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Development of a Rapid and Sensitive SPE-LC-ESI MS/MS Method for the Determination of Chloramphenicol in Seafood

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A method based on liquid chromatography−tandem mass spectrometry was developed and validated for the qualitative and quantitative detection of chloramphenicol (CAP) in seafood samples. The analysis of CAP residues in seafood is important because CAP can cause serious acute reactions in humans, including aplastic anemia and leukemia. The proposed methodology includes a cleanup solid-phase extraction procedure with high recovery efficiency (>90%). Chromatographic separation of CAP and the internal standard (IS) was carried out on a C_{18} column, followed by mass spectrometric detection using electrospray ionization in the negative-ion mode. The precursor/product ion transitions 321→257 (CAP) and 354→290 (IS) were monitored. Statistical evaluation of this multiple reaction monitoring mass spectrometric procedure reveals good linearity, accuracy, and inter- and intraday precisions. The limit of detection was 0.1 ng/mL, and the limit of quantification for CAP in seafood samples is 0.02 μg/kg. Application in seafood samples allowed the detection of CAP in low parts per billion levels.

KEYWORDS: Chloramphenicol; seafood; liquid chromatography−tandem mass spectrometry; triple quadrupole mass spectrometer; solid-phase extraction

INTRODUCTION

Antibiotics are often used in agriculture, as feed additives, and also as prophylactic and therapeutic agents to avoid and cure animal sickness, respectively. The widespread use of veterinary drugs, and particularly of antibiotics, in food-producing animals represents a potential hazard for human health. The main risk arises from the danger of increasing bacterial resistance and the appearance of allergic reactions to antibiotics. To protect human health from the potentially harmful antibiotic residues, the European Union (EU) has established safe maximum residue limits (MRLs) for substances authorized for use as veterinary drugs in food-producing animals (Council Regulation EEC 2377/90). Chloramphenicol (CAP) is a broad spectrum antibiotic isolated in 1947 from *Streptomyces venezuelae*. CAP has been used since the 1950s to combat serious human infections. It is effective against Gram-positive and Gram-negative cocci and bacilli (including anaerobes), *Rickettsia*, *Mycoplasma*, and *Chlamydia*. Nevertheless, CAP has been shown to be harmful for humans because it can cause aplastic anemia, which could lead to leukemia (*1*−*5*). The possible mechanism of action involves the biotransformation of CAP by intestinal bacteria to dehydrochloramphenicol and subsequent nitroreduction (*6*). The metabolite formed can cause single-strand breaks of DNA (*7*, *8*) to bone marrow (*9*−*11*) and induction of faulty chromatid exchange (*12*, *13*). The CAP-induced aplastic anemia is irreversible and not dose-dependent. For these reasons the International Agency for Research on Cancer (IARC) in 1990 considered CAP as "probably cargignogenic to humans" (group 2A). CAP can also induce gray baby syndrome, a pathologic situation that could be fatal up to 40% of the time if not properly treated. It can affect newborn babies as it can pass through the placental barrier and is excreted in breast milk.

In view of the highly toxic effects of CAP to humans, it has been banned as a veterinary drug within the United States and EU. Recently the Health and Consumer Protection Directorate of the EU has informed the member states of the presence of traces (parts per billion levels) of CAP in frozen seafood (shrimps, crabs, and crawfish) imported from East Asia. It is, therefore, important to develop a rapid and sensitive method for the detection and confirmation of CAP residues in seafood samples at sufficiently low levels. A number of LC methods have been reported (*14*−*16*) in the literature for the determination of CAP in seafood samples using either a UV or a UV-

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PDA detector. However, these techniques do not fulfill the criteria established by Commission Decision 93/256/EEC for methods used for regulatory purposes, as this states "methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods" (*17*). Various analytical methods have been developed for the determination of CAP by using gas chromatography employing either electron capture (*18*, *19*) or mass spectrometric detection as trimethylsilyl derivatives (*20*−*22*). However, derivatization techniques, in general, are not preferred for residue analysis because they are time-consuming and not reproducible in trace levels. Application of LC-MS to the detection of CAP in swine tissue (*23*), egg and honey (*24*), and water (*25*) has been reported, showing the advantage of high specificity of a mass spectral method without the difficulties arising from the required analyte derivatization. Using a tandem MS-based technique can lead to further improvement in the specificity and selectivity of the CAP residue detection method. This paper describes the development and validation of a rapid, sensitive, and specific analytical method combining a solid-phase extraction (SPE) procedure with liquid chromatography−electrospray ionization (ESI) (*26*) tandem mass spectrometry (LC-ESI MS/MS) for the detection, identification, and quantification of CAP in seafood samples at low concentration levels.

MATERIALS AND METHODS

Chemicals and Stock Solutions. Chlorampenicol (CAP, purity 99%) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and thiamphenicol (TAP) was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium sulfate (anhydrous) pesticide grade was obtained from Riedel-de Haen (Seelze, Germany). Glasswool pesticide grade was obtained from Alltech (Deerfield, IL). All solvents were of HPLC grade and purchased from Merck (Darmstadt, Germany). For the SPE procedure Oasis HLB (hydrophilic lipophilic balance), 3 cm^3, 60 mg cartridges (Waters, Milford, MA) were used. Stock standard solutions of CAP and internal standard (IS) TAP were prepared at the 1 mg/mL level in methanol and were kept refrigerated at −5 °C. Working solutions of CAP were prepared in methanol−water (10:90 v/v) at six concentration levels, 1, 5, 10, 25, 50, and 100 ng/mL, with TAP as internal standard at a concentration 25 ng/mL.

Sample Preparation. Shrimp samples were kept frozen at −20 °C. Homogeneous sample preparation was performed after 100 g of peeled sample had been grounded in a commercial blender. Ten grams of homogeneous sample was blended with 100 mL of ethyl acetate. The resulting suspension was filtered through a funnel, containing a loose plug of glasswool and 50 g of anhydrous sodium sulfate. The filtrate was rotary evaporated to dryness, and the residue was dissolved in 50 mL of HPLC water and applied to Oasis HLB cartridges for the SPE procedure.

Extraction Procedure. Each SPE cartridge was conditioned with 1 mL of methanol (MeOH) followed by 1 mL of H_2O, and then the sample was applied, washed with 1 mL of H_2O, dried, and eluted with 1 mL of MeOH. The eluate was evaporated to dryness under a gentle stream of nitrogen and reconstituted into 200 μL of MeOH/water (10:90 v/v) for LC-MS analysis. The MeOH/water solution gave significantly better chromatographic peak shape than the pure MeOH due to solvent focusing.

Extraction Recovery Experiments. Recovery experiments were carried out at two fortification levels by adding certain amounts of CAP (200 μL of 0.05 μg/mL and 200 μL of 0.001 μg/mL) and a standard amount of internal standard TAP (200 μL of 25 ng/mL) to 10 g of homogenized blank shrimp samples. As blank sample was considered a shrimp sample obtained from the "open sea", which underwent all of the extraction procedure and showed no detectable levels of CAP. Five spiked shrimp samples were subjected to the proposed analysis procedure (homogenizing, SPE cleanup, and LC-MS/MS analysis). Each concentration level was analyzed in replicates of five on three different days. The recovery of the method was assessed as the ratio of peak area A_{CAP}/A_{TAP} (where A_{CAP} and A_{TAP} are the integrated areas of CAP and TAP, respectively) of the spiked sample to that of the standards. The reproducibility of the extraction procedure was determined as percent relative standard deviation (% RSD).

Instrumentation. All LC-MS and LC-MS/MS analyses were performed on a Finnigan TSQ triple-quadrupole LC-MS/MS system. Chromatographic separation was performed on a Finnigan Spectra system P4000 quaternary pump equipped with a Rheodyne 7725i injector with a 20 μL loop and a Finnigan on-line degasser. The chromatographic system was coupled to a Finnigan Spectra system UV2000 dual wavelength detector. A fully end-capped MS grade RP-C_{18} column (Xterra 150 × 2.1 mm 3.5 μm i.d.) was used preceded by an Xterra C_{18} precolumn and a particle filter (Upchurch Scientific 0.5 μm stainless steel frit). An isocratic elution program was used for the separation of the two substances. The mobile phase consisted of a 60:40 (v/v) mixture of an aqueous solution of NH_4OH (2%) and acetonitrile. A flow rate of 0.2 mL/min was maintained while all analyses were conducted at ambient temperature. The degassing of the solvent was achieved through filtering from a Millipore filter and degassed on-line through a semipermeable membrane. It should be noted that UV detection at the absorption maxima 230 and 280 nm of the two analytes provided a significantly higher detection limit than the desired analytical levels for the specific analysis; that prompted the use of a more sensitive detection technique. Therefore, a mass spectral detection was employed using a Finnigan TSQ triple-quadrupole mass spectrometer equipped with an API2 ESI ion source. Full-scan mass spectra in the negative-ion mode were obtained by scanning the first quadrupole (Q1 scan) in the 160−600 amu region with a scan time of 1 s. Typical source parameters used were as follows: capillary temperature, 350 °C; spray voltage, 4.5 kV; capillary voltage, −20 V; tube lens voltage, −95 V. The mass spectral response for CAP and TAP was optimized by infusing the compounds with a Harvard Apparatus Pump II syringe pump at 10 μL/min and tuning the deprotonated molecular ions at m/z 321 (CAP) and 354 (TAP). Argon was used as collisionally induced dissociation (CID) gas, and the collision cell (Q2) pressure was maintained at 2.1 mTorr, which was found to be critical for the stability of the signal. A collision voltage of 21 V was employed in the analyses in order to facilitate effective fragmentation of the selected precursor ions for both CAP and TAP. Multiple reaction monitoring (MRM) of certain ion transitions was used to achieve sensitive and specific compound identification. It should be noted that a source CID voltage offset of 10 V was necessary to minimize the formation of adduct ions with the solvent and yield maximum abundance for the deprotonated molecular ions. The signals of the deprotonated molecular ions $[M - H]^-$ at m/z 321 (CAP) and 354 (TAP) were selected in the first quadrupole and then subjected to CID fragmentation in the second quadrupole. The product ions at m/z 257 and 290 were monitored via the third quadrupole for CAP and TAP, respectively. The LC-MS/MS analysis was controlled by Xcalibur v 1.1 software through a Finnigan SN4000 controller and a local Ethernet LAN.

RESULTS AND DISCUSSION

Cleanup Procedure. CAP is a polar compound (**Figure 1A**) with a low solubility in organic solvents, which makes it difficult to extract and concentrate its residues from seafood samples. Furthermore, the formation of heavy emulsion is observed during extraction with water. On the contrary, extraction of the homogenized sample with ethyl acetate did not give a heavy emulsion and resulted in cleaner extracts after solvent evaporation and dilution with water. In the SPE cleanup procedure, HLB extraction cartridges were used because they show good and reproducible recovery for both polar and nonpolar compounds from complex matrices, possess high capacity, and do not exhibit loss of recovery due to drying, compared to the commonly used C18 cartridges. TAP was used as internal standard on the basis of its structural similarity to CAP (**Figure 1B**). The two compounds are expected to exhibit high recoveries with the SPE procedure, as well as similar mass spectral fragmentation

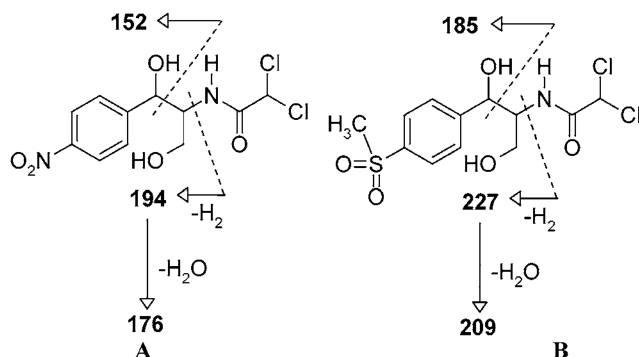

Figure 1. Chemical structures of (**A**) chloramphenicol (CAP) and (**B**) thiamphenicol (TAP) used as internal standard. The proposed fragmentation pathways resulting in the product ions present in the respective tandem mass spectra are also depicted.

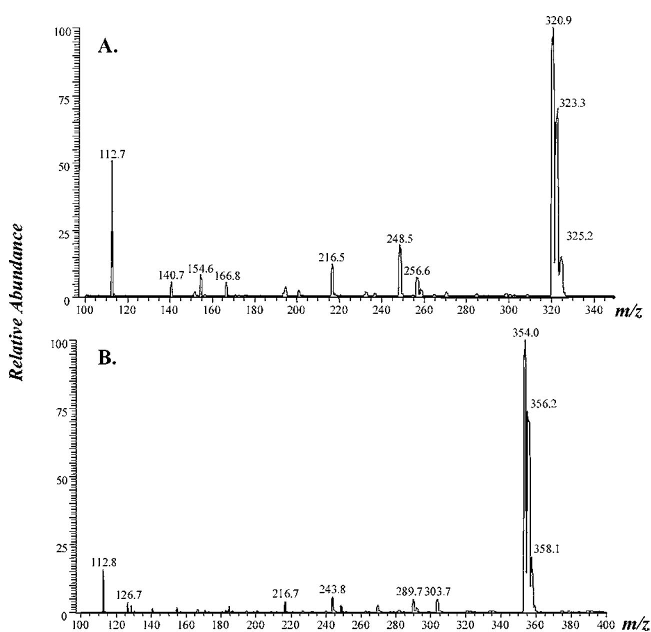

Figure 2. Negative-ion ESI mass spectra of (**A**) CAP and (**B**) TAP obtained by infusion of the standards.

patterns in the MS/MS experiments. The blank shrimp sample did not provide any response in the relevant retention times during the LC-MS/MS analysis.

Liquid Chromatography—Tandem Mass Spectrometry. The concentrated shrimp extracts were analyzed by LC-MS using ESI in the negative-ion mode. The negative-ion ESI mass spectra of CAP and TAP standards exhibited intense deprotonated molecular signals at *m/z* 320.9 and 354.0, respectively (**Figure 2**). The unique isotope pattern due to the presence of two chlorine atoms was evident from the (M + 2) and (M + 4) satellite signals, which accompanied the deprotonated molecular ions. In the product ion mass spectra of the aforementioned ions the predominant ions at *m/z* 151.8 (**Figure 3A**) and 184.7 (**Figure 3B**) corresponded to the loss of the (C_2H_3OH-$NHCOCHCl_2$) group as shown in the fragmentation pattern in **Figure 1**. Other product ions present in the MS/MS spectra of CAP were at *m/z* 194 (loss of $NH_2COCHCl_2$), *m/z* 176 (loss of H_2O from *m/z* 194), and *m/z* 257 (loss of HCl and CO). A similar fragmentation pattern is observed in the MS/MS spectrum of TAP (**Figure 3B**), with the product ion assignments shown in **Figure 1B**. The assignment of the *m/z* 257 ion is corroborated by the observed Cl isotopic pattern of (∼3:1), revealing the presence of only one chlorine atom. The transitions

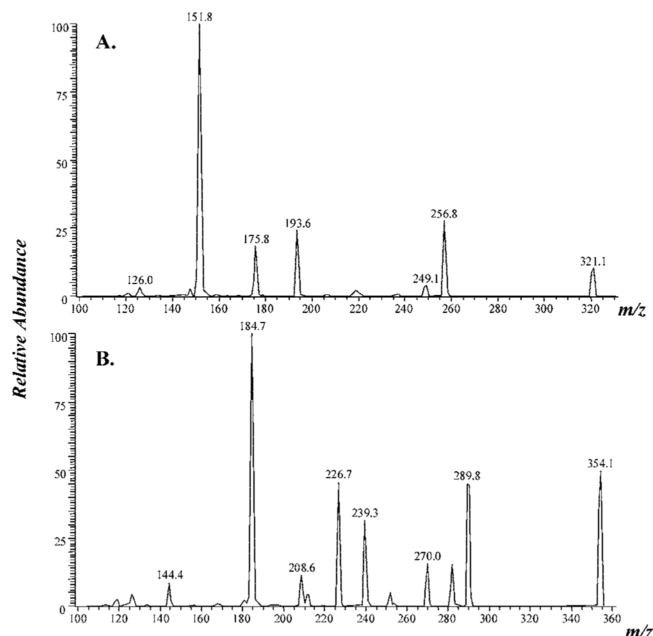

Figure 3. Product-ion mass spectra of (**A**) *m/z* 321 $[M-H]^-$ for CAP and (**B**) *m/z* 354 $[M-H]^-$ for TAP. The proposed fragmentation is shown in **Figure 1**.

321→257 (CAP) and 354→290 (IS) of the ^{35}Cl-containing deprotonated molecular ion signals were selected for the sensitive and specific compound identification in the MRM mode.

Figure 4A shows the total ion (TIC) chromatogram of a CAP/TAP standard solution containing 50 ng/mL CAP and 25 ng/mL TAP; the retention times are 3.7 and 5.5 min, respectively. The MRM chromatogram of the selected precursor/product ion transitions is depicted in **Figure 4B,C**. The combination of chromatographic separation and MRM detection affords the high specificity of the developed assay. A representative MRM chromatogram of a shrimp sample spiked with the internal standard TAP (25 ng/mL) and a standard solution of CAP (1 ng/mL) is shown in **Figure 5A,B**, respectively.

Quantification. The quantification of CAP was performed by LC-MS/MS in the MRM mode using TAP as internal standard. A greater accuracy in the quantification process was achieved by integrating at least 100 scans per chromatographic peak. That was accomplished by selecting a low scan time (only 0.3 s) in order to allow the integration of a high number of scans for the relatively narrow (peak width of 30 s) chromatographic peaks.

The proposed method was validated for its linearity, precision, accuracy, and sensitivity.

Method Validation. *Linearity.* The MRM method for the quantification of CAP in shrimp samples was checked for linearity using six different concentrations of CAP in the range of 1−100 ng/mL. Five samples at each concentration of CAP were analyzed by LC-MS/MS, and the results were used to construct the calibration curve (**Table 1**). Peak integration of the extracted MRM ion chromatograms and the calculation of concentrations were performed using Xcalibur software. Linear regression analysis was performed using no weighting factors. The 0,0 point was neither included nor forced at the determination of the calibration equation. The calibration curve was expressed by

$$y = 73.86 \times 10^{-3} (\pm 0.48 \times 10^{-3})x + 0.074 (\pm 0.024) \quad (1)$$

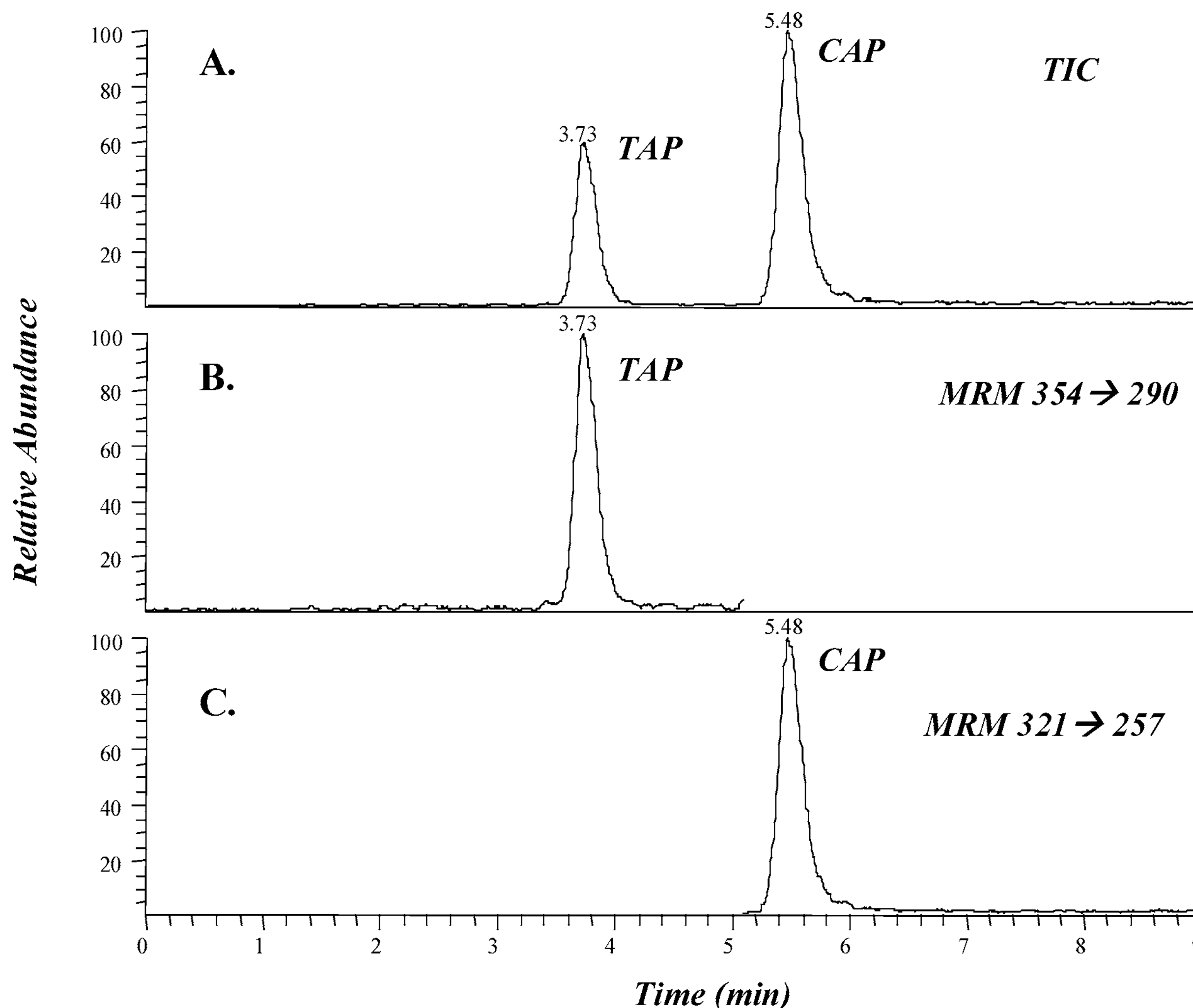

Figure 4. (**A**) Total ion chromatogram of a 2:1 CAP/TAP standard mixture (50 ng/mL/25 ng/mL); MRM chromatograms of (**B**) TAP and (**C**) CAP showing the monitored precursor/product ion transitions.

Table 1. Calibration Data for the Determination of CAP by LC-MS/MS Using TAP as the Internal Standard at a Concentration of 25 ng/mL

concn level (ng/mL)	% RSD[a] ($n = 5$)	mean calcd concn (ng/mL)	% ER[b]
1.0	6.9	0.95	−5.0
5.0	6.2	5.15	3.0
10.0	7.1	10.11	1.1
25.0	1.5	25.27	1.1
50.0	3.1	49.98	−0.04
100.0	1.6	100.26	0.3

[a] % RSD = relative standard deviation. [b] % ER = relative standard error.

where y is the ratio of peak areas [CAP]/[TAP] and x is the concentration of CAP (in ng/mL) and showed an excellent correlation coefficient ($r^2 = 0.9992$). The analytical system repeatability was established by replicate injections ($n = 5$) of standard solutions at six concentration levels (**Table 1**). The RSD values of the peak areas [CAP]/[TAP] at each concentration level did not exceed 7.1%. The accuracy of the measurement expressed as the percent relative standard error (% ER) was estimated. The concentrations of CAP in the standard solutions were back-calculated from the calibration curve, and the % ER was calculated as [(mean calculated concentration − nominal concentration)/nominal concentration] × 100 (**Table 1**). The determined % ER values for all standard solutions in **Table 1** ranged from +1.1 to −5%.

Table 2. Accuracy and Precision Data of the Method[a]

	fortification level (μg/kg)	
μ	0.02	1.0
$\bar{x}$	0.021	0.92
S_r	0.0013	0.066
% RSD_r ($n = 5$)	5.9	7.2
% RSD_R ($n = 15$)	7.1	8.9
% ER	7.1	−8.0
% R	91	116
% RSD	2.9	2.5

[a] μ = true value of the concentration; $\bar{x}$ = average measured value; S_r = standard deviation of the average value; RSD_r = intraday precision; RSD_R = interday precision; % ER = relative standard error; % R = recovery of the method; % RSD = relative standard deviation of the recovery.

Precision and Accuracy. Precision and accuracy data for spiked shrimp samples are reported in **Table 2**. Shrimp samples spiked at two different levels, a low level of 0.02 μg/kg and a high of 1 μg/kg, were analyzed in replicates of five on three different days. The internal standard (TAP) was added at a concentration of 0.5 μg/kg. The concentration of the spiked samples was calculated from the calibration curve, and these data were used for the assessment of the intraday (% RSD_r) and interday (% RSD_R) precisions. The overall precision of the method expressed as the relative standard deviation (% RSD) was better than 8.92%. For both concentration levels (0.02 and 1 μg/kg) the % RSD_r and % RSD_R ranged between 5.88 and

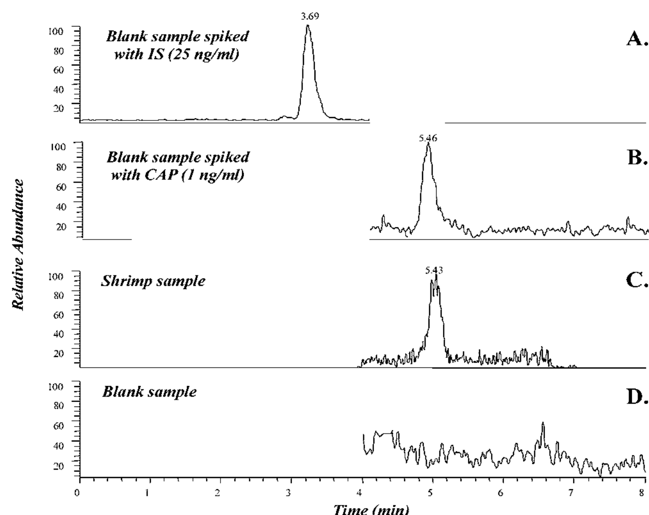

Figure 5. MRM chromatograms of (**A**) blank shrimp sample spiked with the internal standard (TAP, 25 ng/mL), (**B**) blank shrimp sample spiked with CAP (1 ng/mL), (**C**) imported shrimp sample showing a detectable amount of CAP, and (**D**) blank shrimp sample.

7.21 and between 7.05 and 8.92, respectively. The proposed method is under statistical control, as the interday precision did not differ significantly from the intraday precision (**Table 2**).

The accuracy of the proposed method, expressed as the % ER, was evaluated at the two concentration levels of 0.02 and 1 μg/kg of spiked samples. The % ER values obtained were 7.05% for 0.02 μg/kg and −8.03% for 1 μg/kg, which qualify the method as accurate enough for monitoring the CAP levels in seafood samples.

Extraction Recovery. The recovery of the method was expressed as the ratio [CAP]/[TAP] after the SPE to that of the unextracted standard sample, that is, $([CAP]/[TAP])_{SPE}/([CAP]/[TAP])_{unextracted}$, with the numerator being the average value of five replicates. The mean recoveries of CAP at the two fortification levels of 0.02 and 1 μg/kg were 91.18% (% RSD = 2.94) and 116.3% (% RSD = 2.45), respectively (**Table 2**).

Limit of Detection (LOD) and Limit of Quantification (LOQ). Instrumental detection (LOD) and quantification limits (LOQ) were estimated in terms of the baseline noise. The LOD was defined as the CAP concentration yielding signals with a signal-to-noise (S/N) ratio of 3:1, whereas the LOQ was defined as the CAP concentration yielding signals with a S/N ratio of 10:1, that is, peak height 10 times the baseline noise. Therefore, the LOD and LOQ values estimated in this way were 0.1 and 0.33 ng/mL, respectively. The lowest concentration of the analyte in the shrimp sample that can be determined with acceptable precision and accuracy can be considered the LOQ of the method. The validation data shown in **Table 2** imply that the LOQ of CAP in shrimp samples is 0.02 μg/kg. The mean recovery value in that level (91.18%) and the interday precision of the method (% RSD_R = 7.05) are acceptable according to the EPA provisions (*27*). The representative MRM chromatogram obtained from shrimp sample fortified with CAP at the LOQ level (1 ng/mL) is presented in **Figure 5B**.

Application of the Method. The performance of the proposed method was tested on four shrimp samples imported from East Asia, Africa, and South America. Analysis of shrimp samples imported from East Asia gave the MRM chromatogram shown in **Figure 5C**, where a detectable amount of CAP was present. The amount of CAP present in that sample was estimated at low parts per billion (ppb) levels (<0.1 μg/kg). **Figure 5D** shows the MRM chromatogram of a blank sample.

Conclusions. We have described an LC-MS/MS method for the measurement of CAP residues in seafood samples by monitoring the precursor/product ion transitions 321→257 (for CAP) and 354→290 (for the internal standard, TAP). The developed method includes a SPE step with very high recovery efficiency (91−116%) for the antibiotic compound. The method has been validated and has been shown to be accurate, precise, sensitive, and most importantly compound specific due to the combined chromatographic separation and MRM mass spectral detection. Use of this method allowed the detection of CAP in shrimp samples imported from East Asia, with an estimated amount of CAP at low parts per billion (ppb) levels (<0.1 μg/kg). It thus appears that the developed LC-MS/MS assay has the required sensitivity for measuring residues of CAP in seafood samples. This method could serve as a basis toward the development of an LC-MS/MS method for detecting and measuring CAP and possibly other structurally related antibiotic residues in other food matrices.

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