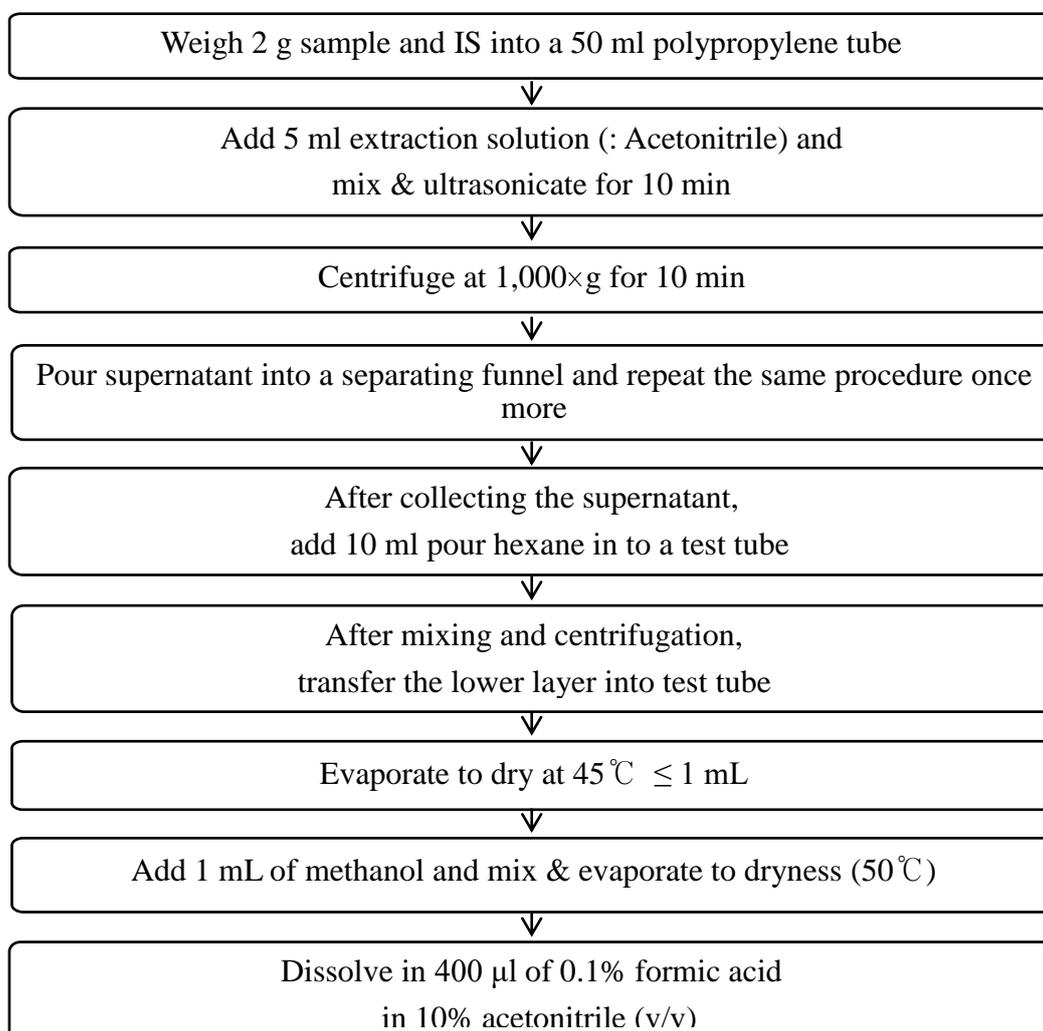


Fig. 3. Schematic diagram of the extraction procedure for the determination of cephalosporins (cefacetrile, cefazolin, cephapirin, cefalexin, cefalexin, desacetyl-cephapirin, cefoperazone, cefuroxime, and cefquinome) in livestock and marine products.

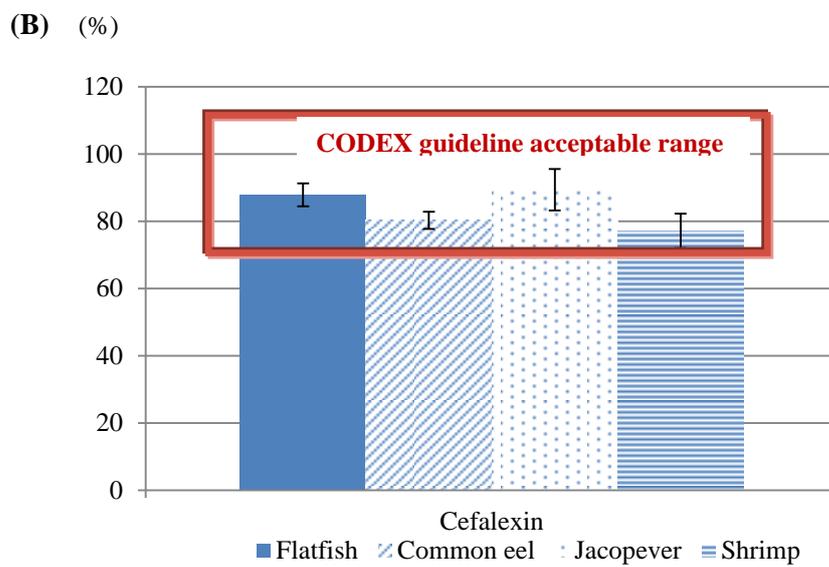
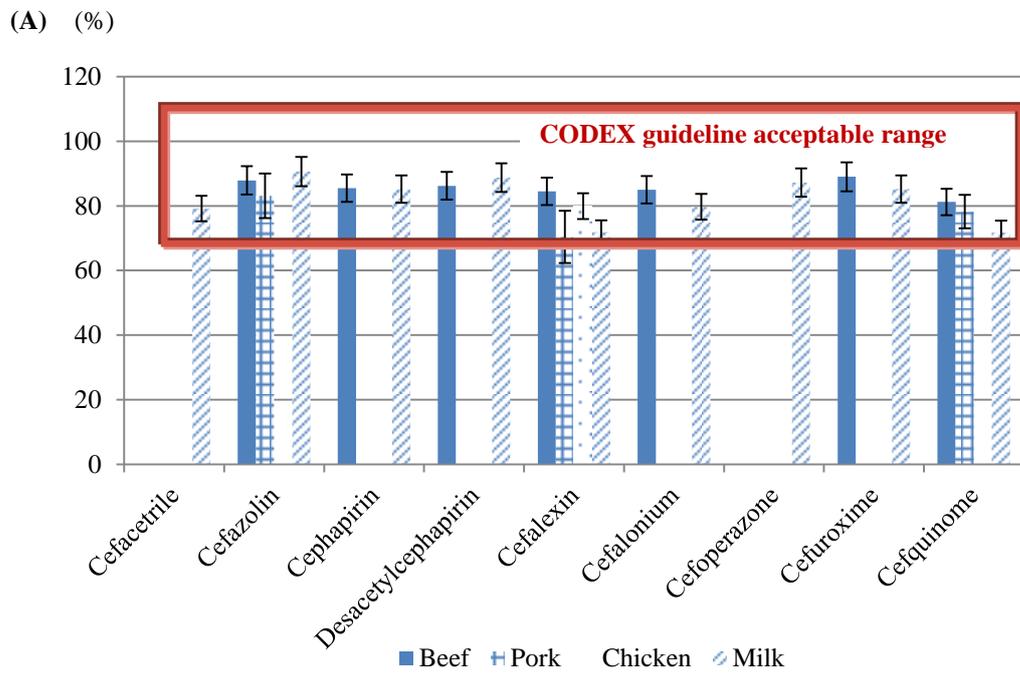


Fig. 4. Average recoveries measured in livestock (A) and marine products (B). The results were within the acceptance criteria of the CODEX guideline.

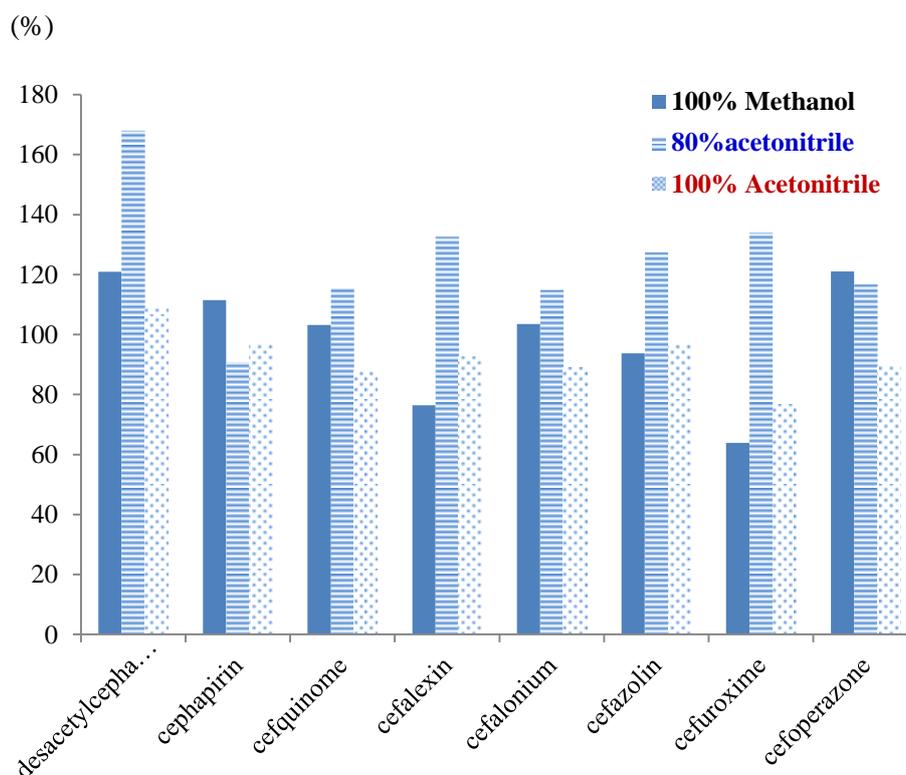


Fig. 5. Comparison of the solvent effects on the recoveries (%) from beef fortified with 8 cephalosporins. Cephalosporins were extracted with solution of 100% methanol (v/v), 100% acetonitrile (v/v), and acetonitrile: DW (80:20, v/v). The recoveries (%) were ranged from 63.9 to 121.1% on extraction of methanol, 90.8 to 168.0% on extraction of 80% acetonitrile (v/v), and 76.7 to 108.5% on extraction of acetonitrile, respectively. As a result, 100% acetonitrile (v/v) was shown to the most effective recoveries.

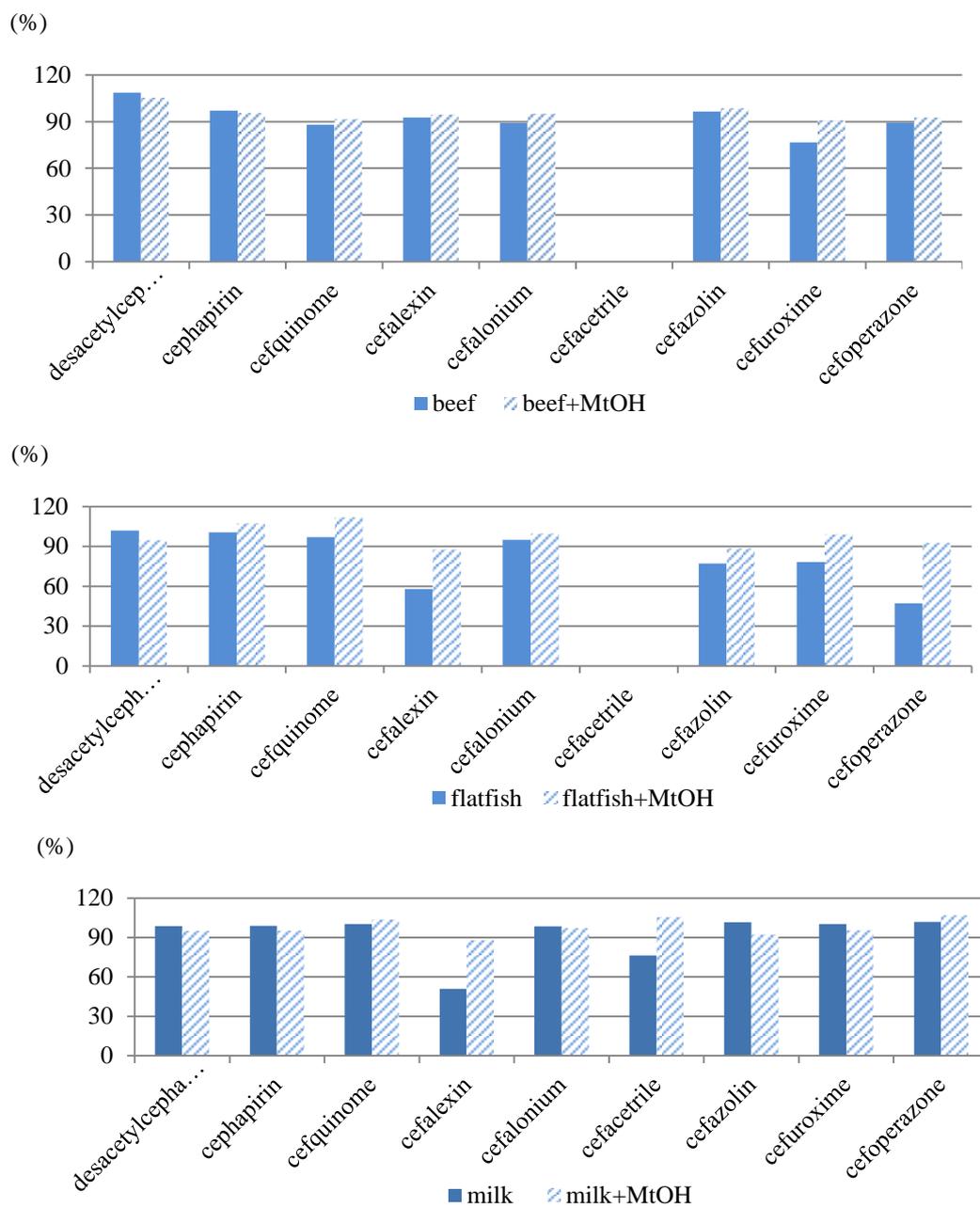


Fig. 6. Optimization of sample preparation in pre-concentration step. Methanol (1mL) was added or not during evaporation under a stream of nitrogen. The addition of methanol played an important role in increasing the recoveries among some cephalosporins.

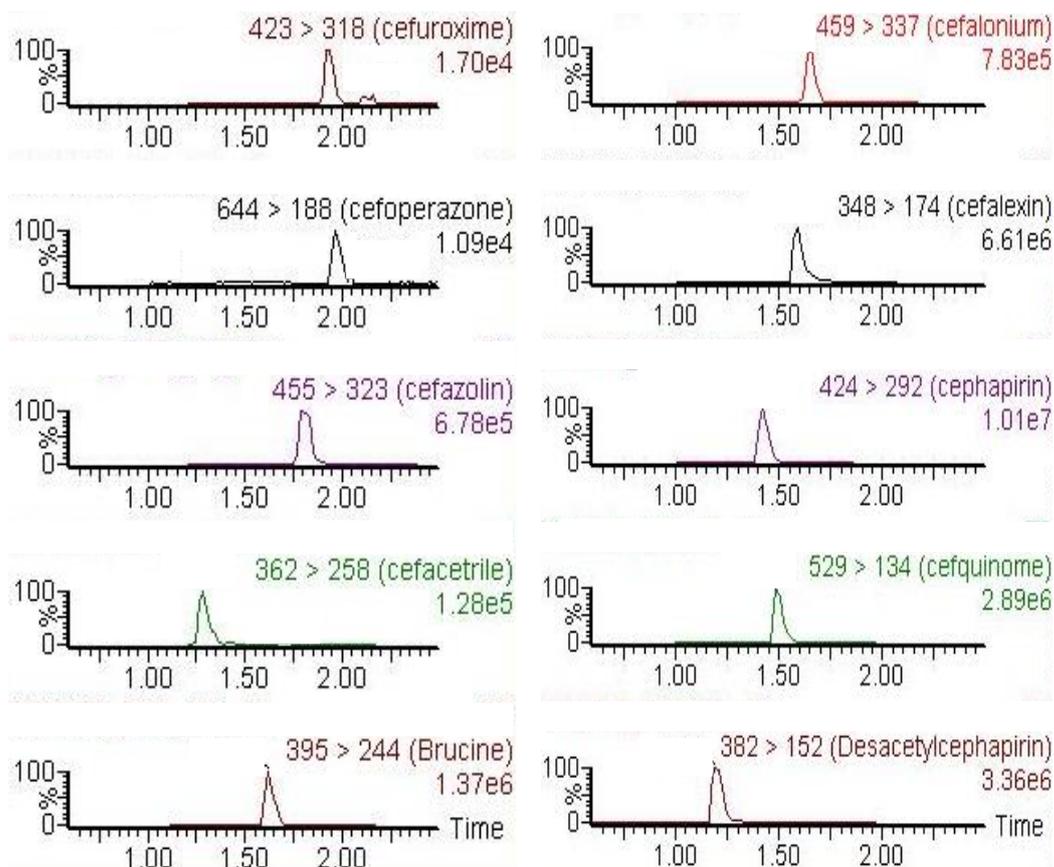


Fig. 7. Chromatograms obtained from a bovine muscle sample fortified at 0.3 mg/kg for cefacetriple, desacetyl-cephapirin, cephapirin, cefoperazone, and cefquinome, 0.6 mg/kg for cefalexin and cefazolin, 0.06 mg/kg for cefalonium, 0.12 mg/kg for cefuroxime, and 0.02 mg/kg for brucine (IS), respectively. The product ions in the MRM mode were produced by collision activated dissociation of selected precursor ions. Two transitions were followed for 9 cephalosporins. The most abundant ions for 9 cephalosporins were presented.

## **CHAPTER III**

### **Development of analytical method for trimethoprim and application to the residue monitoring in livestock and marine products in Korea**

## ABSTRACT

A specific and simple analytical method using ultra performance liquid chromatography with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) was developed to analyze the determination of trimethoprim (TMP) in livestock (beef, pork, chicken, milk, and egg) and marine products (flatfish, jacoever, common eel, and shrimp). This method involved a solid-liquid extraction, ultrasonic-assisted extraction, and a step of solid-phase extraction (SPE) to pre-concentrate and clean up extract. The analytical method was validated according to CODEX guidelines and all results fully complied with CODEX recommendations. The calibration curve was linear from 0 to 50 ng/mL, and the correlation coefficient of calibration curve was 0.999. The LOD ranged from 0.15 to 0.30  $\mu\text{g}/\text{kg}$  and the LOQ ranged between 0.5 and 1.0  $\mu\text{g}/\text{kg}$  depending upon sample species.

A survey for TMP was performed using 369 livestock and marine products distributed in Korea. TMP was detected in 7 marine samples (1.9% incidence) including 5 jacoever, 1 flatfish, and 1 common eel. Residue levels were 1.17 to 16.43  $\mu\text{g}/\text{kg}$  in jacoever, 40.0  $\mu\text{g}/\text{kg}$  in flatfish, and 13.3  $\mu\text{g}/\text{kg}$  in common eel. All the detected levels were below the legal residue limits of 20-50  $\mu\text{g}/\text{kg}$  in the Korean Food Code and the risk value was very safe level of 0.13%.

## INTRODUCTION

Trimethoprim (TMP), a dihydrofolate reductase inhibitor, is an anti-infective agent that is commonly used for treatment of a wide variety of bacteria associated with infections of the middle ear, and the urinary, respiratory, and intestinal tracts (Isabelli et al., 2014) and is also a potentiator when administered in combination with sulfonamides (Mark et al., 2009), such as sulfamethoxazole (as co-trimoxazole, which contains sulfamethoxazole:TMP in a 5:1 ratio), sulfadiazine (Reza et al., 2013), or separately (Renew and Huang, 2004; Chung, 2008).

The presence of antibiotic residues can increase antibiotic resistance of pathogens and can threaten public health. In order to protect consumer health, the European Union set maximum residue limits (MRLs) of 100 µg/kg and 50 µg/kg for TMP for members of family equidae and all other food producing animals, respectively, that supply milk, muscle, fat, liver, and kidney (EU. Commission regulation No. 37/2010, 2010). MRLs of Japan which adopts the Positive List System ranged from 20 to 100 µg/kg in animal and marine products (Ministry of Health, Labour and Welfare of Japan, 2007). In Korea, TMP was licensed for treatment of urinary tract infections and the MRL was established by the Ministry of Food and Drug Safety (MFDS) as shown in Table 1 at 50 µg/kg in muscle, liver, kidney, and fat of cows, pigs, sheep, and chicken, at 50 µg/kg in milk, fish and

crustaceans, at 20 µg/kg in egg, and at 100 µg/kg in muscle, liver, fat, and kidney of horse (Notification No. 2010-51 of the Ministry of Food and Drug Safety, 2010).

Determination of TMP residue at MRL level requires sensitive analytical methods to comply with current regulations. HPLC is a commonly used analytical method (Meiling et al., 2006; Bedor et al., 2008). Most previous works have involved in only one matrix, for instance, blood, plasma, serum, or urine with HPLC (Hiren et al., 2010). Several analytical methods have been described for simultaneous analysis of TMP and sulfonamides in biological fluids (Batziias et al., 2002; Meiling et al., 2006; Hiren et al., 2010). Current studies for TMP analysis with LC-MS/MS are underway for muscle and plasma (Mark et al., 2009; Nhat et al., 2014); however, there was little research for the TMP analysis in livestock and marine products using UPLC-MS/MS.

One of the main problems of TMP analysis of livestock and marine products is the complexity of extraction steps and a need for effective clean-up procedures before instrumental analysis. For this purpose, liquid-liquid extraction (LLE), solid-phase extraction (SPE), pressurized liquid extraction (PLE), and matrix solid-phase dispersion (MSPD) have been used for extraction of TMP from biological fluids (Croubels et al., 2003; Bedor et al., 2008) and wastewater (Renew and Huang, 2004; Nhat et al., 2012).

This paper described the development of a specific and simple analytical method using UPLC-MS/MS for the determination of TMP in livestock and marine products, which involved solid-liquid extraction, followed by simplified solid-phase extraction (SPE) steps. In addition, in this study, TMP residue was monitored in livestock and marine products commercialized in Korea. Koreans enjoy eating flatfish and jacobever sashimi, roasted eel, and Korean style raw minced beef.

## MATERIALS AND METHODS

### *Chemicals and materials*

TMP (Fig. 1) and formic acid were obtained from Sigma-Aldrich (MO, USA). Acetonitrile and methanol of HPLC grade were supplied by Burdick & Jackson (Ulsan, Korea). Acetic acid (99.9%, HPLC grade) was purchased from J.T Baker (NJ, USA). Oasis HLB, MCX, and WAX solid-phase extraction (SPE) cartridges were obtained from Waters (MA, USA).

### *Sample collection*

Sampling area was set in accordance with the 「Sampling with probability proportionate to size」 and species and number of samples were determined on the basis of the consumption of food according to the 「National Health and Nutrition Examination Survey, 2011」. Each sample was purchased at least two and sampling points within the sampling area were not biased.

Therefore, livestock and marine products (n=369) including beef (n=75), pork (n=63), milk (n=77), egg (n=36), chicken (n=21), flatfish (n=23), jacopecover (n=27), common eel (n=22), and shrimp (n=25) were purchased from different markets in Seoul, Busan,

Incheon, Daegu, Daejeon, Gwangju, Ulsan, and Jeju. Details were given in Fig. 2. All solid samples were finely ground with blender and stored in a freezer at -20°C until use.

### ***Preparation of standard solution***

A stock solution of TMP (100 µg/mL) was dissolved in methanol (Burdick & Jackson) and stored at 4°C in the dark. A new stock solution was prepared monthly. A working standard solution (1 µg/mL) was prepared daily diluting in 0.5% formic acid with 20% methanol (v/v). For quantification, a calibration curve for TMP was prepared at 6 concentrations between 0-50 ng/mL (0, 1, 2, 5, 10, and 50 ng/mL) by using 0.5% formic acid in 20% methanol (v/v).

### ***Sample preparation and clean-up procedure***

Homogenized sample (1 g) of beef, pork, egg, chicken, flatfish, jacobever, common eel, and shrimp and 1 mL of milk were placed into a 50 mL disposable polypropylene centrifuge tube and subjected to extraction using 10 mL of acetonitrile (Burdick & Jackson). The mixture was homogenized (Heidolph Reax top, Heidolph, Schwabach, Germany) for 5 min, sonicated (Power sonic 520, Hwashin Tech., Seoul, Korea) for 10 min, and centrifuged (Allegra X-22R, Beckman Coulter, Brea, CA, USA) at 1,000×g for

10 min. The supernatant was filtered through a syringe filter (Whatman<sup>®</sup>, PVDF membrane, pore size 0.45 µm) and evaporated under a gentle stream of nitrogen at 45 °C (EYELA GM-2200, Tokyo, Japan). The residue was dissolved in 2 mL of 20% methanol (v/v).

An Oasis HLB cartridge was conditioned using 5 mL of methanol followed by 5 mL of distilled water. The entire volume of extract was applied to the HLB cartridge, which was subsequently washed with 2 mL of 5% ammonium hydroxide in 10% methanol (v/v). After drying the cartridge for 3 min, the residue was eluted using 2 mL of 2% formic acid in 80% methanol (v/v). The elution fraction obtained from SPE was diluted using deionized water (1:3) and filtered through a syringe filter (Whatman<sup>®</sup>, PVDF membrane, pore size 0.2 µm), after which the diluted eluate was injected into a UPLC-ESI-MS/MS apparatus. A schematic diagram of the sample preparation was presented in Fig 3. Every sample was analyzed in triplicate and results were expressed as a mean value of the total residue concentration ± standard deviation (SD).

### ***UPLC-ESI-MS/MS conditions***

LC analysis was performed using an Acquity Ultra Performance (Waters) and ESI-MS/MS measurement was performed using a Quattro premier XE (Waters). Data were

recorded using MassLynx 4.1 software (Waters) on a personal computer. TMP was separated on an Acquity UPLC BEH C<sub>18</sub> column (2.1 mm × 100 mm, 1.7 μm particle size, Waters). The binary mobile phases composed of 0.1% formic acid in deionized water (A) and 0.1% formic acid in acetonitrile (B) were used. The gradient conditions started at 5% B and increased to 80% B within 3 min, followed by holding at 80% B until 4 min. At 4.1 min, the gradient was programmed to re-equilibrate the column for 1.9 min under initial conditions. The total time of analysis was 6.0 min. The flow rate was 0.3 mL/min and the injection volume was 20 μL in full-loop mode (Table 2).

MS determination was performed with MRM experiments in ESI positive ion mode combined with monitoring of the most abundant MS/MS (precursor→product) ion transitions using a dwell time of 0.04s. Details were given in Table 3. The MS parameters were a capillary voltage of 3.5 kV, a source temperature of 150°C, a desolvation temperature of 350°C, cone gas (N<sub>2</sub>) flow of 50 L/h, and desolvation gas (N<sub>2</sub>) flow of 800 L/h.

## RESULTS & DISCUSSION

### *Method validation*

The proposed method was validated following CODEX guidelines (FAO, 2009) for quantitative method. All results were consistent with the CODEX recommendation, which indicated that the proposed method was suitable for analyzing the TMP antibiotic.

The linearity, limit of detection (LOD), and limit of quantitation (LOQ) for each species of matrices were shown in Table 4. The linearity was calibrated from 0 to 50 ng/mL (6 points) and the squared correlation coefficient ( $r^2$ ) was greater than 0.999. The LOD is the lowest concentration of analyte that an analytical process can reliably differentiate from background levels, while the LOQ is the lowest concentration of analyte that can be quantified. LOD and LOQ values were calculated based on signal-to-noise ratio (S/N) values of 3.3 and 10, respectively. The LOD ranged from 0.15 to 0.30  $\mu\text{g}/\text{kg}$  and the LOQ ranged between 0.5 and 1.0  $\mu\text{g}/\text{kg}$  depending upon species of samples.

Recovery of the analyte was replicated 6x at levels of  $1/2\times\text{MRL}$ ,  $\text{MRL}$ , and  $2\times\text{MRL}$  in accordance with the Food Code in Korea (Table 5). The results of recovery were 76.84-80.52% for beef, 81.13-90.81% for pork, 81.50-85.34% for milk, 83.68-87.04% for egg, 87.92-95.55% for chicken, 72.18-81.07% for flatfish, 85.64-93.13% for jacobever, 75.05-81.99% for common eel, and 70.08-80.03% for shrimp. The results were within the

acceptance criteria (60-120%) of the CODEX for validating analytical method (Fig. 4). Relative standard deviation (RSD, %) results were 3.52-9.60% for beef, 4.27-5.00% for pork, 2.84-5.85% for chicken, 3.53-4.57% for milk, 4.39-6.70% for egg, 2.74-8.29% for flatfish, 2.12-4.70% for jacobever, 2.22-4.33% for common eel, and 1.93-6.16% for shrimp. These values of results did not exceed 9.60%, indicating high precision and reliability of the method (Table 5).

### ***UPLC-ESI-MS/MS optimization***

Chromatographic conditions of UPLC were examined in order to acquire excellent separation and retention for the analytes. Some experiments were performed to test the different mobile phases consisting of acetonitrile and distilled water with formic acid (0.1%) or ammonium formate (5 mM). Therefore, the addition of 0.1% formic acid showed better results than ammonium formate, and improved the ionization efficiency. In addition, formic acid and acetonitrile had a significant impact on improving sharp peak shape and resolution for TMP (Lina et al., 2011; Junza et al., 2014). Other parameters such as gradient profile, flow rate, and injection volume were evaluated in order to acquire a reliable separation. The 6 min of run time was obtained with the optimum conditions for the separation of TMP and the UPLC system showed advantages with

respect to speed, sensitivity, and resolution as an attractive option for analysis of TMP in animal origin samples.

In MS/MS system, the protonated molecular ion  $[M+H]^+$  was selected as precursor ion for TMP, and the product ions were acquired according to the collision energy (eV). The ions in the MRM mode were produced by collision-activated dissociation of selected precursor ions in the collision cell of the triple quadrupole and analyzed with the second analyser of the instrument (Junza et al., 2011). Moreover, MRM mode increased the sensitivity and selectivity of the determination. Two transitions were followed for TMP; one was used for quantification and the other for identification. The most abundant ion was at  $m/z = 230$ . To obtain maximum sensitivity for TMP, MS/MS conditions such as, capillary voltage, source temperature, and desolvation temperature were optimized by direct infusion into the detector of standard solution (10 ng/mL). The acquired chromatogram showed a good resolution for TMP and was described in Fig. 5.

TMP levels were determined using UPLC-ESI-MS/MS in all species of samples to avoid false positive errors due to matrix interference. Comparison with background noise levels in 9 species of matrices showed no interference peaks detected at the expected retention time of TMP. The detected samples were confirmed by the transition ion ratio. As shown in Fig.6, the intensity of quantitative ion (291>230) and qualitative ion

(291>123) were  $1.42 \times 10^6$  (100%) and  $1.09 \times 10^6$  (76.8%) in standard solution, and  $3.69 \times 10^4$  (100%) and  $3.00 \times 10^4$  (81.3%) in detected jacobever sample, respectively. The ion ratios of detected samples were agreed with those of standards.

### ***Optimization of sample preparation and SPE procedure***

Sample preparation was concentrated on the establishment of the most proper conditions used for the extraction of TMP residue from livestock and marine products. This method involved the solid-liquid extraction, ultrasonic-assisted extraction, and a step of SPE to pre-concentrate and clean up extracts; the analytes were extracted with acetonitrile and purified by Oasis HLB cartridge.

Several extraction solvent mixtures were tested. Each species of sample fortified with  $20 \mu\text{g}/\text{kg}$  of TMP was extracted with solutions of 80% and 100% acetonitrile/water (v/v). Additionally trichloroacetic acid in different ratio from 0 to 5% (v/v) was added to extraction solvent. As a result, the extraction of 100% acetonitrile (v/v) without the addition of trichloroacetic acid was shown to the most effective recoveries and lowest RSD (%) in 9 species of matrices. Furthermore, the final concentrated extract was dissolved in 10% methanol and 20% methanol, respectively. Thus, the recoveries were higher when the residue was dissolved in 20% methanol (Fig. 7).

Solvent extraction including centrifugation and syringe filtration was performed, which enabled deproteinization and defatting of samples and also elimination of matrix interference. Ultrasound is also an effective pretreatment because the energy promoted the step of sample preparation, such as dissolution, fusion, and leaching (De Castro and Da Silva, 1997; Marilena and Nikolaos, 2015).

Furthermore, at this initial step of method development, extracts were directly injected into the UPLC-MS/MS apparatus without clean-up in order to confirm whether a clean-up step was required or whether adequate results could be acquired without sample manipulation. Consequently, use of SPE cartridges showed a crucial effect on recovery of the analyte. The SPE cartridge purification step was kept as simple as possible for minimization of ion suppression effects due to the complexity of the sample matrix.

In order to establish the optimum conditions for the SPE procedure we have considered the evaluation of 3 different SPE cartridges to clean up and preconcentrate the TMP in samples. The HLB cartridge is a high-performance, water-wettable copolymer and a hydrophilic-lipophilic balanced reversed-phase sorbent for acid, basic, and neutral compounds. The MCX cartridge is a mixed-mode cation exchange sorbent for bases, and the WAX cartridge is a mixed-mode weak anion exchange sorbent for strong acids. Mixed-mode sorbents provide both reversed-phase and ion exchange modes of retention,

enabling greater cleanup selectivity and sensitivity for both acidic and basic compounds (Waters, 2015).

In this study, SPE clean-up procedures for analyte extracts in blank milk sample spiked with 20 µg/kg TMP was investigated using Oasis HLB, Oasis MCX, and Oasis WAX SPE cartridges. Both recoveries of MCX and WAX cartridge were ranged from 44.0 to 49.1% and HLB cartridge recovery was 86.4%. Thus, the HLB cartridge was used. Besides, the HLB cartridge is commonly available on the market and the type of sorbent covers a large variety of polarities. Therefore, the HLB cartridge is useful for dealing with a wide range of analytes (Perez-Burgos et al., 2012).

Conditioning and equilibration of the SPE cartridge were performed with 100% methanol and 100% distilled water, respectively. Methanol was adequate for elimination of interference and for elution of TMP residues from cartridges. The ratio of organic solvents for washing and elution steps was investigated using basic and acidic solvents by spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan). Consequently both 5% NH<sub>4</sub>OH in 10% methanol (washing solvent) and 2% formic acid in 80% methanol (elution solvent) showed improvement in recoveries (Fig. 8). The proper selection of the extraction conditions was increased the recovery rate and reduced matrix effects.

### ***Application of the proposed method to animal products***

TMP was analyzed in 369 livestock and marine products including 75 beef, 63 pork, 77 milk, 36 egg, 21 chicken, 23 flatfish, 27 jacobever, 22 common eel, and 25 shrimp (Table 6). The results are listed in Table 7. The residues were detected in 7 marine product samples (1.9% incidence) ; 5 out of 27 jacobever samples (18.5% incidence), 1 out of 23 flatfish samples (4.3% incidence), and 1 out of 22 common eel samples (4.3% incidence). Detected concentrations were 1.17 to 16.43  $\mu\text{g}/\text{kg}$  in jacobever, 40.0  $\mu\text{g}/\text{kg}$  in flatfish, and 13.3  $\mu\text{g}/\text{kg}$  in common eel. All detected product samples were shown below the legal residue limit of 20-50  $\mu\text{g}/\text{kg}$  in Korea (Table 4). These residues of samples may be resulted from treatment or prevention of a particular type of infection/disease, or from contamination during production.

Furthermore, the risk value based on the TMP exposure by food intake was calculated using the EDI (estimated daily intake) and ADI (acceptable daily intake). Compared with ADI (0.0042 mg/kg b.w./day), the risk value was safe level of 0.13%. Details were given in Table 8. As shown in Fig. 9, the food contribution for EDI was higher in jacobever (58.0%), flatfish (31.9%), and common eel (10.1%).

Generally, a combination of TMP and sulfadiazine is sold under the name Aqua-Sulprim<sup>®</sup> made by the Korea Thumb Vet Company (Iksan, Korea). The combination of

trimethoprim and sulfadiazine is often medicated for treatment of vibriosis in flatfish, jacoever, common eel, and yellowtail in Korea (Chung, 2008). Furthermore, Won *et al.* (2011) were mentioned that the monitoring of TMP residue was necessary in marine products due to the combination use of sulfadiazine and TMP. It reported that sulfadiazine was found in 1 flatfish (14 µg/kg) and 1 jacoever (26 µg/kg). The level of sulfadiazine residue in marine products exhibited considerable similarity with results reported here in. Therefore, it will be necessary to implement a continuous monitoring of TMP and sulfonamide residues in livestock and marine products.

In this study, the incidence of TMP residues was more common in jacoever. In Korea, the usual dose for fish is 6-30 mg/kg of body weight with a withdrawal period of 30 days (National institute of fisheries science, 2011). As well as, intensive farming, which can lead to a high potential for spread of infectious disease, has been maintained to satisfy an increasing demand for marine products in Korea. Moreover, pharmacokinetics in aquaculture and stockbreeding were affected by species specificity, health conditions, age, size, water temperature, and salinity (Quzhong and Xeumei, 2007). Thus, proper usage of veterinary drugs and observance of withdrawal periods were required.

According to the Korean National Health & Nutrition Examination Survey, intake frequencies per week for livestock and marine products were between 0.5 and 2.6 times

for 12 years or older consumers (Korea Health Statistics 2011: Korea National Health and Nutrition Examination Survey, 2011). Besides, in Korea and other countries in Southeast Asia, many people traditionally consume a variety of livestock byproducts, such as blood, guts, and bones as foods (Choi et al., 2011) and enjoy jacobever and flatfish sashimi. Consequently, the survey of TMP residues in animal origin products is important in Southeast Asia.

## CONCLUSION

A simple and reliable method was developed for the rapid analysis of TMP residues in livestock and marine products using a UPLC-ESI-MS/MS system. The UPLC had a beneficial advantage in speed, sensitivity, and resolution. The sample preparation methods through the ultrasonic-assisted extraction and SPE clean-up procedure were increased the recovery and reduced the matrix effects. The recoveries of TMP in livestock and marine products were higher than 70%, and the LOD and LOQ values were lower than the established MRLs in Korea. Furthermore, acquired validation parameters fully complied with CODEX recommendations.

A large number of animal origin products commercialized in Korea were analyzed and quantified using the proposed method. The residues of TMP were detected in 7 samples (1.9% incidence) but residue levels were below the MRL, and the risk value was safe level of 0.13%. Although TMP residues appeared to be at relatively safe levels in livestock and marine products, the possibility of antibiotic misuse and violation of withdrawal period could occur. Therefore, this monitoring of TMP in livestock and marine products will be valuable information for the national management of antibiotics.

Table 1. Maximum residues limits of TMP in Korea

	Food	mg/kg
Cattle, Pig, Sheep, Goat, Poultry	Muscle, Liver, Fat, Kidney	0.05
Equine	Muscle, Liver, Fat, Kidney	0.1
Egg		0.02
Milk, Fish, Crustacean		0.05

Table 2. Analytical conditions of LC-ESI-MS/MS for analysis of TMP

Items		Conditions		
LC	Instrument	Acquity ultra performance LC		
	Column	Acuity UPLC BEH C <sub>18</sub> (2.1×10 mm, 1.7 um)		
	Mobile phase	A: 0.1% formic acid in DW B: 0.1% formic acid in acetonitrile		
	Gradient	Time (min)	A (%)	B (%)
		Initial	95	5
		3.0	20	80
		4.0	20	80
		4.1	95	5
		6.0	95	5
	Flow rate	0.3 mL/min		
Injection volume	20 µL			
Instrument	Quattro Premier XE			
MS	Ionization mode	ES+		
	Capillary voltage	3.5 kV		
	Cone voltage	35 V		
	Source temperature	150°C		
	Desolvation temperature	800°C		

Table 3. Parameters of LC-ESI-MS/MS for analysis of TMP

Compound name	Transition		Cone voltage (V)	Collision Energy (eV)
	Precursor ion (m/z)	Product ion (m/z)		
TMP	291	230	35	22
		123	35	25

Table 4. Validation parameters for detection of TMP using UPLC-MS/MS

Sample	LOD ( $\mu\text{g}/\text{kg}$ ) <sup>a</sup>	LOQ ( $\mu\text{g}/\text{kg}$ ) <sup>b</sup>	Calibration curve <sup>c</sup>	$R^2$
Beef	0.3	1.0	$y=264.54x+69.95$	0.9994
Pork	0.3	1.0	$y=219.34x+59.21$	0.9991
Chicken	0.15	1.0	$y=256.33x+49.86$	0.9991
Milk	0.3	0.5	$y=269.08x+63.84$	0.9991
Egg	0.3	1.0	$y=226.14x+13.69$	0.9995
Flatfish	0.3	1.0	$y=274.78x+130.61$	0.9999
Jacopever	0.3	1.0	$y=288.73x+96.30$	0.9993
Common eel	0.3	1.0	$y=233.06x+33.75$	0.9997
Shrimp	0.3	1.0	$y=146.32x+104.75$	0.9991

<sup>a</sup> Limit of detection.

<sup>b</sup> Limit of quantification.

<sup>c</sup> x = concentration of TMP ( $\mu\text{g}/\text{kg}$ ), y = intensity.

Table 5. Recovery (%) and RSD (%) of spiked TMP by the proposed method using UPLC-MS/MS

Samples	Spiked conc. (µg/kg)	Mean±SD <sup>a</sup>	RSD <sup>b</sup> (%)
Beef	1/2 MRL	76.84±2.74	3.52
	MRL	78.18±3.77	4.82
	2 MRL	80.52±7.73	9.60
Pork	1/2 MRL	81.13±4.06	5.00
	MRL	90.81±4.49	4.94
	2 MRL	86.15±3.68	4.27
Chicken	1/2 MRL	87.92±2.50	2.84
	MRL	94.44±5.52	5.85
	2 MRL	95.55±3.68	3.19
Milk	1/2 MRL	85.34±3.67	4.30
	MRL	81.50±2.87	3.53
	2 MRL	84.71±3.87	4.57
Egg	1/2 MRL	85.29±3.63	4.25
	MRL	83.68±3.67	4.39
	2 MRL	87.04±5.83	6.70
Flatfish	1/2 MRL	72.18±1.88	2.74
	MRL	77.80±2.26	2.91
	2 MRL	81.07±6.72	8.29
Jacopever	1/2 MRL	85.64±2.98	3.48
	MRL	86.24±3.82	4.70
	2 MRL	93.13±1.97	2.12
Common eel	1/2 MRL	75.05±1.89	2.52
	MRL	81.99±3.55	4.33
	2 MRL	77.10±1.71	2.22
Shrimp	1/2 MRL	70.08±4.32	6.16
	MRL	80.03±2.11	2.64
	2 MRL	75.58±1.46	1.93

<sup>a</sup> Standard deviation

<sup>b</sup> Relative standard deviation

Table 6. Number of samples according to the sampling area

	Seoul	Busan	Incheon	Daegu	Gwangju	Daejeon	Ulsan	Jeju	Total
Beef	33	14	8	8	3	3	4	2	75
Pork	28	11	7	6	3	3	2	3	63
Milk	34	14	9	7	4	4	2	3	77
Chicken	6	3	2	2	2	2	2	2	21
Egg	9	6	3	3	5	4	4	2	36
Flatfish	6	3	2	2	2	2	2	4	23
Common eel	5	4	2	2	3	2	2	2	22
Jacopever	8	4	2	2	2	2	3	4	27
Shrimp	8	4	2	2	2	2	2	3	25
Total	137	63	37	34	26	24	23	25	369

Table 7. Incidence and range of TMP level in 369 livestock and marine products

Sample category	Analyzed samples	Detected sample	Range of TMP level ( $\mu\text{g}/\text{kg}$ )
Beef	n=75	ND <sup>a</sup>	-
Pork	n=63	ND <sup>a</sup>	-
Chicken	n=21	ND <sup>a</sup>	-
Milk	n=77	ND <sup>a</sup>	-
Egg	n=36	ND <sup>a</sup>	-
Flatfish	n=23	1	40.0
Common eel	n=22	1	13.1
Jacopever	n=27	5	1.17-16.43
Shrimp	n=25	ND <sup>a</sup>	-

<sup>a</sup> Non-detection

Table 8. Assessment of dietary intake and risk value of detected antibiotics

Compound	Food	Frequency <sup>1)</sup>	Detected Conc (mg/kg)	Food intake <sup>2)</sup> (kg/person/day)	EDI <sup>3)</sup> (mg/person/day)	EDI/ADI <sup>4)</sup> (%)
	Jacopever	5/27	0.0012-0.0164	36.23	0.0000034	0.08
Trimethoprim	Common eel	1/22	0.0131	54.30	0.0000006	0.01
※ ADI <sup>5)</sup> =0.0042	Flatfish	1/23	0.040	58.93	0.0000019	0.04
					ΣEDI =0.0000059	ΣEDI/ADI =0.13

<sup>1)</sup> Number of detected sample/total sample

<sup>2)</sup> From Korean National Health and Nutrition Examination Survey

<sup>3)</sup> EDI (mg/person/day) = Food intake (kg/person/day) × detected concentration in food (mg/kg)

<sup>4)</sup> Risk value (%)

<sup>5)</sup> ADI is expressed in terms of mg/kg weight/day by a person of 55 kg

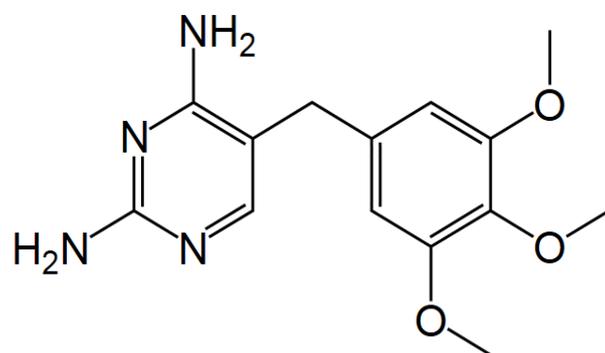


Fig. 1. Molecular structure of TMP (M.W.: 290.32).

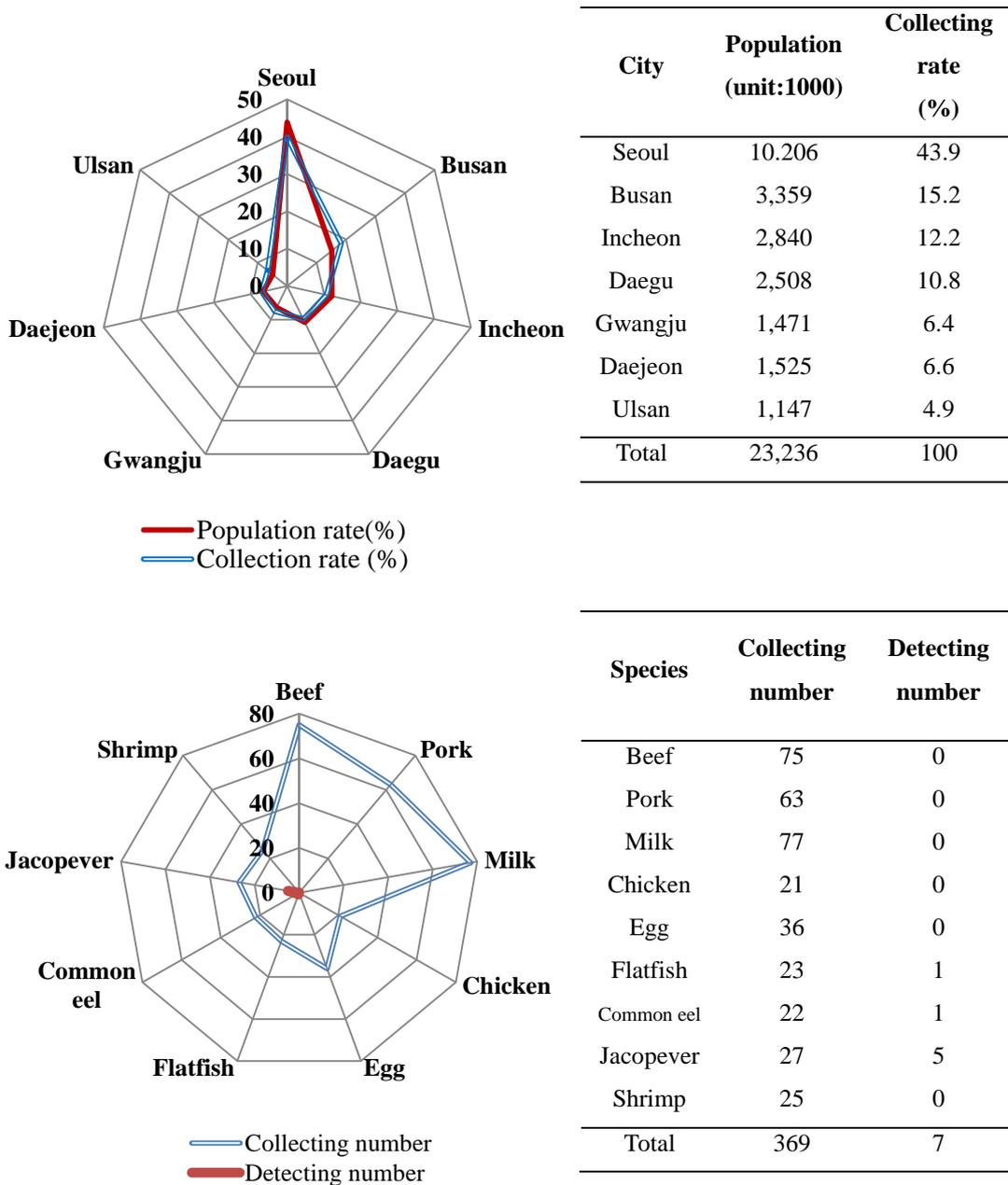


Fig. 2. The sampling number by areas according to the 「Sampling with probability proportionate to size」 (A) and distribution of samples containing TMP residues by region (B).

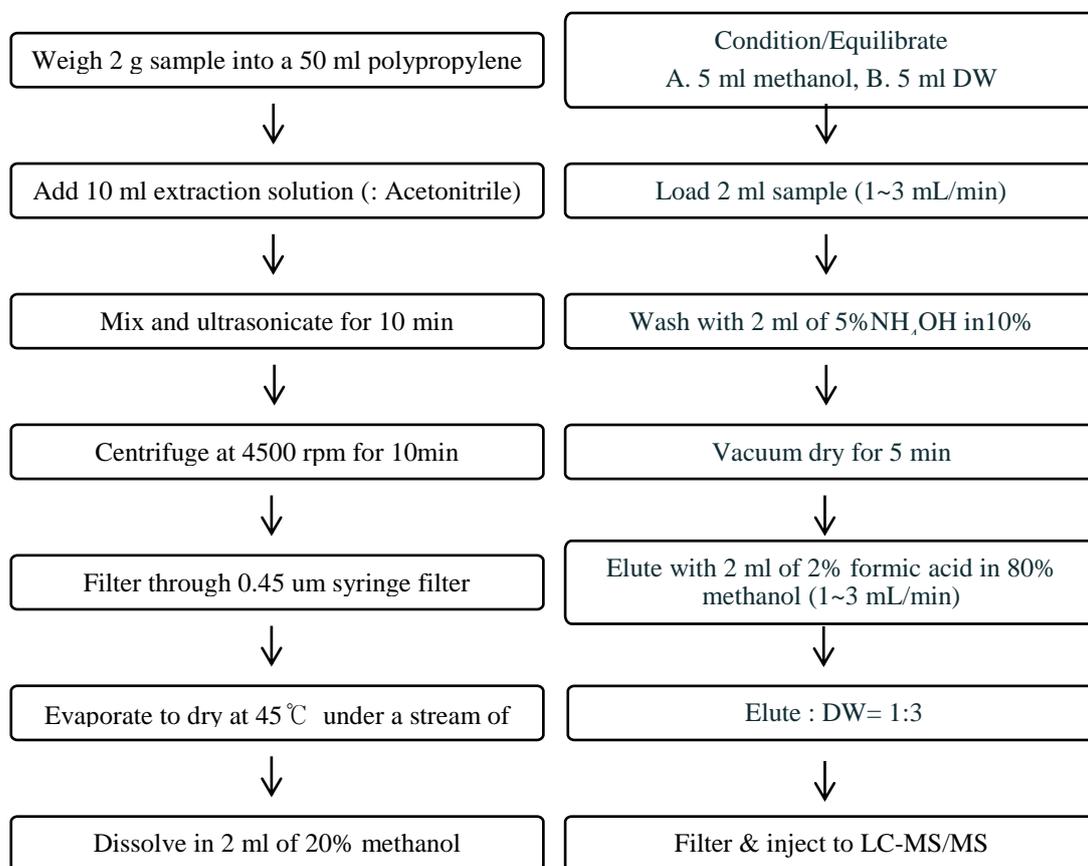


Fig. 3. Schematic diagram of the extraction procedure for determination of TMP in livestock and marine products (Left; preparation of samples, Right; SPE clean-up procedure).

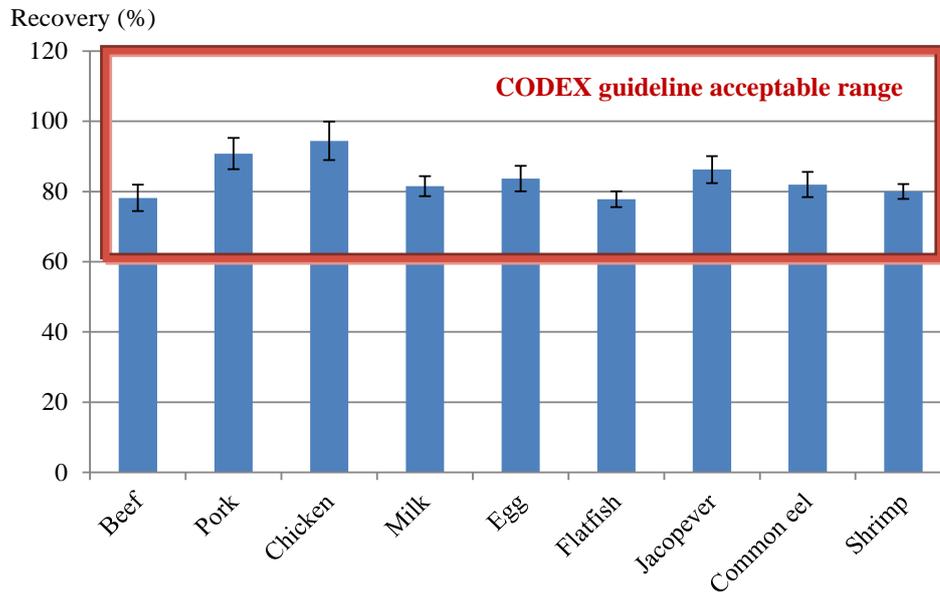


Fig. 4. Average recoveries measured in livestock and marine products. The results were within the acceptance criteria of the CODEX guideline (60-120%).

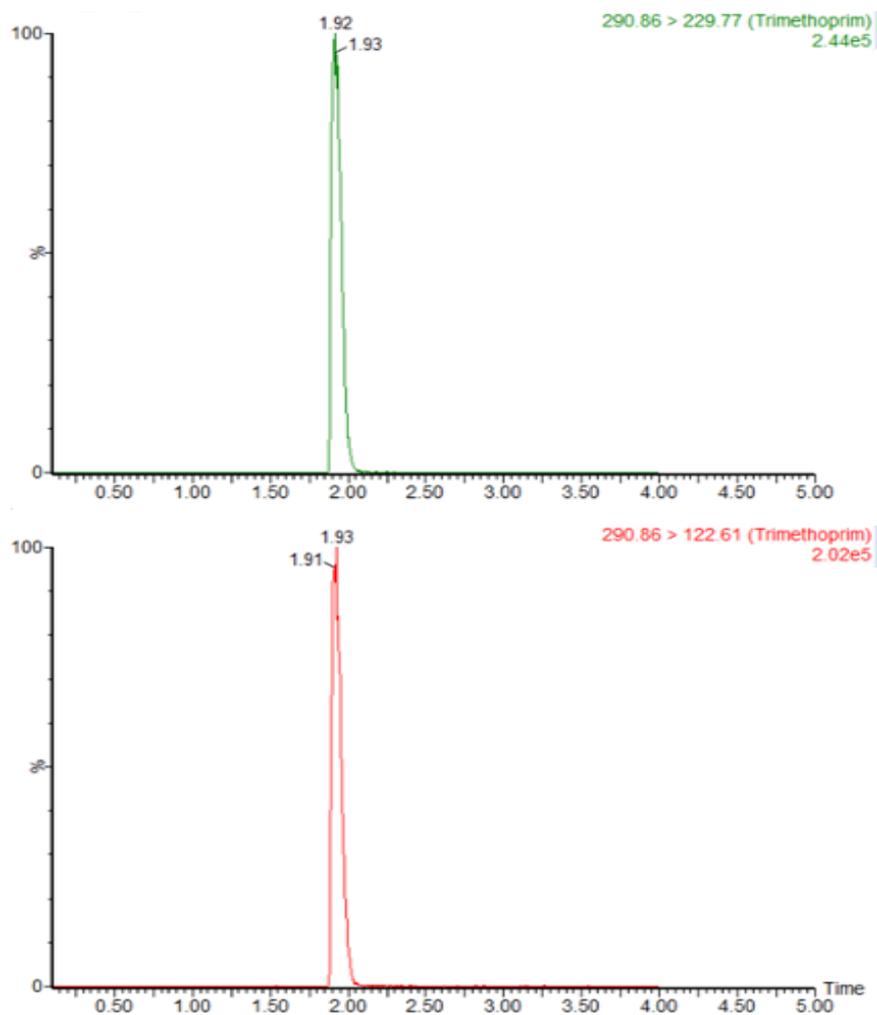


Fig. 5. Chromatograms of TMP obtained from a pork muscle sample fortified at 10  $\mu\text{g}/\text{kg}$  using UPLC-ESI-MS/MS in positive ion mode. For confirmation, 2 characteristic fragmentations of the protonated molecular ion  $[\text{M}+\text{H}]^+$  were monitored. The most abundant fragment ( $m/z$  229.77) was used for quantification, while the other was used as a qualifier ( $m/z$  122.61).

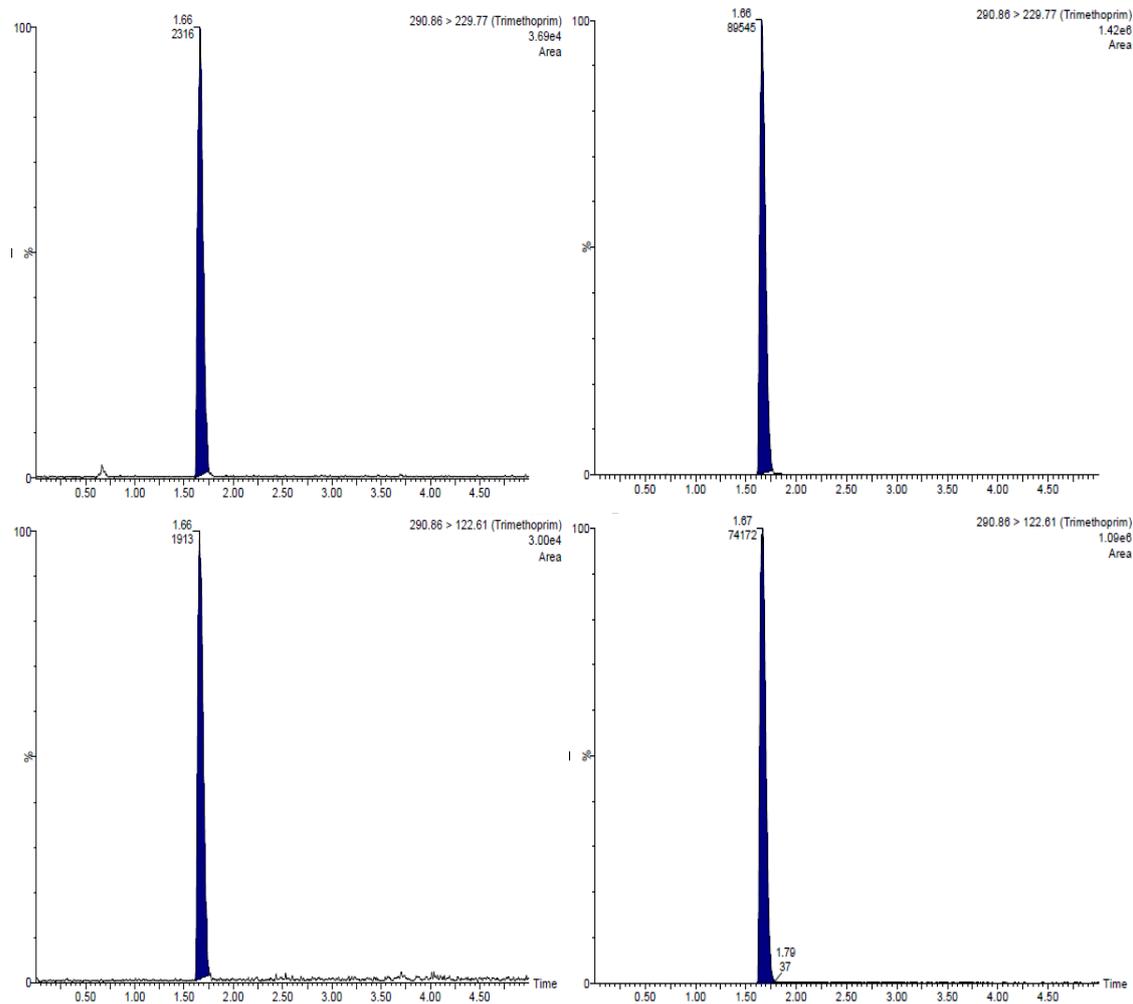


Fig. 6. UPLC-ESI-MS/MS chromatogram of TMP standard solution (left) and detected jacoever samples (right). The detected samples were confirmed by the transition ion ratio. The intensity of quantitative ion (291>230) and qualitative ion (291>123) were  $1.42 \times 10^6$  (100%) and  $1.09 \times 10^6$  (76.8%) in standard solution, and  $3.69 \times 10^4$  (100%) and  $3.00 \times 10^4$  (81.3%) in detected jacoever sample, respectively.

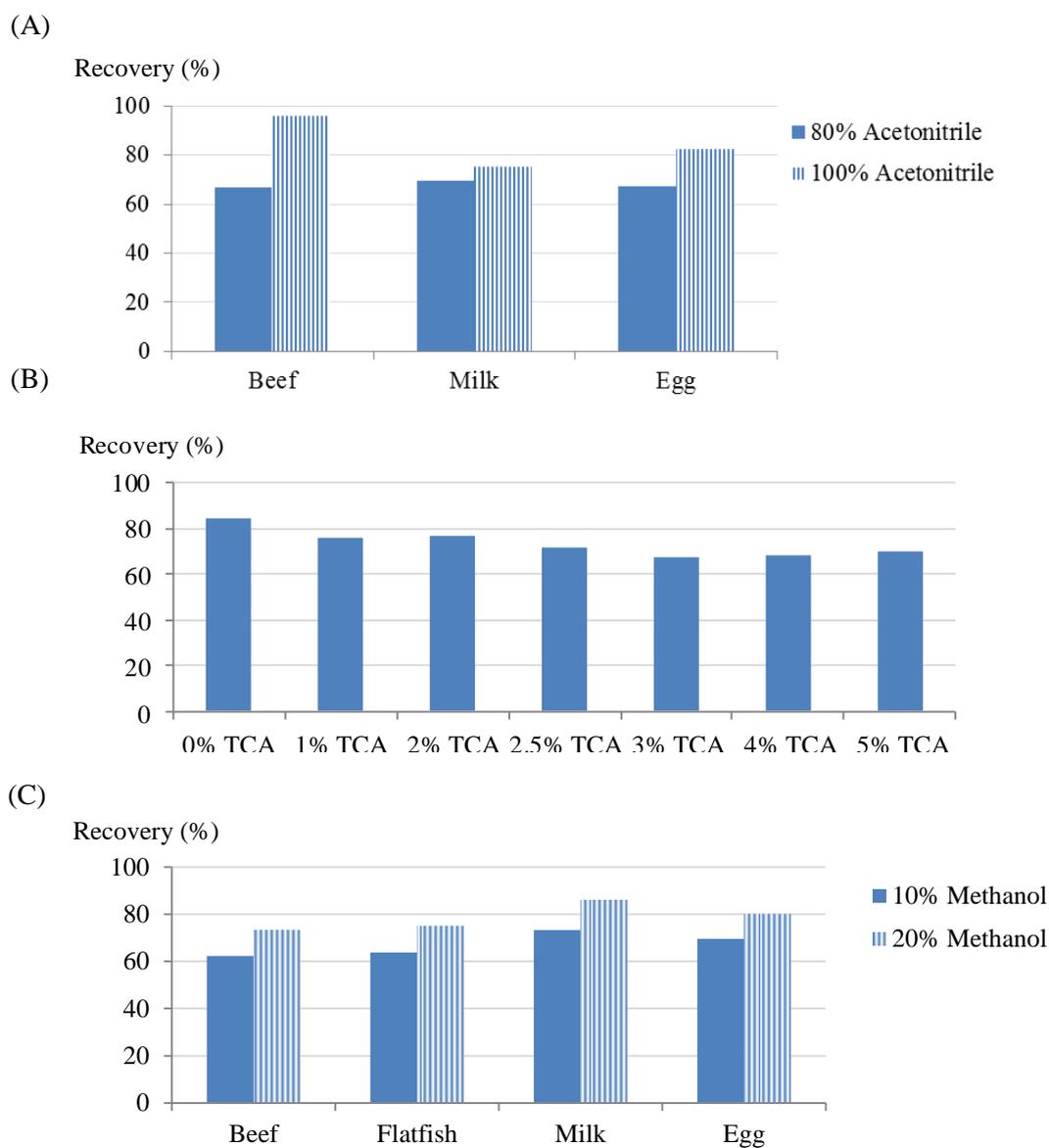


Fig. 7. Optimization of samples preparation. TMP residue was extracted with 80% and 100% acetonitrile, respectively (A). Additionally, trichloroacetic acid in different ratio from 0 to 5% was added to extraction solvent (B). As a result, the extraction of 100% acetonitrile (v/v) without addition of trichloroacetic acid was shown to the most effective recoveries. Furthermore, the recoveries were higher when the residue was dissolved in 20% methanol than 10% methanol (v/v).

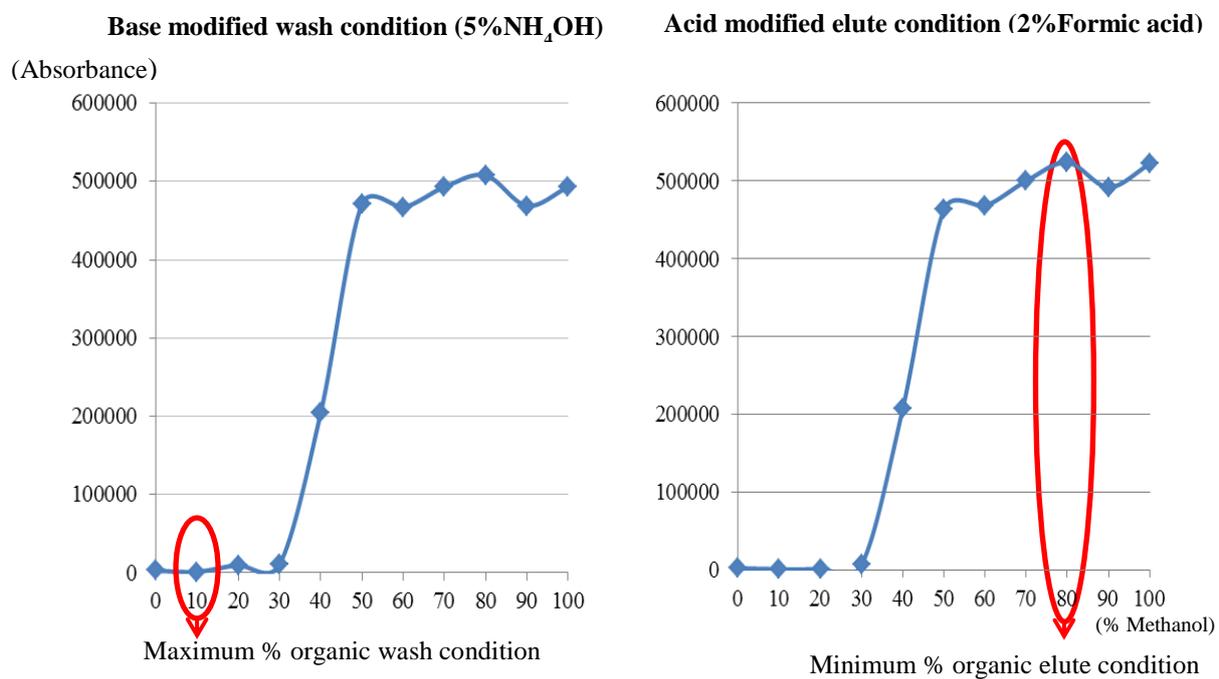


Fig. 8. Optimization of the wash and elution step condition for the SPE procedure obtained by Spectrophotometer (270 nm). The optimal ratio of organic solvent was 5% NH<sub>4</sub>OH in 10% methanol for washing step and 2% formic acid in 80% methanol for elution step.

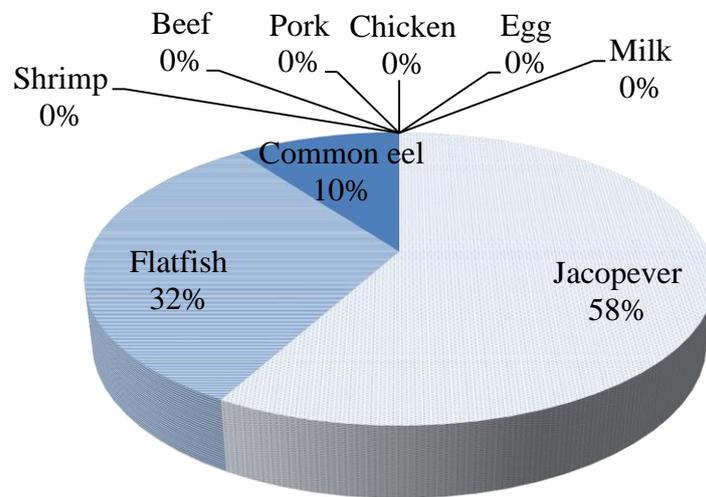


Fig. 9. The food contribution for estimated daily intake from TMP residue. It was higher in jacopever (58.0%), flatfish (31.9%), and common eel (10.1%), respectively. There was no contribution in beef, pork, chicken, egg, milk, and shrimp.

## General conclusion

Three specific and reliable methods were developed for the determination of 13 quinolones, 9 cephalosporins, and TMP in livestock and marine products using UPLC-ESI-MS/MS. The proposed method was validated according to the CODEX guidelines and all results were fully complied with CODEX recommendations. Good linearities were achieved and the correlation coefficients were ranged between 0.9990 and 0.9999. Both the LOD and LOQ were below the MRLs established by MFDS. The analytical methods using UPLC-ESI-MS/MS involved significant advantages with respect to high sensitivity, speed, and resolution, making them an attractive choice for the analysis of antibiotics residues in livestock and marine products. The sample preparation methods through solid-liquid extraction, ultrasonic-assisted extraction, and SPE clean-up reduced the matrix effects and increased the recovery. Moreover, the matrix-matched calibration curves with internal standard played important roles in compensating for the matrix effects.

A survey for 13 quinolones, 9 cephalosporins, and TMP residues was performed using the proposed method. The residues of quinolones were detected in 104 out of 310 (33.5% incidence) livestock and marine products below the MRLs. However, pefloxacin was detected in one common eel above the legal residue limit. Oxolinic acid, enrofloxacin,

and flumequine were the most commonly detected antibiotics. The residues of 9 cephalosporins were detected in 12 out of 333 samples (3.6% incidence) and detected antibiotics were cefalonium in beef and cefquinome in milk. The residue of TMP was detected in only 7 out of 369 samples (1.9% incidence) below the MRLs. The risk values of 13 quinolones, 9 cephalosporins, and TMP were under safe levels. Although the residual concentration appeared to be at within the safety levels, the possibility of antibiotic misuse and violation of withdrawal period could occur. Therefore, the effort to refine and improve national system for monitoring and management of antibiotic residues in livestock and marine products need to be continued.

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## 국문초록

### 축·수산물 중 퀴놀론계·세팔로스포린계 및 트리메토프림 항생제에 대한 동시분석법 개발 및 잔류실태조사

서울대학교 대학원

수의학과 수의생화학 전공

장진욱

(지도교수: 이항)

식생활 문화의 변화로 가축, 가금류 및 어류의 소비가 증가함에 따라, 축·수산업의 형태는 대규모, 밀집사육으로 변하였다. 이로 인해 가축의 치료 및 질병예방과 생산성 촉진을 위한 동물용의약품의 사용증가로, 소비자가 항생제내성에 노출될 위험은 점점 커지고 있다. 또한 동물용의약품의 오남용과 항생제 내성균의 문제는 국민건강에 해로운 영향을 미칠 수도 있다.

국내 동물용의약품에 대한 식품 중 잔류허용기준은 1990년 약 40여종을 설정한 이래 점차 확대 고시되어, 2015년 현재 총 185종에 대한 잔류허용기준이 설정·관리되고 있다. 또한 국제 규제 강화와 분석기술의 발달로 인해 국내에서도 효율성이 낮은 시험법 개선에 대한 요구와 함께 국제적 수준의 분석법의 도입이 이뤄지고 있다.

따라서 본 연구에서는 퀴놀론계, 세팔로스포린계 항생제와 트리메토프림에 대한 국제적 수준의 검사법을 마련하고, 이를 토대로 축·수산물 중 잔류실태 조사를 수행하였다. 검체수집 비율은 「확률비례추출법」에 따라 서울 및 6대

광역시의 인구비율을 감안하여 설정하였고, 조사대상 식품은 「2011년 국민건강영양조사」를 근거로 다소비 축·수산물식품 중 9종을 선정 하였다.

I. 축·수산물 중 퀴놀론계항생제 13종 (시프로플록사신, 다노플록사신, 플루메퀸, 디플록사신, 엔로플록사신, 마보플록사신, 날리딕스산, 옥소린산, 노르플록사신, 오픈플록사신, 오비플록사신, 페플록사신, 사라플록사신)에 대한 다성분 동시분석법을 확립하였다. 시료의 matrix의 영향을 최소화하고 회수율을 높이기 위해 ‘matrix-matched 정량곡선’을 도입하고, 내부표준 물질을 사용하였다. 또한 분석시간의 단축과 시료 중 극미량 잔류하는 퀴놀론계 항생제를 검출하기 위해 UPLC-ESI-MS/MS를 이용하였다.

국제식품규격위원회(CODEX) 지침에 따라 시험법 검증을 수행한 결과, 0.999 이상의 직선성을 얻었으며, 축·수산물 9종에 대한 검출한계와 정량한계는 각각 0.1, 0.4  $\mu\text{g}/\text{kg}$  이하였고, 높은 회수율 (83.0-104.7%)을 보여, CODEX에서 제시한 검증 범위에 적합한 것으로 나타났다.

본 연구에서 개발한 다성분 동시분석법을 토대로 잔류실태조사를 수행 하였다. 유통 중인 축·수산물 310건 중 104건 (축산물 39건, 수산물 65건)의 시료에서 검출되었고, 주로 검출된 퀴놀론계 항생제는 엔로플록사신, 플루메퀸 및 옥소린산 이었다. 검출농도는 장어 1건을 제외하고 모두 국내잔류허용기준 이하로 나타났다. 잔류량이 검출된 사라플록사신, 옥소린산 마보플록사신, 플루메퀸, 엔로플록사신 및 디플록사신에 대한 위해도를 산출한 결과, 일일 섭취허용량 대비 각각 0.201, 0.027, 0.007, 0.09 및 0.004%로 안전한 수준인 것으로 나타났다. 또한 인체노출량에 대한 식품별 기여도는 사라플록사신-우럭(63%), 옥소린산-장어(91%), 마보플록사신-넙치 (69%), 플루메퀸-우럭(45%), 엔로플록사신-닭(71%) 및 디플록사신-새우 (86%)로 나타났다.

II. 세팔로스포린계 항생제 9종(세파세트릴, 세파졸린, 세파피린, 디아세틸세 파피린, 세팔렉신, 세팔로니움, 세푸록심, 세포페라존 및 세프퀴놈)에 대하여

축·수산물 중 다성분 동시분석법을 확립하였다. UPLC-ESI-MS/MS를 이용하였고, MS/MS의 감도향상을 위해 Polarity Switching Mode를 도입하였으며, 세프퀴놈과 브루신은 ESI(-) mode를 적용하여 분석의 특이성을 높였다. CODEX 지침에 따라 시험법검증을 수행한 결과, 우수한 직선성을 나타냈으며, 검출한계와 정량한계는 모든 시료에서 각각 8, 25  $\mu\text{g}/\text{kg}$  이하였다. 또한 matrix의 영향을 줄이기 위해 matrix-matched정량곡선과 내부표준물질을 이용하였다. 이를 통해 CODEX 지침에 따른 적합한 검증결과를 얻을 수 있었다.

본 연구에서 개발한 다성분 동시분석법을 토대로 서울, 6대 광역시 및 제주에서 유통 중인 축·수산물 333건에 대한 잔류실태조사를 수행한 결과, 12건의 시료에서 잔류허용기준 이하로 세팔로스포린계 항생제(세팔로니움 11건, 세프퀴놈 1건)가 검출되었으며, 검출된 시료 모두 축산물(소고기, 우유)이었다. 검출된 세팔로니움과 세프퀴놈에 대한 위해도를 산출한 결과, 일일섭취허용량 대비 각각 0.02%와 0.01%로 안전한 수준으로 나타났다.

Ⅲ. UPLC-ESI-MS/MS를 이용하여 트리메토프림에 대한 분석법을 확립하였다. 시료의 matrix 효과를 감소시키기 위하여 초음파추출과 정제카트리지를 이용하였고, 이를 통해 평균회수율을 70% 이상으로 향상시켰다. 시험법검증을 수행한 결과, 0.999 이상의 직선성을 얻었고, 검출한계와 정량한계는 모든 시료에서 각각 0.3과 1.0  $\mu\text{g}/\text{kg}$  이하로 나타나, CODEX에서 제시한 검증 범위에 적합한 결과를 얻었다.

이를 토대로, 서울, 6대 광역시 및 제주에서 유통 중인 축·수산물 369건에 대한 잔류실태조사를 실시한 결과, 넙치1건, 조피볼락 5건 및 장어 1건에서 잔류허용기준 이하의 농도가 검출되었고, 축산물에서는 검출되지 않았다. 트리메토프림에 대한 위해도를 산출한 결과, 일일섭취허용량 대비 0.13%로 안전한 수준으로 나타났고, 인체노출량에 대한 식품별 기여도를 살펴 본 결과 우육이

58%로 가장 높게 나타났으며, 넙치 32%, 장어 10% 순을 보였다.

이상의 결과를 종합해 볼 때 국내 유통 중인 축·수산물 중 퀴놀론계, 세팔로스포린계 항생제 및 트리메토프림에 대한 잔류량은 국내 잔류허용 기준치 이하로 검출되어, 일일섭취허용량 대비 안전한 수준인 것으로 나타났다. 그러나 세계보건기구에서 3, 4 세대 세팔로스포린계 항생제와 퀴놀론계 항생제를 최우선적으로 관리가 필요한 그룹인 ‘CIA (Critically Important Antimicrobials for human medicine)’ 으로 지정하고 있고, 1 세대 및 2 세대 세팔로스포린계 항생제와 트리메토프림을 ‘HIA (Highly Important Antimicrobials)’로 지정하고 있다. 또한 동물보건기구에서는 퀴놀론계, 세팔로스포린계 항생제 및 트리메토프림 모두 가장 중요한 항생제 그룹인 ‘VCIA (Veterinary Critically Important Antimicrobials)’ 으로 분류하고 있다.

따라서 식품안전 및 국민건강과 국내 동물용의약품의 효율적인 사용을 위한 잔류실태조사와 국제 수준에 맞는 시험법 개발 및 개선 등 제도적인 관리가 지속적으로 필요할 것으로 판단된다.

---

주요어: UPLC-MS/MS, 질량분석기, 모니터링, 항생제잔류

학번: 2007-30455



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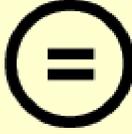
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**A Dissertation of the Degree of Doctor of Philosophy**

**Development of multi-residue analytical methods for  
quinolones, cephalosporins, and trimethoprim and  
application to the residue monitoring in livestock  
and marine products in Korea**

축·수산물 중 퀴놀론계, 세팔로스포린계, 트리메토프림  
항생제에 대한 동시분석법 개발 및 잔류실태조사

2016 년 2 월

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장진욱

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2015년 12월

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위원	(인)
위원	(인)
위원	(인)



## **ABSTRACT**

### **Development of multi-residue analytical method for quinolones, cephalosporin, and trimethoprim and application to the residue monitoring in livestock and marine products in Korea**

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Three reliable analytical methods were developed based on a simple and rapid sample preparation followed by ultra performance liquid chromatography with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) for the determination of 13 quinolones, 9 cephalosporins, and trimethoprim in livestock and marine products (beef, pork, chicken, milk, egg, flatfish, jacopecover, common eel, and shrimp). The proposed methods were validated according to the CODEX guidelines and applied in the survey of in a total of 1012 samples of livestock and marine products commercialized in Korea.

In the first study, a simple and specific analytical method was developed for the simultaneous determination of 13 quinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, ofloxacin,

orbifloxacin, oxolinic acid, pefloxacin, and sarafloxacin) using UPLC-ESI-MS/MS and all results fully complied with CODEX recommendation. Good linearities were achieved and the correlation coefficients were ranged between 0.9992 and 0.9999. The recoveries of 13 quinolones were higher than 80%, the limit of detection (LOD) and limit of quantitation (LOQ) were lower than 0.1 and 0.4 µg/kg, respectively. Besides, the matrix-matched calibration curve and internal standard (IS) played a significant role in compensating for the matrix effects. A survey for 13 quinolones residues was conducted on 310 livestock and marine products. Oxolinic acid, enrofloxacin, and flumequine were the most commonly detected antibiotics. The residues of quinolones were detected on 104 samples (33.5% incidence) but residue levels were below the MRLs. Detected concentrations were 0.1-100.0 µg/kg and the risk value (EDI/ADI, %) were safe levels between 0.0005% and 0.612%. However, pefloxacin was detected in one common eel sample above the legal residue limit and the detected concentration was 62.4 µg/kg.

In the second study, a sensitive and reliable method was developed and validated for the simultaneous determination of 9 cephalosporins (cefacetrile, cefazolin, cephapirin, desacetyl cephapirin, cefalexin, cefalonium, cefoperazone, cefuroxime, and cefquinome)

and all obtained results were satisfied with CODEX recommendation. The use of UPLC-ESI-MS/MS with polarity switching ionization mode improved the sensitivity and reduced analysis time, allowing the identification and quantification of 9 cephalosporins in 5 min. Because of the matrix effects, matrix matched calibration curves with IS were used for quantification to determine cephalosporin residues in samples. Good linearities were acquired and the LOD and LOQ were lower than 8 and 25  $\mu\text{g}/\text{kg}$ , respectively. The survey for the 9 cephalosporins residues was conducted on 333 livestock and marine products. The residues were detected in only 12 livestock products (3.6% incidence) but residue levels were below the MRLs and the detected cephalosporins were cefalonium in beef and cefquinome in milk. Detected concentrations were from 1.10 to 9.77  $\mu\text{g}/\text{kg}$  in beef and 7.50  $\mu\text{g}/\text{kg}$  in milk. The risk value was very safe level of 0.02% in cephalonium and 0.01% in cefquinome.

In the third study, a specific and simple analytical method was developed using UPLC-ESI-MS/MS for the determination of trimethoprim. The sample preparation through the ultrasonic-assisted extraction and SPE clean-up procedure was improved the recovery and reduced the matrix effect. The recoveries were higher than 70% and the LOD and LOQ

were lower than 0.3 and 1.0 µg/kg, respectively. The survey for the trimethoprim residue was conducted on 369 livestock and marine products. The residues of trimethoprim were detected in 7 marine products (1.9% incidence) but residue levels were below the MRL. Detected concentrations were 1.17 to 16.43 µg/kg in jacopeer, 40.0 µg/kg in flatfish, and 13.3 µg/kg in common eel. The risk value was safe level of 0.13%.

In conclusion, the methods developed in this study were more reliable and accurate for screening, quantification, and identification of 13 quinolones, 9 cephalosporins, and trimethoprim residues in livestock and marine products and could be successfully applicable in field samples. The resulting residue level of 13 quinolones, 9 cephalosporins, and trimethoprim appeared to be relatively safe. However, the quinolones and 3<sup>rd</sup> - & 4<sup>th</sup> - generation cephalosporins were classified as critically important antimicrobials for human medicine (CIA) and trimethoprim and 1<sup>st</sup> - & 2<sup>nd</sup> -generation cephalosporins were classified as highly important (HIA) from World Health Organization (WHO). In addition, quinolones, cephalosporins, and trimethoprim were classified as veterinary critically important antimicrobials (VCIA) from the Office International des Epizooties (OIE). Thus, a strict guideline for the use of antibiotics and continuous survey on antibiotic

residues is needed to ensure the safety of animal origin foods. The methods developed in this study will help to implement such guidelines of CODEX on the use of antimicrobials.

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Keywords: UPLC-MS/MS, mass spectrometry, monitoring, antibiotic residue

**Student Number: 2007-30455**

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**Chapter III. Development of analytical method for trimethoprim and application to the residue monitoring in livestock and marine products**

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## ABBREVIATION

ADI	Acceptable daily intake
CIA	Critically important antimicrobials for human medicine
EDI	Estimated daily intake
ESI	Electrospray ionization
HLB	Hydrophile-lipophile balance
IS	Internal standard
LOD	Limit of detection
LOQ	Limit of quantitation
MFDS	Ministry of food and drug safety
MRL	Maximum residue limit
MRM	Multiple reaction monitoring
MSPD	Matrix solid-phase dispersion
OIE	Office international des epizooties
QuEChERS	Quick easy cheap effective rugged and safe
RSD	Relative standard deviation
SPE	Solid-phase extraction
UPLC-ESI-MS/MS	Ultra performance liquid chromatography with electrospray ionization tandem mass spectrometry
VCIA	Veterinary critically important antimicrobials

## **General Introduction**

As the food culture in Korea changes from traditional Korean style to western one, the consumption of foods originated from livestock, poultry, and fish increased dramatically for last decades, and the transition forced livestock and aquaculture industry turned into large and dense breeding system. Antibiotics have been administered to animals for prevention and treatment of infectious diseases and widely used at sub-therapeutic levels for growth promotion of livestock and aquaculture farms. The global consumption of antibiotics for animals has been estimated to be between 100,000 and 200,000 tons/year (Zucatto et al., 2010). Although the annual sales of antibiotic in Korea was greatly decreased from 1,450 ton in 2006 to 630 ton in 2014 (KAHPA, 2015), the sales of phenicols and cephalosporins increased by 1.9 and 2.3 times from 2006 to 2014, respectively (QIA, 2015). Antimicrobial treatments have several benefits, but when large quantities of specific types of antibiotics are supplied, adverse effects, such as appearance of drug resistant microorganisms, can be manifested (Blasco et al., 2009). In Korea, resistance against tetracyclines have remained high ( $\geq 75\%$ ) and resistances against

ciprofloxacin, chloramphenicol, ceftiofur, and trimethoprim/sulfamethoxazole have increased steadily since 2008 (QIA, 2015).

Moreover, misuse and overuse of antibiotics could be caused by livestock owners. Since antibiotics have been used with long-term administration in low concentrations, the presence of antibiotic residues in animal originated foods may increase antibiotic resistance of pathogens and can threaten public health. To minimize the exposure of antibiotics to humans, many countries established maximum residue limits (MRLs) for antibiotics residue in food producing animals. The European Union (EU, Commission regulation No. 37/2010, 2010) and the Joint FAO/WHO Expert Committee on Food additives have set up the MRLs in animals producing milk, muscle, fat, liver, and kidney. The Positive List System of Japan contains MRLs in animal and marine products. In Korea, the MRLs have been established from 1990s by the Ministry of Food and Drug Safety and currently, have been set for 185 kinds of antibiotics. Due to the expansion of international regulations on MRLs in recent years, more sensitive and specific analytical methods are required for the determination of antibiotic residues in animal originated foods.

In these studies, multi-residue analytical methods were developed for the determination of 13 quinolones, 9 cephalosporins, and trimethoprim in livestock and marine products using UPLC-ESI-MS/MS. The high selectivity, sensitivity, and versatility of UPLC-ESI-MS/MS have allowed the successful quantification of antibiotic residues in livestock and marine products. Moreover, these studies were applied to monitoring of antibiotic residues in field samples and risk assessments for multi-antibiotic residues were also performed.

# **CHAPTER I**

## **Development of multi-residue analytical method for 13 quinolones and application to the residue monitoring in livestock and marine products in Korea**

## **ABSTRACT**

The aim of this study was to develop and validate a specific ultra performance liquid chromatography with electrospray ionization tandem mass spectrometric method (UPLC-ESI-MS/MS) for simultaneous determination of 13 quinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, ofloxacin, orbifloxacin, oxolinic acid, pefloxacin, and sarafloxacin) in livestock and marine products. The analytical method involved the sample preparation based on solvent extraction without further clean up procedure. All samples were deproteinized with acidified acetonitrile, followed by defatting with acetonitrile-saturated hexane. Because of matrix effects, matrix matched calibrations with internal standard were used for quantification during the determination of the quinolones residues in samples.

The proposed method was validated according to the CODEX guidelines and all results fully complied with CODEX recommendation. Good linearities were achieved and the correlation coefficients ranged between 0.9992 and 0.9999 depending upon antibiotics.

Both the limit of detection and limit of quantitation for this assay were lower than 0.1 and 0.4 µg/kg, respectively, and these values were below the maximum residue limits (MRLs) established by the Korean Food Code. Recoveries (%) were in the ranged of 87.5 to 104.7% for livestock products and 83.0 to 100.9% for marine products, respectively. The relative standard deviations (RSD, %) of repeatability ranged from 0.33 to 3.72 and reproducibility ranged from 0.71 to 6.75 depending upon sample species. This quantitative method has many advantages including simple preparation step, rapid determination, and high sensitivity, which could be applied to the determination and quantification of quinolones residues in livestock and marine products. The monitoring results revealed that quinolones were detected in 104 out of 310 livestock and marine products (33.5% incidence) but residue levels were below the MRLs in the Korean Food Code and the risk value was very safe level. Of samples, 1 common eel containing pefloxacin above the legal residue limit was found, and the detected concentration was 62.4 µg/kg.

## INTRODUCTION

The quinolones and fluoroquinolones are a family of synthetic broad spectrum antibiotics and the most frequently employed. These drugs are administered at therapeutic doses to treat bacterial infections in food-producing animals, such as cattle, swine, turkey, and chicken (Junza et al., 2011) and also used at sub-therapeutic doses as prophylactics. They are active against Gram-negative bacteria as well as Gram-positive bacteria by inhibiting DNA topoisomerase (Scortichini et al., 2009). Nalidixic acid is the first generation of the quinolones approved on 1963, by Food and Drug Administration for the treatment of urinary tract infections (Eleni et al., 2007) and structural modifications have resulted in the second, third, and fourth generations of the (fluoro)quinolones which have improved coverage of Gram-positive organisms (Roberta et al., 2009). Nowadays, they are widely used in the treatment of respiratory diseases and enteric bacterial infections in humans and food-producing animals and aqua-cultured fish (Hermo et al., 2006). Since 2000 there has been a significant progressive increase in the use of the quinolones in animal production (Strolker and Brinkman, 2005; Di Corcia and Nazzari, 2002). It has produced more chance of their residue in the animal origin foods and led to the

emergence of fluoroquinolone-resistant bacterial strains in animals (Rodriguez et al., 2011). It could cause many health problems in humans, such as the transmission of fluoroquinolone-resistant bacteria (e.g. fluoroquinolone-resistant *Campylobacter* and *Salmonella*) to humans since the early 1990s (Fabrega et al., 2011; Okeke et al., 2005).

To minimize the exposure of antibiotics to humans, many countries have been establishing MRLs for quinolones residue in foodstuffs (Table 1). The European Union (EU) has established the MRLs of veterinary drugs in animal origin foods, and some of them are quinolones (Council Regulation No. 2377/90). In addition, the EU and the Joint FAO/WHO Expert Committee on Food additives have set up the MRLs in several animal tissues, milk, and eggs (Rubies et al., 2007). The Italian National Residue Control Plan is very precise: samples taken at slaughterhouse are screened for the presence of residues/metabolites on the basis of MRLs (Luca et al., 2015). According to the Australia and New Zealand Food Standards Code, residues of quinolones cannot be detected in any foodstuff for domestic consumption and the only exception is set for oxolinic acid in pacific salmon at 0.01 mg/kg (Lesley et al., 2002). In Korea, the 13 quinolones are

permitted for prevention and treatment of animal disease. The MRLs and the withdrawal period were established by the Ministry of Food and Drug Safety (MFDS) (Table 2).

As the MRLs of veterinary drugs are very low level, they have been demanded to improve the specific and sensitive analytical methods for the multi-residue analysis. Many published papers have been reported for analysis of the (fluoro)quinolones residues in livestock and marine products. Liquid chromatography (LC) is the most frequently used for separation (Belal et al., 1999; Hernandez-Arteseros et al., 2002), mainly coupled with ultraviolet (UV) (Eleni et al., 2007; Bailac et al., 2004; Pecorelli et al., 2003), or fluorescence detector (Herranz et al., 2007; Zeng et al., 2005). However, some methods either covered only several compounds of (fluoro)quinolone or were time-consuming for sample preparation. Immunoassay (Ashwin et al., 2009; Anne-Catherine et al., 2006) have been sometimes used for analysis of (fluoro) quinolones and provided a rapid screening test. But it is difficult to apply a multi-residual detection. Thin-layer chromatography (TLC) (Juhel-Gaugain and Abjean, 1998), gas chromatography (GC) (Asami et al., 2000), and capillary electrophoresis (CE) (Ana et al., 2006) have been rarely applied.

Recently, several reports are focused on accurate and sensitive confirmatory quantification methods, such as liquid chromatography combined to tandem mass spectrometry (LC-MS/MS). These procedures included only one sample of interest, for instance, milk (Junza et al., 2014; Lina et al., 2011; Hong et al., 2009; Sara Bogialli, 2008), gilthead sea bream (Romero-Gonzalez et al., 2007), egg (Anna et al., 2012; Antonia et al., 2010; Sara et al., 2009), shrimp (Christine et al., 2007), bovine muscle (Rubies et al., 2007), turkey (Lorena et al., 2015), and swine plasma (Garces et al., 2006). Besides, compared to LC, UPLC increased resolution and sensitivity, as well as considerably decreased sample analysis time and mobile phase solvent consumption (Junza et al., 2011; Stolker et al., 2005).

Therefore, the purpose of this study was to develop a rapid and reliable analytical method for the simultaneous determination of 13 quinolones in 9 species of livestock and marine products (beef, pork, chicken, egg, milk, flatfish, jacopecover, common eel, and shrimp) using UPLC-ESI-MS/MS. Furthermore, this paper was investigated the residues of 13 quinolones in livestock and marine products commercialized in Korea using UPLC-ESI-MS/MS.

## MATERIALS & METHODS

### *Chemical and materials*

Ciprofloxacin (CIP), danofloxacin (DAN), difloxacin hydrochloride (DIF), enrofloxacin (ENR), flumequine (FLU), marbofloxacin (MAR), nalidixic acid (NAL), norfloxacin (NOR), ofloxacin (OFL), orbifloxacin (ORB), oxolinic acid (OA), pefloxacin mesylate dehydrate (PEF), and sarafloxacin (SAR) were supplied by Sigma-Aldrich (St Louis, MO, USA). Piromidic acid (PIR) from Sigma-Aldrich was used as an internal standard (IS). The molecular structures were described in Fig. 1.

HPLC grade acetonitrile and methanol were obtained from Burdick & Jackson (Ulsan, Korea). Hexane and formic acid (98%) were purchased from Merck (Darmstadt, Germany). Acetic acid (99.9%, HPLC grade) was supplied by J.T Baker (NJ, USA) and trichloroacetic acid (99.0%) by Sigma-Aldrich (St Louis, MO, USA). Ultrapure water was purified with a MILLI-Q system (Milipore, Bedford, MA, USA). All solutions prepared for UPLC-MS/MS were passed through a 0.45 µm nylon filter before use.

### ***Preparation of standard solutions***

Each individual standard, such as CIP, DAN, DIF, ENR, FLU, MAR, NAL, NOR, OFL, ORB, OA, PEF, and SAR, was made by dissolving in methanol. Stock standard solutions (50 µg/mL) were obtained and stored at 4 °C in the dark for no longer than 2 months. Working standard solutions (1 µg/mL) were prepared daily by mixing individual stock solutions and diluted in 0.1% formic acid in 10% acetonitrile (v/v). Piromidic acid (IS) was prepared by dissolving solution at the concentration of 100 ng/mL.

### ***Instrumentation***

An AT 261 analytical balance (Mettler Toledo, Greifensee, Switzerland) was used in the preparation of standard solutions. A REAX TOP vortex mixer (Heidolph, Schwabach, Germany), POWER SONIC 520 sonicator (Hwashin Tech., Seoul, Korea), and Allegra X-22R centrifuge (Beckman Coulter, Alle, USA) were used in sample extraction.

UPLC equipment was an Acquity ultra performance LC (Waters, MA, USA) and ESI-MS/MS measurements were performed using a Quattro premier XE (Waters). Data was collected using MassLynx 4.1 software (Waters) on a personal computer.

### ***Sample collection***

Sampling was set in accordance with the 「Sampling with probability proportionate to size」 and species and number of samples were determined on the basis of the consumption of food according to the 「National Health and Nutrition Examination Survey, 2011」. Each sample was purchased at least two and sampling points within the sampling area were not biased.

As a result, livestock and marine products (n=310) including beef (n=59), pork (n=49), milk (n=29), egg (n=27), chicken (n=34), flatfish (n=26), jacobever (n=29), common eel (n=27), and shrimp (n=30) were purchased from different markets in Seoul, Busan, Incheon, Daegu, Daejeon, Gwangju, and Ulsan. Details were given in Fig. 2. All solid samples were finely ground with blender and stored in a freezer at -20°C until use.

### ***Preparation of spiked samples and standard***

One sample of each matrix was repeatedly measured to confirm that no 13 quinolones were present and was used for the preparation of matrix-matched calibration standards and fortified samples for the validation of the proposed method.

Blank samples of beef, pork, chicken, egg, milk, flatfish, jacopeer, common eel, and shrimp were homogenized and stored at -20°C until use. Fortified samples were prepared by spiking 100 µL of diluted working solutions at the concentration of 0.2, 0.4, and 10 µg/kg for DIF, ENR, FLU, NAL, OFL, and SAR, and 0.4, 0.8, and 10 µg/kg for CIP, DAN, MAR, NOR, ORB, OA, and PEF by using 0.1% formic acid in 10% acetonitrile (v/v).

### ***Matrix-matched calibration curves***

Quantification was carried out using matrix-matched calibration curves with IS. The blank samples (beef, pork, chicken, milk, egg, flatfish, jacopeer, common eel, and shrimp) were used as matrix and fortified with working standard solutions.

The seven point matrix-matched calibration curves were prepared with the blank samples spiked with 0, 0.2, 0.4, 1.0, 2.0, 4.0, and 10.0 µg/kg for DIF, ENR, FLU, NAL, OFL, and SAR, and 0, 0.4, 0.8, 2.0, 4.0, 8.0, 10.0, and 20.0 µg/kg for CIP, DAN, MAR, NOR, ORB, OA, and PEF. Piromidic acid (IS) was spiked in all of these samples at the concentration of 4 µg/kg. Each sample was submitted to the full extraction procedures.

The calibration curves for 13 quinolones were constructed by plotting the response factor (the ratio of peak area of analyte vs. peak area of internal standard) as a function of the analyte concentration. Separate calibration curves were used for each sample.

### ***Extraction procedure***

Homogenized sample (1 g) of beef, pork, egg, chicken, flatfish, jacobever, common eel, and shrimp and 1 mL of milk were accurately weighed and taken into a 50 mL disposable polypropylene centrifuge tube. Selected quinolones standard solutions (only for the matrix-matched calibration curves) and IS were directly spiked. The spiked samples were stood in the dark at room temperature, for at least 10 min, to allow the interaction between the quinolones and the matrix. The samples were added with 2.5% trichloroacetic acid (1 mL) and shaken on a vortex mixer (Heidolph Reax top, Heidolph, Schwabach, Germany) for 1 min. After addition of 1% acetic acid in acetonitrile (15 mL), the samples were mixed again for 1 min and centrifuged (Allegra X-22R, Beckman Coulter, Brea, CA, USA) at 1000×g for 15 min to induce the precipitation of proteins. The supernatant was carefully filtered through a syringe filter (Whatman<sup>®</sup>, PVDF memb-

rane, pore size 0.45  $\mu\text{m}$ ) and poured into a separating funnel. Acetonitrile-saturated hexane (15 mL) was added and intentionally agitated for 5 min. The lower layer was transferred into a 20 mL glass tube and evaporated to dry at 45  $^{\circ}\text{C}$  under a stream of nitrogen (EYELA GM-2200, Tokyo, Japan). The dried residue was dissolved in 1 mL of 0.1% formic acid in acetonitrile and filtered through a syringe filter (Whatman<sup>®</sup>, PVDF membrane, pore size 0.2  $\mu\text{m}$ ) prior to UPLC injection. A schematic diagram of the sample preparation was presented in Fig. 3. All samples were analyzed in triplicate and results were expressed as a mean value of the total residue concentration  $\pm$  standard deviation (SD).

### ***UPLC-ESI-MS/MS conditions***

The quinolones were separated on an Acquity UPLC BEH C<sub>18</sub> column (2.1 mm  $\times$  100 mm; 1.7  $\mu\text{m}$  particle size, Waters). Mobile phase A was purified water containing 0.1% formic acid (v/v), and mobile phase B was acetonitrile. The gradient conditions were initiated with 90% mobile phase A followed by a linear decrease to 30% in 5 min, 0% in 0.5 min, and maintained for 1 min at 0%. At 6.6 min, the gradient was programmed to re-

equilibrate the column for 1.5 min under initial conditions. The run time for each injection was 8 min. The flow rate was 0.3 mL/min and the injection volume was 20  $\mu$ L in full-loop mode. Details were given in Table 3.

MS determination was performed with MRM experiments in ESI positive ion mode combined with monitoring of the most abundant MS/MS (precursor $\rightarrow$ product) ion transitions using a dwell time of 0.03s. Detailed parameters for MRM acquisition were presented in Table 4. The MS parameters were as follow: capillary voltage, 3.0 kV; source temperature, 150 $^{\circ}$ C; desolvation temperature, 350 $^{\circ}$ C; cone gas (N<sub>2</sub>) flow, 50 L/h; and desolvation gas (N<sub>2</sub>) flow, 800 L/h.

### ***Method validation***

The method was validated according to the CODEX guideline (FAO, 2009). The validation parameters to evaluate the method were linearity, LOD, LOQ, precision (repeatability and reproducibility), and accuracy (Table 5).

### ***Linearity***

Linearity was evaluated using the squared correlation coefficients ( $r^2$ ) of 7-points

matrix-matched standards curves, which were based on extracts of blank samples (beef, pork, milk, egg, chicken, flatfish, jacobever, common eel, and shrimp). The calibration ranges were from 0 to 10 µg/kg for DIF, ENR, FLU, NAL, OFL, and SAR, and from 0 to 20.0 µg/kg for CIP, DAN, MAR, NOR, ORB, OA, and PEF, respectively.

### ***Limit of detection and quantitation***

The LOD is the lowest concentration of analyte that an analytical process can reliably differentiate from background levels, while the LOQ is the lowest concentration of analyte that can be quantified. LOD and LOQ values were calculated based on signal-to-noise ratio (S/N) values of 3.3 and 10, respectively.

### ***Precision***

The precision was expressed as a percentage of RSD (%), which was performed in a single laboratory. Intra-day and inter-day precision (repeatability and reproducibility) were performed at 3 different concentrations (1.0, 4.0, and 10.0 µg/kg). The intra-day analysis was achieved through six replicates, and the inter-day analysis was obtained through repeating three consecutive days at each concentration level.

### ***Accuracy***

The accuracy was expressed as a percentage of recovery, which was performed in a single laboratory. Accuracy was evaluated through 3 different fortification levels; LOQ (0.2 or 0.4 µg/kg), 2×LOQ (0.4 or 0.8 µg/kg), and 10 µg/kg. Six replicates per each sample were performed and the results were expressed as a mean value of the total concentration ± SD.

## RESULTS & DISCUSSION

### *Method validation*

One sample of each matrix was repeatedly measured to confirm that no 13 quinolones were present and was used for the preparation of matrix-matched calibration standards and fortified samples for the validation of the method. The proposed method was validated with UPLC-ESI-MS/MS according to the CODEX guideline (FAO, 2009) for quantitative methods. All obtained results were satisfied with CODEX recommendation and indicated the stability of the proposed method.

As shown in Table 6, linearities, LOD, and LOQ for each species of matrices were summarized. The linearity was checked with the matrix-matched calibration curves using 7 calibration points. The linearities were calibrated from 0 to 10 ng/mL for DIF, ENR, FLU, NAL, OFL, and SAR and 0 to 20 ng/mL for CIP, DAN, MAR, NOR, ORB, OA, and PEF, respectively. Good linearities were obtained and the correlation coefficients ranged between 0.9992 and 0.9999 depending upon antibiotics. Furthermore, piromidic acid as IS, which significantly increased the linearity of the matrix-matched calibration

curves of 13 quinolones. Based on these results, a good linearity was proven and allowed coverage of 13 quinolones within the working range.

The LOD ranged from 0.06 to 0.1  $\mu\text{g}/\text{kg}$  and LOQ ranged from 0.2 to 0.4  $\mu\text{g}/\text{kg}$  depending upon species of samples. The results of LOD and LOQ showed that the method were enough to determine the MRLs values in animal origin samples.

The repeatability and reproducibility (the intra-day and inter-day precision) were evaluated in beef and flatfish. The repeatability was evaluated by spiking 2 blank samples (n=6 per each matrix) at the validation level (1, 4, and 10  $\mu\text{g}/\text{kg}$ ) and the reproducibility was carried out on 3 consecutive days. Repeatability and reproducibility results are presented in Table 7. The RSD (%) of repeatability ranged from 0.33 (Danofloxacin) to 3.72 (Orbifloxacin) in beef and from 0.43 (Marbofloxacin) to 3.33 (Danofloxacin) in flatfish, respectively. The RSD (%) of reproducibility ranged from 0.68 (Marbofloxacin) to 4.86 (Oxolinic acid) in beef and from 0.71 (Oxolinic acid) to 6.75 (Flumequine) in flatfish, respectively. These results indicate the good precision and reliability of the developed method. In addition, the repeatability values expressed as RSD (%) were lower

than 30%, which is within the acceptance criteria of the CODEX and satisfied the validating analytical methods (Table 5).

To evaluate method accuracy, recoveries were calculated at 3 different spiking levels. Besides, matrix-matched calibration curves were used with IS. Average recoveries of each analyte were obtained performing the analysis in 6 replicates for each matrix. Accuracy data were recorded in Table 8 and 9.

Satisfactory results were acquired, with the percentage of recoveries (%) was between  $87.5 \pm 5.0$  and  $104.7 \pm 3.7$  in livestock products and between  $83.0 \pm 2.8$  and  $100.9 \pm 1.1$  in marine products, respectively. The results were within the acceptance criteria (60-120%) of the CODEX validating analytical method (Table 5). The RSD (%) ranged from 0.4 to 6.0 and from 0.9 to 5.7, respectively. The maximum recoveries were obtained under acidic conditions and in the presence of a high percentage of acetonitrile (Rodriguez et al., 2011; Marazuela and Moreno-Bondi, 2004). In this work, acetonitrile and trichloroacetic acid played an important role in precipitating proteins, which leading to obtained higher recovery.

In this study, excellent validation parameters were acquired, which indicated that the method was suitable for the analysis of quinolones. Furthermore, the use of matrix-matched calibration curves with IS were able to minimize the difference in the signal output of analyte between sample extract solvent and a pure solvent.

### ***Optimization of sample preparation***

The simple and specific analytical method was developed. Sample preparation was focused on the establishment of the most suitable conditions used for the simultaneous determination of 13 quinolones from livestock and marine products.

Several extraction solvent mixtures were evaluated. Beef and flatfish blank samples fortified with 4 µg/kg of 13 quinolones were extracted with solution of acetonitrile with or without 1% acetic acid, and additionally added the solution of trichloroacetic acid in different ratio from 1% to 5% (v/v). As shown in Table 10, the combination of 1% acetic acid in acetonitrile (v/v) and 2.5% trichloroacetic acid was shown to be the most effective recoveries and the lowest RSD (%) of the 13 quinolones.

The precipitation of proteins is achieved in many cases by adding a strong acid, such as

trichloroacetic acid in combination with organic solvents (Andreia et al., 2013). Furthermore, acetonitrile and methanol are more efficient as extraction solvents as they can simultaneously precipitate the proteins, denature enzymes, and extract the target analyte. Many authors prefer acetonitrile over methanol as extraction solvent. Besides, methanol extracts too many matrix compounds, complicating the following clean-up steps (Marilena and Nikolaos, 2015). In this work, acetic acid and trichloroacetic acid was added to samples and acetonitrile was used as the extracting solvent, which promoted the precipitation of proteins. Thus, it could be assumed that the matrix components responsible for possible interference (such as, signal suppression or enhancement) were removed. The obtained results demonstrated acceptable recoveries of all of the quinolones, ranging from 91.93% to 102.20% in beef and from 94.06% to 110.80% in flatfish.

In order to accomplish the deproteinization and defatting of samples, the extraction with acetonitrile-saturated hexane was performed after centrifugation at 1000×g. Moreover, instead of clean-up step, e.g. using SPE cartridge, the solvent extraction including the steps of syringe filtration, centrifugation, and agitation step was applied to

the samples, thereby simplifying sample handling and reducing the time of sample preparation. Antonia et al (2010) reported that solvent extraction provided the good recovery for quinolones among the result of comparing extraction procedures, for instance solvent extraction, QuEChERS procedure, MSPD, and SPE.

Furthermore, the residue concentration of veterinary drugs could be different in the egg white (albumen) and egg yolk (ovum) (Cornelis and Michael, 2000; Antonia et al., 2010). For accurate results, all samples were sufficiently homogenized (at least 3 min). Besides, because target analytes have different polarities, and consequently a different distribution between albumin and yolk, the method was performed on the whole egg.

### ***UPLC-ESI-MS/MS optimization***

Chromatographic conditions of UPLC were evaluated in order to obtain the good separation and retention for the analytes. Several experiments were conducted testing mobile phases consisting of distilled water with different concentrations of formic acid (0.01, 0.05, and 0.1%, v/v) and acetonitrile (80, 90, 100%, v/v).

As a result, the addition of 0.1% formic acid in distilled water and 100% acetonitrile (v/v)

allowed the best resolution, as well as it enhanced the ionization efficiency. Formic acid and acetonitrile were important roles for the formation of well distributed peak and sharp peak shape (Lina et al., 2011; Junza et al., 2014).

Other parameter such as flow rate, injection volume, and gradient profile were studied in order to obtain a fast and reliable separation. The best results were acquired when 0.30 mL was used as flow rate and 20  $\mu$ L were injected. Several gradient profiles were tested, and good response was obtained with the gradient described in Table 3. Besides, in the first 2 min of the run time, major interferences are presented due to polar matrix constituents that are eluted and co-elution with the analytes would lead to large signal suppression (Marilena et al., 2015). Thus, it was necessary that the 13 quinolones began to elute after the 2 min of the run time.

In this work, 8 min of run time was achieved with the optimum conditions for the separation of 13 quinolones. The optimal chromatographic separation of 13 quinolones was performed with a 2.1 mm  $\times$  100 mm column. The UPLC columns consisting of a smaller particle size are the possibility of having high efficiency in peak separation, sharp peaks, and also a reduction in run time when compared with common HPLC columns

(Andreia et al., 2013). The acquired UPLC chromatograms showed a good resolution for all the quinolones (Fig. 4) and indicated the excellent specificity of the developed method. In addition, the use of an acidic mobile phase (acetonitrile) adjusted with 0.1% formic acid promoted positive ionization and maximized sensitivity and resolution, which improved the detection of 13 quinolones.

In MS/MS system, the protonated molecular ion  $[M+H]^+$  was selected as precursor ion for 13 quinolones, and the product ions were obtained according to the collision energy (eV). The MRM mode was used to increase the sensitivity and selectivity of the determination. To achieve maximum sensitivity for 13 quinolones, MS/MS conditions (for example, capillary voltage, source temperature, and desolvation temperature) were optimized by direct infusion into the detector of working standard solutions. The results of UPLC-ESI-MS/MS ion reconstituted chromatograms for 13 quinolones spiked to bovine muscle were presented in Fig. 4. As showed in Table 4, MRM interferences between FLU and OXO were observed because the precursor ion and one of two product ions were common to FLU and OXO. Therefore, the  $[M + H]^+ \rightarrow [M + H-H_2O]^+$  was applied to transition for quantification of FLU and OXO. Also, the transition  $[M + H]^+ \rightarrow$

$[M + H-OC_3H_8]^+$  ( $m/z$  262  $\rightarrow$ 202) was used for FLU and  $[M + H]^+ \rightarrow [M + H-OCH_2O]^+$  ( $m/z$  262  $\rightarrow$ 216) was used for OXO, respectively (Hermo et al (2006); Rubies et al (2007); Christine et al (2007)). The results of ions transition, obtained from the bovine muscle fortified with 10  $\mu$ g/kg of FLU and 20  $\mu$ g/kg of OXO, were described in Fig. 5. Additionally, for separating FLU and OXO, the gradient condition was increased the percentage of mobile B from 10 to 70%. Therefore, there was no interference to be observed with the monitored MS reactions.

To obtain an adequate number of data acquisition points related to peak shape and reproducible chromatographic signals, different dwell times (between 0.01 and 0.1 s) were evaluated. Good peak shape and reproducible data without a decrease of the sensitivity were acquired using a dwell time 0.03 s in 10 quinolones, whereas FLU, NAL, OA, and PIR (IS) were obtained using a dwell time of 0.1 s.

### ***Application of the proposed method to animal products***

Quinolones were analyzed in 310 livestock and marine products including 59 beef, 49 pork, 34 chicken, 29 milk, 29 egg, 26 flatfish, 29 jacobever, 27 common eel, and 30 shrimp (Table 11). The results are shown in Table 12. The residues of quinolones were

detected in 39 out of 198 livestock product samples (19.7% incidence) and 65 out of 112 marine product samples (58.0% incidence); 7 out of 59 beef samples (11.9% incidence), 7 out of 49 pork samples (14.3%), 22 out of 34 chicken samples (64.7%), 2 out of 29 milk samples (6.9%), 1 out of 27 egg samples (3.7%), 12 out of 26 flatfish samples (46.2%), 16 out of 27 common eel samples (59.3%), 27 out of 29 jacopever samples (93.1%), and 10 out of 30 shrimp samples (33.3%) (Fig. 2). The incidence of 13 quinolones residues followed the order; jacopever > chicken > common eel > flatfish > shrimp > pork > beef > milk > egg.

Oxolinic acid, enrofloxacin, and flumequine were the most common antibiotics found in the detected samples. The kinds of the detected quinolones in livestock and marine products are summarized in Table 13. As seen in Table 13, the detected milk samples contained only one antibiotic, while residues of more than one antibiotic were found in the detected beef, pork, chicken, egg, flatfish, jacopever, common eel, and shrimp. It indicated that quinolones were employed as multiple uses in livestock and marine products.

Detected concentrations were 0.2 to 20.0 µg/kg in beef, 0.6 to 4.1 µg/kg in pork, 0.1 to 47.0 µg/kg in chicken, 0.7 to 0.9 µg/kg in milk, 0.3 to 0.5 µg/kg in egg, 0.3 to 21.1 µg/kg in flatfish, 0.1 to 100.0 µg/kg in common eel, 0.3 to 22.8 µg/kg in jacobever, and 0.2 to 5.1 µg/kg in shrimp, respectively. The risk value based on the quinolones exposure by food intake was calculated using the EDI (estimated daily intake) and ADI (acceptable daily intake). Compared with ADI, the risk value was very safe level of 0.205% in sarafloxacin, 0.007% in marbofloxacin, 0.612% in oxolinic acid, 0.027% in flumequine, 0.090% in enrofloxacin, and 0.004% in difloxacin, respectively. Details were given in Table 14 and the food contribution for EDI was described in Fig. 6.

The detected concentration of pefloxacin in common eel was 62.4 µg/kg. The level of residue concentration was above the MRL (not detected in livestock product, fish, and shellfish) for pefloxacin in Korean Food Code. Fig. 7 showed the UPLC-ESI-MS/MS chromatograms of pefloxacin in detected sample. We assumed that non-observance of the required withdrawal period caused the illegal residue of pefloxacin, or the antibiotics were used during the transporting after shipment. Besides, the regular and massive use of veterinary medicines was seen as a risk factor for direct contamination of products

(Mitema et al., 2001; Won et al., 2011). Thus, treatments were carried out in accordance with proper usage of veterinary drugs and withdrawal periods were observed.

In Korea, the annual per capita consumption of livestock and marine products is growing and reached 53.5 kg in marine products, 11.5 kg in chicken, 20.9 kg in pork, 10.3 kg in beef, and 71.6 kg in milk (MAFRA, 2015). Besides, in Korea, many people commonly consume sashimi of jacobever and flatfish and enjoy roasted eel. Intensive farming, which can lead to a high potential for spread of infectious disease, has been maintained to satisfy an increasing demand for marine products. Therefore, the survey of quinolone residues in livestock and marine products is significant in Korea. In particular, quinolones classified as VCIA (veterinary critically important antimicrobials) from the OIE (Office International des Epizooties), and as CIA (critically important antimicrobials for human medicine) from WHO (World Health Organization). Considering the possibility of antibiotic cross-resistance between human and animal, it requires a proper use of antibiotics and continuous monitoring.

## CONCLUSION

A sensitive and specific method was developed and validated for the simultaneous determination of 13 quinolones in livestock and marine products using UPLC-ESI-MS/MS. The recoveries (%) of 13 quinolones were higher than 80%, the LOD and LOQ values were lower than the established MRLs in Korea, and other validation parameters were also in accordance with CODEX guideline. The proposed method using UPLC-ESI-MS/MS involved significant advantages with respect to simple preparation step, high sensitivity, speed, and resolution, making it an attractive choice for the analysis of quinolones in livestock and marine products. However, the fast gradient used in UPLC promoted matrix effects by reducing chromatographic separation between analytes and endogenous compound. As a result, the matrix-matched calibration and IS were used, which play very important roles in compensating for the matrix effects.

The livestock and marine products (n=310) commercialized in Korea were analyzed and quantified by proposed method. The residues of quinolones were detected in 104 samples (33.5% incidence) but residue levels were below the MRLs in Korea Food Code.

The risk value was safe level of 0.205% in sarafloxacin, 0.007% in marbofloxacin, 0.612% in oxolinic acid, 0.027% in flumequine, 0.090% in enrofloxacin, and 0.004% in difloxacin, respectively. However, pefloxacin was detected in one common eel above the legal residue limit and the detected concentration was 62.4 µg/kg.

Although this survey results appear to be relatively safe levels, antibiotic residues are exposed to consumers. Therefore, we proposed that the monitoring of quinolones in livestock and marine products was continued.

Table 1. Global organization for registration and establishment of MRLs for veterinary drugs

Country	Registration	Establishment of MRLs
Korea	Animal and Plant Quarantine Agency (QIA)	Ministry of Food and Drug Safety (MFDS)
Japan	The Ministry of Agriculture, Forestry and Fisheries (MAFF)	Ministry of Health, Labor and Welfare (MHLW)
USA	Center for Veterinary Medicine (CVM)	United States Food and drug Administration (FDA)
EU	Committee for Medicinal Products for Veterinary use (CVMP)	European Medicines Agency (EMA)
Australia	Australian Pesticides and Veterinary Medicines Authority (APVMA)	Food Standards Australia New Zealand (FSANZ)
New Zealand	New Zealand Food Safety Authority (NZFSA)	Food Standards Australia New Zealand (FSANZ)

Table 2. Maximum residues limits of 13 quinolones in Korea

Compound	Food	mg/kg
Enrofloxacin+Ciprofloxacin	Cattle, Pig, Sheep, Goat, Poultry	0.1~0.3
	Egg	Not detected
	Fish, Crustacean	0.1
	Milk	0.05
Danofloxacin	Cattle, Poultry	0.1~0.4
	Pig	0.05~0.2
	Milk	0.03
Difloxacin	Cattle, Pig, Sheep, Goat	0.4~1.4
	Poultry	0.3~1.9
	Fish, Crustacean	0.3
Flumequine	Cattle, Pig, Sheep	0.2~1.5
	Poultry	0.4~1.0
	Fish, Crustacean	0.5
Marbofloxacin	Cattle, Pig	0.05~0.15
	Milk	0.075
Nalidixic acid	Cattle, Fish	0.03
Orbifloxacin	Cattle, Pig, Milk	0.02
Oxolinic acid	Cattle, Pig	0.05
	Chicken	0.15~0.1
	Fish, Crustacean	0.1
Ofloxacin, Pefloxacin, Norfloxacin	Livestock products, Fish, Crustacean	Not detected
Sarafloxacin	Poultry	0.01~0.08

Table 3. Analytical conditions of UPLC-ESI-MS/MS for analysis of 13 quinolones

Items		Conditions		
LC	Instrument	Acquity ultra performance LC		
	Column	Acuity UPLC BEH C <sub>18</sub> (2.1×10 mm, 1.7 μm)		
	Mobile phase	A: 0.1% formic acid in DW		
		B: Acetonitrile		
		Time (min)	A (%)	B (%)
		Initial	90	10
		5.0	30	70
	Gradient	5.5	0	100
		6.5	0	100
		6.6	90	10
8.0		90	10	
	Flow rate	0.3 mL/min		
	Injection volume	20 μL		
MS	Instrument	Quattro Premier XE		
	Ionization mode	ES+		
	Capillary voltage	3.0 kV		
	Source temperature	150 °C		
	Desolvation temperature	350 °C		

Table 4. MRM conditions of the MS/MS for the detection of 13 quinolones

Compound	MRM transition (m/z) (Precursor ion > product ion)	Cone voltage (V)	Collision energy (eV)
Ciprofloxacin	332 > 245	35	26
	332 > 288		18
Danofloxacin	358 > 283	30	22
	358 > 314		18
Difloxacin	400 > 299	35	32
	400 > 356		20
Enrofloxacin	360 > 316	28	20
	360 > 342		22
Flumequine	262 > 202	25	34
	262 > 244		16
Marbofloxacin	363 > 72	30	20
	363 > 320		15
Nalidixic acid	233 > 187	25	26
	233 > 215		14
Norfloxacin	320 > 233	35	24
	320 > 276		18
Ofloxacin	362 > 261	35	28
	362 > 318		20
Orbifloxacin	396 > 295	35	24
	396 > 352		20
Oxolinic acid	262 > 216	25	28
	262 > 244		20
Pefloxacin	334 > 233	35	26
	334 > 290		18
Piromidic acid (IS)	289 > 243	25	30
	289 > 271		20
Sarafloxacin	386 > 299	40	26
	386 > 342		20

Table 5. Accuracy and precision ranges as requirements in CODEX guidelines

Concentration ( $\mu\text{g}/\text{kg}$ )	Coefficient of variability (CV) (Repeatability, %)	Acceptable recovery (%)
$\leq 1$	35	50 - 120
1 to 10	30	60 - 120
10 to 100	20	70 - 120
100 to 1000	15	70 - 110
$\geq 1000$	10	70 - 110

Table 6. Validation parameters for detection of 13 quinolones using UPLC-MS/MS

Compound	Calibration equation	Determination coefficient ( $r^2$ )	LOD ( $\mu\text{g/kg}$ )	LOQ ( $\mu\text{g/kg}$ )
Ciprofloxacin	$y = 0.137x + 0.004$	0.9998	0.1	0.4
Danofloxacin	$y = 0.031x + 0.001$	0.9998	0.1	0.4
Difloxacin	$y = 0.105x + 0.007$	0.9996	0.07	0.2
Enrofloxacin	$y = 0.106x + 0.002$	0.9999	0.06	0.2
Flumequine	$y = 0.322x + 0.077$	0.9992	0.06	0.2
Marbofloxacin	$y = 0.122x + 0.035$	0.9998	0.1	0.4
Nalidixic acid	$y = 0.284x + 0.003$	0.9999	0.06	0.2
Norfloxacin	$y = 0.042x + 0.009$	0.9999	0.1	0.4
Ofloxacin	$y = 0.081x + 0.004$	0.9993	0.07	0.2
Orbifloxacin	$y = 0.218x + 0.050$	0.9995	0.1	0.4
Oxolinic acid	$y = 0.061x + 0.009$	0.9998	0.1	0.4
Pefloxacin	$y = 0.080x + 0.123$	0.9992	0.1	0.4
Sarafloxacin	$y = 0.042x + 0.029$	0.9996	0.07	0.2

Table 7. Intra- and inter-day precision of 13 quinolones at 3 different concentration levels.

Compound	Spiked conc. (µg/kg)	Intra-day precision (n = 6)		Inter-day precision (n = 3)	
		RSD(%) <sup>1)</sup>		RSD (%) <sup>1)</sup>	
		beef	flatfish	beef	flatfish
Ciprofloxacin	1.0	1.06	0.96	3.35	3.75
	4.0	3.36	0.57	2.90	2.91
	10.0	1.19	2.20	3.68	1.74
Danofloxacin	1.0	1.63	1.08	2.07	2.02
	4.0	1.08	3.33	3.18	2.22
	10.0	0.62	1.41	1.45	2.14
Difloxacin	1.0	0.33	0.69	2.12	2.76
	4.0	0.84	0.90	5.25	2.20
	10.0	1.30	1.63	1.12	3.95
Enrofloxacin	1.0	0.52	0.57	1.44	1.51
	4.0	2.00	0.75	2.41	0.95
	10.0	1.69	1.16	1.75	1.29
Flumequine	1.0	1.41	0.49	1.77	2.94
	4.0	0.45	2.23	3.85	2.00
	10.0	1.07	1.59	1.91	2.53
Marbofloxacin	1.0	1.68	2.47	3.97	6.75
	4.0	1.89	1.33	2.20	3.13
	10.0	3.48	0.90	0.68	1.14
Nalidixic acid	1.0	0.79	0.43	2.62	1.01
	4.0	1.64	3.30	4.71	0.80
	10.0	2.35	0.62	1.78	1.94
Norfloxacin	1.0	0.85	0.93	1.38	0.98
	4.0	1.60	0.91	4.13	4.12
	10.0	1.69	1.97	2.28	2.18
Ofloxacin	1.0	1.00	1.44	0.92	1.92
	4.0	3.49	1.22	3.75	1.67
	10.0	1.26	1.16	1.55	1.67
Orbifloxacin	1.0	1.46	0.65	2.80	3.05
	4.0	1.87	2.13	1.84	1.99
	10.0	3.72	1.54	0.96	1.02
Oxolinic acid	1.0	1.78	2.12	2.22	1.10
	4.0	0.49	0.76	4.86	4.54
	10.0	1.12	0.93	2.01	0.98
Pefloxacin	1.0	0.65	0.47	4.60	0.71
	4.0	1.46	0.69	4.27	3.31
	10.0	1.98	1.16	1.00	1.28
Sarafloxacin	1.0	0.52	0.58	2.15	1.06
	4.0	1.18	1.20	3.54	3.80
	10.0	2.48	0.95	3.02	1.43

<sup>1)</sup> Relatively standard deviation

Table 8. Average recoveries and RSD (%) measured in livestock products

Compound	Spiked conc. ( $\mu\text{g}/\text{kg}$ )	Beef		Pork		Chicken		Milk		Egg	
		M <sup>1)</sup>	RSD (%)								
CIP	0.4	94.0 $\pm$ 3.7	3.9	93.8 $\pm$ 3.2	3.5	97.0 $\pm$ 2.4	2.5	96.9 $\pm$ 2.6	2.7	100.4 $\pm$ 2.2	2.2
	0.8	94.2 $\pm$ 3.7	3.8	96.7 $\pm$ 3.8	3.9	97.2 $\pm$ 4.1	4.2	96.9 $\pm$ 2.5	2.6	101.5 $\pm$ 2.9	2.9
	10.0	99.1 $\pm$ 2.8	2.9	97.0 $\pm$ 2.8	2.9	100.2 $\pm$ 1.6	1.6	98.2 $\pm$ 2.7	2.7	100.6 $\pm$ 1.8	1.8
DAN	0.4	98.8 $\pm$ 5.6	5.7	95.8 $\pm$ 4.7	4.9	94.6 $\pm$ 2.2	2.4	97.0 $\pm$ 2.4	2.5	98.3 $\pm$ 3.4	3.5
	0.8	96.0 $\pm$ 2.5	2.6	96.8 $\pm$ 3.7	3.8	93.8 $\pm$ 4.7	5.0	97.0 $\pm$ 3.3	3.4	96.9 $\pm$ 3.1	3.2
	10.0	97.5 $\pm$ 4.3	4.4	91.3 $\pm$ 3.1	3.3	94.2 $\pm$ 2.3	2.5	98.2 $\pm$ 2.1	2.1	100.4 $\pm$ 1.2	1.2
DIF	0.2	95.6 $\pm$ 2.2	2.3	96.1 $\pm$ 3.1	3.2	99.4 $\pm$ 2.0	2.0	96.3 $\pm$ 3.5	3.6	97.8 $\pm$ 2.1	2.1
	0.4	93.3 $\pm$ 2.2	2.4	96.3 $\pm$ 4.8	5.0	97.6 $\pm$ 4.1	4.2	98.2 $\pm$ 2.1	2.2	95.5 $\pm$ 4.1	4.3
	10.0	96.0 $\pm$ 4.6	4.8	97.1 $\pm$ 2.3	2.4	99.5 $\pm$ 1.6	1.6	100.0 $\pm$ 0.5	0.5	102.6 $\pm$ 1.6	1.5
ENR	0.2	91.0 $\pm$ 4.5	5.0	89.0 $\pm$ 3.7	4.2	88.4 $\pm$ 2.9	3.2	101.0 $\pm$ 3.1	3.1	89.2 $\pm$ 5.2	5.9
	0.4	90.0 $\pm$ 3.4	3.8	94.1 $\pm$ 5.6	6.0	95.3 $\pm$ 2.5	2.6	98.6 $\pm$ 3.4	3.4	93.1 $\pm$ 5.0	5.4
	10.0	99.1 $\pm$ 3.1	3.1	97.8 $\pm$ 3.8	3.9	98.0 $\pm$ 2.2	2.3	97.6 $\pm$ 1.0	1.1	100.9 $\pm$ 2.7	2.6
FLU	0.2	100.2 $\pm$ 2.8	2.8	94.4 $\pm$ 3.4	3.6	96.2 $\pm$ 3.1	3.2	96.3 $\pm$ 3.6	3.7	93.0 $\pm$ 2.0	2.2
	0.4	95.6 $\pm$ 3.1	3.3	94.6 $\pm$ 2.3	2.4	98.1 $\pm$ 2.3	2.3	98.1 $\pm$ 2.4	2.5	96.6 $\pm$ 1.8	1.9
	10.0	99.1 $\pm$ 2.3	2.3	100.0 $\pm$ 2.7	2.8	99.0 $\pm$ 1.6	1.6	100.0 $\pm$ 0.8	0.8	99.1 $\pm$ 2.7	2.8
MAR	0.4	95.4 $\pm$ 2.2	2.3	104.3 $\pm$ 5.6	5.4	94.7 $\pm$ 3.5	3.7	98.8 $\pm$ 1.9	1.9	99.3 $\pm$ 2.2	2.2
	0.8	96.6 $\pm$ 3.1	3.2	100.1 $\pm$ 3.3	3.3	90.9 $\pm$ 4.3	4.8	98.7 $\pm$ 1.6	1.6	93.7 $\pm$ 1.6	1.7
	10.0	93.8 $\pm$ 2.3	2.2	100.0 $\pm$ 3.7	3.7	93.5 $\pm$ 2.4	2.5	96.9 $\pm$ 1.1	1.2	99.3 $\pm$ 2.4	2.5

<sup>1)</sup> Mean recoveries (% , n = 6)  $\pm$  Standard deviation

Table 8. (Continued)

Com- pound	Spiked conc. ( $\mu\text{g}/\text{kg}$ )	Beef		Pork		Chicken		Milk		Egg	
		M <sup>1)</sup>	RSD (%)								
NAL	0.2	95.3 $\pm$ 2.0	2.0	100.0 $\pm$ 3.5	3.5	97.3 $\pm$ 1.8	1.9	96.4 $\pm$ 3.4	3.6	96.7 $\pm$ 1.2	1.2
	0.4	96.5 $\pm$ 1.5	1.6	97.4 $\pm$ 3.0	3.1	95.3 $\pm$ 4.6	4.8	98.2 $\pm$ 1.0	1.1	96.3 $\pm$ 3.3	3.5
	10.0	98.0 $\pm$ 1.8	1.9	97.5 $\pm$ 2.7	2.8	97.6 $\pm$ 3.0	3.1	99.2 $\pm$ 3.9	4.0	98.9 $\pm$ 0.4	0.4
NOR	0.4	94.4 $\pm$ 4.6	4.9	93.6 $\pm$ 3.9	4.1	97.1 $\pm$ 4.4	4.9	100.9 $\pm$ 1.9	1.9	95.1 $\pm$ 4.7	5.0
	0.8	96.1 $\pm$ 2.8	2.9	97.3 $\pm$ 4.3	4.4	94.9 $\pm$ 4.0	4.2	96.5 $\pm$ 4.7	4.9	95.2 $\pm$ 2.5	2.7
	10.0	96.2 $\pm$ 4.1	4.3	101.7 $\pm$ 5.1	5.0	98.2 $\pm$ 1.8	1.9	96.9 $\pm$ 3.8	3.9	101.5 $\pm$ 1.7	1.6
OFL	0.2	94.0 $\pm$ 4.4	4.7	102.8 $\pm$ 4.1	4.0	87.5 $\pm$ 5.0	5.8	95.3 $\pm$ 5.7	6.0	92.8 $\pm$ 3.1	3.4
	0.4	90.0 $\pm$ 3.5	3.9	94.6 $\pm$ 4.6	4.9	87.8 $\pm$ 4.7	5.3	91.4 $\pm$ 0.9	1.0	98.8 $\pm$ 4.7	4.8
	10.0	96.1 $\pm$ 3.2	3.3	103.9 $\pm$ 3.1	3.0	96.5 $\pm$ 1.4	1.5	97.7 $\pm$ 2.4	2.4	98.7 $\pm$ 2.6	2.6
ORB	0.4	94.2 $\pm$ 3.0	3.2	93.2 $\pm$ 2.6	2.8	93.1 $\pm$ 2.1	2.2	92.4 $\pm$ 1.5	1.6	92.0 $\pm$ 2.4	2.6
	0.8	93.4 $\pm$ 2.0	2.1	92.9 $\pm$ 2.4	2.6	95.0 $\pm$ 0.7	0.7	92.9 $\pm$ 2.9	3.2	92.5 $\pm$ 2.2	2.4
	10.0	96.8 $\pm$ 2.1	2.2	94.1 $\pm$ 2.9	3.1	93.6 $\pm$ 2.0	2.2	93.8 $\pm$ 1.5	1.6	94.8 $\pm$ 2.7	2.9
OXO	0.4	96.5 $\pm$ 5.4	5.6	92.5 $\pm$ 4.3	4.6	90.8 $\pm$ 3.6	4.0	98.8 $\pm$ 4.1	4.2	97.5 $\pm$ 4.9	5.0
	0.8	94.9 $\pm$ 2.3	2.4	94.7 $\pm$ 3.7	3.9	88.9 $\pm$ 3.2	3.6	97.8 $\pm$ 0.6	0.6	98.9 $\pm$ 4.2	4.3
	10.0	99.2 $\pm$ 1.5	1.6	97.5 $\pm$ 2.4	2.5	97.0 $\pm$ 3.0	3.1	99.4 $\pm$ 3.0	3.0	103.2 $\pm$ 0.8	0.7
PEF	0.4	97.3 $\pm$ 1.7	1.7	96.9 $\pm$ 4.6	4.7	88.7 $\pm$ 4.3	4.9	90.8 $\pm$ 3.9	4.3	94.7 $\pm$ 3.0	3.2
	0.8	92.8 $\pm$ 4.0	4.3	95.4 $\pm$ 3.2	3.4	96.0 $\pm$ 1.9	2.0	93.4 $\pm$ 5.3	5.7	97.4 $\pm$ 3.5	3.6
	10.0	91.0 $\pm$ 2.3	2.6	99.4 $\pm$ 2.5	2.5	97.2 $\pm$ 5.6	2.7	93.5 $\pm$ 5.5	5.9	103.3 $\pm$ 1.3	1.3
SAR	0.2	95.9 $\pm$ 5.7	5.9	104.7 $\pm$ 3.7	3.6	102.2 $\pm$ 3.4	3.3	98.2 $\pm$ 4.8	4.9	91.4 $\pm$ 4.9	5.2
	0.4	94.5 $\pm$ 5.5	5.8	96.1 $\pm$ 4.1	4.3	99.5 $\pm$ 3.6	3.7	99.3 $\pm$ 4.4	4.5	96.2 $\pm$ 5.3	5.5
	10.0	95.8 $\pm$ 5.3	5.6	93.1 $\pm$ 4.2	4.5	96.3 $\pm$ 2.7	2.8	96.9 $\pm$ 2.1	2.2	101.6 $\pm$ 3.4	3.3

<sup>1)</sup> Mean recoveries (%; n = 6)  $\pm$  Standard deviation

Table 9. Average recoveries and RSD (%) measured in marine products

Com- pound	Spiked conc. (µg/kg)	Flatfish		Common eel		Jacopever		Shrimp	
		M <sup>1)</sup>	R <sup>2)</sup>						
CIP	0.4	89.9±3.7	4.0	98.9±0.8	0.8	89.6±3.8	4.3	92.4±1.6	1.8
	0.8	93.4±4.5	4.8	97.8±1.3	1.3	92.1±2.3	2.5	95.7±1.0	1.1
	10.0	95.0±2.6	2.7	95.9±3.4	3.5	98.2±2.1	2.1	97.2±2.2	2.3
DAN	0.4	88.9±1.7	1.9	93.7±3.2	3.4	95.0±1.7	1.8	93.0±1.9	2.0
	0.8	92.8±1.2	1.3	95.0±1.6	1.7	92.9±2.8	3.1	92.7±3.0	3.2
	10.0	97.7±3.8	3.9	97.4±2.7	2.8	96.6±2.0	2.0	96.0±3.3	3.5
DIF	0.2	90.4±4.5	4.9	97.3±3.9	4.0	90.9±3.8	4.2	95.0±2.5	2.6
	0.4	92.7±4.4	4.8	97.5±3.3	3.3	89.2±3.5	3.9	96.5±3.0	3.1
	10.0	92.7±1.3	1.4	95.2±2.4	2.7	88.3±1.1	1.3	96.9±3.6	3.7
ENR	0.2	88.5±4.3	4.8	94.7±3.2	3.3	90.6±4.1	4.5	96.9±3.4	3.5
	0.4	90.3±4.9	5.5	95.1±3.0	3.2	94.8±4.4	4.6	97.3±2.6	2.7
	10.0	91.1±1.2	1.3	96.2±2.5	2.6	94.9±1.4	1.5	97.7±2.9	3.0
FLU	0.2	86.0±2.7	3.1	94.8±2.7	2.9	97.3±2.9	3.0	95.8±2.3	2.4
	0.4	93.5±3.8	4.1	95.2±3.2	3.4	93.4±3.4	3.6	96.5±2.5	2.6
	10.0	96.9±1.8	1.9	96.7±2.3	2.4	98.7±1.8	1.8	98.8±2.5	2.5
MAR	0.4	98.4±3.2	3.3	97.1±1.7	1.7	87.7±3.0	3.4	96.0±2.6	2.7
	0.8	93.1±3.7	3.9	97.1±1.5	1.6	93.7±2.1	2.2	96.1±3.5	3.7
	10.0	96.8±2.2	2.3	97.1±3.0	3.1	91.7±2.7	3.0	96.1±2.6	2.7
NAL	0.2	87.6±2.2	2.6	93.5±2.2	2.3	88.3±3.3	3.7	93.3±1.2	1.3
	0.4	90.9±4.3	4.7	94.1±1.4	1.5	88.8±2.4	2.7	95.8±2.4	2.5
	10.0	94.6±1.6	1.7	95.1±2.9	3.1	92.6±3.0	3.2	96.8±2.7	2.8
NOR	0.4	89.9±2.9	3.2	95.8±1.8	1.9	89.2±2.7	3.0	93.7±3.3	3.5
	0.8	83.0±2.8	3.0	96.4±2.5	2.6	94.6±2.7	2.9	97.4±3.5	3.6
	10.0	95.3±2.4	2.5	95.6±0.9	0.9	96.5±1.1	1.1	97.4±2.7	2.7
OFL	0.2	87.3±3.9	4.5	94.2±1.5	1.6	88.7±5.0	5.6	97.8±2.4	2.4
	0.4	90.9±5.2	5.7	95.1±1.4	1.5	88.7±3.2	3.6	96.9±2.5	2.6
	10.0	94.9±3.6	3.8	97.0±2.8	2.9	92.2±1.8	2.0	97.2±3.0	3.1
ORB	0.4	87.7±2.4	2.8	93.5±3.1	3.3	89.6±4.9	5.4	93.2±1.8	1.9
	0.8	88.1±3.2	3.7	94.2±3.0	3.2	93.2±2.3	2.5	93.2±1.7	1.8
	10.0	94.3±2.0	2.1	94.2±1.3	1.4	94.0±2.1	2.2	95.1±2.1	2.2
OXO	0.4	90.1±4.2	4.7	96.2±2.0	2.1	94.1±1.7	1.8	97.0±2.8	2.9
	0.8	93.6±3.4	3.6	93.6±1.7	1.8	93.7±2.8	3.0	96.3±2.5	2.6
	10.0	97.1±2.2	2.3	94.2±1.8	1.9	100.9±1.1	1.0	96.8±2.2	2.2
PEF	0.4	91.3±3.8	4.1	98.3±2.3	2.3	92.9±3.8	4.1	93.4±0.8	0.9
	0.8	93.3±4.4	4.7	97.7±0.9	0.9	93.4±1.8	2.0	97.3±2.4	2.4
	10.0	95.7±1.8	1.9	95.8±1.9	1.9	92.1±1.4	1.5	97.9±2.6	2.7
SAR	0.2	87.9±4.3	4.8	95.9±2.9	3.1	88.6±4.2	4.7	94.5±2.1	2.2
	0.4	92.6±4.9	5.3	96.1±3.5	3.7	89.2±3.2	3.6	96.2±3.8	4.0
	10.0	93.9±2.2	2.3	95.4±2.1	2.2	88.2±3.9	4.4	98.3±1.5	1.5

<sup>1)</sup> Mean recoveries (% , n = 6) ± Standard deviation

<sup>2)</sup> Relative standard deviation (%)

Table 10. Comparison of the solvent extraction effects on the recoveries (%) from beef and flatfish fortified with 4 µg/kg of 13 quinolones.

Sample	TCA <sup>1)</sup>	Acetonitrile					1% acetic acid in acetonitrile				
		Min. (%)	Max. (%)	Aver. <sup>2)</sup> (%)	SD <sup>3)</sup>	RSD <sup>4)</sup>	Min. (%)	Max. (%)	Aver. <sup>2)</sup> (%)	SD <sup>3)</sup>	RSD <sup>4)</sup>
Beef	1%	77.00	105.30	90.21	7.05	7.82	96.94	109.90	104.60	3.59	3.43
	2%	85.8	111.80	98.58	8.66	8.78	98.85	117.20	104.20	4.63	4.44
	2.5%	89.21	115.10	101.02	6.92	6.85	91.93	102.20	96.10	3.20	3.33
	3%	69.73	112.30	88.62	12.58	14.20	103.70	116.80	110.25	3.72	3.38
	4%	66.72	113.00	94.27	14.35	15.22	96.35	112.70	103.35	4.12	3.99
	5%	87.14	124.80	107.35	8.68	8.09	92.75	121.20	102.76	7.00	6.81
Flatfish	1%	86.08	117.10	100.98	8.26	8.18	91.02	159.6	106.62	17.33	16.25
	2%	92.18	113.70	102.38	7.08	6.92	93.56	124.90	102.43	8.42	8.23
	2.5%	92.28	113.80	103.66	5.95	5.74	94.06	110.80	100.51	4.80	4.78
	3%	113.10	139.40	127.03	8.33	6.56	89.04	114.00	100.96	6.98	6.91
	4%	95.54	131.40	108.34	8.62	7.96	91.03	116.30	99.45	6.93	6.97
	5%	90.23	117.40	105.61	7.57	7.17	98.98	112.40	106.37	5.30	4.98

<sup>1)</sup> Trichloroacetic acid

<sup>2)</sup> Average of recovery rate (%)

<sup>3)</sup> Standard deviation

<sup>4)</sup> Relative standard deviation (%)

Table 11. Number of samples according to the sampling area

	Seoul	Busan	Incheon	Daegu	Gwangju	Daejeon	Ulsan	Total
Beef	21	8	5	7	6	6	6	59
Pork	17	6	7	5	4	5	5	49
Chicken	13	5	5	3	3	2	3	34
Milk	7	6	4	3	3	2	4	29
Egg	5	5	4	2	3	4	4	27
Flatfish	5	5	4	2	3	3	4	26
Common eel	6	5	3	3	4	3	3	27
Jacopever	6	5	4	3	4	4	3	29
Shrimp	7	5	4	4	3	4	3	30
Total	87	50	40	32	33	33	35	(n=310)

Table 12. Incidence and range of 13 quinolones levels in 310 livestock and marine products

Sample category	Number of analyzed samples	Number of detected samples	Below LOQ <sup>1)</sup>	Range of quinolone level (µg/kg)	Incidence of quinolone (%)
Beef	59	7	8	0.2~20.0	11.9
Pork	49	7	5	0.6~4.1	14.3
Chicken	34	22	3	0.1~47.0	64.7
Milk	29	2	8	0.7~0.9	6.9
Egg	27	1	4	0.3~0.5	3.7
Flatfish	26	12	1	0.3~21.1	46.2
Common eel	27	16	5	0.1~100.0	59.3
Jacopever	29	27	1	0.3~22.8	93.1
Shrimp	30	10	11	0.2~5.1	33.3

<sup>1)</sup> LOQ: Limit of quantification

Table 13. The kinds of the detected quinolones in livestock and marine products

Number of detected samples	Beef	Pork	Chicken	Milk	Egg	Flatfish	Common eel	Jacopever	Shrimp
Difloxacin	-	-	-	-	1	-	-	-	5
Enrofloxacin	-	-	8	-	-	2	-	7	1
Ciprofloxacin	-	-	-	-	-	1	-	11	-
Enrofloxacin+ciprofloxacin	-	-	11	-	-	-	-	7	-
Flumequine	-	2	6	-	1	4	3	8	-
Marbofloxacin	-	-	-	1	-	2	-	-	-
Nalidixic acid	-	-	-	-	-	-	-	1	-
Orbifloxacin	4	-	-	1	-	1	-	1	-
Oxolinic acid	1	4	1	-	-	7	14	11	7
Pefloxacin	-	-	-	-	-	-	1 <sup>1)</sup>	-	-
Sarafloxacin	2	2	1	-	-	1	2	1	1

<sup>1)</sup> Above the legal residue limit

Table 14. Assessment of dietary intake and risk value of detected antibiotics

Compound	Food	Frequency <sup>2)</sup>	Detected Conc. (mg/kg)	Food intake <sup>3)</sup> (kg/person/day)	EDI <sup>4)</sup> (mg/person/day)	EDI/ADI <sup>5)</sup> (%)
Sarafloxacin ※ ADI <sup>1)</sup> =0.0004	Beef	2/59	0.0002-0.0007	30.36	0.00000002	0.004
	Pork	2/49	0.007-0.0041	56.19	0.0000002	0.050
	Chicken	1/34	0.0014	78.44	0.00000006	0.014
	Flatfish	1/26	0.0003	58.93	0.00000001	0.003
	Common eel	2/27	0.0007-0.0036	54.30	0.00000013	0.003
	Jacopever	1/29	0.0228	36.23	0.00000052	0.129
	Shrimp	1/30	0.0006	18.45	0.00000001	0.002
					∑EDI=0.00000077	∑EDI/ADI=0.205
Marbofloxacin ※ ADI=0.0045	Milk	1/29	0.0007	217.61	0.0000001	0.002
	Flatfish	2/26	0.0011-0.0015	58.93	0.0000002	0.005
	Shrimp	2/30	0.0003-0.0004	18.45	0.00000002	0.0003
					∑EDI=0.00000033	∑EDI/ADI=0.0073

<sup>1)</sup> ADI is expressed in terms of mg/kg weight/day by a person of 55 kg

<sup>2)</sup> Number of detected sample/total sample

<sup>3)</sup> From Korean National Health and Nutrition Examination Survey

<sup>4)</sup> EDI (mg/person/day) = Food intake (kg/person/day) × detected concentration in food (mg/kg)

<sup>5)</sup> Risk value (%)

Table 14. (Continued)

Compound	Food	Frequency <sup>2)</sup>	Detected Conc. (mg/kg)	Food intake <sup>3)</sup> (kg/person/day)	EDI <sup>4)</sup> (mg/person/day)	EDI/ADI <sup>5)</sup> (%)
Oxolinic acid ※ ADI <sup>1)</sup> =0.0004	Beef	1/59	0.002	30.36	0.00000002	0.00008
	Pork	3/49	0.001-0.003	56.19	0.00000004	0.0002
	Chicken	1/34	0.0009	78.44	0.00000004	0.0002
	Flatfish	7/26	0.0008-0.021	58.93	0.0000092	0.036
	Common eel	14/27	0.0001-0.1	54.30	0.000138	0.553
	Jacopever	10/29	0.0003-0.005	36.23	0.0000045	0.018
	Shrimp	7/30	0.001-0.005	18.45	0.00000102	0.004
					ΣEDI=0.00015	ΣEDI/ADI=0.612
Flumequine ※ ADI=0.00825	Pork	2/49	0.0004-0.0006	56.19	0.000000042	0.0005
	Egg	1/27	0.0005	28.90	0.000000010	0.0001
	Chicken	6/34	0.00003-0.0005	78.44	0.000000242	0.003
	Flatfish	3/26	0.0006-0.0035	58.93	0.000000618	0.007
	Jacopever	7/29	0.0002-0.0037	36.23	0.000000986	0.012
	Common eel	3/27	0.0004-0.001	54.30	0.000000362	0.004
					ΣEDI=0.0413	ΣEDI=0.027

Table 14. (Continued)

Compound	Food	Frequency <sup>2)</sup>	Detected Conc. (mg/kg)	Food intake <sup>3)</sup> (kg/person/day)	EDI <sup>4)</sup> (mg/person/day)	EDI/ADI <sup>5)</sup> (%)
Enrofloxacin ※ADI <sup>1)</sup> =0.0062	Chicken	8/34	0.0004-0.0037	78.44	0.000004	0.064
	Flatfish	2/26	0.0012	58.93	0.0000002	0.003
	Jacopever	7/29	0.0006-0.0037	36.23	0.0000014	0.022
	Shrimp	1/30	0.0004	18.45	0.000000004	0.0001
					∑EDI=0.000006	∑EDI/ADI=0.0895
Difloxacin ※ADI=0.001	Egg	1/27	0.0003	28.90	0.000000006	0.0006
	Shrimp	3/30	0.0002-0.0006	18.45	0.000000037	0.0037
					∑EDI=0.000000043	∑EDI=0.0043

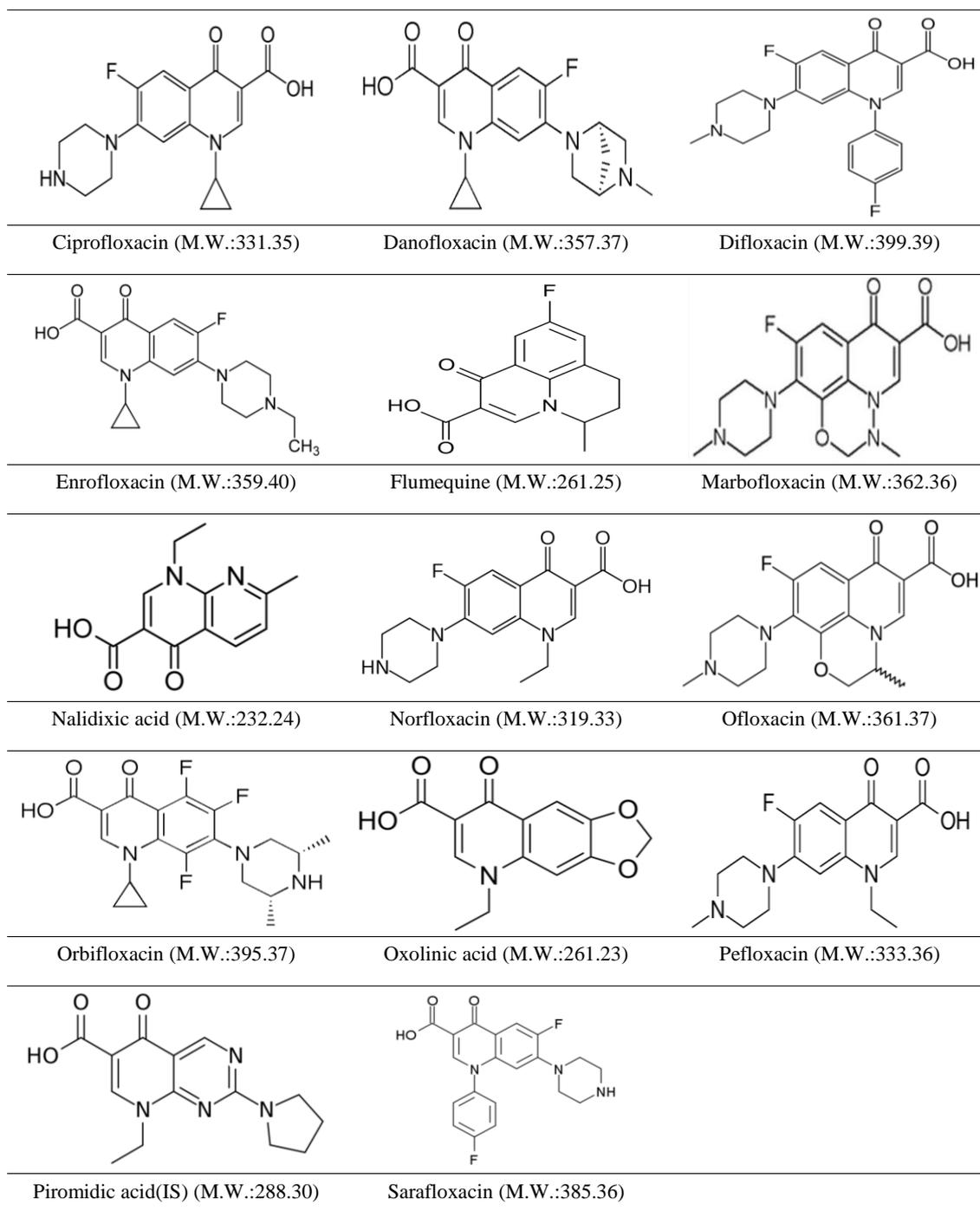
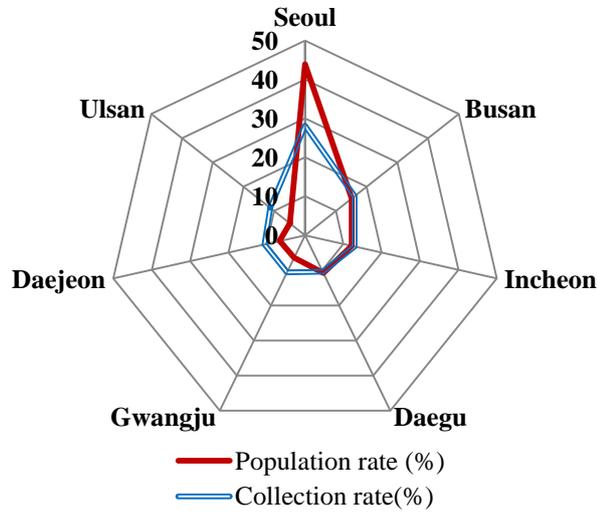


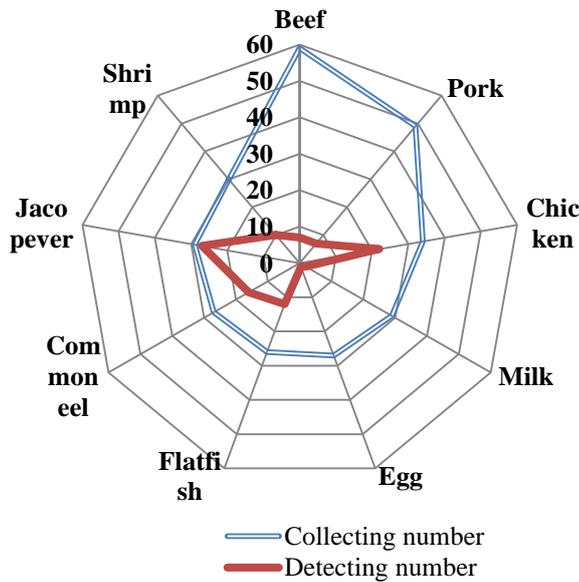
Fig. 1. Molecular structure of 13 quinolones and piromidic acid (IS).

(A)



City	Population (unit:1000)	Collecting rate (%)
Seoul	10,206	43.9
Busan	3,359	15.2
Incheon	2,840	12.2
Daegu	2,508	10.8
Gwangju	1,471	6.4
Daejeon	1,525	6.6
Ulsan	1,147	4.9
Total	23,236	100

(B)



Species	Collecting number	Detecting number
Beef	59	7
Pork	49	7
Milk	34	22
Chicken	29	2
Egg	27	1
Flatfish	26	12
Common eel	27	16
Jacopever	29	27
Shrimp	30	10
Total	310	104

Fig. 2. The sampling number by area according to the 「Sampling with probability proportionate to size」 (A) and distribution of samples containing quinolones residues by region (B).

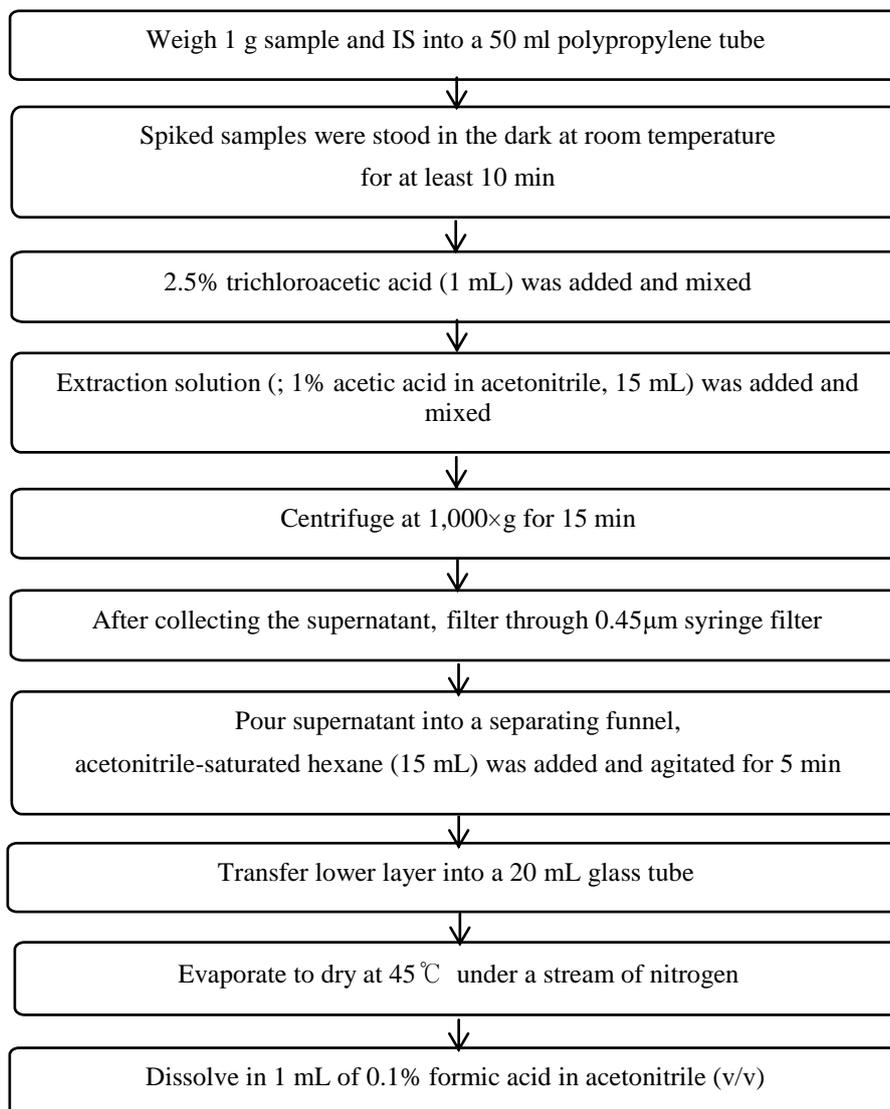


Fig. 3. Schematic diagram of the extraction procedure for the determination of quinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, ofloxacin, orbifloxacin, oxolinic acid, pefloxacin, and sarafloxacin) in livestock and marine products.

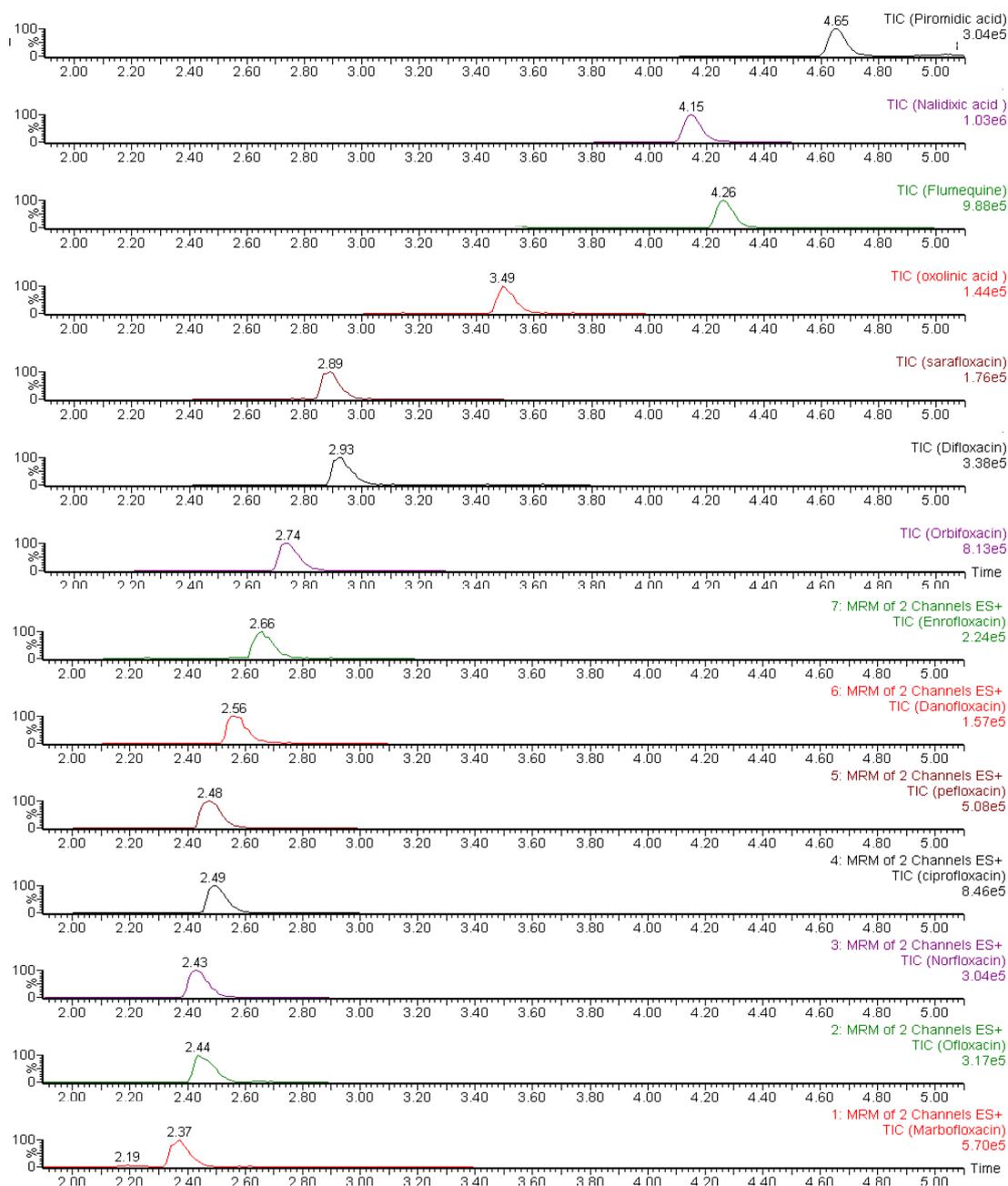


Fig. 4. UPLC-ESI-MS/MS chromatograms of a blank bovine muscle sample spiked at 10  $\mu\text{g}/\text{kg}$  for DIF, ENR, FLU, NAL, OFL, and SAR, 20  $\mu\text{g}/\text{kg}$  for CIP, DAN, MAR, NOR, ORB, OA, and PEF, and 4  $\mu\text{g}/\text{kg}$  for PIR (IS).

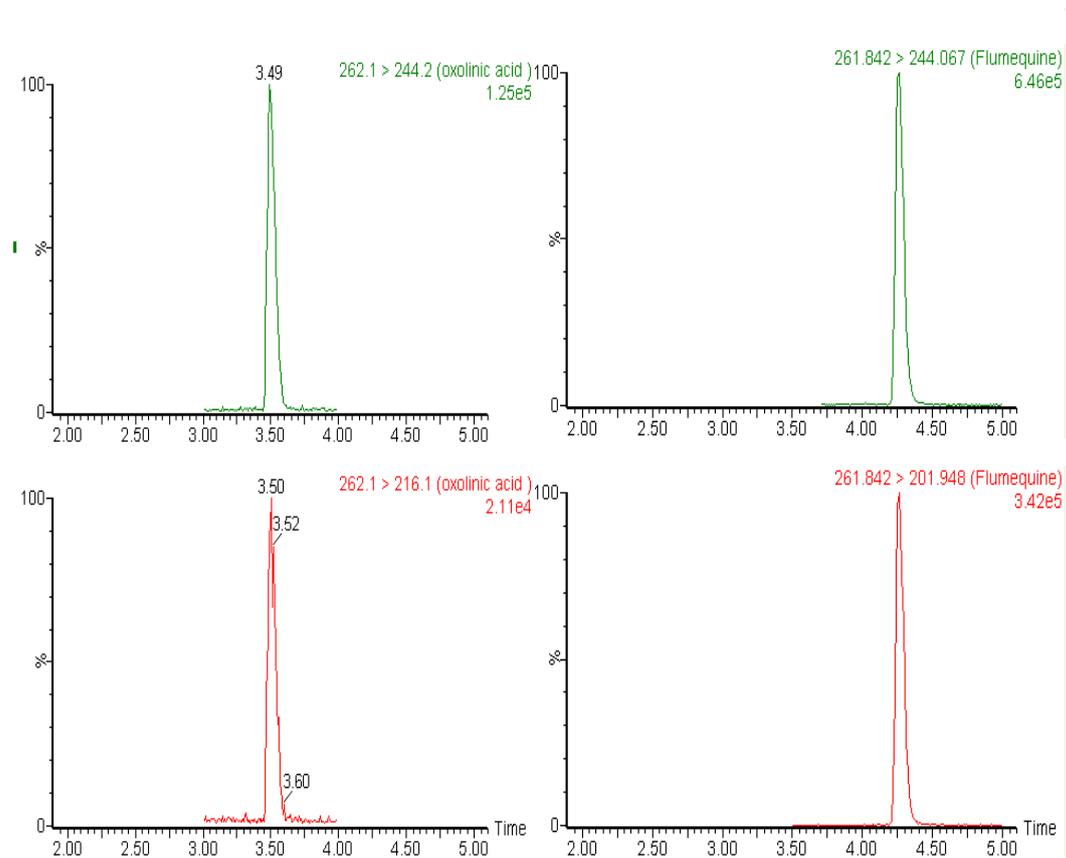
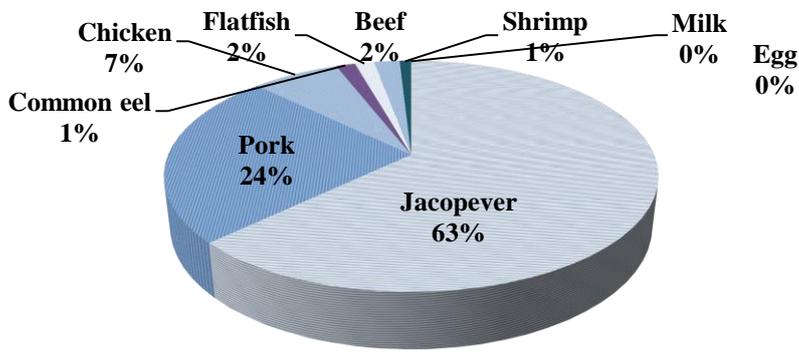
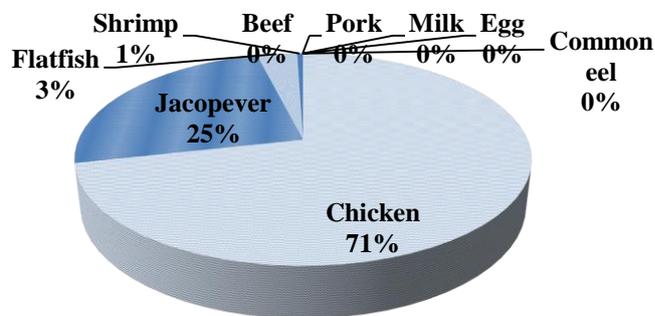


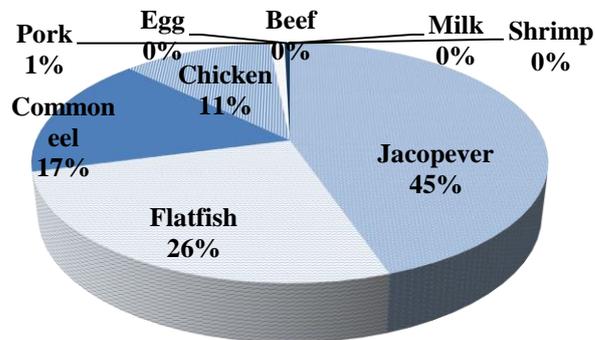
Fig. 5. Comparison of ion transition between flumequine and oxolinic acid. Chromatograms, obtained from the bovine muscle fortified with 10  $\mu\text{g}/\text{kg}$  of flumequine and 20  $\mu\text{g}/\text{kg}$  of oxolinic acid, were described. The transition  $[\text{M} + \text{H}]^+ \rightarrow [\text{M} + \text{H}-\text{OC}_3\text{H}_8]^+$  ( $m/z$  262  $\rightarrow$  202) was used for flumequine and  $[\text{M} + \text{H}]^+ \rightarrow [\text{M} + \text{H}-\text{OCH}_2\text{O}]^+$  ( $m/z$  262  $\rightarrow$  216) was used for oxolinic acid, respectively.



(A) Sarafloxacin

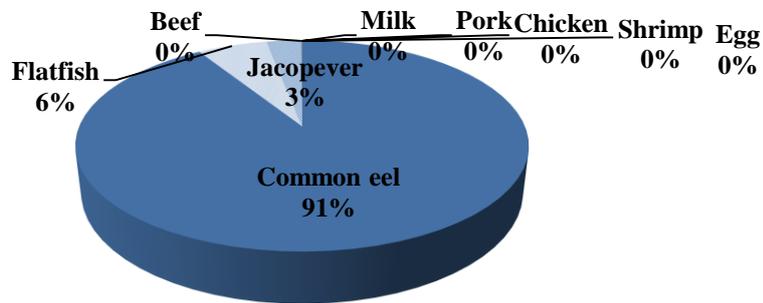


(B) Enrofloxacin

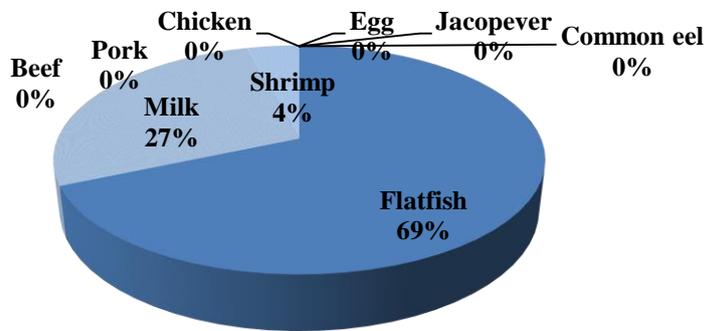


(C) Flumequine

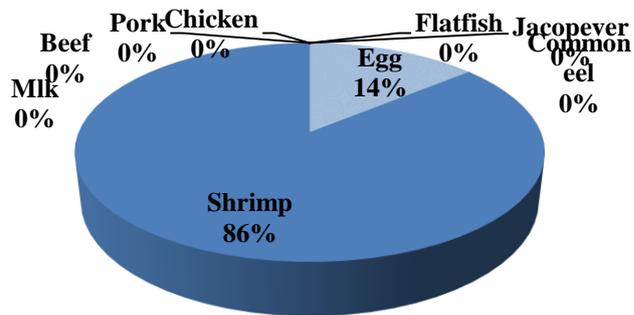
Fig. 6. The food contribution for estimated daily intake from quinolones residues. (A) Sarafloxacin, (B) Enrofloxacin, (C) Flumequine, (D) Oxolinic acid, (E) Marbofloxacin, (F) Difloxacin



(D) Oxolinic acid



(E) Marbofloxacin



(F) Difloxacin

Fig. 6. (Continued)

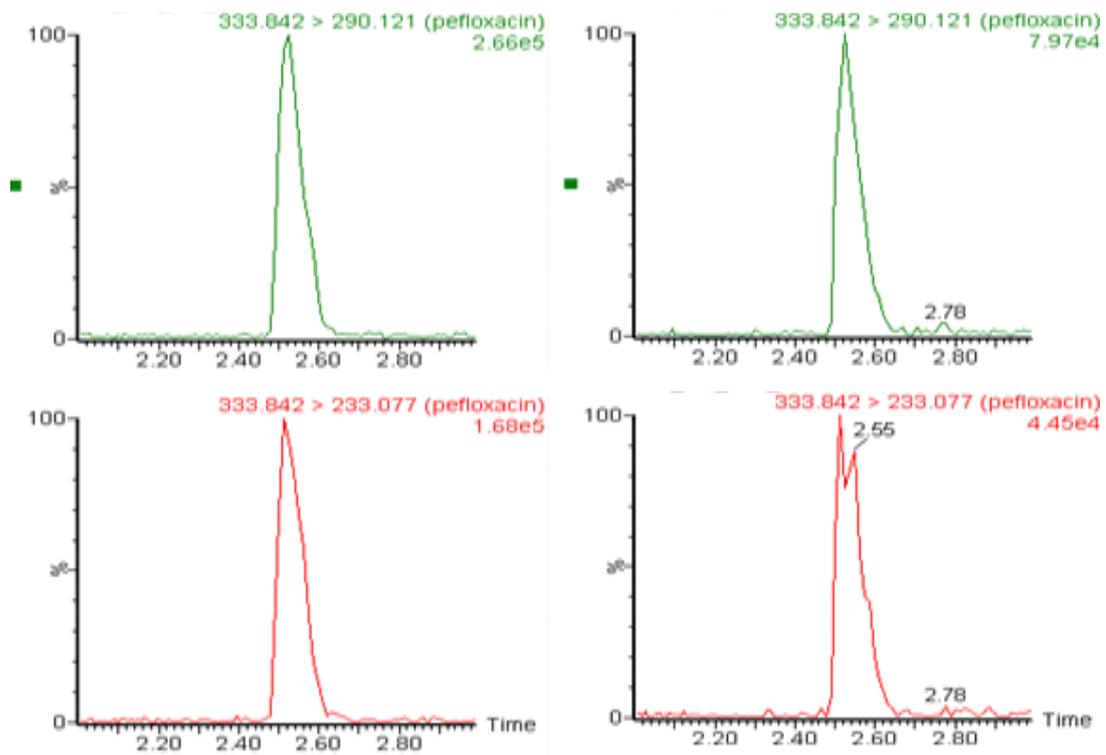


Fig. 7. UPLC-ESI-MS/MS chromatogram of pefloxacin standard solution (left) and detected common eel (right). The detected concentration of pefloxacin was 62.4  $\mu\text{g}/\text{kg}$ .

## **CHAPTER II**

### **Development of multi-residue analytical method for 9 cephalosporins and application to the residue monitoring in livestock and marine products in Korea**

## ABSTRACT

A sensitive ultra performance liquid chromatography with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) method was developed for the simultaneous quantitative analysis of 9 cephalosporins in 8 species of livestock and marine products (beef, pork, chicken, milk, flatfish, jacoever, common eel, and shrimp). Multiple reaction monitoring scanning was performed for quantification with switching electrospray ion source polarity between positive and negative modes in a single run time of 5 min. Highest sensitivity for the analytes was obtained when cefazolin, cephapirin, desacetyl-cephapirin, cephalixin, cefalonium, cefoperazone, and cefuroxime were measured in the positive electrospray ionization mode (ESI(+)) and cefquinome and brucine (as internal standard) were measured in the negative electrospray ionization mode (ESI(-)). The analytical method involved the sample preparation based in a combination of solvent extraction and ultrasonic-assisted extraction. Besides, matrix matched calibration curve with internal standard was used for quantification during the determination of the cephalosporin residues in samples.

The proposed method was validated according to the CODEX guideline and all results fully complied with CODEX recommendation. Good linearities were obtained and the

correlation coefficients ranged from 0.9990 to 0.9997 depending upon antibiotics. The limit of detection and limit of quantitation were lower than 8 and 25  $\mu\text{g}/\text{kg}$ , respectively.

A survey for 9 cephalosporins was conducted using 333 livestock and marine products distributed in Korea. Cefalonium was detected in 11 beef samples and cefquinome was detected in 1 milk sample. Detected concentrations were from 1.10 to 14.7  $\mu\text{g}/\text{kg}$  in beef and 7.50  $\mu\text{g}/\text{kg}$  in milk. All the detected levels were below the legal residue limits of 10-600  $\mu\text{g}/\text{kg}$  in the Korean Food Code and the risk values were very safe levels.

## INTRODUCTION

Cephalosporins are  $\beta$ -lactamic antibiotics that consist of the 7-amino cephalosporonic acid nucleus and a six membered dihydrothiazine ring fused to the  $\beta$ -lactam portion and they act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls (Hou et al., 2013). They are most widely used in veterinary medicine for livestock farming and bovine milk production and used for the prevention and treatment of bacterial infection, such as respiratory, urinary, and skin infections (Hermo et al., 2013). However, it is well known that  $\beta$ -lactam antibiotics possess an allergic potential in pre-sensitized people and therefore it cannot completely be excluded that high residue levels in food of animal origin will evoke allergic reactions in highly sensitive individuals. For these reasons, no feeding before shipping, the proper dosage, the observance of withdrawal period, and the accurate pharmaceutical feeding are major factors to prevent possible resistances.

The EU has established maximum residue limits (MRLs) for cephalosporins in foodstuffs of animal origin (EU, 2009). The MRLs ranges are between 20 and 1,000  $\mu\text{g}/\text{kg}$  for mammalian food producing species, such as milk, muscle, fat, and so on. In Korea, cefacetrile, cefazolin, cephapirin, cefalexin, cefalonium, cefoperazone, cefuroxime, cefquinome, and ceftiofur were licensed for the treatment of bacterial infections of animals used in livestock farming. The MRLs of cephalosporins were established by the

Ministry of Food and Drug Safety (MFDS) (Table 1) and ranged between 0.1 and 1 mg/kg for mammalian food producing species, such as cattle, pig, sheep, etc (MFDS, 2014). The residue levels below the MRLs are considered as safe. In this sense, the simultaneous determination of cephalosporins residues at MRL levels requires reliable and efficient analytical methods to comply with current legislations.

Several analytical methods have been described for the determination of cephalosporins included capillary electrophoresis (Solangi et al., 2007), luminescence (Yunjuan and Jiuru, 2006), spectrophotometry (Saleh et al., 2001; Saleh et al., 2003), and liquid chromatography (Virginia et al., 2000; De Diego et al., 2005; Zhu and Row, 2009; Karaqeorqou and Samanidou, 2010). However, the problems of the methods related to specificity and selectivity. Analytical detection limits of HPLC-UV method are generally limited by significant signal interference associated with UV spectral overlaps with other food constituents (Hui et al., 2013). Nowadays, LC-MS/MS has become the major analytical method for identifying cephalosporins due to its higher selectivity and sensitivity than other methods. Several methods have been described applying LC-MS/MS in bovine milk (Bruno et al., 2001; Hou et al., 2013), muscle (Matthias et al., 2004; Perez-Burgos et al., 2012), and kidney (Fagerquist and Lightfield, 2003; Clifton et al., 2005; Katerina and Alan, 2008). Although other mass spectrometers can be applied,

triple quadrupole mass spectrometers are excellent for quantification because they isolate and target biomolecules of interest, exclude signal from the background matrix, and are sensitive, fast-scanning and reproducible with high dynamic range. For this reason, triple quadrupoles are routinely used for developing mass spectrometry-based quantitative assays via MRM (Min et al., 2012). However, there is still a need for multi-residue methods capable of confirming and quantifying most of cephalosporins for which MRL values were set in different matrices. Moreover, compared to LC, UPLC improved resolution and sensitivity, as well as significantly reduced sample analysis time and mobile phase solvent consumption (Junza et al., 2011; Stolker et al., 2005).

Therefore, the purpose of this work was to develop a simultaneous method based in a combination of solvent extraction and ultrasonic-assisted extraction followed by UPLC-ESI-MS/MS for the detection and quantification of 9 cephalosporins (cefacetrile, cefazolin, cephalixin, desacetylcephalexin, cefalexin, cefalonium, cefoperazone, and cefuroxime) in livestock and marine products. Moreover, the proposed method was applied to the monitoring on residues of 9 cephalosporins in livestock and marine products commercialized in Korea.

## MATERIALS & METHODS

### *Chemicals and materials*

Cefacetrile and desacetyl-cephapirin were obtained from Toronto Research Chemical (Ontario, Canada). Cephalirin, cefalexin, cefalonium, cefoperazone, cefuroxime, and cefquinome were purchased from Fluka and, cefazolin was supplied by Sigma-aldrich (MO, USA) (Fig. 1). Brucine from Sigma-aldrich was used as internal standard (IS). Acetonitrile, hexane, and methanol were HPLC grade and obtained from Merck (Darmstadt, Germany).

### *Sample collection*

Sampling area was set in accordance with the 「Sampling with probability proportionate to size」 and species and number of samples were determined on the basis of the consumption of food according to the 「National Health and Nutrition Examination Survey, 2011」. Each sample was purchased at least two and sampling points within the sampling area were not biased.

As a result, a total of 333 livestock products including beef (n=75), pork (n=63), milk (n=77), chicken (n=21), flatfish (n=23), jacoever (n=27), common eel (n=22), and

shrimp (n=25) were purchased from different markets in Seoul, Busan, Incheon, Daegu, Daejeon, Gwangju, Ulsan, and Jeju. Details were given in Fig. 2. All solid samples were finely ground with blender and stored in a freezer (-20°C) until use.

### ***Preparation of standard solution***

Each individual standard, for example, cefacetriple, desacetyl-cephapirin, cephapirin, cefalexin, cefalonium, cefoperazone, cefuroxime, and cefquinome was dissolved in methanol. Stock standard solution (100 µg/mL) were obtained and stored at 4°C in the dark for no longer than 2 months. Working standard solutions were prepared daily by mixing individual stock solutions and diluted in distilled water. Brucine (IS) was diluted in distilled water at the concentration of 1 µg/ mL.

### ***Preparation of spiked samples and standard***

One sample of each matrix was repeatedly measured to confirm that no 8 cephalosporins were present and was used for the preparation of matrix-matched calibration standards and fortified samples for the validation of the proposed method.

Each blank sample of beef, pork, chicken, milk, flatfish, jacobever, common eel, and shrimp was homogenized and stored at -20°C until use. Fortified samples were prepared

by spiking 100  $\mu$ L of diluted working solutions at the concentration of 1/2 MRL, MRL, and 2 MRL for cephalosporins.

### ***Matrix-matched calibration curves***

For quantification, matrix-matched calibration curves with IS were performed. The blank samples (beef, pork, chicken, milk, flatfish, jacopecover, common eel, and shrimp) were used as matrix and fortified with working standard solutions. Each sample was submitted to the full extraction procedure. The six-point matrix-matched calibration curves in livestock and marine products (except milk) were prepared with the blank samples spiked with 0, 0.25, 0.05, 0.1, 0.2, and 0.3 mg/kg for cefacetile, desacetyl-cephapirin, cephapirin, cefoperazone, and cefquinome, and 0, 0.05, 0.1, 0.2, 0.4, and 0.6 mg/kg for cefalexin and cefazolin, and 0, 0.005, 0.01, 0.02, 0.04, and 0.06 mg/kg for cefalonium, and 0, 0.01, 0.02, 0.04, 0.08, and 0.12 mg/kg for cefuroxime, respectively.

In milk, the curves were prepared with the blank samples spiked with 0, 0.025, 0.05, 0.1, 0.2, and 0.3 mg/kg for cefacetile and cefazolin, and 0, 0.015, 0.03, 0.06, 0.12, and 0.18 mg/kg for cephapirin, desacetyl-cephapirin, and cefoperazone, and 0, 0.05, 0.1, 0.2, 0.4, and 0.6 mg/kg for cefalexin, and 0, 0.005, 0.01, 0.02, 0.04, and 0.06 mg/kg for cefalonium, and 0, 0.01, 0.02, 0.04, 0.08, 0.12 mg/kg for cefuroxime and cefquinome,

respectively. Brucine (IS) was spiked in all of these samples at the concentration of 20  $\mu\text{g}/\text{kg}$ . The calibration curves for 9 cephalosporins were constructed by calculating the ratio of each peak area relative to the corresponding IS. Separate calibration curves were used for each sample.

### ***Extraction procedure***

Homogenized sample (2 g) of beef, pork, chicken, flatfish, jacoever, common eel, and shrimp and 2 mL of milk was taken into a 50 mL disposable polypropylene centrifuge tube and IS solution (100  $\mu\text{L}$ ) was directly spiked. 5 mL of acetonitrile was added to the sample and followed by shaking during 2 min on a vortex mixer (Heidolph Reax top, Heidolph, Schwabach, Germany). The samples were homogenized again for 10 min with ultra-sonicator (Power sonic 520, Hwashin, Seoul, Korea) and centrifuged (Allegra X-22R, Beckman Coulter, Brea, CA, USA) at  $1,000\times g$  for 10 min to induce the precipitation of proteins. The supernatant was poured into a separating funnel and repeat the same step once more. The supernatant obtained two times was moved in a 50 mL centrifuge tube, 10 mL of hexane was added to the supernatant extract in order to remove the lipid. After vortex-mixing for 1 min and centrifugation at  $1,000\times g$  for 10 min, the lower layer was filtered through a syringe filter (Whatman<sup>®</sup>, PVDF membrane, pore size 0.45 $\mu\text{m}$ ) and

transferred into the 15-mL glass test tube. The extract was evaporated to a proper volume (about 1 mL) under a stream of nitrogen (EYELA GM-2200, Tokyo, Japan) and subsequently methanol (1 mL) was added and evenly mixed. After that, the extract was evaporated until dryness under a gentle nitrogen stream at 45°C. The residue was reconstituted with 400 µL of 0.1% formic acid in 10% acetonitrile (v/v) and filtered through a syringe filter (Whatman®, PVDF membrane, pore size 0.2 µm) prior to UPLC-ESI-MS/MS injection (Fig. 3). Every sample was analyzed three times and the result was expressed as the mean of the total residue concentration.

### ***UPLC-ESI-MS/MS conditions***

UPLC equipment was an Acquity ultra performance LC-Waters system equipped with an autosampler (Waters, MA, USA) and ESI-MS/MS measurements were performed using a Quattro premier XE (Waters, MA, USA). Cephalosporins were separated on an Acquity UPLC BEH C<sub>18</sub> column (2.1 mm × 100 mm, 1.7 µm particle size, Waters). Data was controlled by MassLynx 4.1 software (Waters) in a personal computer.

The binary mobile phase of UPLC-MS/MS composed of deionized water and acetonitrile with 0.1% formic acid in both solvents was used. The gradient conditions were initiated with 95% mobile phase A followed by a linear decrease to 10% in 3 min,

and maintained for 0.5 min at 10%. Then mobile phase A was increased to 95% in 0.1 min and holding at 95% for 1.4 min. The run time for each injection was 5 min, the flow rate was 0.35 mL/min, and the injection volume was 20  $\mu$ L in the full-loop mode. Details were given in Table 2.

The mass spectrometer was operated with ESI source in the polarity switching mode. MS determination was performed with MRM experiments combined with monitoring of the most abundant MS/MS (precursor $\rightarrow$ product) ion transitions using a dwell time of 0.03s. Detailed parameters for MRM acquisition are presented in Table 3. The MS parameter were as follow: capillary voltage, 3.5 kV; source temperature, 120 $^{\circ}$ C; desolvation temperature, 350 $^{\circ}$ C; cone gas flow, 50 L/h; and desolvation gas flow, 800 L/h.

### ***Method validation***

The method was validated according to the CODEX guideline (FAO, 2009) for a quantitative confirmation method. The validation parameters to evaluate the method were linearity, limit of detection (LOD), limit of quantitation (LOQ), precision (repeatability and reproducibility), and accuracy.

## RESULTS & DISSCUSION

### *Method validation*

Each matrix was repeatedly measured to confirm that no 9 cephalosporins were detected and was used for the preparation of matrix-matched calibration standards and fortified samples for the validation of the method. The developed method was validated with UPLC-ESI-MS/MS according to the CODEX guidelines (FAO, 2009) for quantitative methods. All obtained results were satisfied with CODEX recommendation and indicated the stability of the proposed method.

The linearities, LOD, and LOQ for each species of matrices were shown in Table 4. The linearity was measured with the matrix-matched calibration curves using 6 calibration points. Good linearities were obtained and the correlation coefficients ranged between 0.9990 and 0.9997 depending upon antibiotics. Besides, brucine as IS, which considerably increased the linearity of the matrix-matched calibration curves of 9 cephalosporins.

The LOD and LOQ values were measured based on signal-to-noise ratio (S/N) values of 3.3 and 10, respectively. The LOD ranged from 0.3 to 6  $\mu\text{g}/\text{kg}$  and LOQ ranged from 1 to 20  $\mu\text{g}/\text{kg}$  in livestock (except milk) and marine products, respectively. In milk, the LOD ranged from 0.8 to 8  $\mu\text{g}/\text{kg}$  and LOQ ranged from 8 to 25  $\mu\text{g}/\text{kg}$ , respectively. The results

of LOD and LOQ demonstrated that the method were enough to determine the MRLs values in animal origin products.

The repeatability and reproducibility (the intra-day and inter-day precision) were evaluated in 8 livestock and marine products. These validation parameters are expressed as relative standard deviation (RSD, %). The repeatability was evaluated by spiking 8 blank samples (n=6, for each matrix) at the MRL level and the reproducibility was carried out on 3 consecutive days. Repeatability and reproducibility results were presented in Table 5 and 6. The RSD (%) of repeatability ranged between 3.07 (Cefuroxime) in pork and 11.68 (Desacetyl-cephapirin) in jacopever. The RSD (%) of reproducibility ranged between 0.88 (Cefalexin) in beef and 10.92 (Desacetyl-cephapirin) in shrimp. It could be observed that RSD (%) were always lower than 12%. The repeatability values expressed as RSD (%) were lower than 15%, which is within the acceptance criteria of CODEX for validating analytical methods.

The accuracy of the method was estimated through recovery studies. The percentage of recoveries were calculated at 3 different spiking levels ( $1/2 \times \text{MRL}$ , MRL, and  $2 \times \text{MRL}$ ). The matrix-matched calibration curves were used with IS. Average recoveries (%) of each analyte were obtained performing the analysis in 6 replicated for each matrix. The recovery data were presented in Table 7 and 8. The acquired results were satisfied, with

the percentage of recoveries (%) ranged from  $63.82 \pm 8.00$  (cefacetrile) to  $92.89 \pm 4.09$  (cefalonium) in livestock products and from  $73.16 \pm 2.63$  to  $95.13 \pm 7.24$  (cefalexin) in marine products. The RSD (%) ranged from 3.27 to 12.54 in livestock products and from 3.06 to 7.61 in marine products. The results were within the acceptance criteria of the CODEX validating analytical method (Fig. 4).

In this study, good validation parameters were obtained indicating the suitability of the proposed method for the multi-residue analysis of 9 cephalosporins. Besides, the use of matrix-matched calibration curves with IS enabled to reduce the difference in the signal output of analyte between a sample extract solvent and a pure solvent.

### ***Optimization of sample preparation***

In this work, the reliable and specific method for livestock and marine products was developed. Extraction procedure was focused on the establishment of the most suitable conditions used for the simultaneous determination of 9 cephalosporins from animal origin products.

Several extraction solvents were evaluated in order to maximize the recovery and minimize preparation time. Beef, flatfish, and milk blank samples fortified with 9 cephalosporins were extraction with solution of 100% acetonitrile (v/v), 100% methanol

(v/v), and acetonitrile: distilled water (80:20, v/v) and consecutively extracted with hexane or not. As a result, 100% acetonitrile (v/v) and additional hexane extraction were shown to the most effective recoveries and lowest RSD (%) in 3 species of matrices (Fig. 5). Acetonitrile was preferred than methanol as an extraction solvent. Because acetonitrile could simultaneously precipitate the proteins, denature enzymes, and extract the target analytes. The extraction of hexane and syringe filtration was useful to achieve the deproteinization and defatting of samples. Moreover, after the solvent extraction of acetonitrile, a continuative ultrasonic-assisted extraction was conducted in order to acquire higher recovery. Ultrasound could be regarded as a useful alternative for solid sample pretreatment because the energy accelerated the steps of sample preparation, such as dissolution, fusion, and leaching (De Castro and da Silva 1997; Marilena and Nikolaos, 2015).

In addition, a pre-concentration step was evaluated. To carry out this step, methanol (1 mL) was added or not during evaporation under a stream of nitrogen. Consequently, when the methanol (1 mL) was added to tube in pre-concentration step, the recoveries (%) were improved by 14% in beef (cefuroxime), 45.7% in flatfish (cefoperazone), and 37% in milk (cefalexin), respectively. Details were given in Fig. 6.

When ESI is used as ionization technique, one of the main problems is the signal suppression or enhancement of the analytes due to the other components present in the matrix (Rocui et al., 2014). Because the existence of matrix effects, matrix matched calibrations were used for quantification during the determination of the 9 cephalosporins in livestock and marine products. Besides, quantification by matrix-matched calibration curves using IS allowed to monitor the efficiency of the extraction procedure and also to correct possible matrix effects (Andreia et al., 2014). Thus, this proposed method could be applied to the diverse matrices.

### ***Optimization of UPLC-ESI-MS/MS***

Chromatographic conditions of UPLC were studied in order to achieve the best separation and retention for the analytes, minimizing analysis time. Several experiments were conducted testing different mobile phases consisting of acetonitrile as organic phase and water with different concentrations of formic acid (0.05 and 0.1%) or ammonium formate (5 mM). As a result, the addition of 0.1% formic acid allowed better results than ammonium formate, and it enhanced the ionization efficiency. Especially, formic acid and acetonitrile were significant roles for the formation of well distributed peak and sharp peak shape (Lina et al., 2011; Junza et al., 2014). Moreover, 5 min of run time was

acquired with the optimum conditions for the separation of 9 cephalosporins. Good response was obtained with the gradient described in Table 2. Furthermore, in the first 1 min of the analysis time, main interference are presented due to polar matrix constituents that are eluted and co-elution with the analytes would lead to large signal suppression (Marilena et al., 2015). As a result, it was important that 9 cephalosporins began to elute after the 1 min of the analysis time. Other parameter such as flow rate, and injection volume were studied in order to obtain a fast and reliable separation. The best results were acquired when 0.35 mL was used as flow rate and 20  $\mu$ L were injected, respectively. The chromatographic separation of 9 cephalosporins was performed with a 2.1 mm $\times$  100 mm column, which was consisting of a smaller particle size than common HPLC column (Andreia et al., 2013). Thus, it could be acquired the high efficiency in peak separation and good peak resolution.

In MS/MS system, at first, the selection and tuning of the precursor and product ions were performed. For polarity switching ionization mode, direct infusion of each standard solution (1 $\mu$ g/mL) was carried out, depending on if the determination of the analyte is achieved in positive or negative ionization mode, respectively. As a result, the protonated ( $[M+H]^+$ ) or deprotonated ( $[M-H]^-$ ) molecular ions were selected as the precursor ion. Most of cephalosporins antibiotics could be detected as protonated molecular ions

$[M+H]^+$  using the positive electrospray ionization mode (ESI(+)). However, the negative electrospray ionization mode (ESI(-)) was found to be the most sensitive electrospray condition. Consequently, in this study, 8 cephalosporins (cefacetrile, cefazolin, cephalirin, desacetyl-cephapirin, cefalexin, cefalonium, cefoperazone, and cefuroxime) had better sensitivity and reproducibility in the positive ion mode, and negative ion mode was suitable for the analysis of cefquinome and brucine. Especially, the ESI(-) mode resulting in a much better signal to noise ratio of the molecular ions (Matthias et al., 2004).

Then, collision energies were evaluated in order to trace the most abundant product ions, selecting the most sensitive transition for quantification purposed and a second one for confirmation. Table 3 presented MS/MS transitions as well as cone voltage and collision energy values optimized for the 9 cephalosporins.

The system was operated in the MRM mode acquiring two diagnostic product ion transitions for each cephalosporin. Two product ion transitions (quantifier ion, qualifier ion) are monitored and the ratio between the monitored fragment ions is calculated and compared with the ratio obtained from the analytical standard. To achieve maximum sensitivity for all analytes, sufficiently long dwell times are required for each daughter ion transition measured. As a result, the chromatography gradient conditions were

adjusted to obtain a nearly complete separation of 9 cephalosporins. Chromatograms of 9 cephalosporin antibiotics were presented in Fig. 7.

### ***Application of the proposed method to animal products***

Cephalosporins were analyzed in 333 livestock and marine products including 75 beef, 63 pork, 77 milk, 21 chicken, 23 flatfish, 27 jacopecover, 22 common eel, and 25 shrimp (Table 9). The results were described in Table 10. The residues of cephalosporins were detected in only 12 livestock product samples (3.6% incidence), such as, beef and milk. 11 out of 75 beef samples (14.7% incidence) and 1 out of 77 milk samples (1.3% incidence) showed cephalosporins residues (Fig. 2). The detected cephalosporins in livestock products were cefalonium in beef and cefquinome in milk, respectively. Detected concentrations were from 1.10 to 9.77  $\mu\text{g}/\text{kg}$  in beef and 7.50  $\mu\text{g}/\text{kg}$  in milk.

Cephapirin is rapidly and quantitatively metabolized to desacetylcephapirin and acetic acid in spiked muscle and kidney homogenate. Thus, only desacetyl cephapirin could be determined and validated in tissue homogenate (Matthias et al., 2004). This metabolism was not observed for spiked raw milk. However, it has to be considered that after intramammary infusion of cephapirin, cephapirin can be detected in milk. The council regulation (EEC) 2377/90 and the Korea Food Code set MRLs for the sum of cephapirin

and desacetylcephapirin. Thus, the proposed method has been developed so that cephapirin and desacetylcephapirin are simultaneously determined in all samples.

Furthermore, there are no MRLs for the fisheries and marine products in Korea, except for cefalexin. In veterinary field, cephalosporin antibiotics are mostly applied to the livestock, and especially cefalonium was widely used as a therapeutic agent for bovine mastitis (Yim, 2014). In this study, among cephalosporin antibiotics, only cefalonium and cefquinome were detected in livestock products below the MRL. Thus, the risk value based on the cephalosporins exposure by food intake was calculated using the EDI (estimated daily intake) and ADI (acceptable daily intake). Compared with ADI (0.0153 mg/kg b.w./ day in cephalonium and 0.0038 mg/kg b.w./ day in cefquinome), the risk value was safe level of 0.02% in cephalonium and 0.01% in cefquinome, respectively. Details were given in Table 11.

However, cephalosporin were classified as veterinary critically important antimicrobials (VCIA) from the Office International des Epizooties (OIE) and also 1- and 2-generation cephalosporins were classified as highly important antimicrobials (HIA), and 3- and 4-generation cephalosporin was classified as critically important antimicrobials (CIA) for human medicine) from the World Health Organization (WHO). Although cephalosporin residues appeared to be at very save levels in livestock and marine products, the

possibility of antibiotic misuse, such as violation of withdrawal period, pharmaceutical feed by mistake, and excess dosage, could occur. Therefore, since cephalosporin antibiotic resistance is very important in terms of public health, continuous management and monitoring are required.

## CONCLUSION

A specific and reliable method was developed and validated for the simultaneous determination of 9 cephalosporins in livestock and marine products using UPLC-ESI-MS/MS. The use of UPLC-ESI-MS/MS improved the sensitivity and reduced analysis time, allowing the identification and quantification of 9 cephalosporins in 5 min. Acquired validation parameters fully complied with CODEX recommendation. Besides, the matrix-matched calibration curves and internal standard play significant roles in compensating for the matrix effects.

In this work, a large number of animal origin products (n=333) commercialized in Korea were analyzed and quantified. The residues of 9 cephalosporins were detected in 12 samples (3.6% incidence) but residue levels were below the MRLs. The detected cephalosporins were cefalonium in beef and cefquinome in milk, respectively. Furthermore, the risk value was safe level of 0.02% in cephalonium and 0.01% in cefquinome. Even though the monitoring results appear to be considerably safe levels, antibiotic residues are still exposed to consumers. Therefore, the survey of cephalosporins residues in livestock and marine products was continued.

Table 1. Maximum residues limits of cephalosporins in Korea

Compound	Food	mg/kg
Cefacetrile	Milk	0.05
Cefazolin	Cattle, Pig, Sheep, Goat, Milk	0.05
Cephapirin	Cattle	0.05~0.1
+desacetylcephapirin	Milk	0.03
Cefalexin	Cattle, Pig, Sheep, Goat, Equine, Chicken	0.2~1.0
	Fish	0.2
	Milk	0.1
Cefalonium	Cattle	0.01
Cefoperazone	Milk	0.03
Cefuroxime	Cattle, Milk	0.02
Cefquinome	Cattle, Pig, Equine	0.05~0.2
	Milk	0.02
Ceftiofur	Cattle, Pig	1.0~6.0
	Milk	0.1

Table 2. Analytical conditions of LC-ESI-MS/MS for analysis of cephalosporins

	Items	Conditions	
LC	Instrument	Acquity ultra performance LC	
	Column	Acuity UPLC BEH C <sub>18</sub> (2.1×10 mm, 1.7 um)	
	Mobile phase	A: 0.1% formic acid in DW B: 0.1% formic acid in acetonitrile	
	Gradient	Time(min)	A (%)      B (%)
		Initial	95          5
		3.0	10          90
		3.5	10          90
3.6		95          5	
5.0	95          5		
MS	Flow rate	0.35 mL/min	
	Injection volume	20 µL	
	Instrument	Quattro Premier XE	
	Ionization mode	Polarity switching mode	
	Capillary voltage	3.5 kV	
	Cone voltage	17 V	
	Source temperature	120 °C	
	Desolvation temperature	800 °C	

Table 3. Multiple reaction monitoring conditions of the MS/MS for the detection of 9 cephalosporins

Analyte	Ionization mode	Transition		Cone volt. (V)	Collision E. (eV)
		Precursor ion (m/z)	Product ion (m/z)		
Cefacetile	ESI(+)	362	178	20	13
			258		12
Cefazolin	ESI(+)	455	156	15	11
			323		17
Cephapirin	ESI(+)	424	292	20	15
			152		23
Desacetyl- cephapirin	ESI(+)	382	152	25	25
			292		14
Cefalexin	ESI(+)	348	174	14	12
			158		8
Cefalonium	ESI(+)	459	152	15	11
			337		19
Cefoperazone	ESI(+)	644	188	18	20
			528		10
Cefuroxime	ESI(+)	423	207	15	8
			318		15
Cefquinome	ESI(-)	529	134	20	15
			125		65
Brucine (IS)	ESI(-)	395	244	49	35
			170		65

Table 4. Validation parameters on the correlation coefficient ( $r^2$ ), LOD, and LOQ of 9 cephalosporins

Compound	Determination coefficient ( $r^2$ )	Livestock & marine products		Milk	
		LOD <sup>1)</sup> (µg/kg)	LOQ <sup>2)</sup> (µg/kg)	LOD <sup>1)</sup> (µg/kg)	LOQ <sup>2)</sup> (µg/kg)
Cefacetrole	0.9990	-	-	8	25
Cefazolin	0.9994	3	10	1.5	5
Cephapirin	0.9997	6	20	1.5	5
Desacetyl- cephapirin	0.9992	1.5	5	1.5	5
Cefalexin	0.9991	6	20	1.5	5
Cefalonium	0.9995	0.3	1	0.8	2.5
Cefoperazone	0.9996	-	-	3	10
Cefuroxime	0.9994	3	10	4	12.5
Cefquinome	0.9991	1.5	5	1.5	5

<sup>1)</sup> Limit of detection

<sup>2)</sup> Limit of quantitation

Table 5. Intra-day precision of 9 cephalosporins at MRL levels

Compound	Intra-day precision (n = 6), RSD (%) <sup>1)</sup>							
	Beef	Pork	Milk	Chicken	Flatfish	Jacopever	Common eel	Shrimp
Cefacetrile			7.91					
Cefazolin	7.40	8.29	6.83	6.62	9.37	6.61	9.47	8.86
Cephapirin	8.26	5.70	8.74	9.93	11.39	9.93	7.76	6.34
Desacetylcephapirin	8.21	5.54	6.27	9.13	5.66	11.68	7.63	6.50
Cefalexin	6.28	10.50	3.74	5.10	3.93	6.92	3.21	6.65
Cefalonium	4.73	5.82	9.51	3.20	10.76	8.96	8.43	8.26
Cefoperazone	6.23	6.95	6.97	8.79	8.39	9.67	10.97	8.48
Cefuroxime	7.57	3.07	8.74	10.15	9.99	8.85	4.92	9.93
Cefquinome	5.80	4.95	6.76	2.40	9.85	9.01	7.87	10.63

<sup>1)</sup> Relatively standard deviation (%)

Table 6. Inter-day precision of 9 cephalosporins at MRL levels

Compound	Inter-day precision (n = 3), RSD (%) <sup>1)</sup>							
	Beef	Pork	Milk	Chicken	Flatfish	Jacopever	Common eel	Shrimp
Cefacetrile	-	-	8.16	-	-	-	-	-
Cefazolin	4.83	4.01	8.42	5.28	4.63	3.59	10.38	9.50
Cephapirin	3.21	4.55	4.70	4.90	5.83	5.27	4.82	5.75
Desacetylcephapirin	3.83	4.65	5.58	1.10	3.58	7.47	6.00	10.92
Cefalexin	0.88	4.35	3.62	4.73	3.88	2.10	0.92	4.61
Cefalonium	4.83	2.95	9.15	8.92	4.22	5.70	2.94	5.81
Cefoperazone	6.52	4.78	4.84	8.37	5.12	7.62	10.52	5.99
Cefuroxime	6.49	8.30	4.70	9.61	9.29	2.80	5.25	6.14
Cefquinome	4.71	4.97	8.02	5.82	6.21	5.17	4.83	6.12

<sup>1)</sup> Relatively standard deviation (%)

Table 7. Average recoveries and RSD (%) measured in livestock products

Com-pound	Spiked conc. (µg/kg)	Beef		Pork		Chicken		Milk	
		Mean±SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>
Cefacetrile	1/2 MRL	-	-	-	-	-	-	63.82±8.00	12.54
	MRL	-	-	-	-	-	-	79.17±6.26	7.91
	2 MRL	-	-	-	-	-	-	76.28±6.28	8.23
Cefazolin	1/2 MRL	86.35±4.68	5.42	78.84±3.04	3.86	-	-	90.60±7.59	8.37
	MRL	87.91±6.50	7.40	83.16±6.90	8.29	-	-	90.55±6.19	6.83
	2 MRL	78.87±5.09	6.45	78.87±4.01	5.09	-	-	72.99±6.42	8.80
Cephapirin	1/2 MRL	79.28±4.55	5.74	-	-	-	-	76.13±4.00	5.25
	MRL	85.48±7.06	8.26	-	-	-	-	85.20±7.45	8.74
	2 MRL	83.85±4.30	5.13	-	-	-	-	82.83±4.06	4.90
Desacetyl-cephapirin	1/2 MRL	78.51±7.47	9.52	-	-	-	-	77.58±5.42	6.98
	MRL	86.16±7.07	8.21	-	-	-	-	88.72±5.56	6.27
	2 MRL	82.71±4.75	5.74	-	-	-	-	82.52±2.62	3.17
Cefalexin	1/2 MRL	80.26±4.56	6.25	70.04±2.99	4.27	87.42±7.15	8.18	78.24±7.03	8.99
	MRL	84.99±4.02	7.57	70.42±8.10	10.50	79.91±4.08	5.10	71.87±2.69	3.74
	2 MRL	81.49±2.66	3.27	72.31±8.19	10.33	83.02±3.62	4.36	72.07±5.71	7.92

<sup>1)</sup> Standard deviation

<sup>2)</sup> Relatively standard deviation (%)

Table 7. (Continued)

Compound	Spiked conc. (µg/kg)	Bovine		Swine		Chicken		Milk	
		Mean±SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>	Mean± SD <sup>1)</sup>	RSD(%) <sup>2)</sup>
Cefalonium	1/2 MRL	80.44±4.56	5.67	-	-	-	-	71.62±6.23	8.69
	MRL	84.99±4.02	4.73	-	-	-	-	79.72±7.58	9.51
	2 MRL	91.22±6.59	7.23	-	-	-	-	92.89±4.09	4.41
Cefoperazone	1/2 MRL	-	-	-	-	-	-	77.50±4.27	5.52
	MRL	-	-	-	-	-	-	87.17±6.07	8.74
	2 MRL	-	-	-	-	-	-	82.60±3.57	4.90
Cefuroxime	1/2 MRL	76.05±4.76	6.25	-	-	-	-	76.13±4.00	5.25
	MRL	88.98±6.74	7.57	-	-	-	-	85.20±7.45	8.74
	2 MRL	81.49±2.66	3.27	-	-	-	-	82.83±4.06	4.90
Cefquinome	1/2 MRL	90.59±5.11	5.64	76.20±2.87	3.77	-	-	88.39±7.62	8.62
	MRL	81.24±4.71	5.80	78.19±5.18	6.63	-	-	71.88±4.86	6.76
	2 MRL	84.87±5.24	6.17	79.93±3.95	4.95	-	-	89.12±8.66	9.72

<sup>1)</sup> Standard deviation

<sup>2)</sup> Relatively standard deviation

Table 8. Average recoveries and RSD (%) measured in marine products

Com-pound	Spiked conc. (µg/kg)	Flatfish		Common eel		Jacopever		Shrimp	
		Mean±SD <sup>1)</sup>	RSD(%) <sup>2)</sup>						
	1/2 MRL	80.03±2.44	3.05	75.70±4.33	5.71	78.57±4.17	5.31	73.16±2.63	3.60
Cefalexin	MRL	87.87±3.45	3.93	80.27±2.58	3.21	89.36±6.18	6.92	77.12±5.13	6.65
	2 MRL	94.77±5.49	5.79	84.65±5.76	6.80	95.13±7.24	7.61	81.53±5.74	7.06

<sup>1)</sup> Standard deviation

<sup>2)</sup> Relatively standard deviation

Table 9. Number of samples according to the sampling area

	Seoul	Busan	Incheon	Daegu	Gwangju	Daejeon	Ulsan	Jeju	Total
Beef	33	14	8	8	3	3	4	2	75
Pork	28	11	7	6	3	3	2	3	63
Milk	34	14	9	7	4	4	2	3	77
Chicken	6	3	2	2	2	2	2	2	21
Flatfish	6	3	2	2	2	2	2	4	23
Common eel	5	4	2	2	3	2	2	2	22
Jacopever	8	4	2	2	2	2	3	4	27
Shrimp	8	4	2	2	2	2	2	3	25
Total	128	57	34	31	21	20	19	23	333

Table 10. The incidence and detection levels of 9 cephalosporins in 333 livestock and marine products

Sample category	Analyzed samples	Detected sample	Below LOQ <sup>1)</sup>	Range of TMP level. (µg/kg)	Incidence of cephalosporins (%)	Kinds of cephalosporins
Beef	n=75	11	5	1.10-9.77	14.7	cefalonium
Pork	n=63	-	-			
Chicken	n=21	-	-			
Milk	n=77	1	-	7.50	1.30	cefquinome
Flatfish	n=23	-	-			
Common eel	n=22	-	-			
Jacopever	n=27	-	-			
Shrimp	n=25	-	-			

Table 11. Assessment of dietary intake and risk value of detected antibiotics

Compound	ADI <sup>1)</sup>	Food	Frequency <sup>2)</sup>	Detected Conc. (mg/kg)	Food intake <sup>3)</sup> (kg/person/day)	EDI <sup>4)</sup> (mg/person/day)	EDI/ADI <sup>5)</sup> (%)
Cephalonium	0.00153	Beef	11/75	0.0011-0.0098	30.36	0.0000030	0.02
Cefquinome	0.0038	Milk	1/77	0.0075	217.61	0.0000004	0.01

<sup>1)</sup> ADI is expressed in terms of mg/kg weight/day by a person of 55 kg.

<sup>2)</sup> Number of detected sample/total sample

<sup>3)</sup> From Korean National Health and Nutrition Examination Survey

<sup>4)</sup> EDI (mg/person/day) = Food intake (kg/person/day) × detected concentration in food (mg/kg)

<sup>5)</sup> Risk value (%)

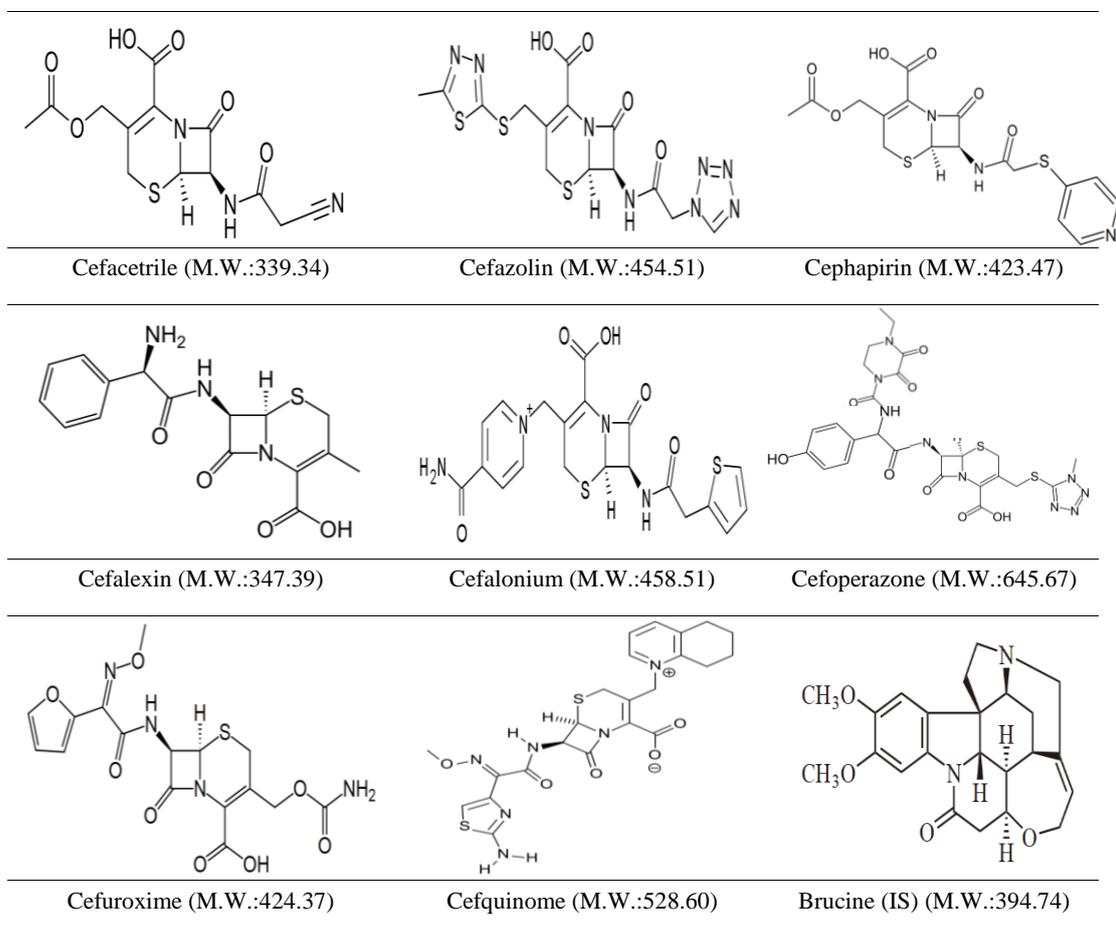
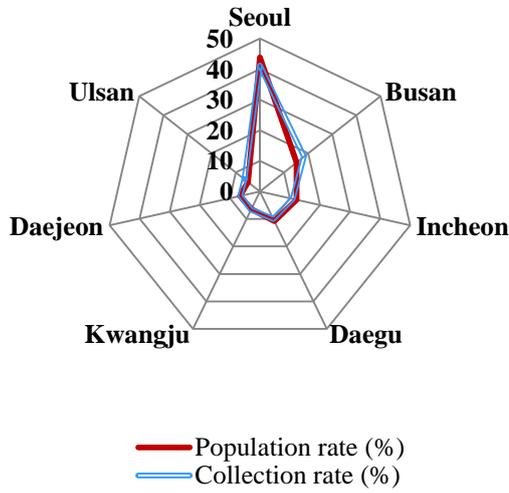


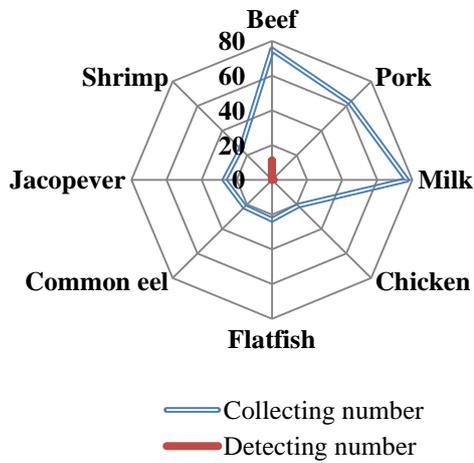
Fig. 1. Molecular structure of cephalosporins and brucine (IS).

(A)



City	Population (unit:1000)	Collecting rate (%)
Seoul	10,206	43.9
Busan	3,359	15.2
Incheon	2,840	12.2
Daegu	2,508	10.8
Gwangju	1,471	6.4
Daejeon	1,525	6.6
Ulsan	1,147	4.9
Total	23,236	100

(B)



Species	Collecting number	Detecting number
Beef	75	11
Pork	63	0
Milk	77	1
Chicken	21	0
Flatfish	23	0
Common eel	22	0
Jacopever	27	0
Shrimp	25	0
Total	333	12

Fig. 2. The number of collecting number according to the 「Sampling with probability proportionate to size」 (A) and distribution of samples containing cephalosporins residues by region (B).

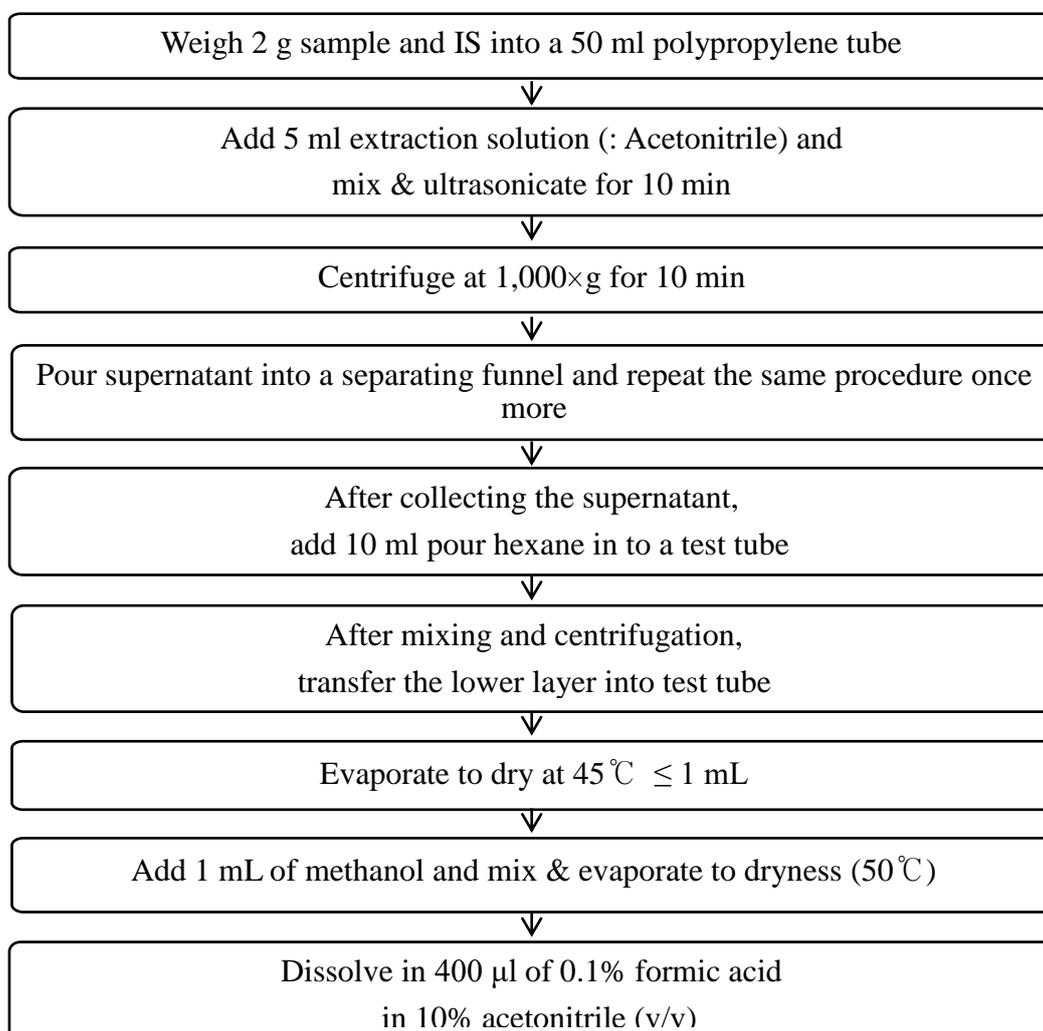


Fig. 3. Schematic diagram of the extraction procedure for the determination of cephalosporins (cefacetrile, cefazolin, cephapirin, cefalexin, cefalexin, desacetyl-cephapirin, cefoperazone, cefuroxime, and cefquinome) in livestock and marine products.

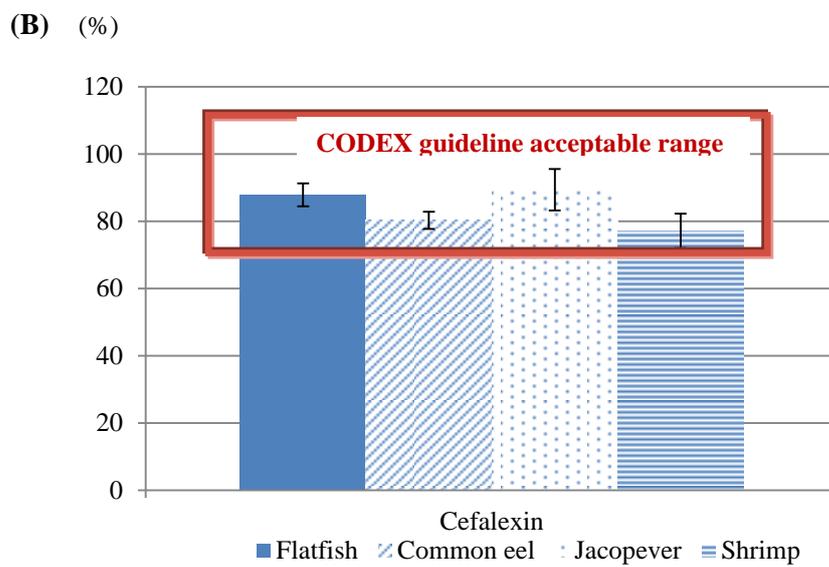
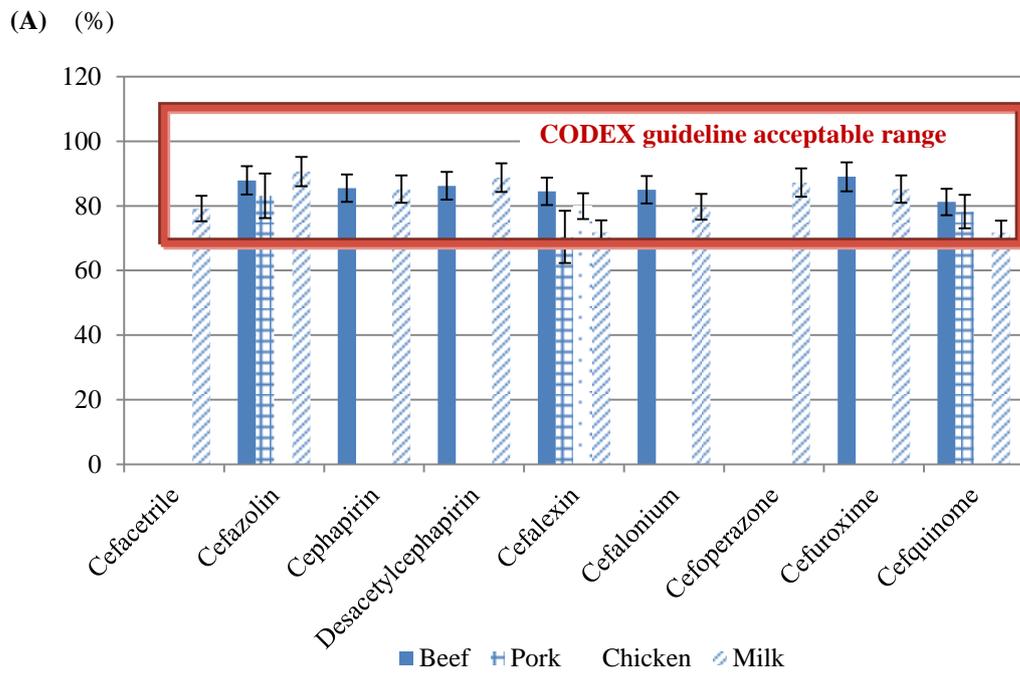


Fig. 4. Average recoveries measured in livestock (A) and marine products (B). The results were within the acceptance criteria of the CODEX guideline.

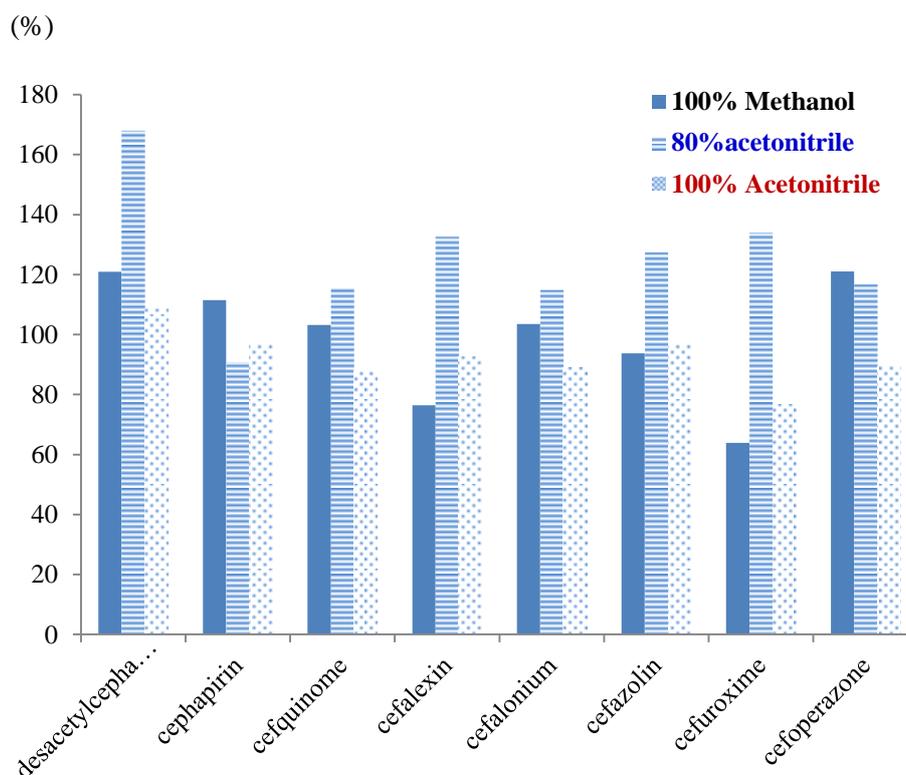


Fig. 5. Comparison of the solvent effects on the recoveries (%) from beef fortified with 8 cephalosporins. Cephalosporins were extracted with solution of 100% methanol (v/v), 100% acetonitrile (v/v), and acetonitrile: DW (80:20, v/v). The recoveries (%) were ranged from 63.9 to 121.1% on extraction of methanol, 90.8 to 168.0% on extraction of 80% acetonitrile (v/v), and 76.7 to 108.5% on extraction of acetonitrile, respectively. As a result, 100% acetonitrile (v/v) was shown to the most effective recoveries.

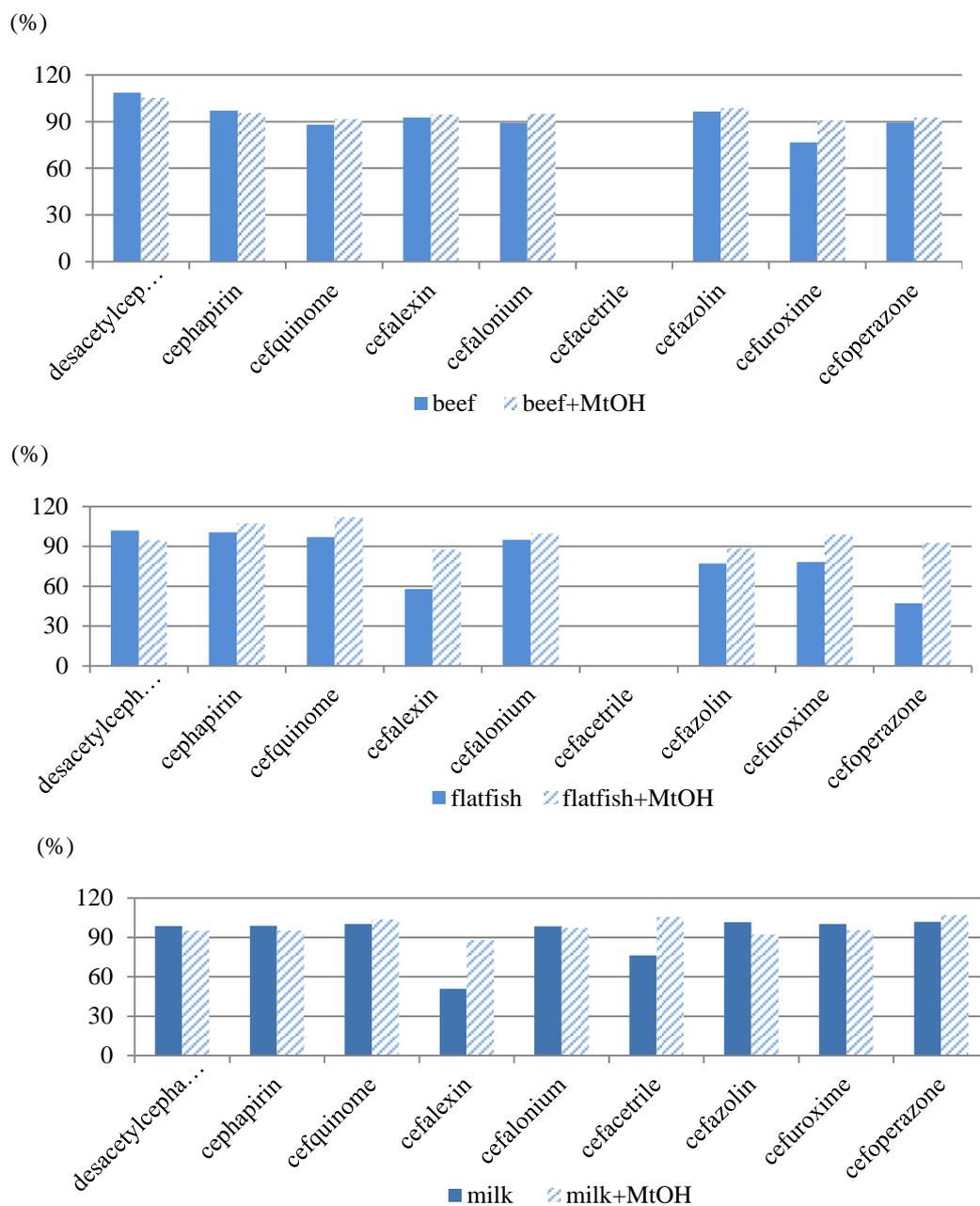


Fig. 6. Optimization of sample preparation in pre-concentration step. Methanol (1mL) was added or not during evaporation under a stream of nitrogen. The addition of methanol played an important role in increasing the recoveries among some cephalosporins.

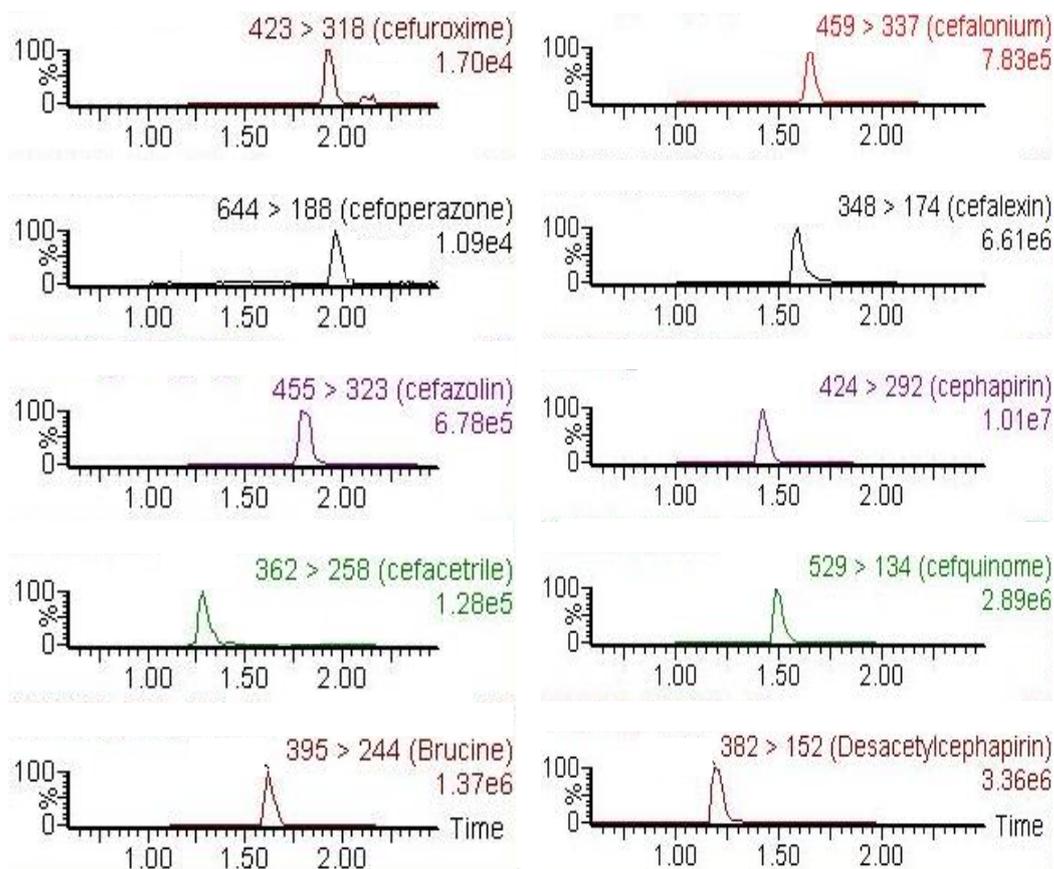


Fig. 7. Chromatograms obtained from a bovine muscle sample fortified at 0.3 mg/kg for cefacetile, desacetyl-cephapirin, cephapirin, cefoperazone, and cefquinome, 0.6 mg/kg for cefalexin and cefazolin, 0.06 mg/kg for cefalonium, 0.12 mg/kg for cefuroxime, and 0.02 mg/kg for brucine (IS), respectively. The product ions in the MRM mode were produced by collision activated dissociation of selected precursor ions. Two transitions were followed for 9 cephalosporins. The most abundant ions for 9 cephalosporins were presented.

## **CHAPTER III**

### **Development of analytical method for trimethoprim and application to the residue monitoring in livestock and marine products in Korea**

## ABSTRACT

A specific and simple analytical method using ultra performance liquid chromatography with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) was developed to analyze the determination of trimethoprim (TMP) in livestock (beef, pork, chicken, milk, and egg) and marine products (flatfish, jacoever, common eel, and shrimp). This method involved a solid-liquid extraction, ultrasonic-assisted extraction, and a step of solid-phase extraction (SPE) to pre-concentrate and clean up extract. The analytical method was validated according to CODEX guidelines and all results fully complied with CODEX recommendations. The calibration curve was linear from 0 to 50 ng/mL, and the correlation coefficient of calibration curve was 0.999. The LOD ranged from 0.15 to 0.30  $\mu\text{g}/\text{kg}$  and the LOQ ranged between 0.5 and 1.0  $\mu\text{g}/\text{kg}$  depending upon sample species.

A survey for TMP was performed using 369 livestock and marine products distributed in Korea. TMP was detected in 7 marine samples (1.9% incidence) including 5 jacoever, 1 flatfish, and 1 common eel. Residue levels were 1.17 to 16.43  $\mu\text{g}/\text{kg}$  in jacoever, 40.0  $\mu\text{g}/\text{kg}$  in flatfish, and 13.3  $\mu\text{g}/\text{kg}$  in common eel. All the detected levels were below the legal residue limits of 20-50  $\mu\text{g}/\text{kg}$  in the Korean Food Code and the risk value was very safe level of 0.13%.

## INTRODUCTION

Trimethoprim (TMP), a dihydrofolate reductase inhibitor, is an anti-infective agent that is commonly used for treatment of a wide variety of bacteria associated with infections of the middle ear, and the urinary, respiratory, and intestinal tracts (Isabelli et al., 2014) and is also a potentiator when administered in combination with sulfonamides (Mark et al., 2009), such as sulfamethoxazole (as co-trimoxazole, which contains sulfamethoxazole:TMP in a 5:1 ratio), sulfadiazine (Reza et al., 2013), or separately (Renew and Huang, 2004; Chung, 2008).

The presence of antibiotic residues can increase antibiotic resistance of pathogens and can threaten public health. In order to protect consumer health, the European Union set maximum residue limits (MRLs) of 100 µg/kg and 50 µg/kg for TMP for members of family equidae and all other food producing animals, respectively, that supply milk, muscle, fat, liver, and kidney (EU. Commission regulation No. 37/2010, 2010). MRLs of Japan which adopts the Positive List System ranged from 20 to 100 µg/kg in animal and marine products (Ministry of Health, Labour and Welfare of Japan, 2007). In Korea, TMP was licensed for treatment of urinary tract infections and the MRL was established by the Ministry of Food and Drug Safety (MFDS) as shown in Table 1 at 50 µg/kg in muscle, liver, kidney, and fat of cows, pigs, sheep, and chicken, at 50 µg/kg in milk, fish and

crustaceans, at 20 µg/kg in egg, and at 100 µg/kg in muscle, liver, fat, and kidney of horse (Notification No. 2010-51 of the Ministry of Food and Drug Safety, 2010).

Determination of TMP residue at MRL level requires sensitive analytical methods to comply with current regulations. HPLC is a commonly used analytical method (Meiling et al., 2006; Bedor et al., 2008). Most previous works have involved in only one matrix, for instance, blood, plasma, serum, or urine with HPLC (Hiren et al., 2010). Several analytical methods have been described for simultaneous analysis of TMP and sulfonamides in biological fluids (Batziias et al., 2002; Meiling et al., 2006; Hiren et al., 2010). Current studies for TMP analysis with LC-MS/MS are underway for muscle and plasma (Mark et al., 2009; Nhat et al., 2014); however, there was little research for the TMP analysis in livestock and marine products using UPLC-MS/MS.

One of the main problems of TMP analysis of livestock and marine products is the complexity of extraction steps and a need for effective clean-up procedures before instrumental analysis. For this purpose, liquid-liquid extraction (LLE), solid-phase extraction (SPE), pressurized liquid extraction (PLE), and matrix solid-phase dispersion (MSPD) have been used for extraction of TMP from biological fluids (Croubels et al., 2003; Bedor et al., 2008) and wastewater (Renew and Huang, 2004; Nhat et al., 2012).

This paper described the development of a specific and simple analytical method using UPLC-MS/MS for the determination of TMP in livestock and marine products, which involved solid-liquid extraction, followed by simplified solid-phase extraction (SPE) steps. In addition, in this study, TMP residue was monitored in livestock and marine products commercialized in Korea. Koreans enjoy eating flatfish and jacobever sashimi, roasted eel, and Korean style raw minced beef.

## MATERIALS AND METHODS

### *Chemicals and materials*

TMP (Fig. 1) and formic acid were obtained from Sigma-Aldrich (MO, USA). Acetonitrile and methanol of HPLC grade were supplied by Burdick & Jackson (Ulsan, Korea). Acetic acid (99.9%, HPLC grade) was purchased from J.T Baker (NJ, USA). Oasis HLB, MCX, and WAX solid-phase extraction (SPE) cartridges were obtained from Waters (MA, USA).

### *Sample collection*

Sampling area was set in accordance with the 「Sampling with probability proportionate to size」 and species and number of samples were determined on the basis of the consumption of food according to the 「National Health and Nutrition Examination Survey, 2011」. Each sample was purchased at least two and sampling points within the sampling area were not biased.

Therefore, livestock and marine products (n=369) including beef (n=75), pork (n=63), milk (n=77), egg (n=36), chicken (n=21), flatfish (n=23), jacopecover (n=27), common eel (n=22), and shrimp (n=25) were purchased from different markets in Seoul, Busan,

Incheon, Daegu, Daejeon, Gwangju, Ulsan, and Jeju. Details were given in Fig. 2. All solid samples were finely ground with blender and stored in a freezer at -20°C until use.

### ***Preparation of standard solution***

A stock solution of TMP (100 µg/mL) was dissolved in methanol (Burdick & Jackson) and stored at 4°C in the dark. A new stock solution was prepared monthly. A working standard solution (1 µg/mL) was prepared daily diluting in 0.5% formic acid with 20% methanol (v/v). For quantification, a calibration curve for TMP was prepared at 6 concentrations between 0-50 ng/mL (0, 1, 2, 5, 10, and 50 ng/mL) by using 0.5% formic acid in 20% methanol (v/v).

### ***Sample preparation and clean-up procedure***

Homogenized sample (1 g) of beef, pork, egg, chicken, flatfish, jacobever, common eel, and shrimp and 1 mL of milk were placed into a 50 mL disposable polypropylene centrifuge tube and subjected to extraction using 10 mL of acetonitrile (Burdick & Jackson). The mixture was homogenized (Heidolph Reax top, Heidolph, Schwabach, Germany) for 5 min, sonicated (Power sonic 520, Hwashin Tech., Seoul, Korea) for 10 min, and centrifuged (Allegra X-22R, Beckman Coulter, Brea, CA, USA) at 1,000×g for

10 min. The supernatant was filtered through a syringe filter (Whatman<sup>®</sup>, PVDF membrane, pore size 0.45 µm) and evaporated under a gentle stream of nitrogen at 45 °C (EYELA GM-2200, Tokyo, Japan). The residue was dissolved in 2 mL of 20% methanol (v/v).

An Oasis HLB cartridge was conditioned using 5 mL of methanol followed by 5 mL of distilled water. The entire volume of extract was applied to the HLB cartridge, which was subsequently washed with 2 mL of 5% ammonium hydroxide in 10% methanol (v/v). After drying the cartridge for 3 min, the residue was eluted using 2 mL of 2% formic acid in 80% methanol (v/v). The elution fraction obtained from SPE was diluted using deionized water (1:3) and filtered through a syringe filter (Whatman<sup>®</sup>, PVDF membrane, pore size 0.2 µm), after which the diluted eluate was injected into a UPLC-ESI-MS/MS apparatus. A schematic diagram of the sample preparation was presented in Fig 3. Every sample was analyzed in triplicate and results were expressed as a mean value of the total residue concentration ± standard deviation (SD).

### ***UPLC-ESI-MS/MS conditions***

LC analysis was performed using an Acquity Ultra Performance (Waters) and ESI-MS/MS measurement was performed using a Quattro premier XE (Waters). Data were

recorded using MassLynx 4.1 software (Waters) on a personal computer. TMP was separated on an Acquity UPLC BEH C<sub>18</sub> column (2.1 mm × 100 mm, 1.7 μm particle size, Waters). The binary mobile phases composed of 0.1% formic acid in deionized water (A) and 0.1% formic acid in acetonitrile (B) were used. The gradient conditions started at 5% B and increased to 80% B within 3 min, followed by holding at 80% B until 4 min. At 4.1 min, the gradient was programmed to re-equilibrate the column for 1.9 min under initial conditions. The total time of analysis was 6.0 min. The flow rate was 0.3 mL/min and the injection volume was 20 μL in full-loop mode (Table 2).

MS determination was performed with MRM experiments in ESI positive ion mode combined with monitoring of the most abundant MS/MS (precursor→product) ion transitions using a dwell time of 0.04s. Details were given in Table 3. The MS parameters were a capillary voltage of 3.5 kV, a source temperature of 150°C, a desolvation temperature of 350°C, cone gas (N<sub>2</sub>) flow of 50 L/h, and desolvation gas (N<sub>2</sub>) flow of 800 L/h.

## RESULTS & DISCUSSION

### *Method validation*

The proposed method was validated following CODEX guidelines (FAO, 2009) for quantitative method. All results were consistent with the CODEX recommendation, which indicated that the proposed method was suitable for analyzing the TMP antibiotic.

The linearity, limit of detection (LOD), and limit of quantitation (LOQ) for each species of matrices were shown in Table 4. The linearity was calibrated from 0 to 50 ng/mL (6 points) and the squared correlation coefficient ( $r^2$ ) was greater than 0.999. The LOD is the lowest concentration of analyte that an analytical process can reliably differentiate from background levels, while the LOQ is the lowest concentration of analyte that can be quantified. LOD and LOQ values were calculated based on signal-to-noise ratio (S/N) values of 3.3 and 10, respectively. The LOD ranged from 0.15 to 0.30  $\mu\text{g}/\text{kg}$  and the LOQ ranged between 0.5 and 1.0  $\mu\text{g}/\text{kg}$  depending upon species of samples.

Recovery of the analyte was replicated 6x at levels of  $1/2\times\text{MRL}$ , MRL, and  $2\times\text{MRL}$  in accordance with the Food Code in Korea (Table 5). The results of recovery were 76.84-80.52% for beef, 81.13-90.81% for pork, 81.50-85.34% for milk, 83.68-87.04% for egg, 87.92-95.55% for chicken, 72.18-81.07% for flatfish, 85.64-93.13% for jacobever, 75.05-81.99% for common eel, and 70.08-80.03% for shrimp. The results were within the

acceptance criteria (60-120%) of the CODEX for validating analytical method (Fig. 4). Relative standard deviation (RSD, %) results were 3.52-9.60% for beef, 4.27-5.00% for pork, 2.84-5.85% for chicken, 3.53-4.57% for milk, 4.39-6.70% for egg, 2.74-8.29% for flatfish, 2.12-4.70% for jacobever, 2.22-4.33% for common eel, and 1.93-6.16% for shrimp. These values of results did not exceed 9.60%, indicating high precision and reliability of the method (Table 5).

### ***UPLC-ESI-MS/MS optimization***

Chromatographic conditions of UPLC were examined in order to acquire excellent separation and retention for the analytes. Some experiments were performed to test the different mobile phases consisting of acetonitrile and distilled water with formic acid (0.1%) or ammonium formate (5 mM). Therefore, the addition of 0.1% formic acid showed better results than ammonium formate, and improved the ionization efficiency. In addition, formic acid and acetonitrile had a significant impact on improving sharp peak shape and resolution for TMP (Lina et al., 2011; Junza et al., 2014). Other parameters such as gradient profile, flow rate, and injection volume were evaluated in order to acquire a reliable separation. The 6 min of run time was obtained with the optimum conditions for the separation of TMP and the UPLC system showed advantages with

respect to speed, sensitivity, and resolution as an attractive option for analysis of TMP in animal origin samples.

In MS/MS system, the protonated molecular ion  $[M+H]^+$  was selected as precursor ion for TMP, and the product ions were acquired according to the collision energy (eV). The ions in the MRM mode were produced by collision-activated dissociation of selected precursor ions in the collision cell of the triple quadrupole and analyzed with the second analyser of the instrument (Junza et al., 2011). Moreover, MRM mode increased the sensitivity and selectivity of the determination. Two transitions were followed for TMP; one was used for quantification and the other for identification. The most abundant ion was at  $m/z = 230$ . To obtain maximum sensitivity for TMP, MS/MS conditions such as, capillary voltage, source temperature, and desolvation temperature were optimized by direct infusion into the detector of standard solution (10 ng/mL). The acquired chromatogram showed a good resolution for TMP and was described in Fig. 5.

TMP levels were determined using UPLC-ESI-MS/MS in all species of samples to avoid false positive errors due to matrix interference. Comparison with background noise levels in 9 species of matrices showed no interference peaks detected at the expected retention time of TMP. The detected samples were confirmed by the transition ion ratio. As shown in Fig.6, the intensity of quantitative ion (291>230) and qualitative ion

(291>123) were  $1.42 \times 10^6$  (100%) and  $1.09 \times 10^6$  (76.8%) in standard solution, and  $3.69 \times 10^4$  (100%) and  $3.00 \times 10^4$  (81.3%) in detected jacobever sample, respectively. The ion ratios of detected samples were agreed with those of standards.

### ***Optimization of sample preparation and SPE procedure***

Sample preparation was concentrated on the establishment of the most proper conditions used for the extraction of TMP residue from livestock and marine products. This method involved the solid-liquid extraction, ultrasonic-assisted extraction, and a step of SPE to pre-concentrate and clean up extracts; the analytes were extracted with acetonitrile and purified by Oasis HLB cartridge.

Several extraction solvent mixtures were tested. Each species of sample fortified with  $20 \mu\text{g}/\text{kg}$  of TMP was extracted with solutions of 80% and 100% acetonitrile/water (v/v). Additionally trichloroacetic acid in different ratio from 0 to 5% (v/v) was added to extraction solvent. As a result, the extraction of 100% acetonitrile (v/v) without the addition of trichloroacetic acid was shown to the most effective recoveries and lowest RSD (%) in 9 species of matrices. Furthermore, the final concentrated extract was dissolved in 10% methanol and 20% methanol, respectively. Thus, the recoveries were higher when the residue was dissolved in 20% methanol (Fig. 7).

Solvent extraction including centrifugation and syringe filtration was performed, which enabled deproteinization and defatting of samples and also elimination of matrix interference. Ultrasound is also an effective pretreatment because the energy promoted the step of sample preparation, such as dissolution, fusion, and leaching (De Castro and Da Silva, 1997; Marilena and Nikolaos, 2015).

Furthermore, at this initial step of method development, extracts were directly injected into the UPLC-MS/MS apparatus without clean-up in order to confirm whether a clean-up step was required or whether adequate results could be acquired without sample manipulation. Consequently, use of SPE cartridges showed a crucial effect on recovery of the analyte. The SPE cartridge purification step was kept as simple as possible for minimization of ion suppression effects due to the complexity of the sample matrix.

In order to establish the optimum conditions for the SPE procedure we have considered the evaluation of 3 different SPE cartridges to clean up and preconcentrate the TMP in samples. The HLB cartridge is a high-performance, water-wettable copolymer and a hydrophilic-lipophilic balanced reversed-phase sorbent for acid, basic, and neutral compounds. The MCX cartridge is a mixed-mode cation exchange sorbent for bases, and the WAX cartridge is a mixed-mode weak anion exchange sorbent for strong acids. Mixed-mode sorbents provide both reversed-phase and ion exchange modes of retention,

enabling greater cleanup selectivity and sensitivity for both acidic and basic compounds (Waters, 2015).

In this study, SPE clean-up procedures for analyte extracts in blank milk sample spiked with 20 µg/kg TMP was investigated using Oasis HLB, Oasis MCX, and Oasis WAX SPE cartridges. Both recoveries of MCX and WAX cartridge were ranged from 44.0 to 49.1% and HLB cartridge recovery was 86.4%. Thus, the HLB cartridge was used. Besides, the HLB cartridge is commonly available on the market and the type of sorbent covers a large variety of polarities. Therefore, the HLB cartridge is useful for dealing with a wide range of analytes (Perez-Burgos et al., 2012).

Conditioning and equilibration of the SPE cartridge were performed with 100% methanol and 100% distilled water, respectively. Methanol was adequate for elimination of interference and for elution of TMP residues from cartridges. The ratio of organic solvents for washing and elution steps was investigated using basic and acidic solvents by spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan). Consequently both 5% NH<sub>4</sub>OH in 10% methanol (washing solvent) and 2% formic acid in 80% methanol (elution solvent) showed improvement in recoveries (Fig. 8). The proper selection of the extraction conditions was increased the recovery rate and reduced matrix effects.

### ***Application of the proposed method to animal products***

TMP was analyzed in 369 livestock and marine products including 75 beef, 63 pork, 77 milk, 36 egg, 21 chicken, 23 flatfish, 27 jacoever, 22 common eel, and 25 shrimp (Table 6). The results are listed in Table 7. The residues were detected in 7 marine product samples (1.9% incidence) ; 5 out of 27 jacoever samples (18.5% incidence), 1 out of 23 flatfish samples (4.3% incidence), and 1 out of 22 common eel samples (4.3% incidence). Detected concentrations were 1.17 to 16.43  $\mu\text{g}/\text{kg}$  in jacoever, 40.0  $\mu\text{g}/\text{kg}$  in flatfish, and 13.3  $\mu\text{g}/\text{kg}$  in common eel. All detected product samples were shown below the legal residue limit of 20-50  $\mu\text{g}/\text{kg}$  in Korea (Table 4). These residues of samples may be resulted from treatment or prevention of a particular type of infection/disease, or from contamination during production.

Furthermore, the risk value based on the TMP exposure by food intake was calculated using the EDI (estimated daily intake) and ADI (acceptable daily intake). Compared with ADI (0.0042 mg/kg b.w./day), the risk value was safe level of 0.13%. Details were given in Table 8. As shown in Fig. 9, the food contribution for EDI was higher in jacoever (58.0%), flatfish (31.9%), and common eel (10.1%).

Generally, a combination of TMP and sulfadiazine is sold under the name Aqua-Sulprim<sup>®</sup> made by the Korea Thumb Vet Company (Iksan, Korea). The combination of

trimethoprim and sulfadiazine is often medicated for treatment of vibriosis in flatfish, jacoever, common eel, and yellowtail in Korea (Chung, 2008). Furthermore, Won *et al.* (2011) were mentioned that the monitoring of TMP residue was necessary in marine products due to the combination use of sulfadiazine and TMP. It reported that sulfadiazine was found in 1 flatfish (14 µg/kg) and 1 jacoever (26 µg/kg). The level of sulfadiazine residue in marine products exhibited considerable similarity with results reported here in. Therefore, it will be necessary to implement a continuous monitoring of TMP and sulfonamide residues in livestock and marine products.

In this study, the incidence of TMP residues was more common in jacoever. In Korea, the usual dose for fish is 6-30 mg/kg of body weight with a withdrawal period of 30 days (National institute of fisheries science, 2011). As well as, intensive farming, which can lead to a high potential for spread of infectious disease, has been maintained to satisfy an increasing demand for marine products in Korea. Moreover, pharmacokinetics in aquaculture and stockbreeding were affected by species specificity, health conditions, age, size, water temperature, and salinity (Quzhong and Xeumei, 2007). Thus, proper usage of veterinary drugs and observance of withdrawal periods were required.

According to the Korean National Health & Nutrition Examination Survey, intake frequencies per week for livestock and marine products were between 0.5 and 2.6 times

for 12 years or older consumers (Korea Health Statistics 2011: Korea National Health and Nutrition Examination Survey, 2011). Besides, in Korea and other countries in Southeast Asia, many people traditionally consume a variety of livestock byproducts, such as blood, guts, and bones as foods (Choi et al., 2011) and enjoy jacobever and flatfish sashimi. Consequently, the survey of TMP residues in animal origin products is important in Southeast Asia.

## CONCLUSION

A simple and reliable method was developed for the rapid analysis of TMP residues in livestock and marine products using a UPLC-ESI-MS/MS system. The UPLC had a beneficial advantage in speed, sensitivity, and resolution. The sample preparation methods through the ultrasonic-assisted extraction and SPE clean-up procedure were increased the recovery and reduced the matrix effects. The recoveries of TMP in livestock and marine products were higher than 70%, and the LOD and LOQ values were lower than the established MRLs in Korea. Furthermore, acquired validation parameters fully complied with CODEX recommendations.

A large number of animal origin products commercialized in Korea were analyzed and quantified using the proposed method. The residues of TMP were detected in 7 samples (1.9% incidence) but residue levels were below the MRL, and the risk value was safe level of 0.13%. Although TMP residues appeared to be at relatively safe levels in livestock and marine products, the possibility of antibiotic misuse and violation of withdrawal period could occur. Therefore, this monitoring of TMP in livestock and marine products will be valuable information for the national management of antibiotics.

Table 1. Maximum residues limits of TMP in Korea

	Food	mg/kg
Cattle, Pig, Sheep, Goat, Poultry	Muscle, Liver, Fat, Kidney	0.05
Equine	Muscle, Liver, Fat, Kidney	0.1
Egg		0.02
Milk, Fish, Crustacean		0.05

Table 2. Analytical conditions of LC-ESI-MS/MS for analysis of TMP

Items		Conditions		
LC	Instrument	Acquity ultra performance LC		
	Column	Acuity UPLC BEH C <sub>18</sub> (2.1×10 mm, 1.7 μm)		
	Mobile phase	A: 0.1% formic acid in DW B: 0.1% formic acid in acetonitrile		
	Gradient	Time (min)	A (%)	B (%)
		Initial	95	5
		3.0	20	80
		4.0	20	80
		4.1	95	5
		6.0	95	5
	Flow rate	0.3 mL/min		
Injection volume	20 μL			
Instrument	Quattro Premier XE			
MS	Ionization mode	ES+		
	Capillary voltage	3.5 kV		
	Cone voltage	35 V		
	Source temperature	150°C		
	Desolvation temperature	800°C		

Table 3. Parameters of LC-ESI-MS/MS for analysis of TMP

Compound name	Transition		Cone voltage (V)	Collision Energy (eV)
	Precursor ion (m/z)	Product ion (m/z)		
TMP	291	230	35	22
		123	35	25

Table 4. Validation parameters for detection of TMP using UPLC-MS/MS

Sample	LOD ( $\mu\text{g}/\text{kg}$ ) <sup>a</sup>	LOQ ( $\mu\text{g}/\text{kg}$ ) <sup>b</sup>	Calibration curve <sup>c</sup>	$R^2$
Beef	0.3	1.0	$y=264.54x+69.95$	0.9994
Pork	0.3	1.0	$y=219.34x+59.21$	0.9991
Chicken	0.15	1.0	$y=256.33x+49.86$	0.9991
Milk	0.3	0.5	$y=269.08x+63.84$	0.9991
Egg	0.3	1.0	$y=226.14x+13.69$	0.9995
Flatfish	0.3	1.0	$y=274.78x+130.61$	0.9999
Jacopever	0.3	1.0	$y=288.73x+96.30$	0.9993
Common eel	0.3	1.0	$y=233.06x+33.75$	0.9997
Shrimp	0.3	1.0	$y=146.32x+104.75$	0.9991

<sup>a</sup> Limit of detection.

<sup>b</sup> Limit of quantification.

<sup>c</sup>  $x$  = concentration of TMP ( $\mu\text{g}/\text{kg}$ ),  $y$  = intensity.

Table 5. Recovery (%) and RSD (%) of spiked TMP by the proposed method using UPLC-MS/MS

Samples	Spiked conc. ( $\mu\text{g}/\text{kg}$ )	Mean $\pm$ SD <sup>a</sup>	RSD <sup>b</sup> (%)
Beef	1/2 MRL	76.84 $\pm$ 2.74	3.52
	MRL	78.18 $\pm$ 3.77	4.82
	2 MRL	80.52 $\pm$ 7.73	9.60
Pork	1/2 MRL	81.13 $\pm$ 4.06	5.00
	MRL	90.81 $\pm$ 4.49	4.94
	2 MRL	86.15 $\pm$ 3.68	4.27
Chicken	1/2 MRL	87.92 $\pm$ 2.50	2.84
	MRL	94.44 $\pm$ 5.52	5.85
	2 MRL	95.55 $\pm$ 3.68	3.19
Milk	1/2 MRL	85.34 $\pm$ 3.67	4.30
	MRL	81.50 $\pm$ 2.87	3.53
	2 MRL	84.71 $\pm$ 3.87	4.57
Egg	1/2 MRL	85.29 $\pm$ 3.63	4.25
	MRL	83.68 $\pm$ 3.67	4.39
	2 MRL	87.04 $\pm$ 5.83	6.70
Flatfish	1/2 MRL	72.18 $\pm$ 1.88	2.74
	MRL	77.80 $\pm$ 2.26	2.91
	2 MRL	81.07 $\pm$ 6.72	8.29
Jacopever	1/2 MRL	85.64 $\pm$ 2.98	3.48
	MRL	86.24 $\pm$ 3.82	4.70
	2 MRL	93.13 $\pm$ 1.97	2.12
Common eel	1/2 MRL	75.05 $\pm$ 1.89	2.52
	MRL	81.99 $\pm$ 3.55	4.33
	2 MRL	77.10 $\pm$ 1.71	2.22
Shrimp	1/2 MRL	70.08 $\pm$ 4.32	6.16
	MRL	80.03 $\pm$ 2.11	2.64
	2 MRL	75.58 $\pm$ 1.46	1.93

<sup>a</sup> Standard deviation

<sup>b</sup> Relative standard deviation

Table 6. Number of samples according to the sampling area

	Seoul	Busan	Incheon	Daegu	Gwangju	Daejeon	Ulsan	Jeju	Total
Beef	33	14	8	8	3	3	4	2	75
Pork	28	11	7	6	3	3	2	3	63
Milk	34	14	9	7	4	4	2	3	77
Chicken	6	3	2	2	2	2	2	2	21
Egg	9	6	3	3	5	4	4	2	36
Flatfish	6	3	2	2	2	2	2	4	23
Common eel	5	4	2	2	3	2	2	2	22
Jacopever	8	4	2	2	2	2	3	4	27
Shrimp	8	4	2	2	2	2	2	3	25
Total	137	63	37	34	26	24	23	25	369

Table 7. Incidence and range of TMP level in 369 livestock and marine products

Sample category	Analyzed samples	Detected sample	Range of TMP level ( $\mu\text{g}/\text{kg}$ )
Beef	n=75	ND <sup>a</sup>	-
Pork	n=63	ND <sup>a</sup>	-
Chicken	n=21	ND <sup>a</sup>	-
Milk	n=77	ND <sup>a</sup>	-
Egg	n=36	ND <sup>a</sup>	-
Flatfish	n=23	1	40.0
Common eel	n=22	1	13.1
Jacopever	n=27	5	1.17-16.43
Shrimp	n=25	ND <sup>a</sup>	-

<sup>a</sup> Non-detection

Table 8. Assessment of dietary intake and risk value of detected antibiotics

Compound	Food	Frequency <sup>1)</sup>	Detected Conc (mg/kg)	Food intake <sup>2)</sup> (kg/person/day)	EDI <sup>3)</sup> (mg/person/day)	EDI/ADI <sup>4)</sup> (%)
	Jacopever	5/27	0.0012-0.0164	36.23	0.0000034	0.08
Trimethoprim ※ ADI <sup>5)</sup> =0.0042	Common eel	1/22	0.0131	54.30	0.0000006	0.01
	Flatfish	1/23	0.040	58.93	0.0000019	0.04
					ΣEDI =0.0000059	ΣEDI/ADI =0.13

<sup>1)</sup> Number of detected sample/total sample

<sup>2)</sup> From Korean National Health and Nutrition Examination Survey

<sup>3)</sup> EDI (mg/person/day) = Food intake (kg/person/day) × detected concentration in food (mg/kg)

<sup>4)</sup> Risk value (%)

<sup>5)</sup> ADI is expressed in terms of mg/kg weight/day by a person of 55 kg

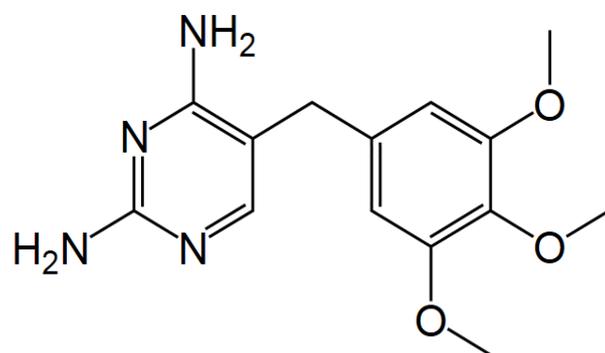


Fig. 1. Molecular structure of TMP (M.W.: 290.32).

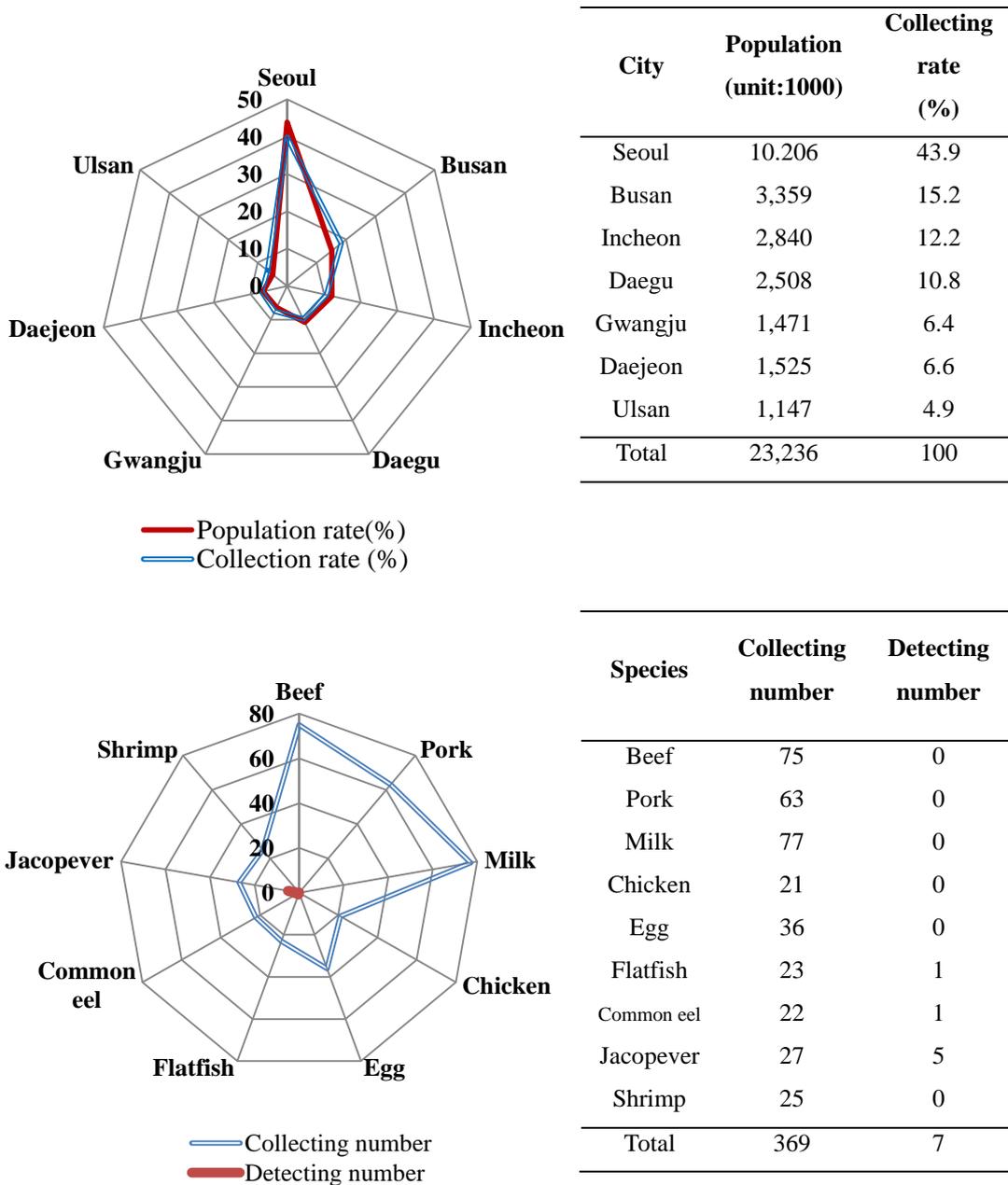


Fig. 2. The sampling number by areas according to the 「Sampling with probability proportionate to size」 (A) and distribution of samples containing TMP residues by region (B).

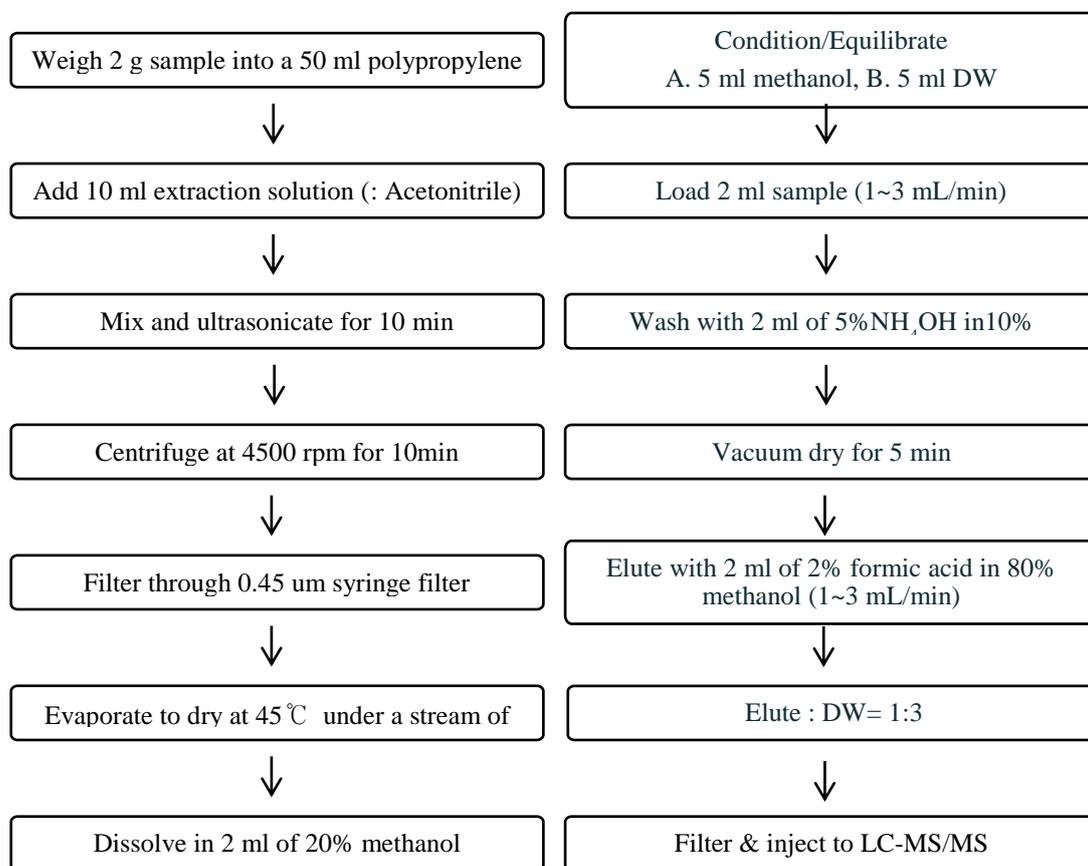


Fig. 3. Schematic diagram of the extraction procedure for determination of TMP in livestock and marine products (Left; preparation of samples, Right; SPE clean-up procedure).

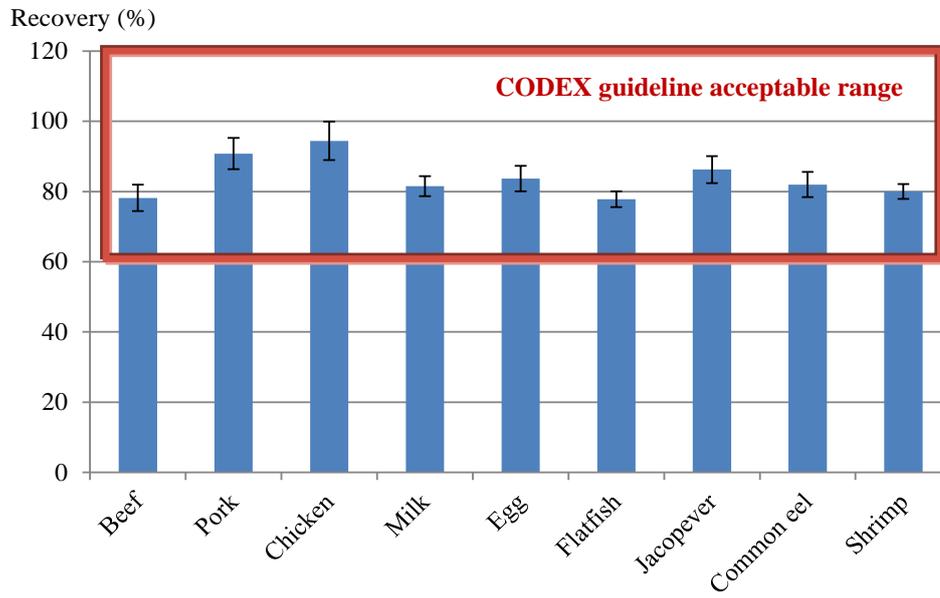


Fig. 4. Average recoveries measured in livestock and marine products. The results were within the acceptance criteria of the CODEX guideline (60-120%).

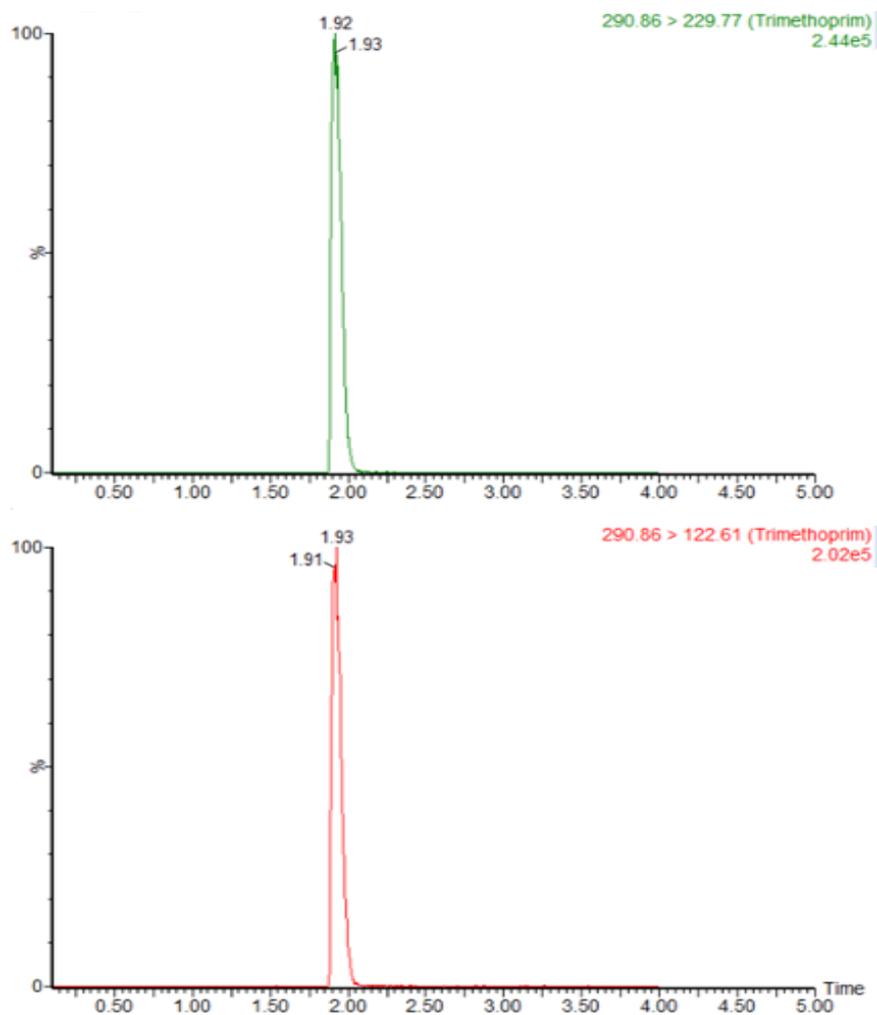


Fig. 5. Chromatograms of TMP obtained from a pork muscle sample fortified at 10  $\mu\text{g}/\text{kg}$  using UPLC-ESI-MS/MS in positive ion mode. For confirmation, 2 characteristic fragmentations of the protonated molecular ion  $[\text{M}+\text{H}]^+$  were monitored. The most abundant fragment ( $m/z$  229.77) was used for quantification, while the other was used as a qualifier ( $m/z$  122.61).

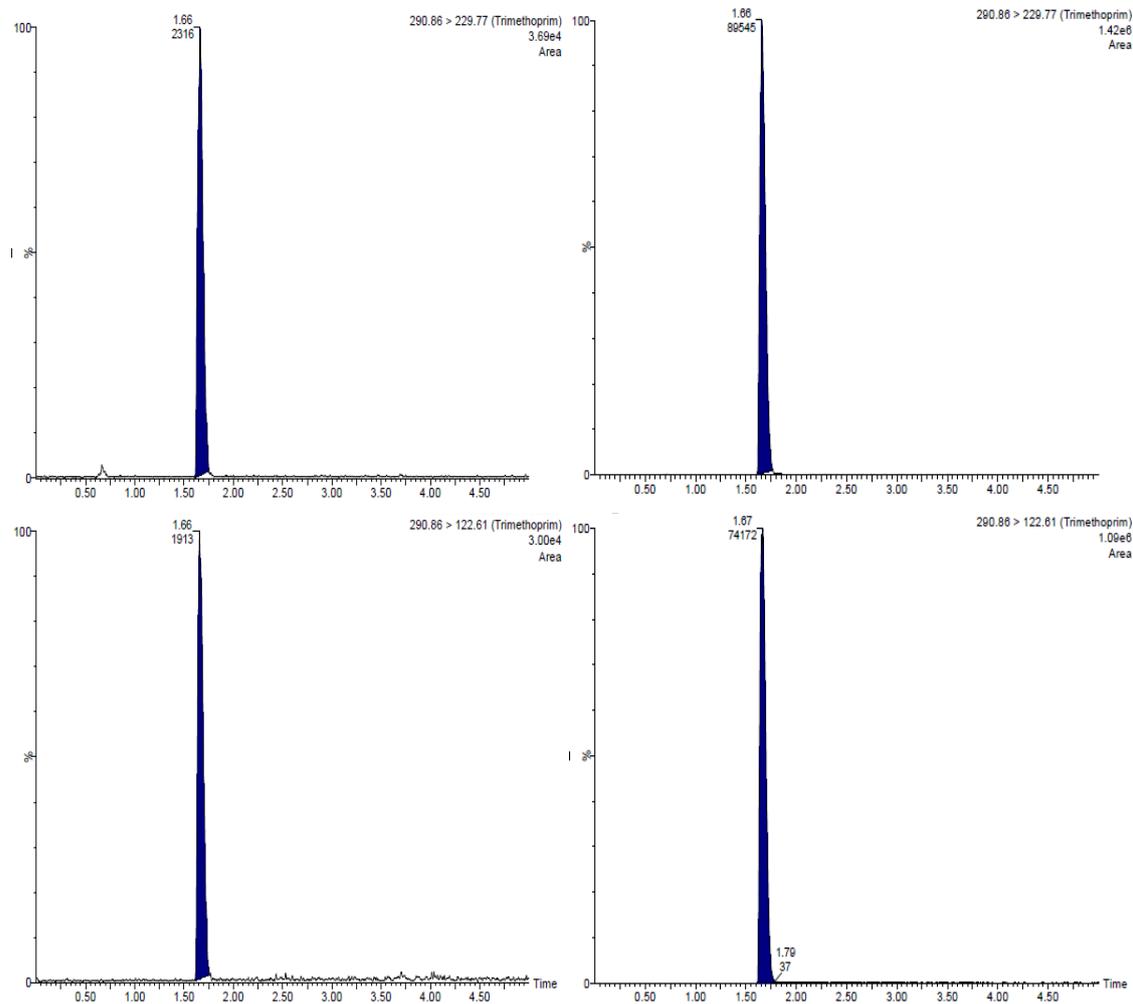


Fig. 6. UPLC-ESI-MS/MS chromatogram of TMP standard solution (left) and detected jacoever samples (right). The detected samples were confirmed by the transition ion ratio. The intensity of quantitative ion (291>230) and qualitative ion (291>123) were  $1.42 \times 10^6$  (100%) and  $1.09 \times 10^6$  (76.8%) in standard solution, and  $3.69 \times 10^4$  (100%) and  $3.00 \times 10^4$  (81.3%) in detected jacoever sample, respectively.

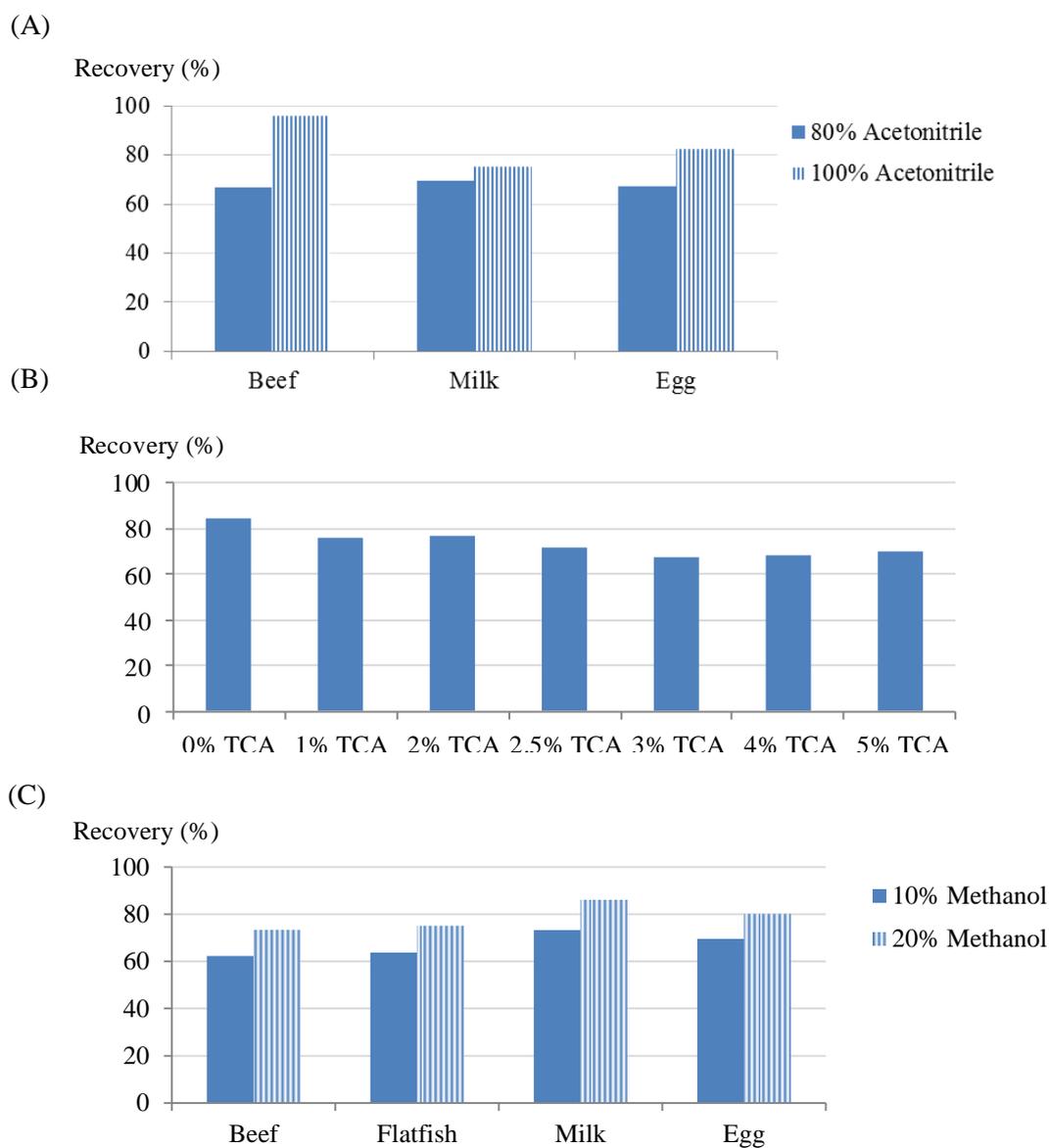


Fig. 7. Optimization of samples preparation. TMP residue was extracted with 80% and 100% acetonitrile, respectively (A). Additionally, trichloroacetic acid in different ratio from 0 to 5% was added to extraction solvent (B). As a result, the extraction of 100% acetonitrile (v/v) without addition of trichloroacetic acid was shown to the most effective recoveries. Furthermore, the recoveries were higher when the residue was dissolved in 20% methanol than 10% methanol (v/v).

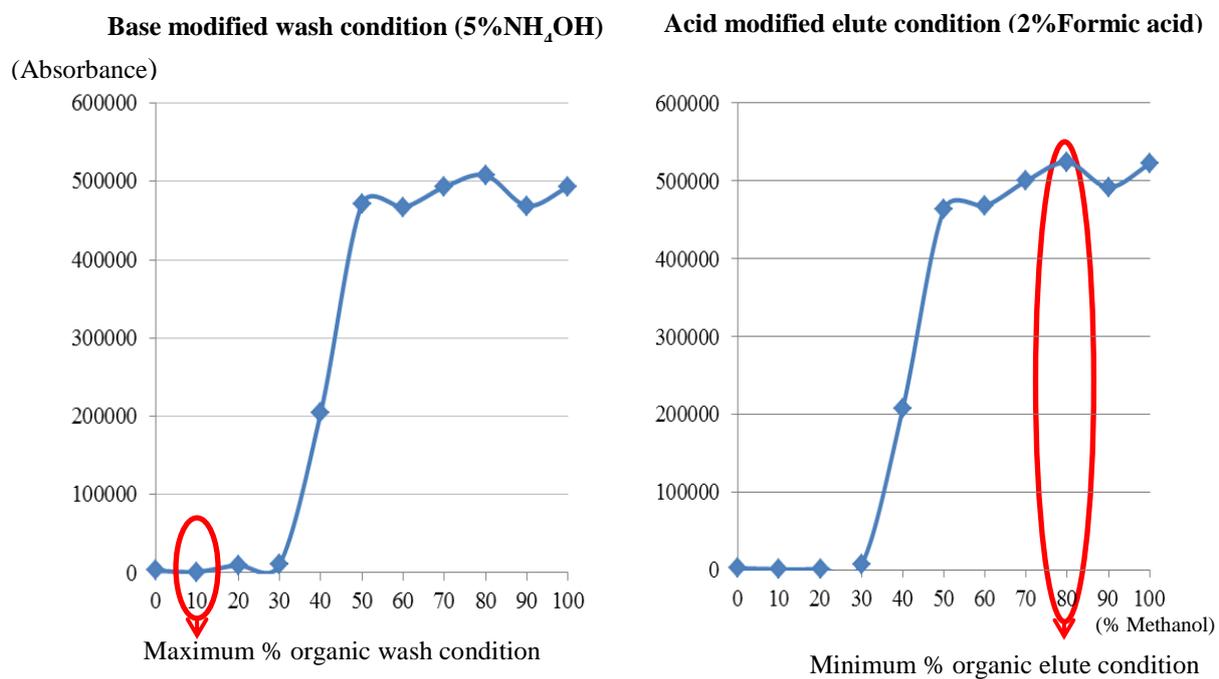


Fig. 8. Optimization of the wash and elution step condition for the SPE procedure obtained by Spectrophotometer (270 nm). The optimal ratio of organic solvent was 5% NH<sub>4</sub>OH in 10% methanol for washing step and 2% formic acid in 80% methanol for elution step.

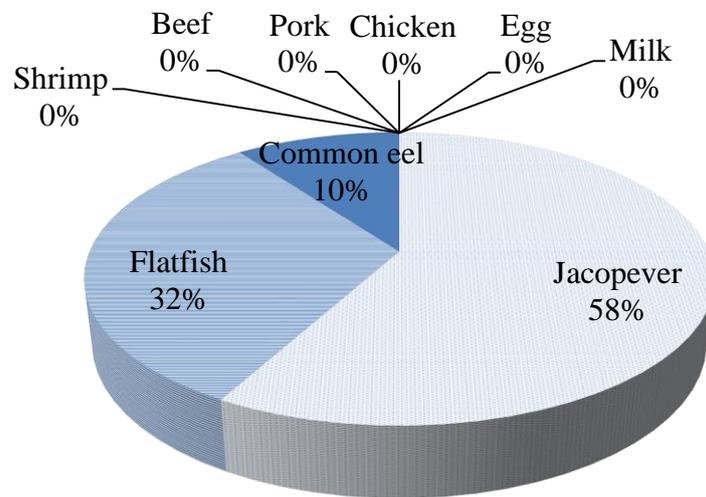


Fig. 9. The food contribution for estimated daily intake from TMP residue. It was higher in jacopever (58.0%), flatfish (31.9%), and common eel (10.1%), respectively. There was no contribution in beef, pork, chicken, egg, milk, and shrimp.

## General conclusion

Three specific and reliable methods were developed for the determination of 13 quinolones, 9 cephalosporins, and TMP in livestock and marine products using UPLC-ESI-MS/MS. The proposed method was validated according to the CODEX guidelines and all results were fully complied with CODEX recommendations. Good linearities were achieved and the correlation coefficients were ranged between 0.9990 and 0.9999. Both the LOD and LOQ were below the MRLs established by MFDS. The analytical methods using UPLC-ESI-MS/MS involved significant advantages with respect to high sensitivity, speed, and resolution, making them an attractive choice for the analysis of antibiotics residues in livestock and marine products. The sample preparation methods through solid-liquid extraction, ultrasonic-assisted extraction, and SPE clean-up reduced the matrix effects and increased the recovery. Moreover, the matrix-matched calibration curves with internal standard played important roles in compensating for the matrix effects.

A survey for 13 quinolones, 9 cephalosporins, and TMP residues was performed using the proposed method. The residues of quinolones were detected in 104 out of 310 (33.5% incidence) livestock and marine products below the MRLs. However, pefloxacin was detected in one common eel above the legal residue limit. Oxolinic acid, enrofloxacin,

and flumequine were the most commonly detected antibiotics. The residues of 9 cephalosporins were detected in 12 out of 333 samples (3.6% incidence) and detected antibiotics were cefalonium in beef and cefquinome in milk. The residue of TMP was detected in only 7 out of 369 samples (1.9% incidence) below the MRLs. The risk values of 13 quinolones, 9 cephalosporins, and TMP were under safe levels. Although the residual concentration appeared to be at within the safety levels, the possibility of antibiotic misuse and violation of withdrawal period could occur. Therefore, the effort to refine and improve national system for monitoring and management of antibiotic residues in livestock and marine products need to be continued.

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## 국문초록

### 축·수산물 중 퀴놀론계·세팔로스포린계 및 트리메토프림 항생제에 대한 동시분석법 개발 및 잔류실태조사

서울대학교 대학원

수의학과 수의생화학 전공

장진욱

(지도교수: 이항)

식생활 문화의 변화로 가축, 가금류 및 어류의 소비가 증가함에 따라, 축·수산업의 형태는 대규모, 밀집사육으로 변하였다. 이로 인해 가축의 치료 및 질병예방과 생산성 촉진을 위한 동물용의약품의 사용증가로, 소비자가 항생제내성에 노출될 위험은 점점 커지고 있다. 또한 동물용의약품의 오남용과 항생제 내성균의 문제는 국민건강에 해로운 영향을 미칠 수도 있다.

국내 동물용의약품에 대한 식품 중 잔류허용기준은 1990년 약 40여종을 설정한 이래 점차 확대 고시되어, 2015년 현재 총 185종에 대한 잔류허용기준이 설정·관리되고 있다. 또한 국제 규제 강화와 분석기술의 발달로 인해 국내에서도 효율성이 낮은 시험법 개선에 대한 요구와 함께 국제적 수준의 분석법의 도입이 이뤄지고 있다.

따라서 본 연구에서는 퀴놀론계, 세팔로스포린계 항생제와 트리메토프림에 대한 국제적 수준의 검사법을 마련하고, 이를 토대로 축·수산물 중 잔류실태 조사를 수행하였다. 검체수집 비율은 「확률비례추출법」에 따라 서울 및 6대

광역시의 인구비율을 감안하여 설정하였고, 조사대상 식품은 「2011년 국민건강영양조사」를 근거로 다소비 축·수산물식품 중 9종을 선정 하였다.

I. 축·수산물 중 퀴놀론계항생제 13종 (시프로플록사신, 다노플록사신, 플루메퀸, 디플록사신, 엔로플록사신, 마보플록사신, 날리딕스산, 옥소린산, 노르플록사신, 오플록사신, 오비플록사신, 페플록사신, 사라플록사신)에 대한 다성분 동시분석법을 확립하였다. 시료의 matrix의 영향을 최소화하고 회수율을 높이기 위해 ‘matrix-matched 정량곡선’을 도입하고, 내부표준 물질을 사용하였다. 또한 분석시간의 단축과 시료 중 극미량 잔류하는 퀴놀론계 항생제를 검출하기 위해 UPLC-ESI-MS/MS를 이용하였다.

국제식품규격위원회(CODEX) 지침에 따라 시험법 검증을 수행한 결과, 0.999 이상의 직선성을 얻었으며, 축·수산물 9종에 대한 검출한계와 정량한계는 각각 0.1, 0.4  $\mu\text{g}/\text{kg}$  이하였고, 높은 회수율 (83.0-104.7%)을 보여, CODEX에서 제시한 검증 범위에 적합한 것으로 나타났다.

본 연구에서 개발한 다성분 동시분석법을 토대로 잔류실태조사를 수행 하였다. 유통 중인 축·수산물 310건 중 104건 (축산물 39건, 수산물 65건)의 시료에서 검출되었고, 주로 검출된 퀴놀론계 항생제는 엔로플록사신, 플루메퀸 및 옥소린산 이었다. 검출농도는 장어 1건을 제외하고 모두 국내잔류허용기준 이하로 나타났다. 잔류량이 검출된 사라플록사신, 옥소린산 마보플록사신, 플루메퀸, 엔로플록사신 및 디플록사신에 대한 위해도를 산출한 결과, 일일 섭취허용량 대비 각각 0.201, 0.027, 0.007, 0.09 및 0.004%로 안전한 수준인 것으로 나타났다. 또한 인체노출량에 대한 식품별 기여도는 사라플록사신-우럭(63%), 옥소린산-장어(91%), 마보플록사신-넙치 (69%), 플루메퀸-우럭(45%), 엔로플록사신-닭(71%) 및 디플록사신-새우 (86%)로 나타났다.

II. 세팔로스포린계 항생제 9종(세파세트릴, 세파졸린, 세파피린, 디아세틸세 파피린, 세팔렉신, 세팔로니움, 세푸록심, 세포페라존 및 세프퀴놈)에 대하여

축·수산물 중 다성분 동시분석법을 확립하였다. UPLC-ESI-MS/MS를 이용하였고, MS/MS의 감도향상을 위해 Polarity Switching Mode를 도입하였으며, 세프퀴놈과 브루신은 ESI(-) mode를 적용하여 분석의 특이성을 높였다. CODEX 지침에 따라 시험법검증을 수행한 결과, 우수한 직선성을 나타냈으며, 검출한계와 정량한계는 모든 시료에서 각각 8, 25  $\mu\text{g}/\text{kg}$  이하였다. 또한 matrix의 영향을 줄이기 위해 matrix-matched정량곡선과 내부표준물질을 이용하였다. 이를 통해 CODEX 지침에 따른 적합한 검증결과를 얻을 수 있었다.

본 연구에서 개발한 다성분 동시분석법을 토대로 서울, 6대 광역시 및 제주에서 유통 중인 축·수산물 333건에 대한 잔류실태조사를 수행한 결과, 12건의 시료에서 잔류허용기준 이하로 세팔로스포린계 항생제(세팔로니움 11건, 세프퀴놈 1건)가 검출되었으며, 검출된 시료 모두 축산물(소고기, 우유)이었다. 검출된 세팔로니움과 세프퀴놈에 대한 위해도를 산출한 결과, 일일섭취허용량 대비 각각 0.02%와 0.01%로 안전한 수준으로 나타났다.

Ⅲ. UPLC-ESI-MS/MS를 이용하여 트리메토프림에 대한 분석법을 확립하였다. 시료의 matrix 효과를 감소시키기 위하여 초음파추출과 정제카트리지를 이용하였고, 이를 통해 평균회수율을 70% 이상으로 향상시켰다. 시험법검증을 수행한 결과, 0.999 이상의 직선성을 얻었고, 검출한계와 정량한계는 모든 시료에서 각각 0.3과 1.0  $\mu\text{g}/\text{kg}$  이하로 나타나, CODEX에서 제시한 검증 범위에 적합한 결과를 얻었다.

이를 토대로, 서울, 6대 광역시 및 제주에서 유통 중인 축·수산물 369건에 대한 잔류실태조사를 실시한 결과, 넙치1건, 조피볼락 5건 및 장어 1건에서 잔류허용기준 이하의 농도가 검출되었고, 축산물에서는 검출되지 않았다. 트리메토프림에 대한 위해도를 산출한 결과, 일일섭취허용량 대비 0.13%로 안전한 수준으로 나타났고, 인체노출량에 대한 식품별 기여도를 살펴 본 결과 우육이

58%로 가장 높게 나타났으며, 넙치 32%, 장어 10% 순을 보였다.

이상의 결과를 종합해 볼 때 국내 유통 중인 축·수산물 중 퀴놀론계, 세팔로스포린계 항생제 및 트리메토프림에 대한 잔류량은 국내 잔류허용 기준치 이하로 검출되어, 일일섭취허용량 대비 안전한 수준인 것으로 나타났다. 그러나 세계보건기구에서 3, 4 세대 세팔로스포린계 항생제와 퀴놀론계 항생제를 최우선적으로 관리가 필요한 그룹인 ‘CIA (Critically Important Antimicrobials for human medicine)’ 으로 지정하고 있고, 1 세대 및 2 세대 세팔로스포린계 항생제와 트리메토프림을 ‘HIA (Highly Important Antimicrobials)’로 지정하고 있다. 또한 동물보건기구에서는 퀴놀론계, 세팔로스포린계 항생제 및 트리메토프림 모두 가장 중요한 항생제 그룹인 ‘VCIA (Veterinary Critically Important Antimicrobials)’ 으로 분류하고 있다.

따라서 식품안전 및 국민건강과 국내 동물용의약품의 효율적인 사용을 위한 잔류실태조사와 국제 수준에 맞는 시험법 개발 및 개선 등 제도적인 관리가 지속적으로 필요할 것으로 판단된다.

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주요어: UPLC-MS/MS, 질량분석기, 모니터링, 항생제잔류

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