ELISA screening and liquid chromatography-tandem mass spectrometry confirmation of chloramphenicol residues in chicken muscle, and the validation of a confirmatory method by liquid chromatography-tandem mass spectrometry

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ABSTRACT In the period December 2008 to August 2009, 180 chicken meat samples, including 90 thigh and 90 breast meats in Bursa province, Turkey, were collected. The determination of chloramphenicol (CAP) residues in the samples was screened by ELISA, and a confirmatory method based on liquid chromatography coupled with tandem mass spectrometry was described and validated. The ELISA screening of the samples was performed after extraction with ethyl acetate and defatting with n-hexane. The results showed that 15 (8.3%) of the chicken meat samples were positive for CAP residues from 12.64 to 226.22 ng/kg, with a mean of 45.32 ng/kg. Confirmatory analysis of the results from ELISA was practiced after an extraction with ethyl acetate. Chromatographic seperation was carried out by using a Synergy MAX-RP 80A column and the mixture of acetic acid-water as a mobile phase. The mass spectral acquisition was done in the negative-ion mode applying selective reaction monitoring with the following ions (mass-to-charge ratio, *m/z*): *m/z* 321 → 152 and *m/z* 321 → 194 for CAP. By liquid chromatography-tandem mass spectrometry, CAP was confirmed in 2 of 15 ELISA positive samples and 1 of 45 negative samples, with concentration levels that varied between 150 and 361 ng/kg. The method was validated according to Commission Decision 2002/657/EC. The calibration curves were linear with a typical r^2 value of 0.9966. The recovery values ranged from 97.3 to 104.0% and within-laboratory repeatability was lower than 5%. The decision limit was 0.10 μg/kg and detection capability was 0.11 μg/kg. To evaluate the presence of CAP residues, this method was successfully implemented in chicken meat samples.

Key words: chloramphenicol, chicken muscle, ELISA, liquid chromatography-tandem mass spectrometry, validation

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INTRODUCTION

Chloramphenicol (**CAP**) is an effective antibiotic widely used in the past to treat several diseases in humans and animals (Rocha Siqueira et al., 2009). In human medicine, however, its use is limited to the treatment of typhoid fever, bacterial meningitis, and for the treatment of conjuctivitis because it is often associated with serious side effects, such as the development of aplastic anemia, resulting in the failure of bone marrow to produce blood cells (Ferguson et al., 2005). In veterinary medicine, CAP has been shown to be a highly effective and well-tolerated antibiotic, as the potentially fatal side effects in humans have not been reported in animals (Guy et al., 2004). However, illegal use of this antibiotic can increase the risk of introducing harmful residues into the human food chain (Penney et al., 2005; Chen et al., 2011). There are many concerns regarding the human consumption of food products contaminated with drug residues. Among these is the risk of chemical poisoning or allergic reaction. In addition, the overuse of antibiotics in livestock could lead to the development of resistant bacterial strains, the transmission of which to humans will result in a significant decrease in the efficacy of antibiotics (Ferguson et al., 2005; Nisha, 2008).

Even low doses of administrated CAP may result in residues in edible tissues from treated food-producing animals; therefore, consumers of milk, meat, aquaculture products, honey, and eggs may be exposed to potentially harmful levels of drug residues (Rocha Siqueira et al., 2009). For these reasons, it has been prohib-

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ited from use in food-producing animals in the European Union (**EU**) member states, the United States of America, Turkey, and many other countries. No maximum residue limit (**MRL**) has been established for CAP in animal-derived foods, as its toxic effects are not dose dependent. The EU has defined a minimum required analytical methods performance limit (**MRPL**) for CAP in food of animal origin at a level of 0.3 μg/kg (European Commission, 2003). In addition, CAP is included in Annex IV of Council Decision 2077/90, which comprises the drugs with an established zero-tolerance level in edible tissues (Council of the European Communities, 1990). Nevertheless, this antibiotic is still illegally used in livestock production.

Thus, specific and sensitive analytical methods are required for concrete monitoring of CAP at residual levels in animal foods. Several different methods for determination of CAP residues in animal tissues have been reported, such as liquid chromatography (**LC**; Tajik et al., 2010), gas chromatography (GC; Zhang et al., 2006), and immunoassay (Mehdizadeh et al., 2010 Tajik et al., 2010). However, according to the Commission Decision 2002/657/EC (European Commission, 2002), the confirmation of suspect positive samples must be carried out by mass spectrometry (**MS**) coupled to adequate chromatographic separation. This is the most reliable analytical method for the unambiguous confirmation of zero-tolerance residue limit substances in products of animal origin. Several authors have reported the analysis of CAP by liquid chromatography-tandem mass spectrometry (**LC-MS/MS**) in chicken muscle (Mottier et al., 2003; Rønning et al., 2006; Zhang et al., 2008; Rocha Siqueira et al., 2009), seafood (Impens et al., 2003; Mottier et al., 2003; Santos et al., 2005; Rønning et al., 2006), milk (Nicolich et al., 2006; Rønning et al., 2006), and honey (Ashwin et al., 2005; Forti et al., 2005; Rodziewicz and Zawadzka, 2007).

In this study, an analytical method for screening and confirmation of CAP residues in chicken meat samples is described. The ELISA was carried out to screen chicken meats, and LC/MS-MS was applied to confirm suspect samples. The method has been validated according to the criteria of the 2002/657/EC Decision.

MATERIALS AND METHODS

Samples

From December 2008 to August 2009, a total of 180 chicken muscle samples (90 thigh and 90 breast meats) were collected in Bursa province, Turkey. Samples were purchased from large supermarkets and smaller units including butcher shops and poulterer shops, and transported to the laboratory immediately after sampling.

ELISA Analysis

To measure the amount of CAP in chicken meats, a commercial ELISA kit (Ridascreen, R1505; R-Biopharm AG, Darmstadt, Germany) was used. The kit had a specificity of 100% for CAP. The detection limit (**LOD**) of the Ridascreen chloramphenicol test was 12.5 ng/kg and the recovery rates were >80% for all samples. The ELISA technique was performed according to manufacturer's instructions. The samples were homogenized using an ultra meat homogenizer (Janke and Kunkel; IKA-Werke GmbH & Co. KG, Staufen, Germany). Afterward, 3 g of the homogenized samples was transferred into a plastic centrifuge tube, mixed with 3 mL of distilled water, and 6 mL of ethyl acetate was added. The suspension was vortexed for 10 min and centrifuged at 3,000 × *g* for 10 min at room temperature (20–25°C). Following centrifugation, 4 mL of ethyl acetate supernatant (corresponding to 2 g of sample) was transferred into a new centrifuge tube and dried at 60°C under a weak stream of N_2. The residue was redissolved in 1 mL of n-hexane and 0.5 mL of the CAP buffer was added to this solution and vortexed for almost 1 min. The solution was centrifuged at 3,000 × *g* for 10 min at room temperature (20–25°C) and 50 μL of the aqueous (upper) layer was used for analysis. The absorbance was measured at 450 nm using an ELISA plate reader (Rayto RT-2100C; Rayto Corp., Shenzhen, China). The concentrations of CAP were calculated according to the percentage of their mean absorbance divided by the absorbance of the maximum binding (B/BO%) using the standard curve. The values were multiplied by the dilution factor (0.25) as suggested by the kit manual.

LC/MS-MS Analysis

Chemicals. The analytical standard CAP was obtained from Riedel-de Haën AG (Seelze, Germany) and CAP-d5, supplied by Cambridge Isotope Laboratories (CIL, Andover, MA) was used as internal standard. All other reagents and solvents were of analytical quality and provided by Merck KGaA (Darmstadt, Germany). Deionized water used in all procedure steps was prepared by the Milli-Q Ultra Pure System (Millipore Corp., Bedford, MA).

Standard Solutions. Stock solutions of CAP were prepared by dissolving the compound in methanol at a concentration of 1 mg/mL. Working standard solutions were made by appropriate dilution of the stock standard solutions with methanol. Working solutions of the internal standard were prepared in methanol at 20 ng/mL. All standard solutions were kept at 4°C.

Sample Preparation. The procedure was performed as described previously (Bogusz et al., 2004), with some minor modifications. Briefly, 2.0 g of homogenized chicken meat sample was weighed into a 50-mL polypropylene centrifuge tube and fortified with 50 μL of the internal standard CAP-d5. Six milliliters of ethyl acetate was added and the mixture was homogenized using a shaker incubator (KS-15; Edmund Bühler GmbH, Hechingen, Germany) at 200 rpm for 15 min. After that, the samples were centrifuged at 4,000 × *g*

for 15 min at 4°C. Five milliliters of upper phase was transferred to another tube, and dried under a nitrogen stream at 40°C. The residues were dissolved in 500 μL of mixture solution (water/methanol, 85/15, vol/vol) and vortexed at 2,000 rpm for 5 min. The volume of sample injected into the LC-MS/MS system was 50 μL.

Instrumental Conditions. The LC-MS/MS analysis was carried out on a TSQ Tandem Gold Triple Quadrapole mass spectrometer (Zivak Technologies, Kocaeli, Turkey). The chromatographic separation was performed on a Synergy MAX-RP 80A column (150 × 2.0 mm inside diameter, 4.0-μm particle size; Phenomenex Inc., Torrance, CA). Mobile phase A consisted of 0.2% acetic acid in water, and mobile phase B was methanol. The flow rate was 0.25 mL/min with a linear gradient at the following conditions: 0 to 2 min with 85% A, 2 to 10 min with 90% A, 10 to 13 min with 90% A and 13 to 20 min with 85% A. The injection volume was set at 50 μL and the column temperature at 40°C.

The mass spectrometer detector was operated in negative-ion mode. The source block temperature was 350°C and the electrospray capillary voltage was −5,000 V. Nitrogen was used as the collision gas. The retention time of CAP and CAP-d5 was 7.95 ± 0.2 min. The collision energies were separately optimized for the selected ion transitions of both CAP and CAP-d5 (Table 1). The data were acquired in the negative selective reaction monitoring (SRM) mode, using the following transitions (mass-to-charge ratio, $\boldsymbol{m/z}$): m/z 321 → 152 and m/z 321 → 194 for CAP and m/z 326 → 157 for CAP-d5.

Calibration Curves. Quantification of CAP was carried out using matrix-matched calibration curves. For preparation of matrix-matched calibration standards, 50, 100, 150, and 200 μL of the 0.1 μg/mL CAP working solution were added to 4 separate 2.0-g portions of a previously tested-negative sample. Afterward the samples prepared were subjected to the entire extraction procedure to obtain calibration standards with matrix equivalent concentrations of 0.1, 0.2, 0.3, and 0.4 ng/μL, respectively.

Method Validation. The method validation was carried out according to criteria set by the European Commission Decision 2002/657/EC (European Commission, 2003). During the validation process, the following parameters were established: specificity, linearity, recovery, within-laboratory repeatability, decision limit (**CCα**), and detection capability (**CCβ**). Specificity of the method was evaluated by the analysis of 20 different blank samples to investigate possible interferents eluting on CAP retention time. Linearity was established through the analytical curve obtained by duplicate analysis at 4 concentration levels: 0.1, 0.2, 0.3, and 0.4 μg/kg, and prepared by fortifying blank samples of each matrix before extraction. A fixed amount (0.5 ng/g) of CAP-d5 was added to all samples. The linearity was expressed as the correlation coefficient (r). Recovery of analytes was determined by comparison of peak areas from blank chicken meat samples spiked with 4 known fortification amounts of CAP and CAP-d5 before the preparation procedure to peak areas from matrix extracts spiked after it. Six replicates were conducted at each level. The within-laboratory repeatability was determined by the analysis of 6 blank chicken meats fortified with CAP at each of the 4 specified fortification levels. The CCα and CCβ were calculated by applying the calibration curve procedure described in Commission Decision 2002/657/EC. The CCα was expressed as the concentration corresponding to the γ-intercept plus 2.33 times the within-laboratory SD of the lowest calibration level (0.3 μg/kg). The CCβ was calculated as CCα + 1.64 times the SD of the within-laboratory reproducubility at 0.3 μg/kg.

Table 1. Ion transitions and their corresponding collision energies used for chloramphenicol (CAP) quantification[1]

Analyte	Ion transitions (m/z)	Collision energy (eV)
CAP	321 → 152	20
	321 → 194	16
CAP-d5	326 → 157	20

[1] m/z = mass-to-charge ratio; CAP-d5 is from Cambridge Isotope Laboratories (Andover, MA).

RESULTS AND DISCUSSION

Screening of CAP in Chicken Meats by ELISA

Chloramphenicol is a broad-spectrum antibiotic that is effective against both gram-positive and gram-negative organisms, and has been widely used in the past to treat several diseases in humans and animals. However, it is well known that this drug has many side effects (Impens et al., 2003). The present study was undertaken for the screening of CAP residues in chicken meat samples by using an ELISA method and for confirmation of the results by an LC-MS/MS method.

A commercial ELISA kit was used for presumptive CAP detection and quantification. From 180 samples, including 90 thigh and 90 breast meats, sold in Bursa markets, 15 (8.3%) presented the residues of this antibiotic. The mean concentration of CAP residues in the samples ranged from 12.64 to 226.22 ng/kg, with a mean value of 45.32 ng/kg. In 165 (91.6%) of the samples, the residual levels were below the LOD of 12.5 ng/kg and evaluated as negative by ELISA. The levels and the distribution of CAP determined by ELISA are given in Tables 2 and 3, respectively. There are several reports suggesting the presence and the incidence of CAP residues in chicken meats and other animal-origin foods. Mehdizadeh et al. (2010) previously recorded a 54.8% incidence of CAP in broiler chicken kidney, liver, and muscle samples analyzed by ELISA. A study conducted by Tajik et al. (2010) showed that 22 liver, 21 kidney,

Table 2. Chloramphenicol residues (ng/kg) in the chicken meat samples determined by ELISA

Sample	n	Number of positive samples (%)	Mean concentration ± SD	Minimum	Maximum
Thigh	90	10 (11.1)	31.32 ± 15.23	12.64	56.45
Breast	90	5 (5.5)	73.34 ± 88.60	13.32	226.22
Total	180	15 (8.3)	45.32 ± 53.03	12.64	226.22

and 14 muscle samples were positive for CAP, and the minimum and maximum levels of 0.54 and 155.2 ng/g were detected in the kidney and liver, respectively. In comparison with the present investigation, some other studies reported lower levels of CAP residues. Antibiotic contamination levels found by Kadim et al. (2010) and Mahgoub et al. (2006) ranged between 5.0 and 74.0 ng/kg (mean 14.38 ng/kg) in broiler chicken meats and 0.0 and 0.08 ng/kg (mean 0.02 ng/kg) in goat and sheep meats, respectively.

Confirmation of CAP in Chicken Meats by LC-MS/MS

Because of the potential health risks posed by residues of CAP, in 1994, the European Commission prohibited its use for the treatment of animals destined for food production (Council of the European Communities, 1996). No MRL has been established for CAP in animal-derived foods, as its toxic effects are not dose-dependent. Thus, EU has defined a 0.3-μg/kg minimum required performance limit for CAP in foods of animal origin (European Commission, 2003). Likewise, in Turkey, the use of CAP in animals produced for food was banned (Türk Gıda Kodeksi, 2002a) and an MRL for CAP in animal-origin foods was not established (Türk Gıda Kodeksi, 2002b). For these reasons, to confirm the results obtained with the ELISA, LC-MS/MS analysis was performed on the samples with high and low CAP residues. Fifteen ELISA positive (residual level of >12.5 ng/kg, LOD) and 45 negative (residual level of <12.5 ng/kg, LOD) chicken meat samples were subjected to LC-MS/MS assay. Two of the samples found positive and one of the samples found negative by ELISA were confirmed by LC-MS/MS (Table 4). The LC-MS/MS chromatogram obtained for an ELISA positive sample is represented in Figure 1. No CAP was found in the remaining 57 chicken samples. In the samples evaluated as positive by LC-MS/MS, the levels of CAP ranged from 150 ng/kg to 361 ng/kg. False-positive results were obtained by ELISA in 13 of 15 chicken meat samples and false-negative results in 1 of 45 samples. False-positive results by using an ELISA method were also indicated by Impens et al. (2003).

Table 3. Frequency of chloramphenicol (CAP) contents according to ELISA

CAP level (ng/kg)	n	Frequency (%)
<12.5[1]	165	91.6
12.5–37.5	9	5
37.5–62.5	4	2.2
62.5–87.5	1	0.5
>87.5	1	0.5

[1]The limit of detection (LOD).

Method Validation

The validation method was carried out according to the criteria of the European Commission Decision 2002/657/EC (European Commission, 2003). The specificity of the method was evaluated by the analysis of 20 blank samples to investigate potential interfering compounds at CAP retention time. The LC-MS/MS chromatograms of a CAP-free chicken muscle sample and a chicken muscle sample fortified with 0.1 μg/kg of CAP are presented in Figures 2 and 3, respectively. No interference was observed with the monitored MS reactions, indicating that our proposed method is highly specific for the determination of CAP. Response linearity was evaluated by calibration curves prepared at 4 concentration levels, ranging from 0.1 to 0.4 μg/kg of CAP in chicken meat samples. Six replicates were made for each level to produce the calibration curve. The concentration of the CAP-d5 was 0.5 μg/kg in all samples. A good linear relation with a correlation coefficient (r^2) of 0.9966 between the different concentration levels (measured by area ratio between CAP and CAP-d5) could be observed (Table 5). Recovery experiments were carried out using fortified blank chicken meat samples at 4 known concentration levels in 6 replicates and analyzed on 3 different days. The results show a good recovery, ranging between 97 and 104%. The within-laboratory repeatabilities were calculated at 4 fortification levels signified above, and variation coefficients (CV%) were 2%, except for a spike level of 0.2 μg/kg in which the CV% was 4%. According to the European Commission Decision 2002/657/EC, CV from a method should not exceed the value calculated by the Horwitz equation: $CV = 2^{(1-0.5 \log C)}$, where C is the mass fraction expressed in a power of 10. However, for mass fractions below 1 ng/kg, this equation gives unacceptably high CV values. Therefore, 2002/657/EC recommends that the CV should be as low as possible in these cases. Our results indicate that the method has adequate precision. The results for recovery and repeatability are summarized in Table 6. Because no MRL

Table 4. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay results for chloramphenicol

Assay	No. of negative samples	No. of positive samples	Residue level (ng/kg)
ELISA	45		<12.5[1]
		14	12.5–100
		1	>100
LC-MS/MS		1	150
		1	259
		1	361

[1]The limit of detection (LOD).

exists for CAP, CCα and CCβ were calculated using the calibration curve procedure described in Commission Decision 2002/657/EC and replaced the LOD and the limit of quantitation (LOQ). As shown in Table 5, calculated CCα and CCβ values were 0.10 and 0.11 μg/kg, respectively, and below the MRPL established by the EU, which is 0.3 μg/kg. Thus, these values appeared very satisfying.

In conclusion, our data indicated the presence of CAP residues in commercial chicken meat samples purchased from supermarkets and retail stores in Bursa province, Turkey. Furthermore, the present results also demonstrated that ELISA assay for screening of drug residues could be useful as a first attempt to gain some insight in the illegal use of CAP by some poultry meat producers, but confirmation of the results should always be

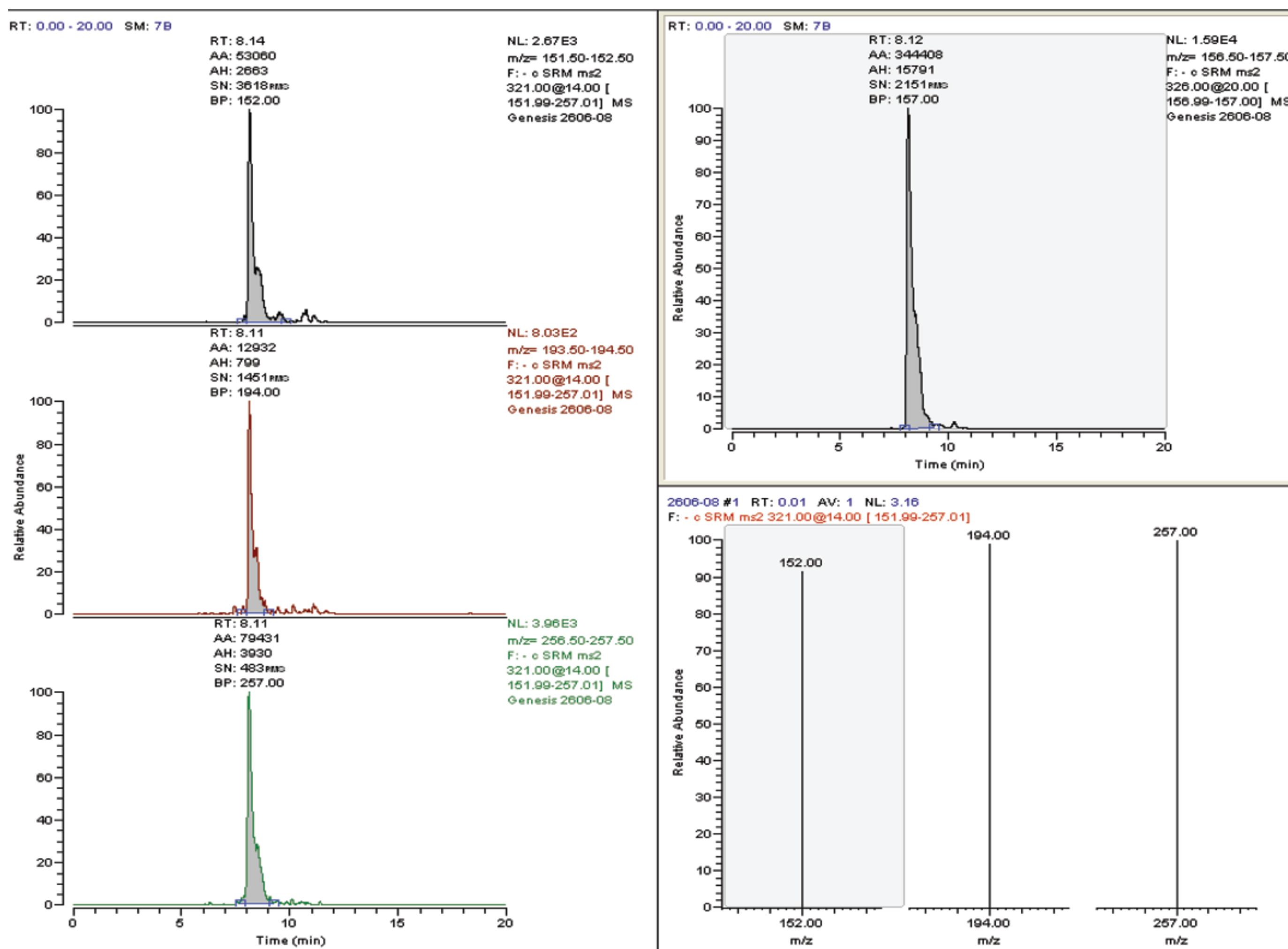

Figure 1. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) chromatogram of an ELISA positive (30 ng/kg) sample for chloramphenicol (CAP). m/z = mass-to-charge ratio; SRM = selective reaction monitoring (run here in the negative mode); RT = retention time; AA = peak area; AH = absolute height of the chromatogram peak; SN = signal-to-noise ratio at the peak maximum; BP = base peak mass; NL = neutral loss; F = scan filter; and AV = averaged (followed by the number of averaged scans). Color version available in the online PDF.

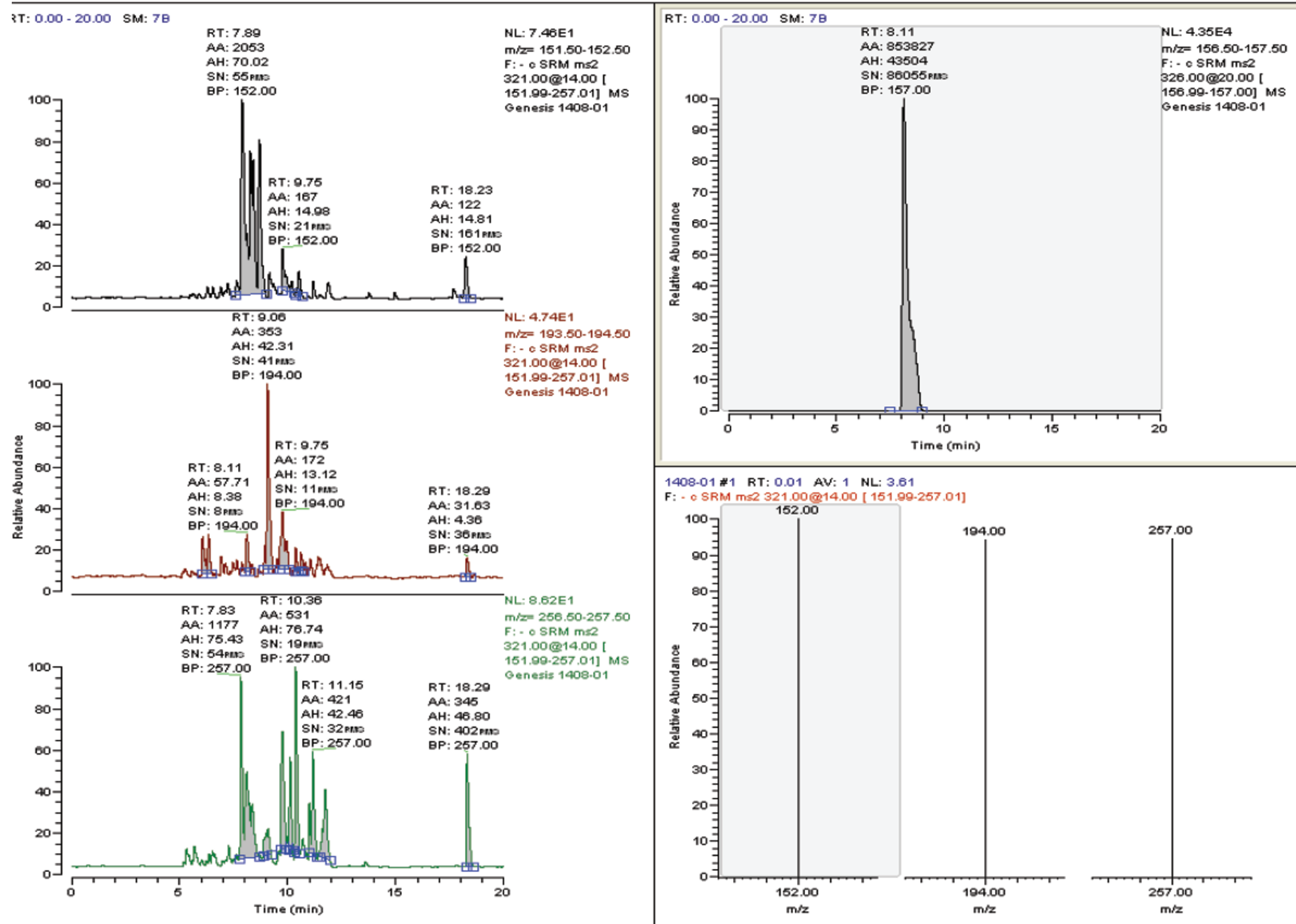

Figure 2. Typical chromatogram of a blank chicken muscle sample. m/z = mass-to-charge ratio; SRM = selective reaction monitoring (run here in the negative mode); RT = retention time; AA = peak area; AH = absolute height of the chromatogram peak; SN = signal-to-noise ratio at the peak maximum; BP = base peak mass; NL = neutral loss; F = scan filter; and AV = averaged (followed by the number of averaged scans). Color version available in the online PDF.

carried out by chromatographic methods coupled with spectrometric methods as well as LC-MS/MS. The validation method was based on the EU criteria for determination of CAP in chicken meats. The CCα (0.10 µg/kg) and CCβ (0.11 µg/kg) were lower than the MRPL value (0.3 µg/kg) set by the EU. The recovery and within-laboratory repeatibilities showed values between 97.3 and 104.0% and below 5%, respectively, which demonstrates reliability of the method for the intended purpose. Finally, the validation results were in accordance with the performance method of the European Commission Decision 2002/657/EC and the method was successfully applied to confirm and quantify CAP in chicken meats.

Table 5. Correlation coefficient (r^2), decision limit (CCα), and detection capability (CCβ) for chloramphenicol

Item	Value
r^2 of calibration curve	0.9966
CCα (µg/kg)	0.10
CCβ (µg/kg)	0.11

Table 6. Recovery and repeatability data of the method (n = 6)

Performance criterion	Validation data
Overall mean ± SD	
0.1 µg/kg	0.098 ± 0.001
0.2 µg/kg	0.194 ± 0.006
0.3 µg/kg	0.312 ± 0.005
0.4 µg/kg	0.394 ± 0.007
Recovery (% ± SD)	
0.1 µg/kg	98.5 ± 1.76
0.2 µg/kg	97.3 ± 3.38
0.3 µg/kg	104.0 ± 1.67
0.4 µg/kg	98.5 ± 1.87
Within-laboratory repeatability (% ± SD)	
0.1 µg/kg	2 ± 0.002
0.2 µg/kg	4 ± 0.007
0.3 µg/kg	2 ± 0.006
0.4 µg/kg	2 ± 0.007

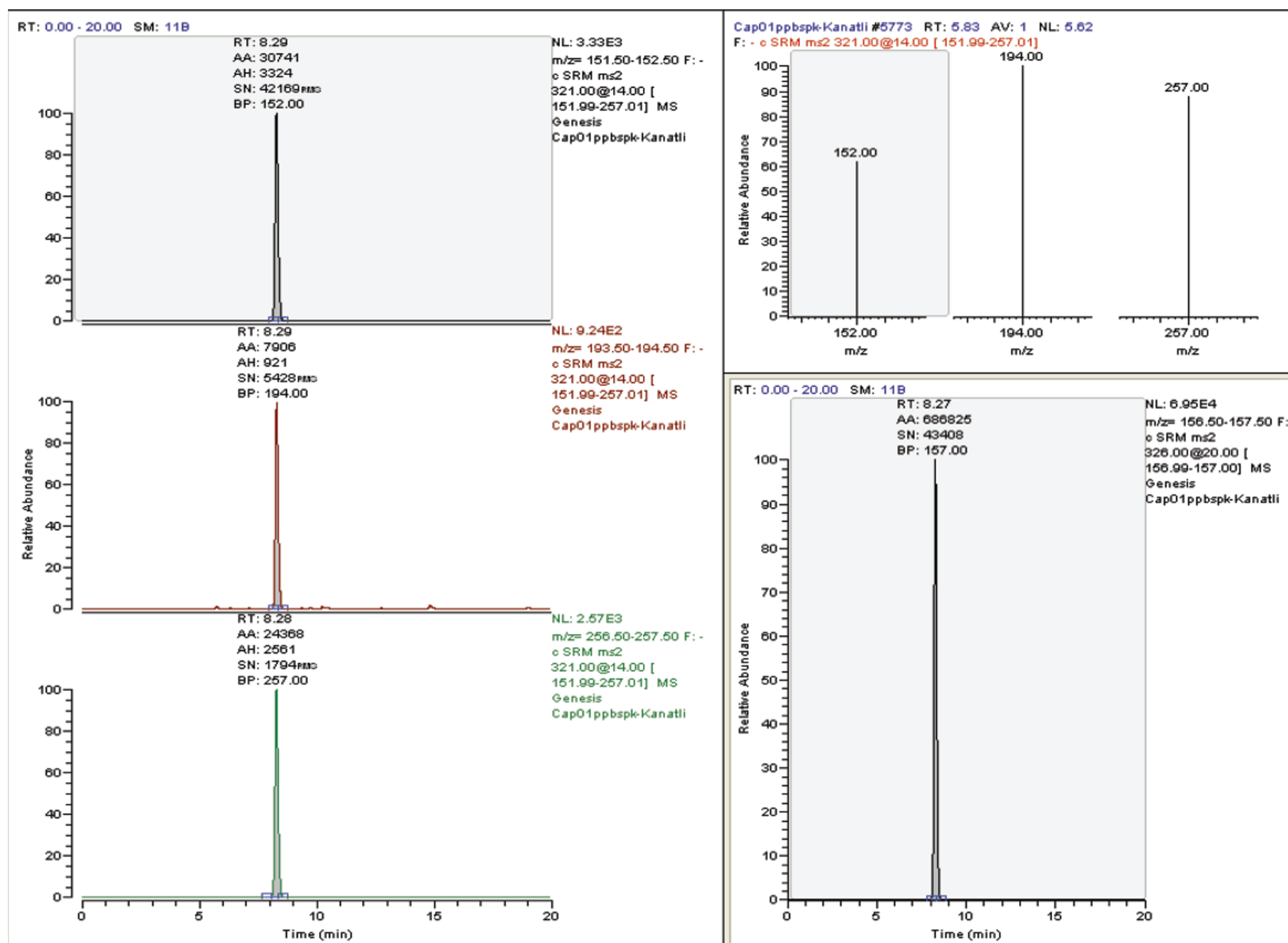

Figure 3. Typical chromatogram of a chicken muscle sample fortified with chloramphenicol (CAP) at 0.1 µg/kg and CAP-d5 (Cambridge Isotope Laboratories, Andover, MA) at 0.5 µg/kg. m/z = mass-to-charge ratio; SRM = selective reaction monitoring (run here in the negative mode); RT = retention time; AA = peak area; AH = absolute height of the chromatogram peak; SN = signal-to-noise ratio at the peak maximum; BP = base peak mass; NL = neutral loss; F = scan filter; and AV = averaged (followed by the number of averaged scans). Color version available in the online PDF.

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