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Determination of Ceftiofur Residues by Simple Solid Phase Extraction Coupled with Liquid Chromatography-Tandem Mass Spectrometry in Eel, Flatfish, and Shrimp

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Abstract : The aim of this study was conducted to develop an analytical method to determine the concentration of ceftiofur residue in eel, flatfish, and shrimp. For derivatization and extraction, the sample was hydrolyzed with dithioerythritol to produce desfuroylceftiofur, which was then derivatized by iodoacetamide to obtain desfuroylceftiofur acetamide. For purification, the process of solid phase extraction (Oasis HLB) was used. The target analytes were confirmed and quantified in C_{18} column using liquid chromatography-tandem mass spectrometry with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as the mobile phase. The linearity of the standard calibration curve was confirmed by a correlation coefficient, $r^2 > 0.99$. The limit of quantification for ceftiofur was 0.002 mg/kg; the accuracy (expressed as the average recoveries) was 80.6-105%; the precision (expressed as the coefficient of variation) was below 6.3% at 0.015, 0.03, and 0.06 mg/kg. The validated method demonstrated high accuracy and acceptable sensitivity to meet the Codex guideline requirements. The developed method was tested using market samples. As a results, ceftiofur was detected in one sample. Therefore, it can be applied to the analysis of ceftiofur residues in fishery products.

Keywords : ceftiofur, residue, analytical method, fish, shrimp, LC-MS/MS

Introduction

Ceftiofur is a third-generation cephalosporin antibiotic that has been widely used in the livestock industry as a treatment for mastitis and respiratory diseases owing to its efficient broad antibacterial activity.[1,2] However, over and improper use of cephalosporin antibiotics in livestock and fishery farms has not only resulted in the generation of antibiotic-resistant bacteria but has also led to a public health issue in the treatment of human and animal disease. Thus, World Health Organization (WHO) and the World Organization for Animal Health (OIE) have raised health concerns owing to treatment issues arising from antibiotic resistance.[3]

To prevent antimicrobial resistance in livestock and fisheries, the Korean government has banned the addition of antibiotics in formulated feed since July 2011. In addition, the Aquatic Animal Disease Control Act introduced in 2013, allows permitted aquaculture drugs such as 51 antibiotics, unregulated veterinary drugs, and/or livestock-use-only drugs like ceftiofur, danofloxacin, etc with a veterinarian's prescription.[4] Nevertheless, in 2015, a large amount of ceftiofur was sold and used at a flatfish farm in Jeju without the prescription or approval.[5] This incident is a representative example of the lack of safety management for aquatic products and has raised concerns over the safety of domestic aquaculture products. Therefore, suitable analytical methods are required to determine the ceftiofur residues of in fishery products.

Several studies have been reported to determine ceftiofur

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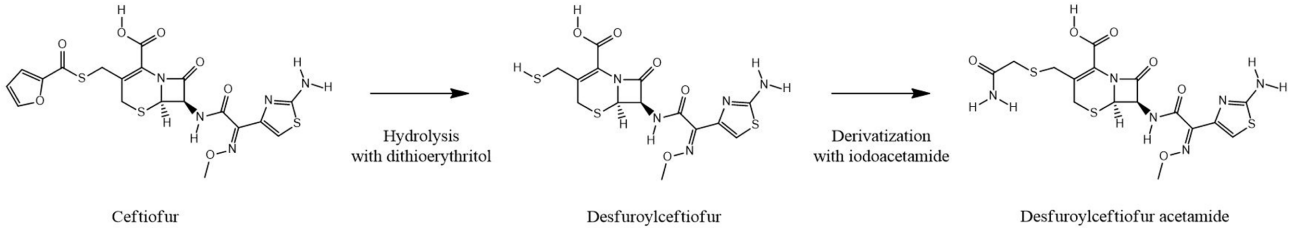

Figure 1. Molecular structure of ceftiofur, desfuroylceftiofur, and desfuroylceftiofur acetamide.

and its metabolites in animal tissue using liquid chromatography and/or coupled with mass spectrometry. The stable derivative-desfuroylceftiofur acetamide of ceftiofur was analyzed by using HPLC in duck and horse.[6,7] Ceftiofur and desfuroylceftiofur was determined in bovine kidney tissue using liquid chromatography-time-of-flight mass spectrometry (LC-TOF/MS).[8] Ceftiofur metabolite, desfuroylceftiofur cysteine disulfide was confirmed in bovine kidney tissue using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with SPE.[9] Ceftiofur and desfuoylceftiofur have been quantified by using multi-residue analytical method in fishery products.[10] However, ceftiofur, desfuroylceftiofur and desfuroylceftiofur cysteine disulfide were not suitable for screening or confirmation for ceftiofur residues because those are discharged rapidly as well and desfuroylceftiofur cysteine disulfide is been at concentrations lower than desfuroylceftiofur.[11] Thus, desfuroylceftiofur acetamide derivatized from desfuroylceftiofur was used for stable maker in chicken meat by LC-MS/MS.[12] Taking into consideration, the stable form of de-conjugated desfuroylceftiofur was analyzed by hydrolysis and derivatization with iodoacetamide into stable form as desfuroylceftiofur acetamide (Figure 1).

LC-MS/MS techniques can provide the sensitive, reliable and quantitative results to monitor veterinary drug residues in fishery products, and most commonly use in residue analysis in food.[13] Therefore, desfuroylceftiofur acetamide was selected and analyzed as the target compound in fishery products by using LC-MS/MS. The study was performed to develop a simple, sensitive analytical method for detection of ceftiofur in fishery products using LC-MS/MS. Moreover, fishery samples were investigated residues of ceftiofur to confirm the application of this method in real samples in the domestic market.

Experimental

Reagents and materials

Ceftiofur (98.8%) and desfuroylceftiofur acetamide (95%) standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Toronto Research Chemical (Ontario, Canada), respectively. Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany) and iodoacetamide (≥ 99%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Other reagents and solvents, such as dithioerythritol (≥ 99%), formic acid (≥ 95%), sodium tetraborate (≥ 99%), potassium chloride (≥ 98%), potassium phosphate monobasic (≥ 99%) and potassium hydroxide solution (≥ 85%) were purchased from Sigma-Aldrich (St. Louis, MO, USA); all these chemicals were analytical grade. For the extraction process, a vortex mixer (Taitec SR-2w, Japan) was used. A pH meter (iSTEK pH meter pH-200 L, Korea) was used to adjust the pH of the extract. For solid phase extraction (SPE), the hydrophilic-lipophilic balance (HLB, 6 mL, 200 mg) was purchased from Waters (Milford, MA, USA) and used in the elution process after adsorption of the extract through the activation process. Only the homogenized muscle portion of the samples of flatfish, eel, and shrimp, sourced from the local market, was used. The homogenized samples were then stored in a freezer (-20°C) until analyzed. The samples were used after confirming that ceftiofur and desfuroylceftiofur acetamide (a derivative of ceftiofur) were not present through the blank test.

Solution preparation

An individual standard solution (ceftiofur and desfuroylceftiofur acetamide) was prepared at a concentration of 100 mg/L (100 ppm) in methanol and was stored at 4°C, in a brown glass bottle. Ceftiofur intermediate and working solutions were prepared immediately before usage by dilution of the standard solution with a 0.025 M phosphate buffer solution (pH 7.0). Calibration standards for matrix-matched calibration were obtained by spiking the standard solution of an appropriate concentration with samples and treating the calibration sample with the identical procedure as that of the sample preparation.

To prepare solvent for the derivatization and extraction step, 0.05 M boric acid buffer solution (pH 9.0); sodium tetraborate decahydrate (19 g) and potassium chloride (3.7 g) were dissolved in water in a 1000 mL glass flask, 0.025 M phosphate buffer solution; potassium phosphate monobasic (3.4 g) was added in water and adjusted the pH to 7.0 using potassium hydroxide solution in a 1000 mL glass flask, 0.4% dithioerythritol solution; dithioerythritol (1 g) was added in 0.05 M boric acid buffer solution

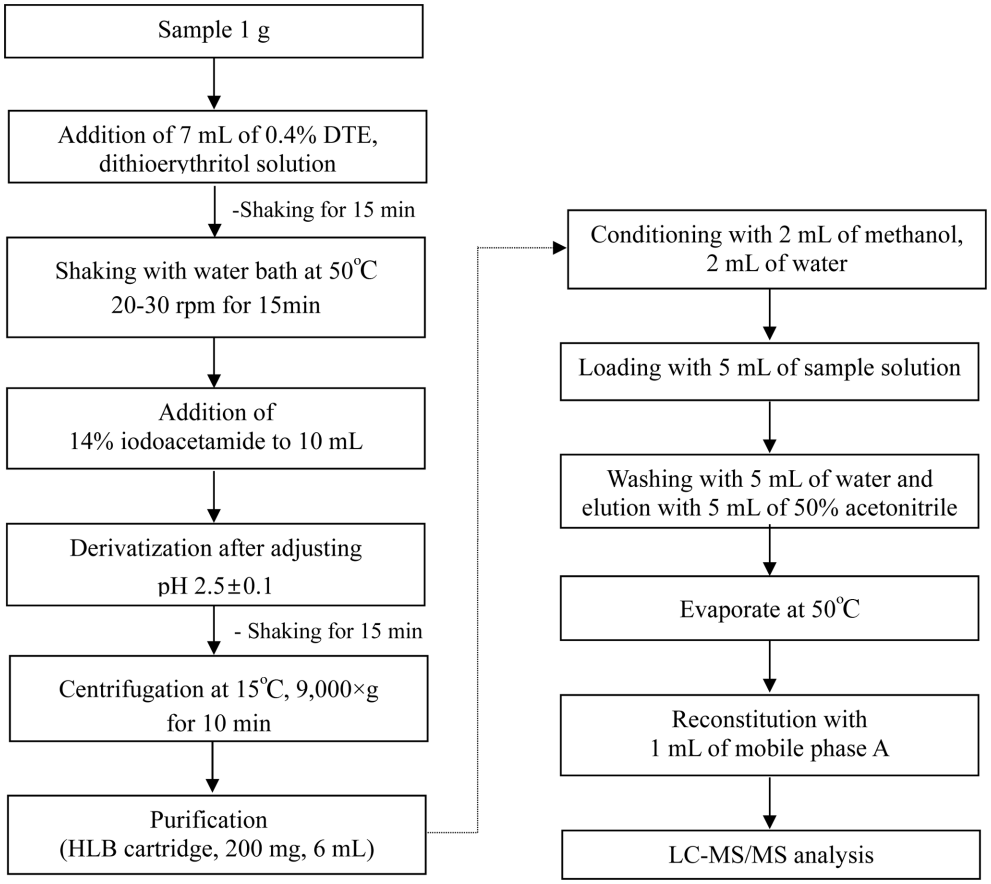

Figure 2. Analytical procedure for ceftiofur (desfuroylceftiofur acetamide) in samples.

(250 mL), and 14% iodoacetamide solution; iodoacetamide (7 g) was dissolved in 0.025 M phosphate buffer solution (50 mL).

Sample preparation

The homogenized sample (1 g) was precisely weighed and placed into a 50 mL centrifuge tube. 7 mL of 0.4% dithioerythritol solution was added and mixed with the sample in a water bath at 50°C for 15 min. After the sample was cooled to room temperature, 14% iodoacetamide solution was added as appropriate to adjust the volume to 10 mL and homogenized for 15 min. The sample was standed at room temperature for 30 min for the derivatization of the analytes. Thereafter, the pH of the mixture was adjusted to 2.5 ± 0.1 with a phosphoric acid solution and centrifuged at 9,000×*g* for 10 min. The supernatant was acquired as the extract and cleaned by using SPE, HLB cartridges (conditioning: 5 mL of methanol, 5 mL of water). After the extract loading (5 mL), the cartridges were washed with 5 mL of water. The extract was eluted with 5 mL of 50% acetonitrile in water and then vacuum-evaporated at 50°C. The residue was then reconstituted with 1 mL of 0.1% formic acid (mobile phase A) solution. The solution was filtered through a 0.2 μm membrane filter (polyvinylidene difluoride, PVDF), and used as a test solution in a glass vial (Figure 2).

LC-MS/MS analysis

A LC-MS/MS (US/Xevo TQ-S, Waters, Milford, MA, USA) was used for the determination of the ceftiofur in the fishery product samples. A reversed phase column, ACQUITY UPLC® BEH C_{18} (2.1 × 100 mm, 1.7 mm, Dublin, Ireland) was used at a column temperature of 40°C. The mobile phase consisted of water containing 0.1% formic acid (v/v) for the mobile phase A, and acetonitrile containing 0.1% formic acid (v/v) for mobile phase B, and applied with an optimized gradient elution. The flow rate of the mobile phase was 0.3 mL/min and the injection volume was 10 μL. To establish the conditions for the mass spectrometer, a standard solution of the desfuroylceftiofur acetamide (100 ng/mL) was used and combined with the mobile phase for analysis. Ionization of the analytes was performed using the positive ion mode of electrospray ionization (ESI) to select the precursor ion. The voltage and the collision energy were tuned to

Table 1. LC-MS/MS parameter for the analysis of ceftiofur (desfuroylceftiofur acetamide).

LC system	Waters, UPLC				
Column	Waters, ACQUITY UPLC® BEH C_{18} (2.1 mm × 100 mm, 1.7 μm)				
Column temperature	40°C				
Injection volume	10 μL				
Flow rate	0.3 mL/min				
Mobile phase	A = 0.1% formic acid				
	B = 0.1% formic acid in acetonitrile				
	Time (min)	A(%)	B(%)		
	0	95	5		
	1	95	5		
	9	65	35		
	10	5	95		
	11	5	95		
	12	95	5		
	13	95	5		
Mass spectrometry	Waters, Xevo TQ-S				
Ionization mode	ESI positive				
Capillary temp.	350°C				
Spray voltage	3.5kV				
Collision gas MRM parameters	Ar				
Desfuroylceftiofur acetamide	Exact mass (m/z)	Precursor (m/z)	ion Confirmation ion (m/z)	Collision Energy (eV)	Retention time (min)
	486.5	487.4	125.2	70	4.4
			167.1	33	
			241.2*	25	

*Quantification ion

optimize the precursor ion and achieve the most abundant product ions. Thus, multiple reaction monitoring (MRM) was established to determine the quantitative and qualitative ions. The LC-MS/MS analysis conditions are shown in Table 1.

Method validation

The established method is validated based on the accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), and recovery according to the Codex guideline CAC/GL-71.[17] Because the maximum residue limits (MRL) of ceftiofur has not been established in fishery products, the target concentration was set to 0.03 mg/kg in accordance with the principle of application of the antibiotic standard in the Food Code. Matrix-matched calibration curves were obtained by calibration standards at different concentrations (0.0075, 0.015, 0.03, 0.06, 0.12 mg/kg), and their linearity was determined. Accuracy and precision were evaluated by measuring the average recovery and coefficient variation (CV, %) through the blank samples spiked with the standard solution causing the concentrations to be 0.5, 1, and 2 times the target concentration (0.03 mg/kg). These values were evaluated by spiking five blank samples for flatfish, eel and shrimp. The concentration levels with the signal-to-noise ratio (S/N ratio) ≥ 3 were defined as LOD, (S/N ratio) ≥ 10 were defined LOQ.

Results and Discussion

Sample preparation

Ceftiofur administed into the animal's body is easily metabolized in a way that thioester (R-S-CO-R') bond and the disulfide (S-S) bond in ceftiofur were converted into desfuroylceftiofur.[14] Desfuroylceftiofur was used the marker residue of ceftiofur in order to analyze ceftiofur level in food matrix. However desfuroylceftiofur may immediately bind to proteins and amino acids and forms other compounds such as desfuroylceftiofur cysteine disulfide, desfuroylceftiofur glutathione and dimers of desfuroylceftiofur.[12] Therefore, this study selected a stabilized form of desfuroylceftiofur to deterimine ceftiofur

residues taking into consideration the unstable nature of desfuroylceftiofur. Then, the thioester bonds and/or the disulfide were cut from proteins in the ceftiofur to convert into desfuroylceftiofur and which was derivatized with iodoacetamide.[15] After that desfuroylceftiofur acetamide was analyzed by LC-MS/MS. These treatments convert those metabolites containing intact β-lactam structure to a single stable derivative of desfuroylceftiofur acetamide. Specifically, this study used a dithioerythritol solution serving as a reducing agent, which has a function in the hydrolysis of ceftiofur or desfuroylceftiofur bound to an aquatic product sample composed primarily of protein. The derivatization process was performed by adjusting the pH to 2.5 because cephalosporins, with β-lactam structures, are stable within the range of pH from 2.5 to 10.[11]

For the cleanup procedure, high speed centrifugation was employed to remove the solidified impurities from liquid layer, and organic phase was purified by using SPE.

It was difficult that the QuEChERS methodology which is been applied to sample preparation a lot lately was used as an analysis method of the ceftiofur because of low recovery and high variation in the CV.[10] Many studies have reported that HLB cartridges can be applied for the purification process according to the solid-phase extraction method by considering the molecular structure of the analyte and the extraction solvent.[16] The HLB cartridge was chosen and used for the water-soluble and liposoluble samples and was employed to analyze both polar and nonpolar target materials. After selectively adsorbing the analyte, impurities were removed by rinsing with water and the analytical method was established by eluting the analyte with 50% acetonitrile. Finally, a PVDF syringe filter was used for extracts purification and to protect the instrument.

Optimization of instrumental analysis condition

Many analytical studies have been developed for veterinary drug residues in fishery products using LC-MS/MS which is the most commonly used for detecting multi-residues of compounds in food simultaneously due to high sensitivity and selectivity.[13] Therefore, LC-MS/MS was used to analyze the ceftiofur residues. Three kinds of C_{18} column (Waters BEH, Agilent Eclipse Plus, and Waters Xbridge) were examined. The Waters BEH C_{18} column affords superior separation and intensity. For the mobile phase, 0.1% formic acid in acetonitrile and methanol were compared. The results showed that 0.1% formic acid in acetonitrile gave sharper peaks and highest intensity. The optimum MRM conditions were established by confirming three or more product ions. We optimized the MS parameter by combining a standard solution (10 μL/min) with the mobile phase into the mass spectrometer. Ionization method for instrumental analysis was the positive mode of electric spray afforded intense signals. The mass spectrum of the precursor ion was identified in the full scan mode and the proton (H^+) was attached to the mass value (486.55, exact mass) of desfuroylceftiofur acetamide to form a precursor ion with $[M + H]^+$ and m/z 487.4. The m/z 241.2, a quantification ion, as well as m/z 167.1 and m/z 125.2, qualification ions were obtained by controlling the collision energy during MS/MS analysis.

Validation

The proposed method was validated based on the CODEX guideline (CAC/GL-71) to evaluate its performance.[17] To verify the selectivity, specificity, and linearity of the analytical method, chromatograms of the untreated samples, standard solutions, and the spiked samples in flatfish, eel, and shrimp were used (Figure. 3). The results indicate that no interfering substances with the same retention time as ceftiofur were detected in the blank samples. Thus, it was confirmed that this method for analyzing ceftiofur has high resolution and selectivity.

The LOD and LOQ were 0.0007 and 0.002 mg/kg based on S/N ratio in fish samples. The LOQ of this study is lower than the LOQ (0.009 mg/kg and 0.005 mg/kg) suggested by previous studies.[6,7] Matrix-matched calibration curves were made with blank samples spiked with standard solution to determine the linearity. The calibration curve was drawn at the levels (0.0075, 0.015, 0.03, 0.06, and 0.12 mg/kg) based on the 0.03 mg/kg in accordance with the Korean Food Code. The correlation coefficient was above 0.99 and satisfied when compared to an $r^2 > 0.98$, which is recommended by CODEX.

To evaluate the accuracy and precision of this method, the recovery test was repeated five times for flatfish, eel, and shrimp samples at three different concentrations (0.015, 0.03, and 0.06 mg/kg). As a result, the accuracy (recovery) and precision (variation coefficient) of ceftiofur in the aquatic product samples were 80.6% to 105% and

Table 2. Validation results (n = 5) for the analytical method of ceftiofur (desfuroylceftiofur acetamide) in fishery products.

Concentration (mg/kg)	Eel		Flatfish		Shrimp	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
0.015	105	4.26	86.2	5.19	103	1.95
0.03	80.6	6.31	97.1	3.47	95.7	3.28
0.06	104	1.33	95.8	3.06	98.6	3.40

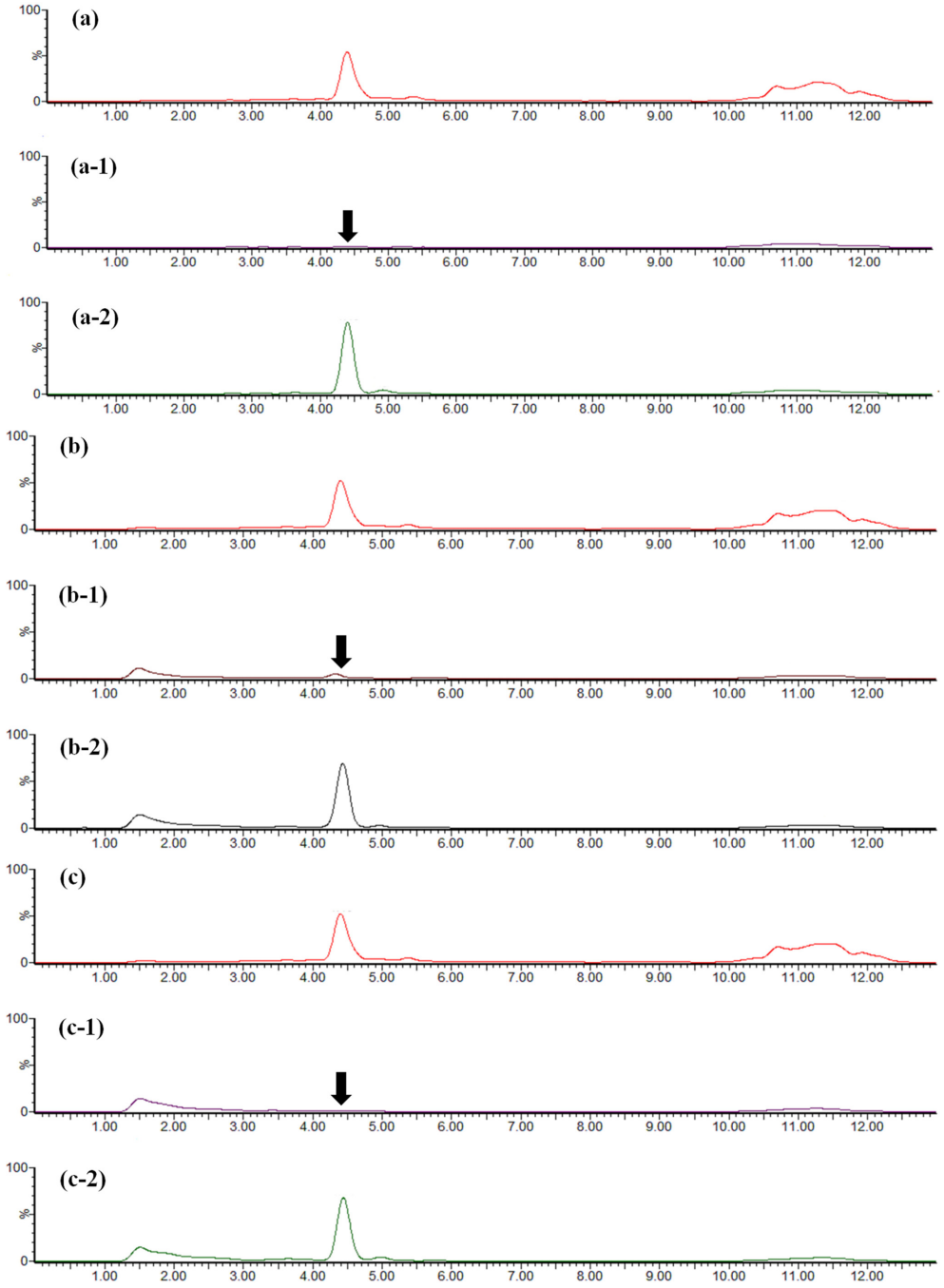

Figure 3. LC-MS/MS chromatograms of ceftiofur (desfuroylceftiofur acetamide, m/z 487.4→241.2) in eel, flatfish and shrimp samples. Chromatogram of desfuroylceftiofur acetamide standard at 0.03 mg/L (a)–(c), blank eel (a-1) and fortified eel sample at 0.03 mg/kg (a-2). Chromatogram of blank flatfish (b-1) and fortified flatfish sample at 0.03 mg/kg (b-2). Chromatogram of blank shrimp (c-1) and fortified shrimp sample at 0.03 mg/kg (c-2).

1.33% to 6.31%, respectively (Table 2). In this case, both the accuracy and precision meet the CODEX Guidelines. Also, the inter-lab test for three labs was conducted to evaluated the ruggedness of which results was average recovery of 97.8~95.2% and average CV of 6.17~9.31% in fishery products at 0.01 and 0.02 mg/kg. Additionally the recovery conducted in other lab was 70% and 85.1% in flatfish at 0.01 and 0.05 mg/kg respectively. These confirm the adequacy of this test method for veterinary drug residues.

Application to real samples

To evaluate the applicability of the proposed method, the fishery products samples were analyzed using proposed method. The fish and shrimp samples were collected between May 2016 and November 2016 from 403 Korean domestic markets to evaluate the applicability of the proposed method. As a result, ceftiofur was only detected in one seabream sample (0.001 mg/kg). In addition, the proposed method was used for the residue deletion study of ceftiofur in fish sample (olive flounders) by national institute of fisheries science. The study was conducted in serum and muscle of olive flounders with the dose levels and water temperature. The residue level of ceftiofur was estimated from that of desfuroylceftiofur acetamide. In results, the maximum blood concentration of ceftiofur was achieved within 30 minutes and then they were quickly lost to a concentration of less than 0.1 mg/kg within 24 hours in muscle under optimum water temperature, 23°C after dosing the effective concentration, 10 mg/kg bw of ceftiofur. In this study, desfuroylceftiofur acetamide can be used as a representative biomarker for quantification, with regard to safety management and the misuse of ceftiofur that can remain in aquatic products. Therefore, the proposed method would be suitable for determining residue of ceftiofur. Further investigation is needed to determine metabolites of ceftiofur in fishery products.

Conclusions

The present method have been developed and validated for the determination of ceftiofur including its metabolites in fishery products. The desfuroylceftiofur acetamide was used for stable form of ceftifor and desfuroylceftiofur. Thus, the proposed method included a hydrolysis and a derivatisation step to convert desfuroylceftiofur acetamide form using a dithioerythritol solution as a reducing agent and extraction solution and iodoacetamide solution for derivatization. Thereafter, SPE cartridge was used for purification. As a result, the method was validated in fishery products on linearity, accuracy, precision, ruggedness, LOD, LOQ, specificity, results of which were consistent with the Codex guidelines. The accuracy (recovery) and precision (coefficient variation) of ceftiofur in eel, flatfish, and shrimp were 80.6% to 105% and 1.33% to 6.31%, respectively. Therefore, this analytical method is reliable to determine ceftiofur and can further contribute to the safety management of fishery products.

Acknowledgments

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